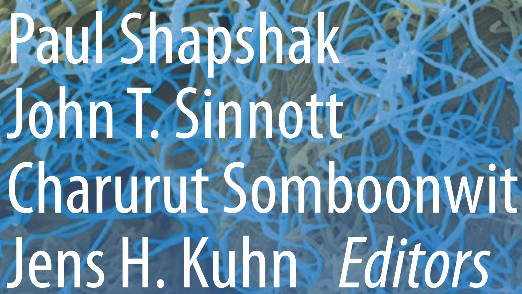

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John T. Sinnott
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Global Virology I

Identifying and Investigating Viral Diseases

Global Virology I - Identifying and Investigating Viral Diseases

Paul Shapshak • John T. Sinnott
Charurut Somboonwit • Jens H. Kuhn
Editors

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Foreword

Viral diseases are spreading globally. Recent changes are accelerating due to concomitant human behaviors including war, violence, poverty, starvation, and contemporaneous vector transmission. Additional factors include global warming, international travel, and encroachment of the prior balance of nature, i.e., invasion of nonhuman ecological domains by humans.

This book for professionals, students, faculty, and the interested reader brings to bear a snapshot of where we are.

We acknowledge and thank Professor Francesco Chiappelli (UCLA, Los Angeles, CA) for help in initiating this book, and Ioanna Panos Morris and Rita Beck of Springer Science+Business Media for help and guidance through the steps leading to the production of this book.

Preface

Global warming, ever-increasing international travel, concomitant changes in human and animal behaviors, and vector transmission all influence and have had a huge impact on the spread of viral diseases. Many excellent and informative books review these topics. To reference a few, Wertheim et al. [1] published a human infectious disease atlas and Petersen et al. [2] published a geographic guide to infectious diseases. Geopolitics is also discussed in these books, as is the involvement of many diseases, including measles, influenza, poliomyelitis, yellow fever, dengue, malaria, smallpox, cholera, leprosy, typhoid, typhus, bubonic plague, tuberculosis, and diseases caused by parasites and protozoa. Historically, of 150 common infections, the most devastating have been 35 diseases caused by bacteria, 28 diseases caused by viruses, and 6 diseases caused by protozoa [3].

This book provides trajectories and illustrations of viruses that have catapulted into the global arena (linked to humans, animals, and vectors) due to human behaviors in recent years, as well as viruses that have already shown expansion among humans, animals, and vectors just a few decades ago. Topics in the current book include vaccines, environmental impact, emerging virus transmission, filoviruses (Ebola virus), hemorrhagic fevers, flaviviruses, dengue evasion, papillomaviruses, hepatitis C, giant viruses, bunyaviruses, encephalitides, West Nile virus, Zika virus, XMRV, henipaviruses, respiratory syncytial virus, influenza, and several aspects of HIV-1 infection.

It should also be noted that among many articles pertaining to public health, lack of hygiene is demonstrably an important element in the spread of disease. Moreover, public education is a key component of what is needed to combat the spread of disease (e.g., hepatitis A) [4, 5].

In conclusion, the eradication of war, human trafficking, drug abuse, and poverty should be major goals toward the suppression of such pestilence. Education is a pillar upon which such eradication is based.

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Chapter 1
Short Peptide Vaccine Design and Development: Promises and Challenges

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Core Message There is a need for novel vaccine technologies where existing viral vaccine types (viruses, killed or inactivated viruses, and conjugate or subunits) are unsuitable against many viruses. Hence, short peptide (10–20 residues) vaccine candidates are considered promising solutions in recent years. These function on the principle of short epitopes developed through the binding of CD8+/CD4+-specific HLA alleles (12542 known so far). Thus, the specific binding of short peptide antigens to HLA alleles is rate limiting with high sensitivity in producing T-cell-mediated immune responses. Identification of HLA allele-specific antigen peptide binding is mathematically combinatorial and thus complex. Therefore, prediction of HLA allele-specific peptide binding is critical. Recent advancement in immune-informatics technologies with the aid of known X-ray-determined HLA-peptide structure data provides solutions for the accurate identification of short peptides as vaccine candidates for further consideration. Thus, we document the possibilities and challenges in the prediction, large-scale screening, development, and validation of short peptide vaccine candidates in this chapter.

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1 Introduction

The types of approved viral vaccines include live attenuated viruses, killed/inactivated viruses, and conjugate/subunits. However, these types of vaccine technologies may prove unsuitable against some viruses. In some cases, there is interest in the development of short peptide vaccines to fill the gaps. For example, the use of live attenuated HIV-1/AIDS vaccines is not as yet approved due to safety concerns [1]. There are several subunit vaccines under consideration and evaluation. However, one of these, the NIAID and Merck Co.-sponsored 2004 STEP (HVTN 502 or Merck V520-023) trial using three recombinant adenovirus-5 (rAD5) vectors containing HIV-1 genes Ad5-gag, Ad5-pol, and Ad5-Nef, did not show promising results [2]. This has led to the development of a multifaceted strategy for HIV-1/AIDS vaccine development. However, encouraging results were observed with four priming injections of a recombinant canary pox vector (ALVAC-HIV) and two booster injections of gp120 subunit (AIDSVAX-B/E) in a community-based, randomized, multicenter, double-blind, placebo-controlled efficacy trial (NCT00223080) in Thailand [3]. The main concern following this study was that this vaccine did not affect the degree of viremia or the CD4 T-cell count in patients who later seroconverted. Further studies indicated that the challenges with the development of an HIV-1/AIDS vaccine are viral diversity and host-virus molecular mimicry [4–6]. Nonetheless, there is considerable amount of interest to develop gp160 (gp120-gp41 complex) TRIMER envelope (ENV) protein as a potential vaccine candidate [4].

The production of an HIV-1 ENV spike protein trimer complex is nontrivial due to protein size, protein type, sequence composition, and residue charge polarity. Therefore, the need for the consideration of alternative approaches for vaccine development such as T-cell-based HLA-specific short peptide vaccines is promising

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[6, 7]. The LANL HIV molecular immunology database provides comprehensive information on all known T-cell epitopes in the literature [8]. Thus, these resources in combination with other predictive advancements described in this chapter are collectively useful for the design, development, evaluation, and validation of short peptide vaccine candidates.

2 Methodology

2.1 Structural Data

A structural dataset of complexes for class I HLA-peptide (Table 1.1) and class II HLA-peptide (Table 1.2) is created from the protein databank (PDB) [9]. The characteristic features of the datasets are presented in Tables 1.1 and 1.2.

2.2 Structural Superposition of HLA Molecules

The peptide-binding grooves of both class I HLA (Fig. 1.1a) and class II HLA (Fig. 1.1c) molecules were superimposed using the molecular overlay option in the Discovery Studio software from Accelrys® [10].

2.3 Molecular Overlay of HLA-Bound Peptides

HLA-bound peptides in the groove of both class I HLA (Fig. 1.1b) and class II HLA (Fig. 1.1d) molecules were overlaid using the molecular overlay option in the Discovery Studio software from Accelrys® [10].

2.4 Accessible Surface Area Calculations

Accessible surface area (ASA) was calculated using the WINDOWS software Surface Racer [12] with Lee and Richard implementation [13]. A probe radius of 1.4 Å was used for ASA calculation.

2.5 Relative Binding Measure

Relative binding measure (RBM) is defined as the percentage ASA $Å^2$ of residues in the peptide at the corresponding positions buried as a result of binding with the HLA groove. This is the percentage change in ASA (ΔASA) of the position-specific peptide residues upon complex formation with the HLA groove (Fig. 1.2).

Table 1.1 Dataset of class 1 HLA-peptide structures downloaded from PDB

S	Code	Allele	Peptide sequence	L	Source	RÅ	Year	Group	Country	State
1	1 W72	A*0101	EADPTGHSY	9	Melanoma related	2.15	2004	Ziegler A	Germany	Berlin
2	3BO8	A*0101	EADPTGHSY	9	Melanoma related	1.8	2008	Ziegler UB	Germany	Berlin
3	3UTS	A*0201	ALWGPDPAAA	10	Insulin	2.71	2012	Andrew SK	UK	Cardiff
4	3UTT	A*0201	ALWGPDPAAA	10	Insulin	2.6	2012	Sewell AK	UK	Cardiff
5	1I4F	A*0201	GVYDGREHTV	10	Melanoma related	1.4	2001	Mabbutt BC	Australia	Sydney
6	1JHT	A*0201	ALGIGILTV	9	Mart-1	2.15	2001	Wiley DC	USA	Cambridge
7	1B0G	A*0201	ALWGFFPVL	9	Human-peptide	2.6	1998	Collins EJ	USA	North Carolina
8	1I7U	A*0201	ALWGFVPVL	9	Synthetic	1.8	2001	Collins EJ	USA	North Carolina
9	1I7T	A*0201	ALWGVFPVL	9	Synthetic	2.8	2001	Collins EJ	USA	North Carolina
10	1I7R	A*0201	FAPGFFPYL	9	Synthetic	2.2	2001	Collins EJ	USA	North Carolina
11	1I1F	A*0201	FLKEPVHGV	9	HIV RT	2.8	2000	Collins EJ	USA	North Carolina
12	1HHI	A*0201	GILGFVFTL	9	Synthetic	2.5	1993	Wiley DC	USA	Massachusetts
13	1AKJ	A*0201	ILKEPVHGV	9	HIV-1 RT	2.65	1997	Jakobsen BK	UK	Oxford
14	1HHJ	A*0201	ILKEPVHGV	9	Synthetic	2.5	1993	Wiley DC	USA	Massachusetts
15	1QRN	A*0201	LLFGYAVYV	9	Tax peptide P6A	2.8	1999	Wiley DC	USA	Massachusetts
16	1QSE	A*0201	LLFGYPRYV	9	Tax peptide V7R	2.8	1999	Wiley DC	USA	Massachusetts
17	1QSF	A*0201	LLFGYPVAV	9	Tax peptide Y8A	2.8	1999	Wiley DC	USA	Massachusetts
18	1AO7	A*0201	LLFGYPVYV	9	HTLV-1 Tax	2.6	1997	Wiley DC	USA	Massachusetts
19	1BD2	A*0201	LLFGYPVYV	9	HTLV-1 Tax	2.5	1998	Wiley DC	USA	Massachusetts
20	1DUZ	A*0201	LLFGYPVYV	9	HTLV-1 Tax	1.8	2000	Wiley DC	USA	Massachusetts
21	1HHK	A*0201	LLFGYPVYV	9	Synthetic	2.5	1993	Wiley DC	USA	Massachusetts
22	1IM3	A*0201	LLFGYPVYV	9	HTLV-1 Tax	2.2	2001	Wiley DC	USA	Boston
23	1HHG	A*0201	TLTSCNTSV	9	HIV-1 gp120	2.6	1993	Wiley DC	USA	Massachusetts
24	1I1Y	A*0201	YLKEPVHGV	9	HIV-1 RT	2.2	2000	Collins EJ	USA	North Carolina
25	3FQN	A*0201	YLDSGIHSGA	10	Beta-catenin	1.65	2009	Purcell AW	Australia	Victoria

26	3FQR	A*0201	YLDGIHSGA	10	Beta-catenin	1.7	2009	Purcell AW	Australia	Victoria
27	3FQT	A*0201	GLLGSPVRA	9	Tyrosine-phosphatase	1.8	2009	Purcell AW	Australia	Victoria
28	3FQU	A*0201	GLLGSPVRA	9	Tyrosine-phosphatase	1.8	2009	Purcell AW	Australia	Victoria
29	3FQW	A*0201	RVASPTSGV	9	Insulin receptor	1.93	2009	Purcell AW	Australia	Victoria
30	3FQX	A*0201	RVASPTSGV	9	Insulin receptor	1.7	2009	Purcell AW	Australia	Victoria
31	1QQD	A*0201	QYDDAVYKL	9	HLA-CW4	2.7	1999	Wiley DC	USA	Massachusetts
32	1P7Q	A*0201	ILKEPVHGV	9	POL polyprotein	3.4	2003	Bjorkman PJ	USA	California
33	2HN7	A*1101	AIMPARFYPK	9	DNA polymerase	1.6	2006	Gajhede M	Denmark.	Copenhagen
34	1X7Q	A*1101	KTFPPTEPK	9	SARS nucleocapsid	1.45	2005	Gajhede M	Denmark.	Copenhagen
35	3BVN	B*1402	RRRWRRLTV	9	Latent membrane	2.55	2009	Ziegler A	Germany	Berlin
36	3BP4	B*2705	IRAAPPPLF	9	Lysosomal	1.85	2008	Ziegler A	Germany	Berlin
37	1HSA	B*2705	ARAAAAAAA	9	N/A	2.1	1992	Wiley DC	USA	Massachusetts
38	1JGE	B*2705	GRFAAAIAK	9	Synthetic (M9)	2.1	2002	Ziegler UB	Germany	Berlin
39	1OF2	B*2709	RRKWRRWHL	9	Intestinal	2.2	2004	Ziegler UB	Germany	Berlin
40	1JGD	B*2709	RRLLRGHNQY	10	s10R	1.9	2003	Ziegler A.	Germany	Berlin
41	1K5N	B*2709	GRFAAAIAK	9	Synthetic (M9)	1.09	2002	Ziegler UB	Germany	Berlin
42	3BP7	B*2709	IRAAPPPLF	9	Lysosomal	1.8	2008	Ziegler A.	Germany	Berlin
43	1ZSD	B*3501	EPLPQGQLTAY	11	BZLF1	1.7	2005	McCluskey J	Australia	Brisbane
44	1A9B	B*3501	LPPLDITPY	9	EBNA-3C	3.2	1998	Saenger W	Germany	Berlin
45	1A9E	B*3501	LPPLDITPY	9	EBV-Ebna3c	2.5	1998	Saenger W	Germany	Berlin
46	3LN4	B*4103	AEMYGSV TEHPSPSPL	16	Ribonucleo protein	1.3	2010	Blasczyk R	Germany	Hannover
47	3LN5	B*4104	HEEAVSVDRVL	11	Thioadenosine	1.9	2010	Blasczyk R	Germany	Hannover
48	3DX6	B*4402	EENLLDFVRF	10	EBV decapeptide	1.7	2009	Rossjohn J	Australia	Victoria
49	3DX7	B*4403	EENLLDFVRF	10	EBV decapeptide	1.6	2009	Rossjohn J	Australia	Victoria
50	1SYS	B*4403	EEPTVIKKY	9	Sorting nexin 5	2.4	2004	McCluskey J	Australia	Victoria
51	3DXA	B*4405	EENLLDFVRF	10	EBV decapeptide	3.5	2009	Rossjohn J	Australia	Victoria

(continued)

Table 1.1 (continued)

S	Code	Allele	Peptide sequence	L	Source	RÅ	Year	Group	Country	State
52	3DX8	B*4405	EENLLDFVRF	10	EBV decapeptide	2.1	2009	Rossjohn J	Australia	Victoria
53	1E27	B*5101	LPPVVAKEI	9	HIV-1 Kml	2.2	2000	Jones EY	UK	Oxford
54	1A1M	B*5301	TPYDINQML	9	HIV-2 gag	2.3	1998	Jones EY	UK	Oxford
55	1A1O	B*5301	KPIVQYDNF	9	HIV-1 Nef	2.3	1998	Jones EY	UK	Oxford
56	3VRJ	B*57:01	LTTKLTNTN	10	Cytochrome c Oxidase	1.9	2012	McCluskey J	Australia	Victoria
57	3UPR	B*57:01	HSITYLLPV	9	Synthetic construct	2	2012	Peters B	USA	Gainesville
58	3VRI	B*57:01	RVAQLEQVYI	10	SNRPD3	1.6	2012	McCluskey J	Australia	Victoria
59	2RFX	B*5701	LSSPVTKSF	9	Synthetic construct	2.5	2008	McCluskey J	Australia	Victoria
60	3VH8	B*5701	LSSPVTKSF	9	Ig kappa chain C region	1.8	2011	Rossjohn J	Australia	Victoria
61	2DYP	B27	RIIPRHLQL	9	Histone H2A.x	2.5	2006	Maenaka K	Japan	Fukuoka
62	2D31	B27	RIIPRHLQL	9	Histone H2A.x	3.2	2006	Maenaka K	Japan	Fukuoka
63	1EFX	Cw*0304	GAVDPLLAL	9	Importin-2	3	2000	Sun PD	USA	Maryland
64	1IM9	Cw*0401	QYDDAVYKL	9	Synthetic	2.8	2001	Wiley DC	USA	Cambridge
65	3CDG	G	VMAPRTLFL	9	Synthetic construct	3.1	2008	Rossjohn J	Australia	Victoria
66	3KYN	G	KGPPAALTL	9	Synthetic construct	2.4	2010	Clements CS	Australia	Victoria
67	3KYO	G	KLPAQFYIL	9	Synthetic construct	1.7	2010	Clements CS	Australia	Victoria

S = Serial number; Code = PDB code; L = Length of peptide; R = Resolution

Table 1.2 Dataset of class 2 HLA-peptide structures downloaded from PDB

S	Code	Allele	Peptide sequence	L	Source	RÅ	Year	Group	Country	State
1	1UVQ	DC1	EGRDSMNLPSTKVSWAA VGGGGSLVPRGSGGGG	33	Human Orexin	1.8	2004	Fugger L	UK	Oxford
2	1S9V	DQ1	LQPFPQPELPY	11	Synthetic	2.2	2004	Sollid LM	USA	Stanford
3	2NNA	DQ8	QQYPSGEGSFQPSQENPQ	18	Gluten	2.1	2006	Anderson RP	Australia	Victoria
4	1JK8	DQ8	LVEALYLVCGERGG	14	Human insulin	2.4	2001	Wiley DC	USA	Boston
5	4GG6	DQ1	QQYPSGEGSFQPSQENPQ	18	MM1	3.2	2012	Rossjohn J	Australia	Victoria
6	1KLG	DR1	GELIGILNAAKVPAD	15	Synthetic	2.4	2001	Mariuzza RA	USA	Maryland
7	1KLU	DR1	GELIGTLNAAKVPAD	15	Synthetic	1.9	2001	Mariuzza RA	USA	Maryland
8	1T5W	DR1	AAYSDQATPLLLSPR	15	Synthetic	2.4	2004	Stern LJ	USA	Massachusetts
9	2IAN	DR1	GELIGTLNAAKVPAD	15	Human	2.8	2006	Mariuzza RA	USA	Maryland
10	2FSE	DR1	AGFKGEQGPKGEPG	14	Collagen	3.1	2006	Park HW	USA	Memphis
11	1SJH	DR1	PEVIPMFSALSEG	13	HIV1	2.2	2004	Stern LJ	USA	Cambridge
12	2Q6W	DR1	AWRSDEALPLGS	12	Integrin	2.2	2007	Stern LJ	USA	Cambridge
13	1ZGL	DR2	VHFFKNIVTPRTPGG	15	Myelin	2.8	2005	Mariuzza RA	USA	Maryland
14	1H15	DR2	GGVYHFVKKHVHES	14	EPV related	3.1	2002	Fugger L	UK	Oxford
15	1A6A	DR3	PVSKMRMATPLLMQA	15	Human CLIP	2.7	1998	Wiley DC	USA	Massachusetts
16	2SEB	DR4	AYMRADAAAGGA	12	Collagen	2.5	1997	Wiley DC	USA	Massachusetts

S = Serial number; Code = PDB code; L = Length of peptide; R = Resolution

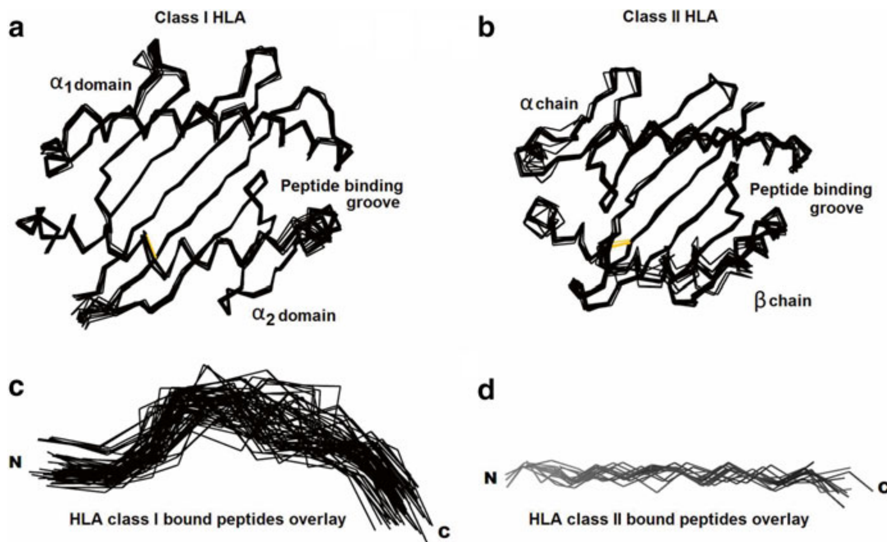

Fig. 1.1 The structural basis for short peptide vaccine design is illustrated. The allele-specific nomenclature defined, ethnicity profiled using known HLA sequences at the IMGT/HLA database [11], and the striking backbone structural similarity of antigen peptides at the HLA binding groove is the bottleneck. This is generated with using a dataset (Tables 1.1 and 1.2) of HLA-peptide complexes (67 class I and 16 class II) retrieved from protein databank (PDB) [9] using with Discovery Studio® (Accelrys Inc.) [10]. (**a**) The peptide-binding groove (superimposed) in class I HLA is structurally similar among known alleles and complexes. (**b**) The peptide-binding groove (superimposed) in class II HLA is structurally similar among known alleles and complexes; (**c**) class I HLA-bound peptides overlay showing structural constraints (bend peptides) at the groove; (**d**) class II bound peptides overlay showing extended conformation at the groove. This clearly suggests that class I (panel **c**) and class II (panel **d**) bound peptides do not have identical binding patterns at the groove

3 Results and Discussion

3.1 HLA-Peptide Binding Prediction for T-Cell Epitope Design

The rate-limiting step in T-cell epitope design is allele-specific HLA-peptide binding prediction. The number of known HLA alleles is over 12542 in number as of March 2015 at the IMGT/HLA database [11]. Hence, a number of methods have been formulated so far and optimized for HLA-peptide binding prediction during the last two decades. Structural information on HLA-peptide complexes has increased our understanding of their binding patterns (Tables 1.1 and 1.2). The HLA-binding groove is structurally similar among class I (Fig. 1.1a) and class II (Fig. 1.1b) alleles. The class I (Fig. 1.1c) and class II (Fig. 1.1d) bound peptides do not show an identical binding pattern at the groove. A detailed illustration of peptide binding patterns (Fig. 1.2) at the groove of class I and class II alleles provides valuable insights using mean and deviation profiles (Fig. 1.3).

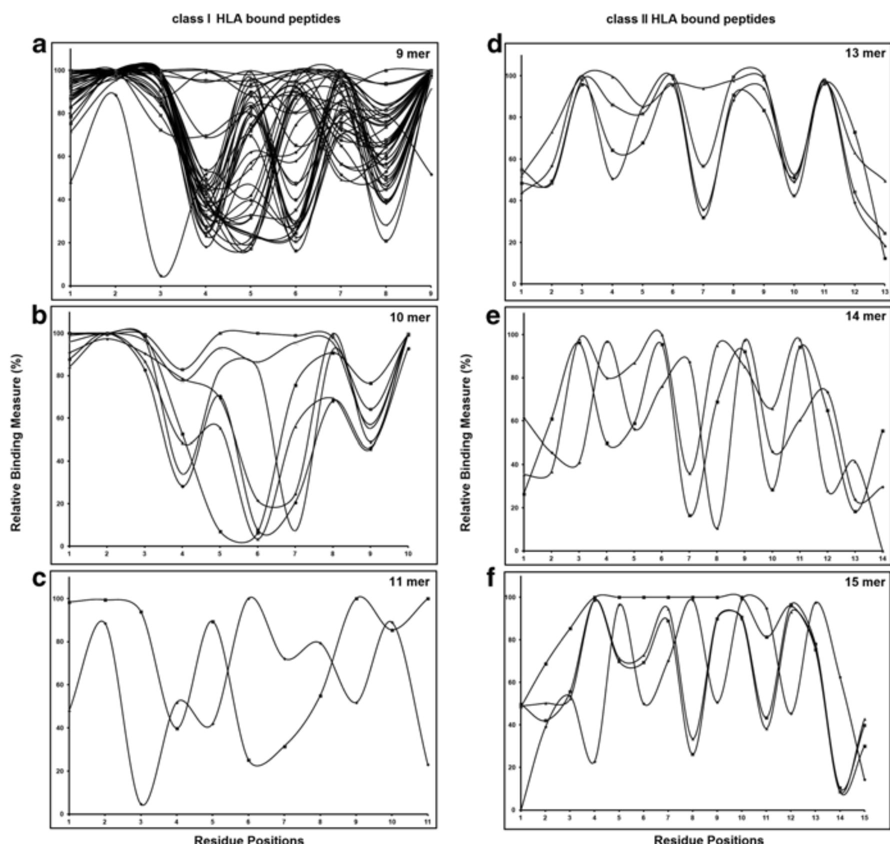

Fig. 1.2 The peptide binding pattern at the groove is illustrated as function of residue position for class I and class II alleles using a dataset (Tables 1.1 and 1.2) of HLA-peptide complexes (67 class I and 16 class II) retrieved from protein databank (PDB). This dataset is represented by several class I and class II alleles (see Tables 1.1 and 1.2). The peptide lengthwise distribution of the binding pattern is shown as relative binding measure using change in solvent-accessible surface area upon complex formation with the HLA groove

A comprehensive description of HLA-peptide binding prediction is documented [14, 15]. Lee and McConnell [16] proposed a general model of invariant chain association with class II HLA using the side-chain packing technique on a known structural template complex with self-consistent ensemble optimization (SCEO) [17, 18] using the program CARA in the molecular visualization/modeling software LOOK (Molecular Application Group (1995), Palo Alto, CA) [16, 19]. This was an important development in the field and the approach was extended to a large dataset of known HLA-binding peptides. Kangueane et al. [20] collected over 126 class I peptides with known IC_{50} values from literature with defined HLA allele specificity. These peptides were modeled using available templates for a large-scale assessment of peptide binding to defined HLA alleles. Thus, a structural framework was estab-

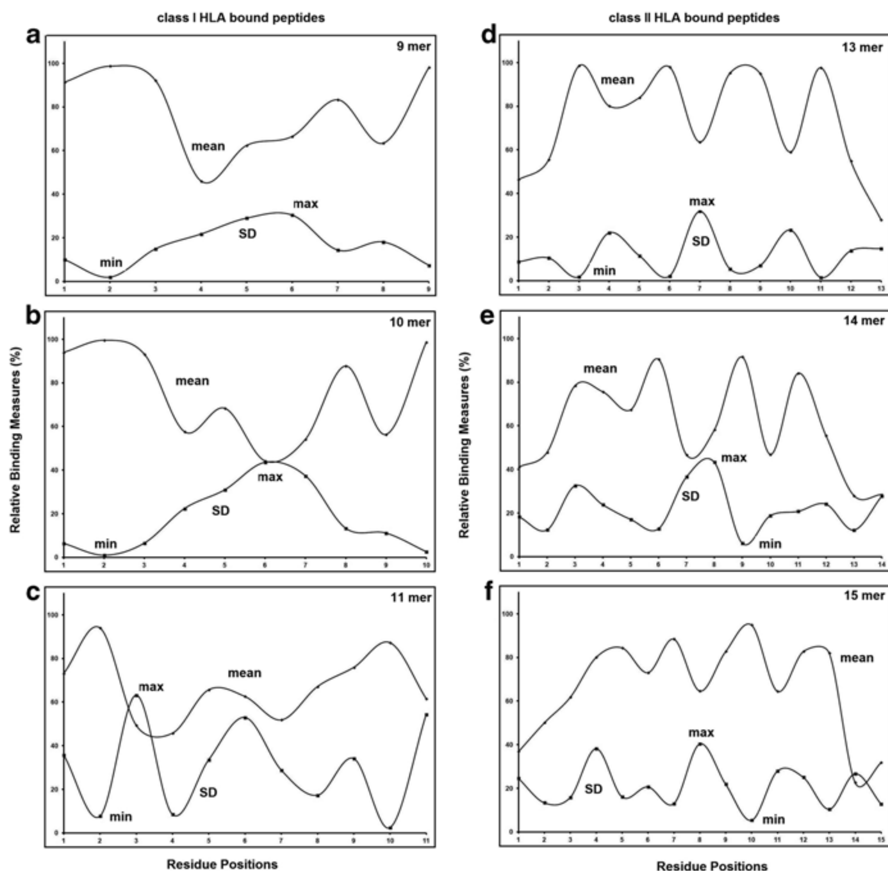

Fig. 1.3 The mean peptide binding pattern with standard deviation (SD) at the groove is illustrated as function of residue position for class I and class II alleles using a dataset (Tables 1.1 and 1.2) of HLA-peptide complexes (67 class I and 16 class II) retrieved from protein databank (PDB). This provides insight into the understanding of the nature of peptide binding at the groove towards the design of an effective T-cell epitope candidate

lished for discriminating allele-specific binders from non-binders using rules derived from a dataset of HLA-peptide complexes. This procedure was promising.

An extended dataset of class 1 and class 2 complexes were manually created, curated, and analyzed for insights into HLA-peptide binding patterns at the groove [21]. These studies lead to a detailed analysis of the HLA-peptide interface at the groove and the importance of peptide side chain and backbone atomic interactions were realized [22]. Meanwhile, the amount of structural data on HLA-peptide complexes was increasing in size leading to the development of an online database [23]. Thus, information gleaned from HLA-peptide structural complexes helped to identify common pockets among alleles in the binding groove and provided insights into

functional overlap among them [24]. The need for a simple, robust, generic HLA-peptide binding prediction was evident. Therefore, a model was formulated by defining virtual pockets at the peptide-binding groove using information gleaned from a structural dataset of HLA-peptide complexes [25]. The model (average accuracy of 60 %) was superior because of its application to any given class I allele whose sequence is clearly defined. The model (53 % accuracy) was then extended for class II prediction using a class II-specific HLA-peptide structural dataset [26].

The techniques thus far established are highly promising towards short peptide vaccine design and development [27, 28]. Nonetheless, it was observed that alleles are covered within few HLA supertypes, where different members of a supertype bind similar peptides, yet exhibiting distinct repertoires [29]. These principles led to the development of frameworks to group alleles into HLA supertypes [30, 31], understand their structural basis [32], and cluster alleles based on electrostatic potential at the groove [33]. These observations should aid in the design of peptide vaccine candidates for viruses including HIV/AIDS [5, 6]. Further, for example, the importance of protein modifications to enhance HIV-1 ENV trimer spike protein vaccine across multiple clades, blood, and brain is discussed [4]. Currently available types of vaccine technology [34, 35], such as live virus, killed virus, and conjugate vaccines, have failed to produce a promising vaccine against several clinically important viruses, including HIV/AIDS [36]. Therefore, short peptide vaccines are promising solutions for viral vaccine development. It should be noted that there are many other viruses for which vaccines are needed. Examples of additional viruses for which there are no vaccines available, vaccines are still under development, vaccine failures occurred, or more effective vaccines are needed include RSV, measles, HBV, WNV, Coronaviruses, H5N1 influenza virus, HCV, Adenovirus, Hantavirus, and Filoviruses [37–47].

4 Conclusion

The design and development of short peptide cocktail vaccines is a possibility in the near future. This function on the principle of short epitopes developed through the binding of CD8+/CD4+-specific HLA alleles. HLA molecules are specific within ethnic populations and are polymorphic with more than 12542 known alleles as of March 2015. Thus, the binding of short peptide antigens to HLA alleles is rate limiting yet specific, with high sensitivity, while producing T-cell-mediated immune responses. Our understanding of this specific peptide binding to HLA alleles has improved using known HLA-peptide complexes. There is a search for superantigen peptides covering major HLA supertypes. Thus, peptide-binding predictions with large coverage, accuracy, sensitivity, and specificity are essential for vaccine candidate design and development. It should be noted that available HLA-peptide binding prediction methods are highly promising in these directions.

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Chapter 2
Human Papillomaviruses

Lynette J. Menezes, Jamie P. Morano, and Leela Mundra

Core Message HPV-associated anogenital and oropharyngeal cancers place an enormous burden on the health of populations globally. The natural progression of HPV infection is potentiated by HIV coinfection. Further investigation into site-specific HPV acquisition is vital given the increasing trend in anal and oropharyngeal cancers and the need to inform prevention and treatment. HPV vaccination for both females and males is a promising strategy to prevent HPV infection and potential oncologic sequelae.

1 Introduction

Human papillomavirus (HPV) is a major cause of infection related malignancies at multiple anatomic entry sites in both men and women globally. As the most common sexually transmitted infection among men and women worldwide, it is estimated that between 50 and 80 % of men and women will acquire an HPV infection in their lifetime [1]. HPV was first discovered to be an infectious etiological agent of cervical cancer by Harald zur Hausen in the late 1970s, when his laboratory isolated HPV-16 and 18 from cervical cancer biopsies [2]. Overall 4.8 % of all incident cancers globally can be attributable to HPV infection [3] although the fraction attributable to HPV varies by anatomic site. HPV infection has been implicated in nearly 100 % of invasive cervical cancers, 88 % of anal cancers, 70 % of oropharyngeal and vaginal cancers, 43 % of vulvar cancers, and 50 % of penile cancers [3, 4].

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Currently, there are more than 100 fully sequenced HPV genomes that infect the skin and mucosal squamous epithelia. Of these, nearly half have been identified in the anogenital tract [5]. A substantial portion of previous research on HPV has been devoted to understanding the epidemiology of HPV infection, carcinogenesis, screening, and immunization in prevention of cervical cancer. As HPV-associated cervical, anal, and oropharyngeal cancers place an enormous burden on the health of populations globally, more attention has been turned to HPV screening and vaccination. This chapter describes HPV viral structure, molecular biology, and immune response. Additionally, it examines the epidemiology, natural history, and risk factors associated with HPV infection of the cervix, anal canal, and oropharynx including the effects of human immunodeficiency virus (HIV) infection with chronic immunosuppression. Lastly, it discusses treatment modalities and prevention strategies, specifically screening and immunization.

2 Virus Structure and Molecular Biology

HPV is a small, circular, double-stranded non-enveloped DNA virus approximately 55 *nm* in diameter [6, 7]. Its DNA genome is approximately 8 kb [8] and contains six nonstructural proteins (E1, E2, E4-E7) that are involved in DNA replication and cell immortalization [1] The virus has two structural proteins, L1 and L2, which are produced late in the infectious cycle; [9] L1 is the major component on the exterior surface of the virion, and L2 is the minor structural protein that typically interacts with L1 and the viral genomic DNA. L1 protein spontaneously self-assembles into capsomeres and virus-like particles (VLPs) when expressed in eukaryotic organisms; thus it is responsible for the initial interaction of the HPV capsid with the host. L2 proteins interact with E2 proteins produced earlier in the viral replication cycle and facilitate transportation of L1 to the nucleus and encapsulation of viral DNA [10–12].

There are more than 100 HPV genotypes that infect the human epidermal or mucosal epithelial cells with varying clinical manifestations and oncogenic potential ranging from benign cutaneous lesions to advanced squamous cell carcinomas of the anogenital and oropharyngeal areas depending on anatomic sites of exposure. These HPV genotypes are divided into low-risk and high-risk categories based on their ability to integrate into host DNA and therefore the potential to produce lesions [13, 14]. Low-risk HPV genotypes such as HPV-6 and 11 do not integrate into the host DNA and are associated with benign warts called condyloma acuminatum, usually found on the oral or genital regions. Other HPV genotypes such as HPV-1, 2, 3, and 10 cause cutaneous lesions, such as common digital warts and flat warts [15]. High-risk genotypes, such as HPV-16, 18, 31, 33, 45, and 56 are commonly found integrated into host DNA and are associated with anogenital lesions that may progress to carcinoma [14]. Currently, there are 13 HPV types, HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 that are designated as carcinogenic [16]. HPV-16 and 18 are the most carcinogenic types, and account for approximately 92 % of anal cancers, 90 % of oropharyngeal cancers, 80 % of vulvovaginal cancers,

70 % of cervical cancers, and 63 % of penile cancers [17]. The attributable fraction of HPV-16 is far greater than HPV-18 at all neoplastic transformation sites [17]. The complex interactions between the HPV genotype, viral genetic variables, host immune response, the phenotype of the infected epithelial cell, and environmental and lifestyle choices impact clinical and microscopic presentation [12, 18].

3 Transmission and Immune Response

HPV is highly transmissible through cutaneous and mucosal contact and has a broad incubation period from weeks to years, depending on the amount (dose) of virus transmitted. The infectious period commences when the virus reaches the basal layer of the epithelium, binds, and enters human cells [14, 19]. (Fig. 2.1) [14]. The

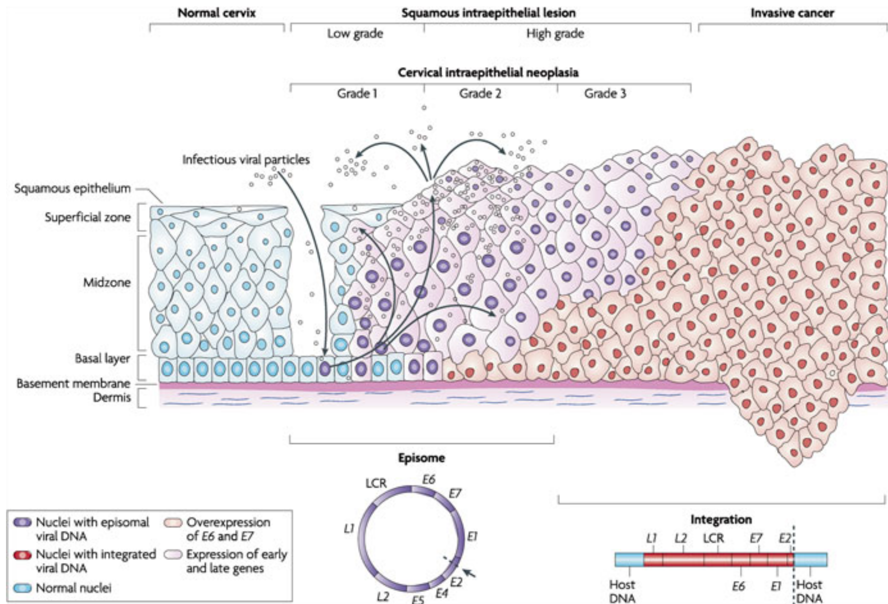

Fig. 2.1 Human papillomavirus (HPV) induced progression to invasive cervical cancer (ICC). HPV gains entry into the basal cells through microabrasions in the cervical epithelium. Following infection, the early HPV genes E1, E2, E4, E5, E6, and E7 are expressed and the viral DNA replicates from episomal DNA. The viral genome undergoes further replication in the upper mid and superficial layers of epithelium where the late genes L1 and L2, and E4 are expressed. L1 and L2 encapsulate the viral genomes to generate progeny virions. If untreated, the shed virus can initiate a new infection. Viral replication continues in low grade intraepithelial lesions. Some high-risk HPV infections progress from low grade to high-grade cervical intraepithelial neoplasias, and if untreated, some of these lesions progress to invasive cancer. This progression occurs when the HPV genome integrates into the host chromosomes (shown above with red nuclei), accompanied by a loss or disruption of E2, and the subsequent upregulation of E6 and E7 oncogene expression. Reproduced with permission from Woodman et al. [14]

HPV cycle is influenced by the maturity of the infected keratinocyte, as the production of virions is limited to the mature suprabasal epithelial cells [8, 12]. Active replication commences when the basal cells penetrate to the suprabasal compartment and initiates the terminal differentiation program. HPV will replicate and be released into the environment for a variable amount of time, causing viral DNA to be detected [1, 14, 19].

The HPV structural and nonstructural viral regulatory proteins are a result of viral gene expression. The E4 protein is expressed in terminally differentiated keratinocytes in the squamous epithelium, while E1 and E2 are associated with the regulation of viral DNA replication, and early transcription. HPV proteins E6 and E7 that induce proliferation, immortalization, and malignant transformation of cells are critical to viral replication [1, 20]. Their interactions with the proteins pRB and p53 result in multiple mutations that are thought to be the mechanisms of oncogenesis [1]. Such continued activity of E6 and E7 increase genomic instability and result in the accumulation of oncogenic mutations, loss of cell-growth control, and eventually cancer formation. The viral genome eventually integrates into the host genome, providing constant level of E6 and E7 protein activity due to stabilization of the mRNA, further developing the tumor [7, 14, 19, 21, 22].

In a normal host, a cell mediated immune (CMI) response will often clear the virus [22, 23]. Most HPV infections are transient and asymptomatic, over 50% of new infections are cleared in 6–18 months, while 80–90 % can be cleared within 2–5 years through the immune system or other mechanisms [1, 24]. However, in approximately 10–20 % of individuals, and more commonly in those with immune compromise, a failure to develop an effective CMI results in chronic and persistent HPV replication in the host nucleus of a differentiating skin or mucosal epithelial cell. For these individuals unable to clear the virus, HPV infection is likely to progress to clinically or histologically significant lesions [25].

When infection involves oncogenic HPV genotypes, a human host is at greater risk of developing high-grade precancerous lesions that may advance to invasive carcinoma. Such lesions can be understood to be along a histologic continuum of: (1) low grade lesions, where HPV continues to replicate in an episomal state, (2) high grade lesions resulting from viral integration into the host genome, and (3) invasion that can represent oncological transformation [14, 26]. For example, women with a history of genital warts are shown to have an increased risk of progression to stages of cervical intraepithelial neoplasia (CIN1, CIN2, CIN3) and cancer [27].

4 Epidemiology of Invasive Cervical Cancer

Invasive cervical cancer (ICC) is the fourth leading cause of cancer in women globally representing 528,000 cases and 266,000 deaths in 2012 [28]. Age-standardized ICC incidence is estimated at 14 per 100,000 women worldwide with incidence rates greater than 30 per 100,000 women in Melanesia and sub-Saharan Africa [28].

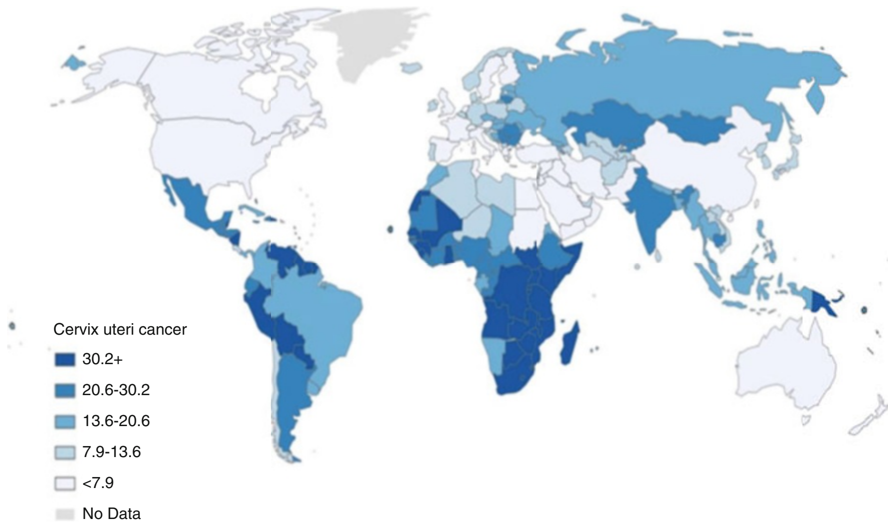

Fig. 2.2 Age-standardized incidence of cervical cancer worldwide in 2012. Reproduced with permission from GLOBOCAN 2012 [28]

In 2012, 86 % of ICC cases and 88 % of ICC deaths occurred in less developed countries (Fig. 2.2) [28]. In a recent meta-analysis of 194 studies with greater than one million cytologically normal women, HPV prevalence varied from 16 % in Latin America to greater than 30 % among women from Eastern Africa and the Caribbean [29]. HPV-16 was the predominant genotype (3.2 %) followed by HPV-18 (1.4 %), HPV-52 (0.9 %), HPV-31 (0.8 %), and HPV-58 (0.7 %) [29]. Another meta-analysis that included studies comprised of women with normal and abnormal cytology found that HPV prevalence increased with severity of cervical abnormalities [30]. HPV prevalence rose from 76 % in women with CIN1 to 90 % in women with CIN3 [30]. HPV-16 was the most common genotype (63 %) in ICC cases followed by HPV-18 (16 %) and HPV-45 (5 %) [30].

4.1 Natural History of Cervical HPV Infection

HPV prevalence varies with age globally. In Europe and North America, the highest HPV prevalence was among women younger than 25 years old, followed by a gradual decline over time with a lower HPV prevalence in women older than 45 years. HPV prevalence was more constant in women from Asia and Africa, whereas among women from Latin America and the Caribbean, HPV prevalence declined followed by a second prevalence peak during middle age [29].

Although studies in several developed countries show that HPV prevalence can reach 40–80 % in young women 18–25 years old [31–33], many prospective studies have demonstrated that nearly 90 % of women clear these asymptomatic infections

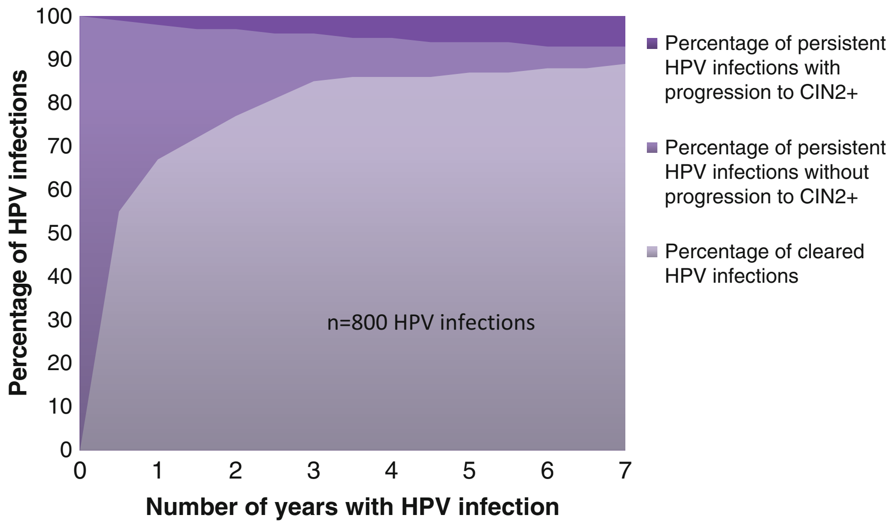

Fig. 2.3 Clearance, persistence, and progression of human papillomavirus (HPV) infections among 599 women, 18 years and older, from Guancaste, Colombia over 7 years of follow-up from 1993 to 2001. Adapted from Rodriguez et al. [36]

within 2 years [34–36]. Of women with prevalent infections, an estimated 4–10 % have persistent infections that may lead to neoplastic disease [31]. In a subset of 599 women from Guanacaste, Costa Rica, with 800 oncogenic HPV infections and a mean 6.7 years of follow-up, 7 % (58) of HPV infections were persistent with progression to CIN2+ (Fig. 2.3) [36]. The cumulative risk for CIN2+ was 21 % for women with infections that persisted beyond 12 months [36]. Women with persistent infections were older (mean 43 years) compared with those who cleared (mean 23 years). Those with persistent infections also had multiple prevalent infections at baseline. Not all women with persistent HPV infections developed neoplastic disease, but these numbers were small [36].

Despite the extensive research on the natural history of HPV and neoplastic disease of the cervix, there are no well-developed, sensitive tools to detect the exact time at which a high-risk HPV infection transforms into a CIN3+ lesion [24]. Moscicki et al. note that the time to development of a CIN3 lesion is shorter than the decades needed for a CIN3 lesion to progress to complete invasion. In a few aggressive cases, however, CIN3 may progress to early invasive disease. While persistence of HPV infection is critical to the development of neoplastic disease, detection of HPV genotype—including some genetic variants within a genotype—is a better predictor of neoplasia for those with persistent infections [37]. Higher viral loads of oncogenic HPV types are predictive of more severe cytologic abnormalities [24, 38]. Currently, severe neoplastic disease as determined by CIN3 or CIN3+ is the best available clinical surrogate marker of cancer risk, since sometimes CIN2 lesions may result from non-oncogenic HPV types and are often not clearly distinguishable from CIN1 or CIN3 [24].

4.2 Risk Factors for Cervical HPV Infection, Persistence, and Progression to Invasive Cancer

Numerous studies have investigated factors that increase risk of HPV acquisition, persistence, and progression to invasive cervical disease. Younger age at first sexual intercourse is linked to increased HPV acquisition [39]. The high prevalence of infections among adolescents and young adults may be due to immature cervical epithelium (a combination of columnar, squamous, and metaplastic epithelium) as compared to adult women with more impervious squamous cervical epithelium [24, 40, 41]. Of note, most infections are transient in younger populations, and studies have shown that prevalent HPV infections are more likely to persist in women older than 40 years [34]. Rodriguez et al. [34] found that new infections in older women were not associated with CIN2 or severe cervical disease, but instead, prevalent infections were highly correlated with progression to CIN2 or more severe disease, suggesting that persistent infections that were acquired at a young age were more likely to invade [24]. The number of sexual partners, both recent and lifetime, as well as male partner sexual behavior have a strong influence on acquisition of all types of cervical HPV [33, 42–44]. In one study, the risk of HPV detection increased sevenfold among women reporting ≥8 sex partners in their lifetime and increased nearly fivefold among women reporting a new sex partner in 90 days before study enrollment [33, 43]. Likewise, HPV infection increased tenfold among young women whose male partner reported ≥6 lifetime partners [45].

Several studies have also documented the association of cigarette smoking with persistence and development of cervical neoplasia [46–49]. Among women 18–35 years old, the duration of HPV infection was longer at 10.7 months for smokers versus 8.5 months for nonsmokers. A dose response relationship was evident, wherein women who had smoked for greater than 6 years were 60 % less likely to clear an HPV infection [46]. Similarly, women who were former or current smokers were two to four times more likely to develop CIN3 or cancer compared to nonsmokers [47–49]. Long-term oral contraceptive (OC) use has been associated with an elevated risk for persistent HPV infection and progression to invasive cancer [48, 50]. Individual data from 35 studies of women with and without cervical cancer globally showed that the relative risk for cervical cancer increased with current use of oral contraceptives. However, this risk decreased on stopping OC use, and by 10 or more years, risk was similar to that of never-users [50].

5 Epidemiology of Anal Cancer

Anal cancers are rare with an age-standardized incidence of less than 2 cases per 100,000 men and women per year, worldwide [51]. They are mostly squamous cell carcinomas arising from the transition zone of the anal mucosa. Almost 90 % of anal cancer cases can be attributed to HPV infection, and HPV-16 is the predominant

genotype (up to 83 %) detected in HPV-positive anal tumors [5]. Globally, in 2002, there were an estimated 99,000 incident cases of anal cancer with slightly more cancers among women (60 %) than men (40 %) [52]. In the USA, there were an estimated 7210 cases of anal cancer and 950 deaths in 2014 [53]. National US data from 2000 to 2010 indicate a rising trend of 2.2 % new cases annually among both men and women [53], with a greater increase among men having sex with men (MSM) and HIV-infected individuals [54]. It has been suggested that the increasing trend in anal cancers is possibly a reflection of the changing sexual practices among men and women and likely potentiated by HIV infection [24]. (Please see section on HPV/HIV coinfection and associated chronic immunosuppression).

Data on anal HPV prevalence among healthy adult HIV-uninfected women and men are limited with most studies reporting anal HPV prevalence in HIV-infected women and men. One of the few studies conducted by Hernandez et al. in Hawaii reported an anal HPV point prevalence of 27 % among 1378 healthy adult women [55]. Other studies of HIV-uninfected women have reported a wide range of anal HPV prevalence from 13 to 56 % [56]. Most of the studies that estimated a high prevalence were among high-risk HIV-uninfected women reporting a history of injection drug use, sexually transmitted infections, and multiple sex partners [56, 57]. Among healthy adult men having sex with women (MSW), findings from two studies, one conducted in the southwest US and the second (HPV in Men (HIM)) study among 1305 heterosexual men from Brazil, Mexico, and the USA revealed that HPV infection in the anal canal was common and anal HPV prevalence ranged from 13 to 24.6 % [56, 58].

5.1 HPV Genotype Distribution

Although most studies have found a similar range of HPV genotypes between the anal canal and cervix, there is relative variation in the frequency of their detection [55, 57]. In the Hawaiian study, among women with concurrent HPV infection, HPV genotypes were slightly more diverse in the anal canal (34 types) than cervix (32 types), but more oncogenic types were detected in the cervix. In contrast, women with only anal and no cervical HPV infection had an almost equal distribution of non-oncogenic (48 %) and oncogenic HPV infections (52 %) with HPV-84 being the most frequently detected genotype followed by 62, 16, 51, and 53. Among women with only cervical HPV infection and no anal HPV infection, HPV-16 was most frequently detected, followed by HPV types 53, 58, 52, and 62. Concurrent anal and cervical HPV infection was observed most commonly in younger women averaging 29 years old [55].

Similar to women, heterosexual men harbored a wide range of HPV types in the anal canal, with up to 34 different genotypes in the HIM study, and HPV 16, 6, and 61 being the most common [58].

5.2 Natural History of Anal HPV Infection in Women and Men

Prospective data assessing the natural history of anal HPV infection among women and men show differences in viral persistence. In the Hawaiian cohort, among women followed for a median 15 months, 50 % had an incident anal HPV infection, the majority of which were transient [59]. Of the incident anal HPV infections that cleared, 87 % had cleared within 1 year. Oncogenic anal HPV infections cleared more quickly (median duration 150 days) than oncogenic cervical HPV infections (median duration 8 months) in the same cohort of women [59]. A more recent study of 75 younger women (mean 23.5 years) with a mean follow-up of 7 years found that >80 % of non-HPV-16 high- and low-risk anal HPV infections cleared within 3 years and nearly 24 % of HPV-16 infections persisted beyond 3 years [60]. Among heterosexual men in the HIM study, incident anal infections are much lower at 8.5 per 1000 person months than women in the Hawaii cohort. Most HPV infections including all HPV-16 infections cleared within 6 months with persistent HPV types detected in 4.2 % of men [61].

Conversely, men who have sex with men (MSM) regardless of HIV status have a much higher incidence and persistence of anal HPV infections. In a one-year prospective study of 94 young MSM (mean age 21 years), incident anal HPV infections were high at 38.5 infections per 1000 person months [62]. Nearly 42 % had persistent any type HPV infection, and 19 % had detectable HPV-16 and/or 18. Of those who had prevalent infections at baseline, 81 % cleared one or more types within 6 months. Lifetime number of male receptive anal sex partners was associated with prevalent, incident, and persistent anal HPV infection [62]. Among slightly older HIV-infected MSM (median age 43 years) in Montreal, Canada, the incidence of anal HPV-16 was 10.8 per 1000 person-months, and the cumulative incidence of HPV-16 over 36 months was 33.2 %. Chronic anal HPV-16 infections were the slowest to clear with a mean duration of 36 months [63].

5.3 Risk Factors for Anal HPV Infection

Substantive evidence in developed countries supports the link between sexual behavior and anal cancers. Early studies on anal cancers in both men and women found a robust association between a history of sexually transmitted infections and incidence of anal cancer [64, 65] Lifetime number of sex partners (>10), anal intercourse (receptive being higher risk than insertive), and having a partner with a sexually transmitted infection have also been strongly associated with prevalent anal HPV infection and cancer incidence, in addition to other risk factors such as HPV-related cervical neoplasia in women and cigarette smoking [62, 65–68]. Among MSW, data from recent studies indicate that genital HPV infection is a risk factor

for prevalent anal HPV infection. Heterosexual men with genital HPV infection were two to four times more likely to have anal HPV infection [69, 70]. These findings may help explain HPV infection in the anal canal in the absence of receptive anal intercourse in men [56]. Of note, several studies have reported prevalent anal HPV infection in the absence of receptive anal intercourse in both men and women. It has been suggested that in women, a possible transmission route is autoinoculation, wherein the cervix acts as a reservoir for the transmission of HPV through cervico-vaginal fluid to the anus [57]. In addition, hand carriage, the use of objects, and other non-penetrative sexual behaviors may also act as potential modes of transmission [56].

6 Epidemiology of Oropharyngeal Cancer

Cancers of the head and neck, specifically oral cavity and oropharyngeal cancers (OPC) that include the tongue base, oropharynx, and tonsils, account for an estimated 400,000 cases and 223,000 deaths in 2008 [71]. HPV has been clearly shown as the causative agent of oropharyngeal squamous cell carcinomas [72] and up to 70 % of OPC are attributed to HPV infection, mostly HPV-16 [4]. In contrast, HPV prevalence is low in the oral cavity, suggesting other etiologic causes [4]. Kreimer et al. suggest that HPV infects the oropharynx because the tonsilar tissue area may resemble the squamous–columnar junction of the cervix with its large, exposed layer of basal epithelial cells [73].

From 1988 to 2004, there was a 225 % increased incidence in HPV-associated OPC in the USA [4]. Similarly, Chaturvedi et al report that from 1983 to 2002, the incidence of OPC increased significantly compared with oral cavity cancers among individuals less than 60 years of age in developed countries most likely due to changes in sexual behavior patterns and thus greater exposure to HPV [74]. OPC incidence was 2–17 times higher in males than in females [74].

Oropharyngeal HPV acquisition and clearance data are limited but initial studies demonstrate that males might be at higher risk than females. Among 5579 male and female participants (14–69 years old) of the US National Health and Nutrition Examination Survey (NHANES) 2009–2010, oral HPV prevalence was significantly higher at 10.1 % in males compared to 3.6 % in females, with an overall population prevalence of 6.9 % and an HPV-16 prevalence of 1 %. Age-specific prevalence showed a bimodal pattern with peak HPV prevalence at 30–34 years and 60–64 years of age [75]. The only prospective study (the HIM Study) showed that for healthy heterosexual men, oral HPV infections are infrequent and transient. Over 1 year, 4.4 % of men acquired a new oral HPV infection, 1.7 % acquired a new oncogenic HPV infection, and 0.6 % acquired a new HPV-16 infection. Oral HPV infections cleared quickly with a median duration of 6.9 months for all HPV, 6.3 months for oncogenic HPV, and 7.3 months for HPV-16, specifically [76].

Risk factors for oral HPV infection include increased lifetime number of vaginal and/or sex partners, oral–anal sexual contact (“rimming”), current tobacco smoking, and immunosuppression as measured by a low CD4 count among HIV patients [72, 75–78].

7 Coinfection with HIV and Associated Immunosuppression

HIV-infected men and women are at elevated risk for HPV-associated malignancies at multiple anatomic sites. HIV-infected women share a disproportionate burden of ICC risk with a 2- to 25-fold increase in incident invasive cervical cancers [79]. Furthermore, HIV-infected individuals have a greater than 25-fold increased risk for anal cancer [54] and a 1.3- to 3-fold increased risk for oropharyngeal cancer than HIV-negative individuals [80].

7.1 Cervical Cancer in HIV-Infected Women

HIV-infected women are two to five times more likely to have a cervical HPV infection, experience increased incidence and persistence of HPV, and experience a rapid progression to cervical lesions compared to HIV-negative women. Further, data also indicate that women with HIV have a higher incidence of cervical lesions, recurrent disease, and progression to invasion at a younger age [81–88]. In the USA, the Centers for Disease Control and Prevention (CDC) have designated invasive cervical cancer as an AIDS defining illness [89]. Thus, cervical cancer screening guidelines for HIV infected men and women suggest more frequent screening than within the general population

Cervical HPV prevalence remains markedly higher in HIV-infected women even in the absence of any cervical disease. For example, in a meta-analysis by Clifford et al. that included 20 studies of 5578 HIV-infected women across 5 continents, any type HPV prevalence was 36.3 % in women without cervical abnormalities and 12 % harbored multiple HPV types [90]. HPV-16 was the most common subtype (4.5 %) followed by HPV-58, 18, 52, 31, and 33. HIV-infected women with cervical abnormalities had a twofold to threefold higher HPV prevalence of any type ranging from 69.4 % in ASCUS/LSIL to 84.1 % in HSIL. HPV-16 was the most common type with a prevalence that was almost threefold higher in women with HSIL (31.9 %) in comparison to women with ASCUS/LSIL (12.0 %). Compared to the general female population with HSIL, HIV-infected women with HSIL were more likely to harbor multiple HPV types (41.9 %), exhibit a higher prevalence of HPV types 11, 18, 33, 51, 52, 53, 58, and 61 but a lower prevalence of HPV-16 [90]. In a more recent multicenter study of frozen tissue biopsies from women with cervical carcinoma in Kenya and South Africa, DeVuyst et al. reported a higher HPV-18 prevalence in HIV-positive compared to HIV-negative cases, but the combined prevalence of HPV-16 and/or 18 was similar [91].

7.2 *Anal Cancer in HIV-Infected Individuals*

HIV-infected men who have sex with men (MSM) have the highest anal cancer incidence rates (131/100,000 person-years) in comparison to HIV-infected men who have sex with women (MSW) (46/100,000 person-years), HIV-infected women (30/100,000 person-years) and HIV-uninfected men (2/100,000 person-years) and women (no cases) (Fig. 2.4) [54]. The growing research on HPV infection in HIV-infected MSM supports the high anal cancer incidence estimates. Data from a meta-analysis of 53 studies indicate that the pooled prevalence of anal HPV infection (any type and oncogenic type) was significantly higher in HIV-infected MSM (92 % and 74 %) compared with HIV-uninfected MSM (64 % and 37 %) [92]. A few research studies with HIV-infected MSWs also, report a comparatively lower anal HPV prevalence ranging from 46 to 68 % [93, 94]. Similar to HIV-infected MSM, studies of HIV-infected women in the developed world report an equally high anal HPV prevalence ranging from 79 to 90 % compared with cervical HPV prevalence (53–83 %) in the same population [57, 95]. However, Gonçalves et al. found a similar HPV prevalence across the cervix (61.6 %) and anus (63.7 %) in 138 HIV-infected women from Brazil [96]. Despite the high prevalence of anal HPV, anal cancer incidence is still far lower than ICC incidence suggesting that the natural history of anal HPV infection varies from the cervix and that the process and pathways of carcinogenesis to invasion might be different for the cervix versus the anal canal.

In HIV-infected women and men, HPV-16 is among the most common genotypes to be detected [57, 95–97]. Other HPV types frequently detected in women are 18, 35, 45, 51, 52, 53, 58, 61, and 70 [57, 95, 96]. Two studies that investigated concurrent anal and cervical infection in HIV-infected women found a high concordance

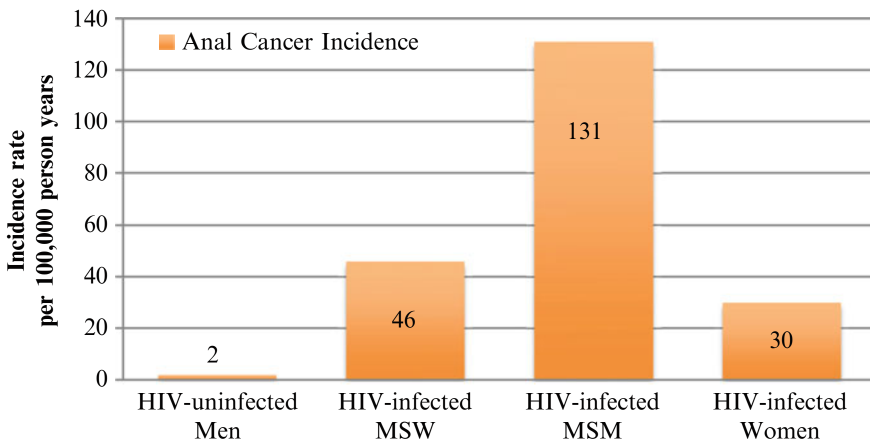

Fig. 2.4 Anal cancer incidence rate (unadjusted) per 100,000 years among HIV-infected and HIV-uninfected individuals. Adapted from Silverberg et al. [54] Note: No cases among HIV-uninfected women. HIV—human immunodeficiency virus; MSW—men who have sex with women; MSM—men who have sex with men

of HPV genotypes across the cervix and anal canal (63 % and 68.6 %) [95, 96], whereas Palefsky et al. found diverse genotypes between the cervix and anus [57]. In the Hawaii cohort of healthy women, concordance of at least one HPV type across the cervix and anus was 86 % [55]. Among MSM, Machalek et al. reported a pooled anal HPV-16 prevalence of 35.4 % for HIV-infected MSM and 12.5 % for HIV-uninfected MSM [92]. Using PCR testing, Palefsky et al. detected 29 diverse anal HPV types from both HIV-infected and un-infected MSM. Some of the HPV types that were isolated are less frequently detected in the cervix [98].

Although there are limited long-term studies of anal disease, anal cytological abnormalities in both HIV-infected women and men are common. Among 99 women followed prospectively for a minimum of 2 visits and a maximum of 3 visits, the prevalence of anal cytological abnormality was 33 %. HPV was isolated from 67 % of the 33 women. Anal cytological abnormalities consisted of only LSIL and ASCUS. However, of 36 women that underwent high-resolution anoscopy (HRA), 12 were diagnosed with AIN2-3. Incidence of anal abnormalities was 13.1 cases per 100 person-years of follow-up [68]. Similarly, in a study of HIV-infected MSM and MSW, the prevalence of anal cytological abnormalities in MSM was twice (40 %) that of MSW (20 %) [99]. Correspondingly, in a study of 450 HIV-infected MSM in the Spanish AIDS network cohort, slightly more than half (54.7 %) were diagnosed with anal cytological abnormalities that did not include ASCUS. Multiple oncogenic HPV ≥5 types was the only risk factor associated with prevalent anal abnormalities [100]. In Machalek et al.'s meta-analysis, histological high grade AIN pooled prevalence was higher at 29.5 % in HIV-infected MSM versus 21.5 % in HIV-uninfected MSM and anal cancer incidence was eightfold higher among HIV-infected at 45.9 per 100,000 men in comparison to 5.1 per 100,000 in HIV-uninfected men [92].

7.3 Oropharyngeal Cancer in HIV-Infected Individuals

HIV-infection and its associated immunosuppression is clearly one of the risk factors for acquiring an oral HPV infection. Similar to HIV-negative individuals, the natural history of HPV infection in HIV-positive individuals also varies by anatomic site and is influenced by immunosuppression. Data from one study indicated that oral HPV prevalence was significantly higher among HIV-infected women (33 %) than HIV-uninfected women (15 %). However, cervical HPV prevalence was much higher at the same time point in both groups (73 % and 51 %) respectively [78]. Additionally, oral HPV incidence rate in HIV-infected women (3.3 per 100 person months) was twice that of HIV-uninfected women (1.7 per 100 person months). More than half of infections in all women persisted to 6 months [78]. Similarly, another study of HIV-infected men and women found that prevalence (28 % vs 84 %), incidence (31 vs 145 per 1000 person months) and persistence (29 % vs 54 %) were significantly lower for oral HPV infections than anal HPV infections [97].

7.4 Antiretroviral Therapy and HPV Infection

Antiretroviral therapy (ART) has had a dramatic impact on morbidity and mortality due to HIV globally. A similar effect has not yet been evident in HIV-infected individuals coinfected with HPV, though theoretically HIV virological suppression and elevated CD4 counts would contribute to a better immune response to increase HPV clearance and decrease acquisition of new infections. Reviews indicate that cervical and anal cancers are not currently declining despite the introduction of ART, but there is insufficient data on the influence of ART on HPV infection and cancer among HIV-infected individuals in the developing world [101–103]. Inconsistent results have been published regarding the effect of ART on the incidence, prevalence, and progression of cervical and anal HPV infections and disease. Some studies show regression of cervical lesions and neoplasias associated with receipt of antiretroviral therapy [104–106], other studies show no such effect [103, 107–109]. Additionally, incidence of anal cancers [110, 111] and oral warts [112] are on the rise among both HIV-positive men and women on ART. Franceschi and Jaffe note that ART-induced immune reconstitution has a modest effect on HPV infection and that the incidence of cervical cancers has not declined in developed nations [113].

The reasons for the limited impact of ART on HPV-related carcinogenesis in HIV-infected individuals are unclear. Palefsky suggests that HIV patients might suffer enough genetic destruction in the epithelium allowing cells to proliferate despite restoration of HPV specific immunity with ART [114]. However, given the relatively recent history of successfully, virologically suppressed HIV and widely available ART, more studies are needed to examine the relationship between immune reconstitution and HPV progression, especially with the confounding factors of ongoing, high-risk behaviors often concurrent in HIV infection. The introduction of the HPV vaccine among HIV infected individuals is also expected to decrease oncologic transformation among this population, with studies forthcoming.

8 Prevention of HPV-Related Malignancies

Currently, the two main strategies for prevention of invasive cervical cancer are cervical screening followed by treatment of abnormalities and HPV vaccination. The adoption of cervical screening using Papanicolaou (Pap) testing for the early detection of cervical abnormalities has dramatically decreased the progression to invasive cervical carcinoma worldwide [115]. The two main modalities for cervical screening include: (1) cytology using the conventional Papanicolaou (Pap) test or the newer liquid-based, thin layer cytology which has largely become the standard of care in developed countries, and (2) high-risk HPV nucleic acid testing. In resource-limited settings, particularly in rural areas, the lack of adequate

infrastructure for Pap cytology testing including trained cytopathologists has created a need for alternative visual inspective techniques with acetic acid (VIA) and Lugol's iodine (VILI) that allow same day screen-and-treat approaches [116, 117]. However, due to the subjective nature, quality control issues, and low specificity, solely using visual inspective techniques should be reserved for resource-limited settings [118–121].

8.1 Cervical Screening

8.1.1 Pap Cytology Screening

The Pap test, first introduced in 1928 by Dr. Georgios Papanikolaou, utilizes inspection of a smeared cell sample directly onto a glass microscope slide for inspection by a trained cytopathologist. Newer liquid-based cytology (Thin Prep® or Sure Path®) utilizes submerged cervical cell samples within a preservative liquid that is later inspected on a glass microscope slide. The liquid medium removes contaminants, can be performed during menses, and allows the cervical cells to be spread more evenly on the slide. This liquid-cell medium is clinically preferred as it can further undergo HPV nucleic acid testing for high-risk HPV genotypes [115, 122, 123].

8.1.2 Screening Using HPV Nucleic Acid Testing

Although Pap cytology has historically been the gold standard for ICC screening, recent evidence indicates that HPV nucleic acid testing is a superior and more cost-effective screening strategy to prevent ICC among women older than 30 years of age, particularly in low and middle-income countries that lack a well-developed infrastructure including well-trained cytopathologists [124–128]. In the USA and other developed countries, HPV nucleic acid testing is recommended in combination with cytology screening and is an invaluable component of cancer screening, management, and treatment. Currently, the FDA has approved four HPV tests: to be used in conjunction with Pap cytology: Hybrid Capture 2®, Cervista®, Cobas, and Aptima® [129]. Aptima® is the only test that detects HPV mRNA, whereas the other three tests detect HPV DNA. These commercially available tests are available from qualitative to semiquantitative platforms and are of two types: (1) tests that detect any of the 12–14 high-risk genotypes and (2) tests that detect HPV-16 or 18 genotypes (Cobas, Cervista™ HR, and most recently Aptima 16,18/45) [130–132]. The Qiagen developed careHPV™ test is a new rapid clinically validated HPV test that can detect any of 14 high-risk genotypes, is able to provide results in a few hours, and is designed specifically for screening women in low-resource settings [124, 133]. It has recently been approved by the China FDA to be used for cervical screening of Chinese women [134].

All of these HPV screening tests are able to detect a small number of HPV genotypes, mainly combined high-risk types or HPV-16/18. For HPV genotyping, other validated molecular assays using type-specific PCR primers that are able to detect a large number of genotypes are mainly used in research facilities [133]. Cuzick et al. note that newer technologies with improved specificity are required to screen high-risk HPV positive women if HPV testing must become an alternate primary modality for screening women [133]. Some potential new approaches could be HPV typing, methylation of host and viral genes, detection of HPV E6 and E7 proteins, and cytologic methods such as p16^{INK4a} staining [133].

8.1.3 Cervical Screening Guidelines

Cervical screening guidelines in developed countries differ from developing nations. In the USA, the US Preventive Services Task Force, the American Society for Colposcopy and Cervical Pathology, the American Cancer Society and the American College of Obstetricians and Gynecologists have set the standard for screening guidelines for US women [115, 135]. For women in resource-limited settings, the World Health Organization (WHO) has set the standard and recently updated their guidelines significantly to included specific guidelines for HIV-infected women [136]. (Table 2.1).

In the USA, HIV-negative women between the ages of 30–65 years with a cervix should receive cytology screening every 3 years, or if receiving HPV DNA testing then screening may be extended to 5 years [115]. Women are screened more frequently if HPV positive or abnormal pathology is detected [115, 137]. Women 21–29 years old should receive screening with cytology alone every 3 years if screening results are normal. Screening is not recommended for HIV-negative women under the age of 21. HPV vaccination regardless of HIV status does not change current screening recommendations. For women above the age of 65 who have undergone hysterectomy and/or have no prior history of high-grade cervical lesions may defer HPV screening [115]. (Table 2.1)

As noted earlier, HIV-positive women are at significantly higher risk of progression to cervical carcinoma and should undergo a cervical Pap test at baseline, month 6 and month 12 of first HIV diagnosis; if normal, yearly screens are recommended thereafter regardless of age or modality of HIV acquisition. Immediate referral to colposcopy is recommended for lesions greater than ASCUS [138, 123, 139]. HIV-positive women and high-risk HIV negative women in developing countries should be screened more frequently within 3 years of each negative screening [136].

8.2 Prevention of HPV-Related Anal and Oropharyngeal Cancers

Given the elevated incidence of anal cancers in HIV-positive MSM anal cytology screening is recommended in this population. with frequency of screening currently at the discretion of the health care provider based pathology results [54, 140].

Table 2.1 Summary of cervical cancer screening guidelines for women by the US Preventive Services Task Force (USPSTF) [135], American Society for Colposcopy and Cervical Pathology (ASCCP) [115], and World Health Organization (WHO) [136]

Population	USPSTF	ASCCP	WHO[a]
Age <21 years	No screening	No screening	No screening for women <30 years unless HIV+ or living in high HIV prevalence area
Age 21–29 years	Cytology screening (Pap smear) alone every 3 years. No HPV screening alone or with cytology.	Cytology screening every 3 years	No screening for women <30 years unless HIV+ or living in high HIV prevalence area
Age 30–65 years	Cytology screening (Pap smear) alone every 3 years Cytology and HPV contesting every 5 years (if women prefer to increase screening interval)	Cytology and HPV co-testing every 5 years (preferred) Cytology screening alone every 3 years (acceptable)	Prioritize women 30–49 years[a] Screening interval with HPV testing should be minimum 5 years (not less) Screening interval with VIA/cytology should be 3–5 years
Age >65 years	No screening, if adequate prior negative screening and not high-risk for cervical cancer	No screening, if previous history of negative screening Women with prior history of ≥ CIN2 must continue routine screening 3–5 years as in women 30–65 years for at least 20 years	
After hysterectomy	No screening for women without cervix and no history of ≥ CIN2 or cervical cancer	No screening for women without cervix and previous negative screening for CIN2	
HPV vaccinated		Same as age-specific recommendations for unvaccinated women	

VIA visual inspection with acetic acid, *CIN2* cervical intraepithelial neoplasia grade 2, *ICC* invasive cervical cancer, *HPV* human papillomavirus

[a]WHO guidelines for resource-limited settings with no organized screening efforts

Although formal recommendations as to frequency of screening have not been codified, annual anal screening and referral to high resolution anoscopy (HRA) among HIV-positive MSM and transgendered individuals is now the standard of care in HIV specialty clinics in the USA [141]. Some US facilities are also offering similar annual screening services to all HIV-infected women and men [142]. Unlike in cervical screening, HPV testing has not been validated as a screening tool for anal

precancerous lesions because of the high-prevalence of HPV in the anal canal of high-risk individuals even in the absence of anal abnormalities.

Prevention of oropharyngeal cancer is challenging because of the lack of visibility of precancerous lesions. Although high-risk individuals can be screened for oropharyngeal lesions to detect invasive cancer by visual inspection and cytology, the low sensitivity and specificity of cytology screening studies does not currently justify widespread screening [143, 144]. Oropharyngeal cytology screening is not currently recommended [4, 145].

8.3 Treatment of HPV-Related Disease

Depending on the HPV subtype and location of infection, manifestations can range from simple, isolated lesions to more extensive, clustered lesions necessitating excision. For uncomplicated, external cutaneous HPV condyloma with very mild dysplasia, recommendations are podophyllotoxin (antimitotic agent), imiquimod cream (topical cytokine inducer), or sinecatechins (green tea catechins). For more extensive lesions, cryotherapy using liquid nitrogen, cauterization using trichloroacetic acid or bichloroacetic acid, or traditional surgical excision such as curettage or electrocautery are recommended. For the latest recommendations, dosages, and duration of therapy please refer to the US Centers for Disease Control and Prevention's "Guidelines for the prevention and treatment of opportunistic infections in HIV-Infected Adults and Adolescents." [146]

For more dysplastic lesions such as cervical intraepithelial neoplasia (CIN), anal intraepithelial neoplasia (AIN), vulvar intraepithelial neoplasia (VIN) and vaginal intraepithelial neoplasia (VAIN), more extensive excision and repeated evaluation is recommended based on the pathological spectrum of atypical cells of uncertain significance (ASC-US), low grade squamous intraepithelial lesion (LSIL), high grade squamous intraepithelial lesion (HSIL), carcinoma in situ, and invasive, squamous cell carcinoma. LSIL includes mild dysplasia (CIN1, AIN1, VAIN1); HSIL includes moderate dysplasia (CIN2, AIN2, VAIN2), severe dysplasia CIN3, AIN3, VAIN3), carcinoma in situ (CIS) [147].

Treatment recommendations depend on pathological grade and patient profile and can include colposcopy (visualization of abnormal cells in the cervical squamocolumnar junction), high resolution anoscopy (HRA), loop electrosurgical excision (LEEP), endocervical curettage (ECC), chemoradiation, or hysterectomy [115, 123, 138]

8.4 HPV Vaccination

A most exciting development for reducing infection-related malignancy are the HPV vaccines (bivalent, quadrivalent and nonavalent), which are estimated to prevent up to 70 % of all cervical and anal cancer cases associated with HPV-16/18

infections and >95 % of genital warts (quadrivalent vaccine) in both women and men globally [31, 148]. A series of clinical trials have shown that both vaccines are highly efficacious in reducing both incident and persistent cervical and vulvovaginal infections and abnormal lesions in women [149, 150], whereas the quadrivalent vaccine has also demonstrated efficacy against persistent genital and anal infection as well as anal lesions in men [148, 151]. The vaccine contains noninfectious and non-oncogenic recombinant L1 proteins that form virus-like proteins (VLPs). These VLPs induce both a humoral response and cell-mediated immune response that is more profound and sustained than natural infection [1, 152].

In 2006, the US Food and Drug Administration (FDA) approved the first quadrivalent (qHPV) vaccine (Gardasil®) that is now recommended for both females and males aged 9 through 26 years to protect against infection from HPV genotypes 6, 11, 16, and 18 [152–155]. Cervarix®) also known as the bivalent HPV vaccine was approved in 2009 by the FDA for the prevention of precancerous lesions and cervical cancer due to HPV types 16 and 18 and is recommended only for females 9 to 26 years old [152, 155]. Both vaccines are administered in three separate shots over 6 months as to ensure high immunogenicity with serum antibodies peaking at 2 years and sustained levels at 5 years [152, 156, 157]. Several clinical trials and other follow-up studies have corroborated that the two vaccines are safe and well-tolerated with limited adverse events, sustained protection of up to 8.4 years and high levels of immunogenicity [31]. Both vaccines have also shown some measure of cross protection against a few HPV types not in the vaccine—HPV 31 for both vaccines and HPV 33 and 45 for Cervarix® [31]. The nonavalent HPV vaccine (Gardasil®9) includes protection against HPV-31, 33, 45, 52 and 58 in addition to the quadrivalent vaccine HPV genotypes of 6, 11, 16 and 18. The Gardasil®9 was recently approved for use in both females and males aged 9–26 years [158]. Recent clinical trial data showed that the nonavalent vaccine was 96 % efficacious against HPV-31, 33, 45, 56, 58 related persistent infection at 6 months, as well as 96.7 % efficacious against related high-grade cervical/vulvar/vaginal disease [159].

8.4.1 Impact of HPV Vaccine and Barriers to Implementation

Given that HPV associated anogenital cancers are slow growing, assessing the true impact on incidence of cancers would require future studies. However, a number of recent studies have documented a decreasing cervical HPV prevalence and incidence of cervical abnormalities. In the USA among females 14–19 years old, the prevalence of vaccine HPV types 6, 11, 16, and 18 had decreased from 11.5 to 5.1 % in the post-vaccine era (2007–2010) [160]. Data from Australia and the USA demonstrate a reduction in genital warts in both women and men having sex with women [161–163]. Two other studies among 18–31-year-olds in the USA and <18-year-olds in Australia have documented a decline in the incidence of high grade cervical lesions [164, 165]. A more recent study in Costa Rica demonstrated a vaccine efficacy of 93 % against oral HPV [166] suggesting that the vaccine may have a protective effect against infection at multiple anatomic sites in men and women.

Serrano et al., estimate that the nonavalent vaccine has the potential to prevent up to 90 % of cervical cancers worldwide [167].

There are several challenges for the HPV vaccine to be effective worldwide. The low coverage of HPV vaccinations in the USA as compared to other developed countries is a major challenge. Current data on immunization rates among teens 13–17 year old indicate that slightly more than half (53.8 %) of girls have received one dose of the HPV vaccine and only a third have completed the full three doses [168]. The coverage for males was only 21 % for one dose of the HPV vaccine [167]. For developing countries where the vaccine has the potential to have the maximum impact because of the large burden of ICC, the vaccine is cost prohibitive. In addition, the lack of awareness about the vaccine and its efficacy, concerns about safety, lack of physician recommendations combined with cultural beliefs that adolescents are not sexually active as well as concerns of safety and fear of promiscuity have become major barriers to vaccine uptake in the USA and worldwide [169, 170, 171].

9 Conclusion

In summary, HPV-associated cancers are a significant global health burden. Persistent HPV infection with HPV-16 and/or other carcinogenic types has been firmly established as a precursor of cervical disease. Thus, understanding the natural history of cervical HPV infection has been essential to the development of screening and treatment guidelines for the prevention and treatment of cervical cancer. Unlike in cervical cancer, there are relatively few studies that have assessed the natural history of anal and oropharyngeal cancer among women and men. The rising trend in these cancers suggests a critical need for such studies to inform the development of screening and treatment. The vast majority of invasive cervical cancers are among women in developing countries where HIV is also a tremendous burden. While the differences in ICC incidence indicate the lack of widespread availability of preventative services or issues with access when available, HIV must be considered when women present with ICC in these resource-limited settings. Additionally, HIV is a crucial cofactor in the rising incidence of anal cancers among the MSM population as well as in heterosexual men and women. Reducing the high incidence and prevalence of HPV-associated cancers worldwide will require several approaches including prevention of HPV infection, newer screening technologies that can detect precancerous lesions early, and new therapeutics. Meanwhile, HPV vaccination seems to be the most promising in terms of preventing incident HPV infection and its sequelae of HPV-related disease.

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Chapter 3
Adaptation of Freshwater Mosquito Vectors to Salinity Increases Arboviral Disease Transmission Risk in the Context of Anthropogenic Environmental Changes

Ranjan Ramasamy

Core Message *Aedes aegypti* and *Aedes albopictus*, the dominant vectors of dengue, chikungunya and yellow fever, have been regarded to undergo pre-imaginal development only in freshwater. Vector control efforts therefore presently target only their freshwater habitats. The two *Aedes* vectors have recently been shown to develop in brackish water with associated biological changes in *Ae. aegypti*. Anthropogenic environmental changes have produced habitats that favour brackish water adaptation in the two *Aedes* and other vector species and increase the risk of arboviral disease transmission. Such changes can also increase disease transmission by other salinity-tolerant arboviral vector mosquitoes. Appropriate strategies are needed to address the associated health risks, particularly in the context of rising sea levels increasing coastal groundwater salinity.

1 Human Arboviral Diseases and Their Mosquito Vectors

Dengue is the most common arboviral disease of humans, with 50–100 million annual cases in more than 100 countries and an increasing incidence and spread worldwide that places 2.5 billion people at risk according to the World Health Organisation (WHO) [1]. Severe dengue or dengue hemorrhagic fever has a case fatality rate of 2.5 % [1]. There is presently no licensed vaccine or specific antiviral drug for dengue [2]. Yellow fever has a zoonotic reservoir, is endemic in Africa and South America with the potential to spread to Asia and is responsible for 200,000 cases and 30,000 deaths in the world every year [3]. An effective live attenuated

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vaccine is however available against yellow fever. Chikungunya, a debilitating arboviral disease, is endemic in Southeast Asia and has produced epidemics in tropical Africa and Asia, and more recently in temperate Europe and the Americas [4–6]. A vaccine against chikungunya is not yet available. Many other arboviruses have animal reservoirs and can be transmitted to humans with serious risk to health. These include viruses causing West Nile fever, Japanese encephalitis, St. Louis encephalitis and Eastern and Western equine encephalitis [7]. However the viremia that develops in humans is typically not high enough to permit human-to-human transmission but this situation can change if the viruses undergo appropriate genetic alterations.

Aedes aegypti (Linnaeus) (Diptera: *Culicidae*) is the principal mosquito vector of arboviruses causing yellow fever, dengue and chikungunya in populated areas of tropical and subtropical regions [7, 8]. *Aedes albopictus* Skuse is however becoming an increasingly important vector of dengue and chikungunya because of its recent global spread, the evolution of an ability to survive winters and a propensity to outcompete *Ae. aegypti*. *Aedes albopictus* has been responsible for the recent transmission of dengue and chikungunya in temperate regions [4, 5, 7, 9]. Both species are able to bite outdoors and during the day making the use of bed nets less effective than for malaria control. Therefore the control of dengue and chikungunya in tropical countries mainly relies on surveillance for *Ae. aegypti* and *Ae. albopictus* larvae, elimination of their pre-imaginal development habitats, use of larvicides and the space spraying of insecticides in areas of high transmission [1, 2, 5].

2 Salinity-Tolerant Mosquito Vectors of Human Arboviral Diseases

While most mosquito species lay eggs and undergo pre-imaginal development in freshwater, approximately 5 % of mosquito species undergo pre-imaginal development in brackish and saline waters. Water with less than 0.5 ppt or parts per thousand, 0.5–30 and greater than 30 ppt of sodium chloride are commonly termed fresh, brackish and saline, respectively [10]. Many mosquitoes that transmit human arboviral diseases, including *Ae. aegypti* and *Ae. albopictus,* have been long considered to undergo pre-imaginal development only in freshwater habitats [1, 2, 8, 11]. However, some salinity-tolerant mosquito species are known to transmit human arboviral diseases [8, 10, 12]. Common examples of salinity-tolerant mosquito vectors and the arboviral diseases that they transmit are presented in Table 3.1. Salinity-tolerant mosquito larvae and pupae have evolved various physiological and structural mechanisms to cope with high osmolarity in their aqueous environment [13–15].

Table 3.1 Salinity-tolerant mosquito vectors of human viral diseases

Species	Distribution	Examples of viruses transmitted
Aedes dorsalis	Pacific coast of North America, Temperate Eurasia	West Nile and Western equine encephalitis viruses
Ae. (Ochlerotatus) taeniorhynchus	North and South America	Eastern equine encephalitis virus
Ae. togoi	North Pacific rim	Japanese encephalitis virus
Ae. (Ochlerotatus) vigilax	Australasia, Southeast Asia	Ross River and Barmah forest viruses
Culex sitiens	Indian Ocean rim countries	Japanese encephalitis and Ross River viruses
Cx. tarsalis	North America	St. Louis encephalitis, Western equine encephalitis and West Nile viruses
Cx. tritaeniorhyncus	Russia, Middle East, Africa, India	Japanese encephalitis virus

Common examples of mosquito vector species that undergo pre-imaginal development in brackish or saline water, their geographical distribution and the viruses that they transmit are listed in this table (modified with permission from Ramasamy and Surendran, BMC Infectious Diseases 11:18 (2011). doi:10.1186/1471-2334-11-18 and Ramasamy and Surendran, Frontiers in Physiology 3:198 (2012). doi: 10.3389/fphys.2012.00198)

3 Recent Evidence Shows That *Ae. aegypti* and *Ae. albopictus* Can Undergo Pre-imaginal Development in Brackish Water

There is now evidence that *Ae. aegypti* and *Ae. albopictus* can also lay eggs and undergo pre-imaginal development into adults in brackish water collections in coastal areas of the tropics [16–21]. Pre-imaginal stages of *Ae. aegypti* and *Ae. albopictus* were observed in brackish water in coastal areas of up to 15 ppt and 14 ppt salinity, respectively, in northern and eastern Sri Lanka [16–18] and up to 8 ppt salinity in Brunei Darussalam [19, 20].

The habitats where the larvae of the two vectors were found in Sri Lanka were brackish water collections in discarded plastic and glass food and beverage containers along beaches, disused fishing boats and coastal domestic wells that were either abandoned or used for domestic purposes other than drinking, e.g., washing clothes and bathing (Fig. 3.1). Discarded food and beverage containers were also found to provide suitable brackish water habitats for the development of *Ae. albopictus* along the South China sea coast of Brunei Darussalam [19].

In the Jaffna peninsula in northern Sri Lanka, recent data show that adaptation to brackish water is accompanied by greater tolerance of *Ae. aegypti* larvae to salinity, which is only partly reversible after transfer to freshwater for five generations [21]. This suggests that genetic and reversible physiological changes are in combination

Fig. 3.1 Typical brackish water habitats of *Aedes* larvae in coastal areas of northern and eastern Sri Lanka. The photographs show the brackish water collections containing larvae in (**a** and **b**) disused boats; (**c** and **e**) abandoned wells; (**d** and **f**) discarded food and beverage containers (reproduced with permission from Ramasamy et al. PLoS Neglected Tropical Diseases 5(11): e1369. doi:10.1371/journal.pntd.0001369)

responsible for the adaptation to brackish water. Brackish water *Ae. aegypti*, unlike freshwater isolates in the Jaffna peninsula, tended to prefer laying eggs in brackish water [21]. But the hatching of eggs was significantly less efficient and the time taken for larvae to develop into pupae was prolonged in 10 ppt brackish water compared to freshwater with both brackish and freshwater-derived *Ae. aegypti* [21]. Brackish and freshwater *Ae. aegypti* isolates from the Jaffna peninsula were however shown to interbreed and produced viable offspring in the laboratory [21]. These findings also showed that there was restricted gene flow between coastal brackish and inland freshwater *Ae. aegypti* isolates even when separated by a distance of only 5 km in the Jaffna peninsula. The results suggest that there is an ongoing adaptive process involving genetic and physiological changes, which could potentially lead to speciation in the future if the two populations were to become reproductively isolated.

4 Significance of the Likely Role of Brackish Water *Aedes* Vectors in Dengue Transmission

The analysis of the relationship between monsoonal rainfall and the incidence of dengue in coastal areas of northern and eastern Sri Lanka has been performed to evaluate the contribution of brackish water developing *Aedes* vectors in dengue transmission in the island [20]. The findings showed that monsoon rains, that allow freshwater to collect and form suitable habitats for pre-imaginal development of

Aedes vectors in the vicinity of populated areas, are the dominant driver for the increased dengue transmission that follows the monsoons in Sri Lanka. This was also found to hold true for the dry zone coastal districts of Jaffna and Batticaloa in northern and eastern Sri Lanka that receive rainfall during the Northeast monsoon [20] and where pre-imaginal development of *Ae. aegypti* and *Ae. albopictus* in brackish water habitats was first observed [16].

However it is likely that brackish water habitats provide a previously unappreciated source of vectors for maintaining dengue transmission in coastal areas that may be particularly important in a local context, e.g., coastal areas of Jaffna city in northern Sri Lanka [16–18]. Some brackish water habitats such as coastal wells constitute perennial habitats that are relatively independent of rainfall. Monsoonal and inter-monsoonal rains in combination with sea spray and tidal movements may also cause brackish water to accumulate in discarded plastic and glass containers and coastal rock pools that provide additional habitats for the dengue vectors. However monsoonal rains rapidly increase the extent of freshwater habitats for the *Aedes* vectors in coastal and inland areas leading to the greater transmission of dengue that follows soon after the rains. It seems possible therefore that brackish water vectors in coastal areas may play a role as a perennial reservoir of virus to support the post-monsoonal amplification of dengue transmission by freshwater vectors. Vertical or transovarial transmission of dengue virus in brackish water-adapted *Aedes* will enhance their capacity to serve a virus reservoir in the period between monsoons. Determining the relative vector competence of brackish and freshwater vectors, changes in adult vector densities and larval indices, temporal and spatial variation in dengue cases and rainfall and other pertinent epidemiological parameters during and between monsoons in selected coastal districts will be needed to confirm this hypothesis.

5 Anthropogenic Environmental Changes That Expand Brackish Water Habitats Increase the Potential for Transmission of Arboviral Diseases

5.1 Rising Sea Levels

Global climate change is caused by long-term changes in common meteorological parameters that can be ascribed to anthropogenic influences, particularly the continuing accumulation of greenhouse gases like carbon dioxide in the atmosphere. Temperature, rainfall and humidity are primary climate changes and these have been studied for their impact on common vector-borne diseases, notably malaria and dengue (reviewed in [10, 12, 20]). These studies predict an expansion of the range of mosquito vectors due to climate change. Primary changes in global climate lead to secondary changes in the biosphere and geosphere, including an altered distribution of animals and plants and a rise in sea levels [10]. It has been proposed

with supporting evidence that a rise in sea levels caused by global warming can potentially increase the transmission of vector-borne diseases [12].

The Intergovernmental Panel on Climate Change in its most recent report predicts a likely sea level rise of up to 82 cm by the year 2100 [22]. A rise in sea levels will produce greater saline intrusion into coastal freshwater aquifers [10, 12, 20, 23]. This will be particularly important for low-lying areas like the Jaffna peninsula in northern Sri Lanka which is largely composed of relatively porous limestone [10, 20]. The Jaffna peninsula also has a high population density and consequently a high rate of groundwater withdrawal rate from freshwater aquifers that are only replenished during the Northeast monsoon in October to December [10, 20]. A rise in sea level will therefore increase the availability of brackish water larval habitats for brackish water-adapted *Ae. aegypti* and *Ae. albopictus* as well as other brackish water mosquito vectors of human disease that are present in the peninsula and outlying islands [16–18, 21].

Tropical South and Southeast Asia have extensive coastlines bordering the Indian and Pacific Oceans and various seas. Southeast Asia also has the largest archipelago in the tropics encompassing populous countries like Indonesia and the Philippines with extensive coastal areas compared to their total land area. A similar situation prevails in the Caribbean countries and the Indian Ocean islands of Seychelles, Singapore and Reunion.

Countries with long coastlines and high coast to land area ratios are particularly vulnerable to the consequences of rising sea levels. One in ten persons worldwide live in coastal localities that are less than 10 m above sea level [24]. Such low-lying areas are prone to greater salinisation caused by rising sea levels. Many densely populated tropical countries have a large proportion of their populations living in such vulnerable areas, e.g., Vietnam, Bangladesh, Thailand and Indonesia [24].

5.2 Expanding Coastal Populations and Beach Litter

Beach container litter provides excellent habitats for brackish water-adapted *Ae. aegypti* and *Ae. albopictus* [16, 19]. Increasing population densities along coastal regions will result in the construction of more wells that may become brackish and a greater tendency to litter the shoreline and beaches with discarded containers that can collect brackish water. Garbage collection and disposal mechanisms managed by local government authorities in coastal areas of resource-poor countries may find it hard to cope with such hazardous environmental changes posed by expanding populations.

Population increase in coastal areas can reduce vegetation cover, thereby driving vector mosquitoes, notably *Ae. albopictus,* to seek other habitats closer to human dwellings for laying eggs. Another likely consequence is a decrease in the relative numbers of animals and birds that can serve as alternative sources of a blood meal. The two factors combined with higher population density will increase the human biting rate and therefore the rate of propagation of arboviral diseases as outlined previously [10].

5.3 Agriculture in Coastal Zones

Intensive agriculture in dry coastal areas promotes salinisation of the land and causes inland water bodies to become more saline, thereby facilitating the expansion of salinity-tolerant mosquito vector habitats. For example, higher densities of *Aedes* (*Ochlerotatus*) *camptorhynchus*, a vector of Ross River virus, are associated with increasing salinisation of inland freshwater bodies caused by wheat farming in Western Australia [25]. Aquaculture, an increasing economic activity along tropical coasts, also creates new brackish water habitats for mosquito vectors. There is presently no data on its ensuing impact on the transmission of arboviral diseases though a consequential increase in malaria vector populations has been recorded [12].

5.4 Use of Insecticides and Larvicides in Inland Areas Can Drive the Adaptation of Freshwater Vector Mosquitoes to Coastal Brackish Water Habitats

Insecticides are used for agricultural pest control in inland areas. Malaria control programmes use indoor residual spraying of insecticides that target houses in endemic areas. Larvicides against malaria vectors are predominantly applied to freshwater collections in most countries and this has been exclusively the case for dengue control targeting *Ae. aegypti* and *Ae. albopictus.* Adaptation of vector mosquitoes to coastal brackish water habitats therefore has a selective advantage. Data from the Jaffna peninsula in northern Sri Lanka recently documented that coastal brackish water isolates of *Ae. aegypti* were significantly more sensitive to the organophosphate insecticide malathion than inland populations [21]. It was proposed that this was caused by the recent use of malathion for malaria control and organophosphate insecticides in general for controlling agricultural pests in inland areas of the Jaffna peninsula [21].

5.5 Increased Coastal Arboviral Disease Transmission Will Impact on Inland Areas

Any increase in the transmission of arboviral diseases in coastal areas due to conducive environmental changes will also act to increase disease incidence in inland areas through bridging freshwater vectors and inland movement of infected persons.

6 Implications for Other Arboviral Diseases Transmitted by Mosquitoes

Ae. aegypti and *Ae. albopictus* are potential vectors for arboviruses other than those causing dengue, chikungunya and yellow fever [8]. *Ae. albopictus*, for example, has been reported to be a vector of 26 different viruses from the Bunyaviridae, Flaviviridae, Nodaviridae, Reoviridae and Togaviridae families [26]. Brackish water-developing *Ae. aegypti* and *Ae. albopictus* may therefore make a hitherto unrecognised contribution to the transmission of many zoonotic arboviruses to humans in coastal areas. Besides *Ae. aegypti* and *Ae. albopictus*, other freshwater arboviral vectors also have the potential to adapt to brackish water and contribute to disease transmission.

The increased extent of brackish water habitats caused by anthropogenic factors outlined in Sect. 5 also heighten the risk of arboviral disease transmission by known salinity-tolerant mosquito vector species.

7 Strategies for Controlling the Transmission of Arboviral Diseases in Coastal Areas

Local government and national and international authorities responsible for concerned sectors, e.g., health, agriculture, coastal planning, environment, irrigation, local government and livestock development, need to be aware of the health risk associated with mosquito vectors developing in brackish water habitats in coastal areas. The potentially greater risk associated with rising sea levels needs to be appreciated and included appropriately in development plans. The WHO in particular needs to take cognizance of the likely role of brackish water mosquitoes in transmitting dengue and chikungunya and incorporate it into their international guidelines. Most countries depend on the WHO recommendations for formulating national dengue control programmes and the WHO guidelines are presently based on the assumption that the *Aedes* vectors of dengue only develop in freshwater habitats [2].

There is a need for more research at all levels into the bionomics of salinity-tolerant mosquito vector populations that has become more important in the context of the documented ability of the most important arboviral vectors to adapt to salinity. The underlying genetic and physiological mechanisms that facilitate salinity adaptation in *Ae. aegypti* and *Ae. albopictus* need to be elucidated. A readily usable molecular marker for salinity tolerance in the two species would be a useful epidemiological tool. Mathematical models of mosquito-borne disease transmission would need to incorporate the consequences of vectors undergoing development in brackish water habitats. Larvicidal formulations with *Bacillus thuringiensis* toxin that were primarily developed for freshwater use are less effective against *Ae. aegypti* in brackish water at high salinities [17]. Controlling pre-imaginal stages of

the vectors in brackish water may therefore require the development of specific larvicides or larvicide formulations. Larvivorous fish and predatory mosquito larvae that are effective in controlling vector larvae in brackish water [18, 27] are other measures that can be utilised in brackish water habitats in coastal areas.

The monitoring of disease incidence in tropical coasts and mosquito vector development in coastal brackish and saline water habitats is a current deficiency that needs to be redressed. The immediate extension of vector source reduction and management programmes to the brackish water habitats of *Ae. aegypti* and *Ae. albopictus* may rapidly improve disease control in coastal areas, e.g., Kurunagar in Jaffna, Sri Lanka, that has a high dengue incidence [16]. Larval source reduction in defined habitats with the cooperation of affected communities has proved effective in controlling dengue and chikungunya in many countries. The likely role in disease transmission of salinity-tolerant mosquito vectors developing in brackish water habitats provided by beach litter and domestic wells need to communicated to coastal communities at risk and their cooperation sought for disease control.

Conflict of Interest The author declares no conflict of interest in this publication.

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Chapter 4
Epidemiology of Henipaviruses

Stephen Luby and Emily Gurley

Core Message The henipaviruses are RNA viruses whose natural reservoir is large fruit bats. People occasionally become infected with these viruses by being exposed to body fluids of bats or other infected animals.

Henipaviruses are a recently discovered genus of paramyxovirus. At the time of drafting this chapter three henipaviruses had been isolated: Hendra virus [1], Nipah virus [2] and Cedar virus [3]. The reservoir for all three isolated henipaviruses is fruit bats of the genus *Pteropus* in the family *Pteropodidae* [3, 4]. Segments of RNA closely related to known henipaviruses, but likely representing different species of henipavirus have been identified from urine and saliva of *Pteropus giganteus* [5], and from feces and tissue samples from *Eidolon helvum*, a native African fruit bat in the family *Pteropodidae* [6, 7].

Neither Nipah nor Hendra virus causes any apparent disease in infected bats [4, 8, 9] and likely coevolved with these bats. The ephrin-B2 and ephrin-B3 molecules which henipaviruses exploit to enter epithelial cells [3, 10] are widely conserved across mammals, and many mammals are therefore susceptible to henipavirus infection [11].

Human infection and severe disease has been recognized occasionally with Hendra virus, repeatedly with Nipah virus, but has not yet been described with Cedar virus. In contrast to Nipah virus and Hendra virus which causes severe illness in laboratory animals that are experimentally infected, ferrets and guinea pigs that were infected with Cedar virus remained clinically well [3]. This chapter updates a previous chapter on the epidemiology of human henipavirus infection by these authors [12].

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1 Hendra Outbreaks

Hendra virus, previously referred to as equine morbillivirus, was first identified in an outbreak in September 1994 in Hendra, a suburb of Brisbane, Queensland, Australia [13, 14]. The first recognized infection occurred in a pregnant mare that was staying in an open paddock when noted to be ill. The mare was moved into a stable in Hendra and died within 2 days. Between 8 and 11 days after the mare's death 18 other horses residing in or near the stable became ill. Affected horses had depression, loss of appetite, fever, ataxia, tachycardia, tachypnea, dyspnea, and a copious frothy nasal discharge. Among 18 horses with clinical illness, 14 died, 12 horses from the Hendra stable, 1 horse staying in the paddock adjoining the stable and 1 horse living on a neighboring property that had very close contact with horses from the Hendra stable. Autopsy findings from the horses were notable on gross pathology for heavy edematous lungs with hemorrhage and froth in the airway. The histopathological findings suggested interstitial pneumonia, with focal necrotizing alveolitis, and syncytial giant cells within the vascular endothelium [1].

Two employees at the stable, a 40-year-old male stable hand and a 49-year-old male horse trainer had particularly close contact with the index mare during the final stages of her fatal illness. The horse trainer, whose hands and arms had abrasions, attempted to force feed the mare by placing his bare hands with food into the sick mare's mouth. Both the stable hand and the horse trainer became ill 5–6 days after the death of the mare with fever, myalgia, headaches, lethargy, and vertigo. The stable hand remained lethargic for several weeks but eventually recovered. The horse trainer developed progressive respiratory failure and died. His autopsy findings were consistent with interstitial pneumonia, with focal alveolitis and syncytial formation [14]. An identical virus which was ultimately named Hendra virus was grown from samples from both the affected horses and the affected people [1].

Since its identification and the first two recognized human infections, five additional human infections with Hendra virus have been recognized, all in Queensland, Australia, though Hendra virus infection of horses has also been identified in New South Wales, Australia [15]. The third person with recognized Hendra infection was a 35-year-old male who lived on a horse stud farm [16]. He had cared for two sick horses, one with acute respiratory distress and the other with a rapid onset of neurological symptoms. Both horses died. He assisted a veterinary surgeon during the necropsy of the two horses. Throughout caring for the horses and the necropsy the assistant never wore gloves, mask or protective eyewear. A few days after assisting with the autopsies he became ill, and sought medical attention. Subsequent PCR evaluation of serum samples from that illness amplified a 500 nucleotide sequence of the matrix gene of Hendra virus.

McCormack and colleagues evaluated people who had contact with Hendra infected humans and horses during these first two recognized outbreaks of Hendra. They collected serum samples from 159 people who had contact with Hendra infected human patients, 16 who participated in necropsies on Hendra virus infected

horses, 6 who had other close contact with Hendra infected horses and 113 who had other variable contact with horses [17]. None of the tested study subjects had neutralizing antibody to Hendra virus.

The fourth recognized human infection with Hendra occurred in a recent veterinary graduate who conducted a limited autopsy on a 10-year-old horse that died of a rapidly progressive respiratory illness with large amounts of blood stained frothy nasal secretions [18]. Although she initially wore gloves, she soon removed them because they were not appropriately designed and had become contaminated inside. She did not use any other personal protective equipment. She reached deep into the carcass to examine internal organs and became heavily contaminated with the horse's body fluids. After completing the autopsy, the veterinarian returned home and showered. Seven days later she developed a dry cough, sore throat, fever, body aches, and fatigue. She recovered after 8 days. Serial serological samples demonstrated seroconversion of IgM and IgG antibodies against Hendra virus. The two autopsy assistants and an adult family member who held the dying animal's head and were exposed to frothy bloody nasal secretions did not develop clinical illness and were seronegative for Hendra virus infection [18].

The fifth and sixth recognized human infections with Hendra virus were a 33-year-old male veterinarian and a 21-year-old female veterinary nurse who worked at a veterinary practice in Thornlands, Queensland during an outbreak of Hendra virus that affected five horses in the practice [19]. Both the veterinarian and the nurse performed nasal cavity lavage to a horse during the 3 days before the horse developed symptoms of ataxia, depression, and disorientation and was later confirmed to be infected with Hendra virus [20]. The veterinarian developed fever, myalgia, and headache which progressed to confusion, ataxia, respiratory failure, and death. The nurse developed fever, confusion, and ataxia. She survived with substantial neurological deficits. Both the veterinarian and nurse had Hendra virus RNA detected by reverse transcription PCR from both serum and nasopharyngeal aspirate specimens. The outbreak investigation identified 83 other people who had contact with the sick horses. Sixteen reported mild symptoms, but none developed a clinical illness. None had Hendra virus RNA or Hendra antibodies. Among the 28 persons who reported contact with potentially infected equine body fluids only the two cases developed Hendra virus infection. One veterinary worker who had a percutaneous blood exposure from an infected horse also had no evidence of infection.

The seventh recognized human infection with Hendra virus was a veterinarian who examined a horse that died the next day. A pony and a horse on the same property died of confirmed Hendra infection in the subsequent 11 days [21].

These seven people infected with Hendra virus were infected through contact with only five Hendra virus infected horses. Most infected horses do not transmit Hendra virus to people. Indeed, of the 84 recognized equine Hendra virus infections through July 2013, only 5 have resulted in human infection [15, 22]. In the originally identified outbreak in the Hendra stable, all of the infected horses developed illness within one incubation period (8–11 days after the death of the index mare). This suggests that the mare was a superspreader [23], though we do not know if this

exceptionally efficient transmission was due to unusual viral shedding in this mare, care practices by its animal handlers or both. The absence of a successive wave of infection among horses, and the low attack rate of Hendra virus among people who had contact with Hendra virus infected horses suggest that such superspreaders are exceptional. All seven recognized human cases of Hendra virus had intimate contact with Hendra virus infected horses, usually with heavy exposure to respiratory secretions and without wearing personal protective equipment. Other people with close contact with these same horses did not develop Hendra virus infection. These observations suggest that Hendra virus is not easily transmitted from horse to human. It apparently requires a horse that is an unusually efficient transmitter and a person with a high exposure to infectious secretions.

All humans confirmed with Hendra virus infections had contact with Hendra virus infected horses. The absence of human cases among healthcare workers and among family members suggests that Hendra virus is not easily transmissible from person to person. Selvey and colleagues identified 128 people who cared for Pteropid bats, the wildlife reservoir of Hendra virus [24]. The bat carers included volunteers who cared for injured or orphaned bats and professionals who cared for captive bats. Bat carers had a median 48 months of bat contact. Seventy-four percent reported daily contact with flying foxes. Seventy-four percent reported having been bitten, 88 % scratched, and 60 % reported exposure to flying fox blood. None of the bat carers tested positive for antibodies to Hendra virus. While, direct transmission of Hendra virus from flying foxes to humans could not be excluded, the study suggested that it was extremely rare.

2 Nipah Virus Outbreaks

2.1 Malaysia/Singapore

Human Nipah virus (NiV) infection was first recognized in a large outbreak in peninsular Malaysia from September 1998 through May 1999 [25–27]. The initial human cases were identified among pig farmers who lived near the city of Ipoh within the state of Perak in northwestern peninsula Malaysia in late September 1998. Patients presented with fever and headache. Over half developed a reduced level of consciousness; 42 % had seizures [28]. Among 28 early cases, 4 had IgM antibodies against Japanese encephalitis. The government declared the outbreak was due to Japanese encephalitis and initiated widespread mosquito control measures. By December 1998 larger clusters of similar cases were reported within the Port Dickson District of Negri Sembilan, 300 km south of Ipoh [29]. In March 1999 a novel paramyxovirus was isolated from the cerebrospinal fluid of a patient from Sungai Nipah village [2] that was confirmed to be the cause of the outbreak [25]. Ultimately the Malaysian Ministry of Health reported 283 cases with 109 (39 %) fatalities [27].

Parashar and colleagues conducted a case–control study to explore the risk factors for human illness with NiV during the outbreak [30]. They enrolled 110 NiV antibody confirmed cases from Port Dickson and two sets of controls, 147 community farm controls from among persons who either lived or worked on pig farms with no reported human encephalitis cases, and 107 case-farm controls who were selected from among NiV antibody negative persons who lived on farms where there was a known case of human NiV infection. Case patients were more likely than community farm controls to report increased numbers of sick or dying pigs, dogs and chickens on their farms. Case patients were more likely than case farm controls to have direct contact with pigs that appeared sick and to have close contact with pigs through feeding pigs, processing baby pigs, assisting in breeding of pigs, assisting in birth of pigs, injecting or medicating pigs, and handling dead pigs.

In contrast to the severe illness manifested by Hendra virus infected horses, most pigs infected with NiV had mild illness. Forty-one percent of human NiV infected cases who worked on pig farms reported no increase in sick or dying pigs on their farm [30]. Case fatality among adult infected pigs was low, ranging from <1 to 5 % [31]. Among three pigs infected with NiV through experimental oral inoculation or sharing a cage with an inoculated pig, all developed asymptomatic infections [32]. A subset of NiV infected pigs were severely affected and developed fever, agitation, trembling, and twitching accompanied by rapid labored respirations, increased drooling and a non-productive loud barking cough [31]. Pathological examination of severely affected pigs demonstrated extensive involvement of the lungs with a giant cell pneumonia with multinucleated syncytial cells containing NiV antigen in the lungs and epithelial cells lining the upper airways [25]. NiV was recovered from respiratory secretions of infected pigs, and NiV antigen was detected in renal tubular epithelial cells [25, 32].

Between March 10 and 19, 1999 eleven workers in one of Singapore's abattoirs developed NiV associated with encephalitis or pneumonia [26]. One worker died. Compared to controls who were also abattoir workers, cases were more likely to be exposed to pig urine or feces from pigs that had been imported from Malaysia during the Malaysian NiV outbreak. NiV RNA recovered from autopsy specimens from the one worker who died, had a nucleotide sequence that was identical to the sequences of NiV isolates from humans and from pigs in Malaysia [26].

The isolation of NiV from pigs' lungs and respiratory secretions combined with the observation that human cases of NiV infection had closer contact with pigs and so more contact with pigs' secretions and excretions than controls suggests that NiV was transmitted from infected pigs to humans through contaminated saliva and possibly urine. The human outbreak of NiV infection ceased after widespread deployment of personal protective equipment to people contacting sick pigs, restriction on livestock movements, and culling over 900,000 pigs [33]. Since the outbreak ended through December 2014 no human or porcine NiV infections have been reported from Malaysia.

Mathematical modeling suggests that multiple spillovers into the pig population were necessary to create a dynamic population with sufficient newly susceptible pigs to sustain NiV transmission within pigs for months [34]. All human NiV

infections in the outbreak in Malaysia/Singapore in 1998–1999, may have been linked to a single NiV transmission from an infected bat to an immunologically primed pig population, leading to a sustained porcine epidemic which in turn led to a human epidemic. NiV isolates from pigs and people were nearly identical [25, 35].

Not all people infected with NiV in Malaysia had contact with pigs. In the Port Dickson case control study, two human NiV infected cases reported no contact with pigs [30]. KS Tan provided details on two additional NiV patients who had no direct contact with pigs [36]. One NiV patient who did not enter or go near a pig farms prior to his illness, worked repairing pig cages. His illness suggests that pig secretions/excretions remain infectious at least for hours and perhaps for days. The Port Dickson case control study noted an increased risk of dying dogs on farms where NiV cases were confirmed [30]. Serological studies in dogs in Malaysia demonstrated that they were commonly infected [37, 38]. One NiV patient who had no pig exposure worked as a cabinet maker and lived near a pig farm. His two pet dogs became seriously ill and died before the patient became ill with NiV infection [36].

There was limited evidence of person-to-person transmission of NiV in Malaysia. Multiple cases in families may have resulted from shared exposures. A large cohort study enrolled healthcare workers from the three hospitals that admitted over 80 % of patients with suspected NiV encephalitis [39]. The study enrolled 363 health care workers who provided direct patient care to encephalitis patients. More than 60 % reported contact with encephalitis patients before the institution of infection control measures on March 19, 1999. Many reported episodes of high risk exposure including skin exposure to body fluids of NiV infected patients ($n=89$), splash of patient body fluids to mucosal membranes ($n=39$), or needle stick injuries ($n=12$). None reported any serious illness, encephalitis or hospital admission. None of the first serum samples were positive by EIA for NiV IgG or IgM antibody. In the second round of antibody testing conducted 30 days later 3 of 293 serum samples (1 %) from exposed health care workers were positive for NiV IgG antibody, though none had detectable IgM and all three were negative for anti-NiV neutralizing antibodies. All three were nurses who cared for outbreak related encephalitis patients for more than 30 days compared with a mean of 10 days in nurses with negative IgG antibodies [39]. One of the nurses with NiV IgG antibody reported a febrile illness before the first serum sample was obtained, and the second reported a febrile illness between the two serum samples. One of the nurses reported a mucosal splash exposure. In a separately reported investigation a nurse who cared for hospitalized NiV infected patients and had antibody against NiV but was asymptomatic, had MRI findings characteristic of NiV infection [40]. Eleven years after the Malaysian outbreak a 32-year-old women presented with characteristic MRI findings of late onset NiV encephalitis and NiV IgG antibody [41]. Her family had stopped pig farming and moved away from the outbreak area 10 years before the outbreak, but she visited her aunt and uncle during the NiV outbreak and cared for her aunt who became ill and died. The woman reported no contact with pigs or other domestic animals.

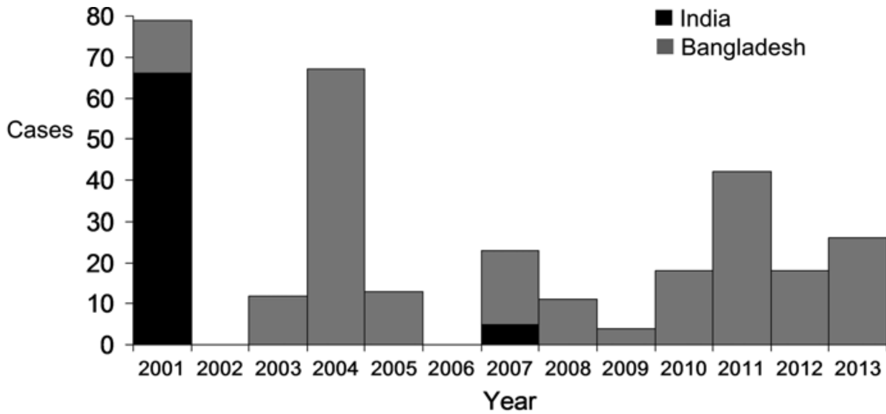

Fig. 4.1 Human infections with Nipah virus in South Asia 2001–2013

2.2 *NiV Epidemiology India/Bangladesh*

The epidemiology of NiV in Bangladesh/India has been quite different than in Malaysia. Since 2001 human infections with Nipah virus have been recognized in South Asia most years (Fig. 4.1). The cases in Bangladesh have largely clustered in western/northwestern Bangladesh (Fig. 4.2). The two recognized Indian outbreaks occurred in West Bengal, remarkably near where cases have been repeatedly identified in Bangladesh (Fig. 4.2).

2.2.1 NiV Transmission Through Date Palm Sap

Outbreak investigations in Bangladesh have identified drinking raw date palm sap as the most common pathway of NiV transmission from *Pteropus* bats to people. In the 2005 outbreak investigation in Tangail, Bangladesh NiV cases were 7.9 times more likely to report drinking raw date palm sap in the 10 days before they developed illness than neighborhood matched controls [42]. Similarly in the 2008 outbreak in Manikgonj and Rajbari districts in Bangladesh cases were 25 times more likely than controls to report drinking raw date palm sap [43]. In outbreaks in Faridpur, Bangladesh in 2010, and in Lalmonirhat in 2011 cases were again significantly more likely than controls to report drinking raw date palm sap in the 2 weeks prior to the onset of illness [44, 45]. The outbreaks of human NIV infection in Bangladesh and India coincide with the date palm sap harvesting season [46].

In Bangladesh date palm sap harvesters collect sap beginning in December with the first cold night and continue collecting most regularly through January and early February, though some harvesters continue to collect in at least a few trees through

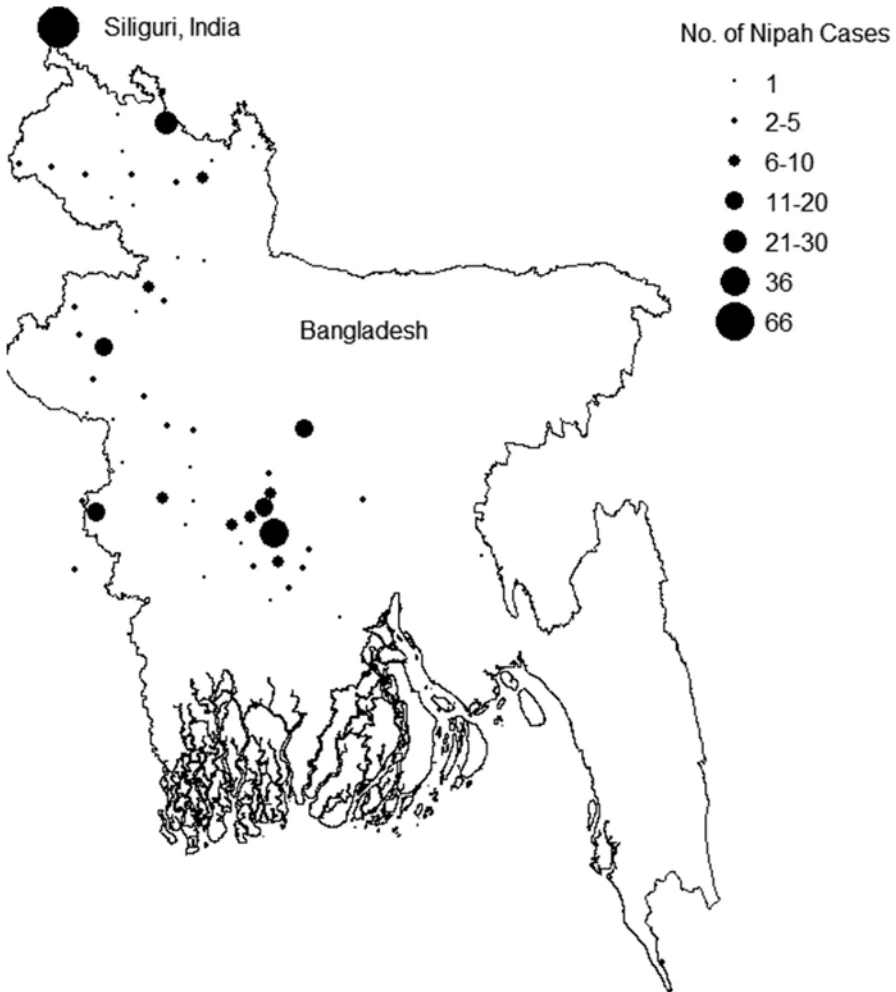

Fig. 4.2 Location of Nipah cases, Bangladesh/India 2001–2013

March and early April. At the beginning of the season, the bark is shaved off of one side of the tree (*Phoenix sylvestris*) near the top in a V shape and a small hollow bamboo tap is placed at the base of the V [47]. In the late afternoon, the date palm sap harvester climbs the tree, scrapes the area where the bark is denuded so the sap can flow freely, and ties a 2–4 l clay pot underneath the tap. During the night as the sap rises to the top the tree, some sap oozes out from where the bark is denuded, flows through the tap and drips into the clay pot. Palm sap collectors climb the trees at daybreak to gather the clay pots.

Most date palm sap in Bangladesh is cooked and made into molasses that is a popular sweetener for cakes and other desserts [47, 48]. A smaller amount of date palm sap is sold fresh for immediate consumption. Indeed, after a few hours, likely

because of fermentation, the date palm sap is less sweet and sap sellers have to lower the price. Collectors will often share fresh sap as a treat with family members and walk house to house near where the sap was collected and offer to sell it to neighbors.

Sap harvesters and villagers report that bats and other animals sometimes visit the trees during sap collection. Sap harvesters commonly find bat excrement outside of the clay pot or floating in the sap and occasionally find drowned dead bats floating in the pots [42, 47]. Infrared wildlife photography confirms that *Pteropus* bats, the presumed reservoir of NiV in Bangladesh, commonly visit date palm trees during collection and lick the sap stream [49]. Infrared cameras placed in the seven trees that were the source of fresh date palm sap drunk by the human NiV cases in the Manikgonj/Rajbari outbreak in 2008, identified an average of four *Pteropus* bat visits per tree where the bat licked the sap stream, per night of observation [43].

Date palm sap is a plausible vehicle for transmission of NiV from *Pteropus* bats to people. *Pteropus* bats occasionally shed NiV in their saliva [8, 50, 51]. The infrared camera studies confirm that *Pteropus* bats directly lick raw date palm sap and occasionally urinate in the sap collection pot [49]. NiV inoculated in mango flesh, mango juice, pawpaw juice, and lychee juice for up to 3 days was recoverable at high concentrations [52]. NiV that was inoculated into a solution of 14 % sucrose and 0.21 % bovine serum albumin to mimic date palm sap, survived for 8 days at 22 °C with no reduction in titer [53]. To date, in outbreak investigations NiV has not been isolated directly from date palm sap [43]. This is not surprising, because *Pteropus* shedding of NiV is intermittent [54], and with the median 10 day incubation period from exposure to date palm sap to illness [43], and the time required to recognize an outbreak of NiV, outbreak investigation teams have only been able to collect sap samples from trees weeks after the likely transmission event.

Some date palm sap in Bangladesh is fermented into palm wine (*tari*). One NiV case in India [55] and an outbreak in Bangladesh [56] have been tied to drinking this fermented date palm sap. Apparently, at least in some cases, the alcohol content of the fermented sent sap is insufficient to inactivate the virus.

Other direct pathways of NiV transmission from *Pteropus* to people have not been confirmed. In the 2004 outbreak in Rajbari District, Bangladesh, cases were more likely to climb trees than controls (83 % versus 51 %, $p=0.025$) [57]. It is possible that children climbing trees had direct contact with NiV contaminated bat urine or bat saliva that subsequently infected their respiratory or gastrointestinal tract and led to infection; however, this pathway of transmission has been assessed but not implicated in any of the subsequent outbreak investigations through 2014. Moreover, 91 % of cases in the Rajbari outbreak reported drinking raw date palm sap [57]. The father of two of the cases was a date palm sap harvester and the outbreak was centered on his friends and family (Emily Gurley personal communication). Although there was insufficient statistical power to implicate date palm sap in the Rajbari outbreak investigation (91 % versus 72 %, $p=0.328$), the subsequent repeated implication of date palm sap as the vehicle of transmission in other outbreaks, and the high level of exposure among cases (91 %) in Rajbari suggests that fresh date palm sap was the primary vehicle of NIV infection in this outbreak.

2.2.2 NiV Transmission from Domestic Animals

A second route of transmission for NiV from bats to people in Bangladesh is via domestic animals. Fruit bats commonly drop partially eaten saliva-laden fruit. Domestic animals in Bangladesh forage for such food. Date palm sap that is contaminated with bat feces and so is unfit for human consumption is also occasionally fed to domestic animals [47]. Animal husbandry practices in Bangladesh are quite different than in Malaysia. In Malaysia, thousands of pigs were raised together on large factory farms. By contrast, in Bangladesh many rural families keep just a few domestic animals. If a domestic animal in Bangladesh contracts NiV, there are few susceptible mammals physically close enough to become infected, so rather than sustained transmission as was observed in the Malaysian outbreak, in Bangladesh the chain of transmission would be expected to be short.

Nevertheless, there have been human NiV cases linked to apparent domestic animal infections in Bangladesh. The index case in the Meherpur District 2001 outbreak developed illness on April 20, the latest post winter onset of any confirmed NIV outbreak in Bangladesh, past the end of the date palm sap season in most communities. NiV cases in Meherpur were eight times more likely to report contact with a sick cow than controls [58]. In the Naogaon outbreak in 2003, NiV cases were six times more likely than controls to report contact with a pig herd that visited the community 2 weeks before the human outbreak [59]. In 2004 a child developed NiV infection 2 weeks after playing with two goats that developed an illness that began with fever, and progressed to difficulty walking, frothing at the mouth and death [60].

2.2.3 NiV Person-to-Person Transmission

In contrast to limited evidence of person-to-person transmission of NiV in Malaysia, person-to-person transmission of NiV has been repeatedly identified in Bangladesh/India. The first NiV outbreak recognized in the Indian subcontinent was a large outbreak affecting 66 people in Siliguri, India in 2001. The outbreak apparently originated from an unidentified patient admitted to Siliguri District Hospital who transmitted infection to 11 additional patients, all of whom were transferred to other facilities. In two of the facilities, subsequent transmission infected 25 staff and 8 visitors [61].

The longest sustained chain of person-to-person transmission of NiV so far identified in Bangladesh occurred in an outbreak in Faridpur District in 2004. Friends and family members who provided direct care to NiV infected patients, or helped to carry them or transport them to health facilities when they were near death, sustained a chain of transmission through five generations [62] (Fig. 4.3). One NiV patient was a popular religious leader who was visited by many of his family members and followers when he became ill. Twenty-two of these visitors developed NiV infection.

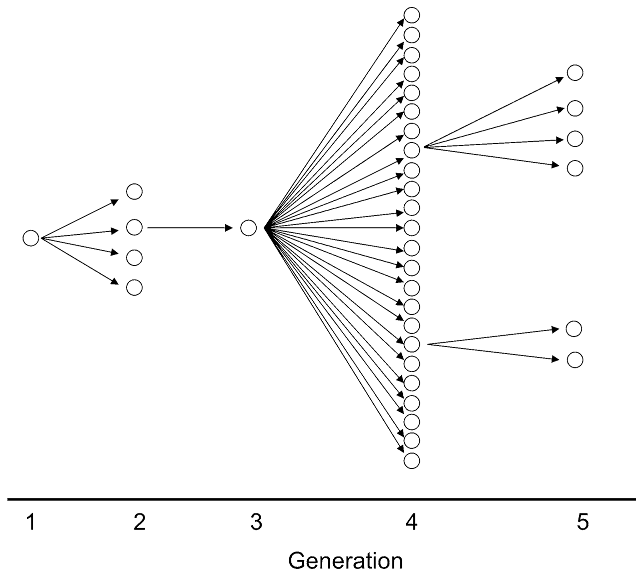

Fig. 4.3 Chain of person-to-person transmission in NiV outbreak in Faridpur, Bangladesh, 2004. (From [60])

While the outbreaks in Siliguri in 2001 and Faridpur in 2004 were the largest examples, person-to-person transmission has been identified in other outbreaks in Bangladesh [45, 63]. In a review of the 122 NiV cases identified in Bangladesh from 2001 through 2007, 62 (51 %) developed illness 5–15 days after close contact with another NiV patient [46].

Outbreak investigations in Bangladesh suggest that respiratory secretions are the primary vehicle of person-to-person transmission of NiV. Patients in Bangladesh were more likely to have respiratory symptoms then were patients in Malaysia. In a review of cases in the first four recognized outbreaks in Bangladesh, 62 % of patients had cough and 69 % had respiratory difficulties [64]. By contrast, in Malaysia only 14 % of patients presented with cough [28]. In the 2004 Faridpur outbreak, cases were more likely than controls to report touching an NiV infected patient who later died (OR 5.5, 95 % CI 2.1, 16) [62]. Similarly, in Thakurgaon in 2007 six family members and friends who cared for an NiV infected patient developed NiV infection. Cases were more likely than controls to have been in the same room when the index case was coughing (100 % versus 0 %, $p=0.04$) [63]. Across all recognized outbreaks in Bangladesh from 2001 through 2007, NiV patients who had difficulty breathing during their illness were more likely to transmit NiV than NiV patients who did not have difficulty breathing (12 % versus 0 %, $p=0.03$) [46].

NiV RNA has been frequently identified in the saliva of NiV patients [65, 66]. In Bangladesh family members and friends without health care or infection control training provide nearly all the hands on care to ill patients both at home and in the hospital [67]. During the Faridpur 2004 outbreak care providers shared eating

utensils, ate leftovers of food offered to NiV patients, commonly slept in the same bed with a sick, coughing NiV patient, and often fed and hugged the dying patient [68]. During an outbreak in Faridpur in 2010 a person whose only contact with an NIV infected patient was cleaning the corpse in preparation for burial became infected with NIV [45].

2.2.4 Other Plausible Pathways of NiV Transmission

There are a number of plausible pathways of NiV transmission from *Pteropus* bats to people that have been explored in outbreak investigations in Bangladesh, but have not been implicated as pathways of transmission. One of these pathways is living underneath a bat roost. Pteropus bats intermittently shed NiV in their urine [54]. Although some homes are located quite close to bat roosts, living near a bat roost has not been identified frequently in outbreak investigations, and has not been found more commonly among cases than controls [60].

Another plausible pathway of transmission is consumption of bat-bitten fruit. Both birds and *Pteropus* bats often drop fruit after taking a single bite. In Bangladesh, where child malnutrition is widespread [69], ripe tasty dropped fruit is commonly picked up and consumed by rural Bangladeshi residents. In each of the outbreak investigations in Bangladesh consumption of dropped fruit has been evaluated as a potential exposure, but in none of the outbreaks have cases been reported to have eaten dropped fruit significantly more commonly than controls [70].

3 Open Questions in Henipavirus Epidemiology

Both Hendra and Nipah virus are widely distributed among *Pteropus* bats, but spill-over occurs in a much more restricted region. Apparently the frequency of a specific human behavior that is uncommon across the human population but more common in these areas provides an opportunity for henipavirus transmission. In Queensland, the popularity of horse racing leads to many horses sharing the natural environment with *Pteropus* bats, and people come in close contact with symptomatic ill horses. In Bangladesh, *Pteropus* bats are present across the entire country, and presumably shed virus throughout the year [54]. We hypothesize that people living in the out-break infected regions in Bangladesh are more likely to drink fresh date palm sap or have other activities that put them in more contact with bat secretions compared with people living in other regions with *Pteropus* bats, but without recognized human NiV cases.

Among the most important open question in Henipavirus epidemiology is esti-mating the magnitude of risk that a strain of Nipah virus would develop sufficient capacity for person-to-person transmission to cause a high mortality global out-break [71]. NIV is a stage III zoonotic disease that is an agent that normally lives in its animal reservoir, but occasionally spills over into people and is capable of

non-sustained person-to-person transmission [72]. Because its basic reproductive number (R_0), i.e., the average number of people who a new case infects is <1, spillovers result in stuttering chains of person-to-person transmission. While stage III zoonotic agents are infecting humans, the virus comes under selection pressure favoring characteristics that facilitate person-to-person transmission [73]. Humanity has a deadly historical example of a different zoonotic paramyxovirus, rinderpest, whose ancestor virus spilled over into humans as measles virus between the eleventh and twelfth century [74] and was subsequently a major cause of human mortality for centuries [75]. The Henipaviruses are widely distributed across species of bats and there is no evidence that they cause illness in bats [4, 8]. Thus, these viruses likely coevolved with the bats. Date palm sap has been collected in the area that is now Bangladesh for centuries [76] and so while NiV disease is newly recognized, there have likely been occasional human infections for a long time, none of which have resulted in pandemic transformation of the virus. Nevertheless, population density in South Asia has reached unprecedented levels, and so there is increased opportunity for sustained person-to-person transmission. Better understanding the frequency of spillover of Henipavirus from bats to other mammals in the environment, and the rate of change in adaptation of those viruses can provide a more precise estimate of the risk of a NIV pandemic, which, in turn, could prioritize and inform policy to reduce risks.

A related question to pandemic risk is how much strain differences in Henipavirus are responsible for observed epidemiological differences. There is substantial heterogeneity among Nipah strains in Bangladesh compared with much less strain heterogeneity associated with the single large Nipah outbreak in Malaysia [77]. Nipah patients in Bangladesh were much more likely to have severe respiratory symptoms and much more likely to transmit Nipah person-to-person compared with Nipah patients in Malaysia [64]. In animal experiments inoculating Syrian hamsters and African green monkeys, animals exposed to a lower dose of Nipah virus were more likely to develop encephalitis; animals exposed to a higher dose of Nipah virus were more likely to develop severe respiratory disease [78, 79]. In human infections it is unclear if dose of exposure increases the proclivity for respiratory infection and subsequent person-to-person transmission. Alternatively, specific risk behaviors, especially the frequency of intimate personal contact with people who are dying in Bangladesh [68] may be the primary determinant of person to person transmission. It is also possible that some strains of Nipah that have characteristics which favor pulmonary tropism or other characteristics that facilitate person-to-person transmission. We do not yet have enough strains of henipavirus, paired with careful epidemiological data to resolve these questions, but continued careful outbreak investigation and collection of additional isolates could provide additional insight. If there are structural differences in viral proteins that facilitate person-to-person transmission, then better understanding the variability of these structures and capacities and their rate of change in different contexts can help to estimate pandemic risk and provide targets for intervention.

4 Conclusion

Careful investigation over the last 20 years have clarified the basic transmission pathways of Hendra and Nipah virus infection, and found evidence of other henipaviruses. These organisms are not easily transmitted to people. When humans do become infected, only occasional superspreaders infected with NiV transmit illness. To date transmission has not been sufficiently efficient to maintain person-to-person transmission. However, these agents are newly recognized. Human infection with either Hendra virus or Nipah virus is commonly fatal and their pandemic potential is poorly defined. Henipaviruses warrant ongoing public health and scientific attention.

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Chapter 5
Respiratory Syncytial Virus

Gary Hellermann and Shyam Mohapatra

Core Message Since its discovery in 1956, respiratory syncytial virus, RSV, has been the subject of extensive research and study; yet it has stubbornly resisted our efforts to create effective vaccines and drugs to prevent or treat RSV infection. At the present time, our knowledge of RSV biology and the intimate ways the virus interacts with its host and bends the immune response to its advantage has matured. That knowledge combined with mind-boggling new advances in nanotechnology, nanomaterials, and delivery platforms makes the current time perhaps the most exciting and promising in RSV history.

1 Introduction

Respiratory syncytial virus, RSV, was discovered nearly 60 years ago as the causative agent of human respiratory tract disease, and it still remains a serious threat to infants, immunocompromised persons, and the aging adult population. Although infants and young children are still the main target of RSV, pneumonia in the elderly is being found more frequently associated with RSV infection and leads to more deaths in that population than among young children [1–3]. Approximately 3.4 million people throughout the world are hospitalized every year with pneumonia or bronchitis attributed to RSV infection and nearly 17,000 die in the USA alone [4, 5].

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The cost of morbidity from RSV in terms of lost productivity and impaired quality of life is astronomical.

Prevention of RSV infection has been the goal of research and clinical trials for many decades, but the virus has proven to be a tough adversary. Vaccination of children with formalin-inactivated RSV was tried unsuccessfully in the 1960s and received a major setback when it was shown that the immune response to the vaccine actually exacerbated the disease. Since then, researchers have proceeded with caution, testing recombinant antigen combinations, adjuvants, and immune system boosters with some success in animal models. Other prophylactic compounds such as the humanized monoclonal antibody palivizumab (Synagis) that binds to an epitope in the viral F protein can be used to reduce RSV pathology in at-risk infants, but it is too costly for routine use and has not been approved for RSV prevention in the elderly [6, 7]. In terms of therapeutic intervention, the only approved antiviral drug in the USA is ribavirin which lowers overall mortality among transplant patients but it has serious side effects at the doses needed to kill RSV and is not that effective [8].

Data on RSV-host interactions have been accumulating from years of study. Today we have a much better idea of potential viral targets such as the virus-cell fusion process for prophylaxis to prevent entry of RSV into host epithelial cells. Here we review the current field of RSV research and highlight some of the more promising current strategies for RSV prophylaxis with a special emphasis on the elderly who are making up an increasingly larger portion of the world's population.

2 Epidemiology

RSV is one of the most important respiratory pathogens targeting all age groups; however, infants (<18 months old), immunosuppressed individuals, and the elderly are the groups who experience the most severe aspects of the disease—bronchiolitis and pneumonia [9]. The greatest number of hospitalizations for complications of RSV infection occur in infants 2–3 months of age [3] and RSV bronchiolitis imposes a severe burden upon healthcare services especially in developing countries. Worldwide, about five million infants are hospitalized annually for severe RSV infection resulting in more infant deaths than influenza [10, 11]. Costs related to emergency department visits amounted to approximately 202 million US dollars between 1997 and 2000 [12].

2.1 Seasonality

Outbreaks of RSV infection are seasonal, lasting for about 4 months with peaks occurring in the northern hemisphere between November and March and in the southern hemisphere between July and September [13, 14]. Records of RSV

morbidity and mortality are not kept consistently, especially in resource-poor developing countries, but it is estimated that RSV infection worldwide results in about 100,000 hospitalizations and 4,500 deaths annually [5]. World mortality estimates range as high as four million deaths annually with no indication of improvement in many areas [11].

2.1.1 USA

The average length of the RSV season is 15 weeks [13] but peak times and rates can vary geographically throughout the country [2]. Southern states tend to show the earliest onset of the RSV season while cities in the Midwest experience the latest. For example, the RSV season in parts of Florida can start as early as July and continue into March.

2.1.2 Europe

The pattern of RSV epidemics in Europe differs from that in the Western hemisphere in that there are cycles of 9 and 15 months that recur every 2 years [15]. This 2-year cycle is also seen in Germany, Switzerland, and Scandinavia, but the pattern is 23–25 months. The incidence of severe RSV infection in children less than 2 years of age in western Russia is similar to that in other European countries, but the peak appears to be later in the season, more towards the spring. Geography again plays a role in RSV epidemics in the UK, where the cycle reverts to the monophasic pattern seen in the USA [16].

2.1.3 Southern Hemisphere

In Australia, the majority of the 100,000 annual cases of RSV lower respiratory tract infections (LRTIs) occur in winter between July and September [17].

2.2 RSV in Developing Countries

Limited record keeping and availability of testing labs hinder the picture of RSV epidemics, incidence, seasonality, and mortality in the developing world. The World Health Organization has sponsored studies in Africa and Asia targeting respiratory tract infections in children but results are far from complete and accurate. Hospital-based studies reported RSV as the main etiological agent in LRTIs in 6–96 % of cases with an average of 65 % of virus infections being RSV and associated with 17 % of the hospitalizations. In one WHO survey of LRTIs in Africa, RSV was identified as the culprit in 36 % of cases in Nigeria but only 6 % in Mozambique,

again emphasizing the importance of geography, climate, regional differences, and social factors in the epidemiology of RSV. Between 66,000 and 199,000 children die from RSV complications every year and the great majority of these deaths occur in resource-poor developing countries [5].

3 RSV Biology: Host vs. Virus

3.1 Taxonomy and Structure of RSV

RSV is classified in the family *Paramyxoviridae* which also includes parainfluenza virus, measles, and mumps virus. Viruses in this family have a genome consisting of a non-segmented negative-sense RNA that must be transcribed by an endogenous RNA polymerase to provide mRNA for translation of the viral proteins. Epithelial cells of the bronchioles and alveolae are the primary targets of RSV and the mechanism of viral recognition and fusion with the host cells has received much attention in recent years as a blocking point for inhibiting viral infection [18–20]. The RSV virion is surrounded by a lipid bilayer acquired from the host plasma membrane in which are spikes of viral G and F glycoproteins. Recent investigations implicate the F protein as key to RSV binding and entry into epithelial cells, hence the relative success of the prophylactic anti-F monoclonal antibody, palivizumab, in preventing pediatric RSV infections.

The nucleocapsid is constructed from the N protein and contains the genomic RNA, the large RNA polymerase subunit L with its cofactor P phosphoprotein, and the anti-termination factor M2-1. The nucleocapsid is linked to the envelope via the M matrix protein and the virion is spherical with a diameter of 140–300 nm, although filamentous forms have also been seen. Viral genes in the negative-sense RNA genome are oriented 3′ to 5′ in the order NS1, NS2, N, P, M, SH, G, F, M2, and L. The first two proteins expressed from the viral RNA, nonstructural proteins 1 and 2 (NS1 and NS2), have been shown to suppress the host's antiviral interferon response which allows RSV to rapidly spread among susceptible lung epithelial cells [21, 22]. The other proteins encoded in order on the genome participate in replication of the viral genome, nucleocapsid formation, encapsidation with the plasma membrane, and entry into host cells.

3.2 Viral Attachment, Fusion, and Entry

RSV virions are only about one-hundredth the size of an epithelial cell and must utilize the cell's surface proteins as docking points for fusion and entry [20]. This is the obvious golden point for suppressing viral infection and research is still being aggressively conducted to achieve this end [23]. The viral envelope G

glycoprotein has two variants known as A and B that account for the difference in RSV strains. The G proteins are thought to interact with specific surface membrane proteins on target cells after which the viral F protein undergoes a molecular rearrangement that promotes fusion of the viral envelope with the plasma membrane [19]. Various membrane-bound host proteins have been proposed over the years as primary candidates for RSV targeting, including ICAM-1, nucleolin, Toll-like receptor 4, annexin 2, and the fractalkine receptor CX3CR1. The viral F protein is the main player in the fusion process. It possesses two heptad repeat domains that interact to form a helical bundle when the virion is in the proper orientation to the host epithelial cell and insertion of the hydrophobic region into the plasma membrane promotes "melting" of the envelope into the cell membrane [24]. The small hydrophobic, SH, protein is also found in the viral envelope but there has been no evidence that it is necessary for attachment or fusion of the virion to the target cell.

Among the paramyxoviruses, the structure of the F protein appears to be highly conserved, leading to the hopeful hypothesis that pharmacological intervention at the fusion event may be an effective strategy to block the infection of several different species of virus in a way that does not permit the development of resistance. Recent comparisons of peptide inhibitors among Nipah, Hendra, and other family members suggest that there is a common fusion mechanism involving the helical bundles that is susceptible to interference [25].

3.3 Cell Surface Receptors for RSV Infection

There does not appear to be a single cell-surface receptor for RSV. Over the years, primary candidates have included intercellular adhesion molecule-1 (ICAM-1), heparin sulfate, chondroitin sulfate, lipid rafts, and nucleolin. The viral envelope proteins G and F are both involved in attachment, but only F is obligatory for infection. That the G protein is important but not mandatory is shown by experiments in which RSV lacking G protein is still able to infect epithelial cells, but at a reduced level [26]. The plasma membrane-bound cell-surface glycoprotein, nucleolin, meets the criteria for an RSV receptor: RSV infection in vitro is inhibited by treating the cells with anti-nucleolin antibody, soluble nucleolin, or small interfering RNA against the nucleolin mRNA, and cells that are normally resistant to RSV infection become permissive when transfected with a plasmid-over-expressing nucleolin [27]. The RSV F protein immunoprecipitates with nucleolin and with ICAM-1 and virus lacking F are noninfective. Lay et al. [28] have proposed a model in which the RSV G protein first attaches to negatively charged cell-surface glycosaminoglycans, which brings the fusion protein F into contact with nucleolin allowing it to undergo the conformational change that causes association with cholesterol-rich lipid rafts.

3.4 RSV Detection and Attack by the Host Immune System

Pattern-recognition receptors such as the Toll-like receptors (TLRs) are one of the molecular sentinels whose particular response signals the presence of a particular molecular signature corresponding to a microbial invader. The intracellular TLRs found primarily in dendritic cells are localized to the endoplasmic membrane where they are exposed to the cytoplasmic milieu and the transcriptional and replicative forms of RNA viruses [29]. Single-stranded viral RNA is recognized by TLR7/8 while TLR3 binds double-stranded viral RNA. TLR3 signaling does not require the adapter protein MyD88 while TLR7/8 signaling does. Binding of RNA to the TLRs precipitates a signal cascade that results in activation of the interferon response factor 3 and production of the type I interferons, IFN-alpha/beta.

In addition to the TLR viral detection system, cells have a pair of cytosolic RNA-sensing proteins, RIG-I (retinoic acid-inducible gene-I) and MDA5 (myeloma differentiation-associated protein 5), that bind double-stranded viral RNA [30]. MDA5 is specific for non-triphosphorylated RNA such as that of the picornaviruses, while RIG-I recognizes the triphosphorylated form, which is found, for example, in the influenza virus [31, 32]. Binding of RNA to RIG-I causes it to interact with the mitochondrial antiviral signaling protein, MAVS, which forms a complex that activates the antiviral defense mechanism with production of IFN-alpha/beta and inflammatory cytokines, and the induction of programmed cell death, apoptosis, of infected cells. There is yet another RNA-sensing molecule in the cytoplasm, LGP2 (“Laboratory of Genetics and Physiology”-2), whose role is apparently to sequester viral RNA and thereby put the brakes on RIG-I/MDA5-triggered signaling [33]. Inhibiting LGP2 synthesis has the effect of increasing production of IFN-alpha/beta after viral infection [34].

3.4.1 RSV Subversion of the Host Antiviral Defense System

Viruses infecting mammalian hosts coevolved with them, inventing new ways to circumvent the immune system’s strategies to inactivate and eliminate the invading microbes. RSV has a number of tactics of its own to blunt the antiviral interferon response and interrupt leukocyte attack. At the 3′ end of the viral genome are two genes encoding nonstructural proteins, NS1 and NS2. These are the key to the subversion of the antiviral defenses by suppressing the interferon-alpha/beta signaling pathways [21]. Once RSV enters the cell, its negative-sense RNA genome is released and viral mRNA is produced. In the cytosol, RIG-I acts as a receptor for the viral RNA, and the complex in turn activates mitochondrial antiviral signaling protein, MAVS. MAVS triggers a signaling cascade that results in activation of the interferon pathway with expression of interferon-alpha/beta, translocation of NF-kappa B to the nucleus and production of inflammatory cytokines, and induction of apoptosis [35]. Transfection of epithelial cells with siRNA against RIG-I or MAVS severely limits IFN-alpha/beta production upon virus infection.

NS1, the viral protein that is translated first in order on the viral mRNA, is localized to mitochondria where it short-circuits the MAVS-RIG-I interaction by binding to MAVS before RIG-I and inhibiting activation of the antiviral system [36]. The second viral nonstructural protein, NS2, contains 124 amino acids and appears to bind to RIG-I but not MAVS. The cooperative role of NS2 in subverting activation of MAVS signaling has not been completely worked out. NS1 and NS2 were shown to associate under some conditions and the presence of NS2 is necessary for full suppression of STAT2 [37]. Normally, MAVS/RIG-I association triggers TRAF3 which results in the downstream activation of the serine/threonine protein kinases, IKK-epsilon and TBK-1. These in turn phosphorylate residues in the C-terminal region of interferon regulatory factor 3 (IRF3) and IRF7 which then translocate to the nucleus where they can promote transcription of the IFN-alpha/beta genes [38]. The secreted IFNs then bind to cell-surface receptors on virus-infected cells and signal the activation of STAT1 and STAT2 which results in expression of the whole panoply of IFN-responsive genes and induction of viral resistance. By preventing MAVS/RIG-I interaction, RSV subverts this defense system and insures its survival and rapid replication. The viral NS1 and NS2 proteins thus constitute logical therapeutic targets for drugs to treat RSV infection. Short interfering RNAs against NS1 and NS2 combined with anti-fusion agents in nanoparticles could produce an RSV prophylactic formulation that is safe, relatively inexpensive, and very effective.

3.4.2 Dendritic Cells: Key Watchdogs in the Host Response to RSV Infections

Dendritic cells or DCs are one of the body's main sentinels on guard against viral attack in the lungs and other organs. They constitute the primary link between the innate and adaptive immune systems and they are often the first responders when we become infected with RSV, producing large amounts of IFN-alpha/beta to eliminate the virus. There are a number of subpopulations of DCs and the type known as plasmacytoid (pDCs), derived from bone marrow precursor cells, is one of the most important in terms of type I IFN production and activation of immune system cells [39]. pDCs are released from the bone marrow into the blood and are found in mucosal associated lymphoid tissue as well as spleen and lymph nodes. Through secretion of IFN and cytokines such as IL12 as well as their usual functions of antigen presentation, pDCs can participate in both innate and adaptive immune responses to RSV and thus may be an important effector cell in the antiviral defense mechanism.

pDCs lack TLR3 and are resistant to RSV infection, so how is the function of these cells regulated? They do possess TLRs-7 and -9 and signaling through these plasma-membrane receptors can induce the IFN-alpha/beta cascade. Another key regulatory factor in pDC-mediated response to RSV appears to be the bone sialoprotein, osteopontin (Opn) [40]. Opn is found in several isoforms, but primarily as a secreted form and an intracellular form (iOpn). The intracellular variant is

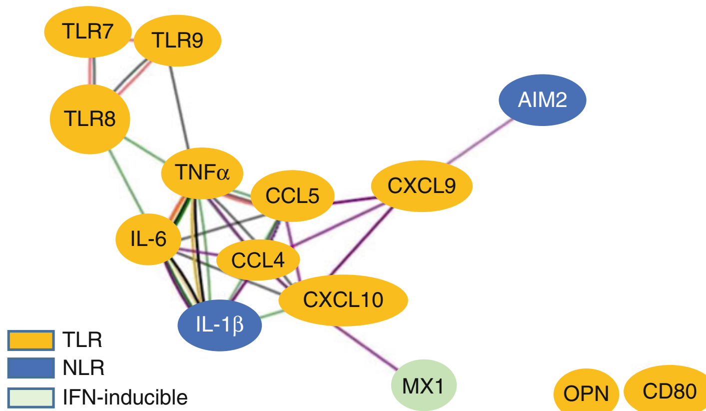

Fig. 5.1 Pathway map illustrates innate immune genes upregulated >2-fold in aged as compared to young [50]

synthesized from the same mRNA as the secreted form, but uses an alternative translation start site. As its name suggests, Opn is produced by bone cells, osteoblasts, and osteocytes, but it is also expressed in DCs and recent evidence suggests that iOpn may regulate expression of TLR9 and thus the IFN-alpha production by pDCs, which affects the adaptive immune response to viral attack through induction of Th1 effector cells and memory cells [41].

The normal, healthy response of the adaptive immune system's cells to viral attack declines with age, but age-related deterioration of the innate antiviral system has not been so well characterized. Analysis of RSV infection in aged mouse models has identified a number of specific changes in gene expression in relation to changes to the activation of proinflammatory cytokine signaling (Fig. 5.1). Expression of 84 antiviral gene targets in RSV, strain A2-infected young and aged mice was compared with RT2-PCR Profiler array analysis. (A) Twenty-seven genes from the PCR array analysis were found upregulated. A GeneMania network map, assembled using signals that were greater than twofold. The pathway illustrating the networks linking genes of different innate immune mechanisms is shown in Fig. 5.1. Also, aged mice infected with RSV showed decreased expression of the antiviral genes, RIG-I, TLR8, IFN-alpha-R1, IL-1β, and osteopontin [50]. Mucus production was lower in aged mice compared to young and the disease symptoms persisted longer. These effects in elderly mice suggest that similar declines in the native immune response occur in humans. Strategies for antiviral prophylaxis and treatment must take these phenotypic changes into consideration in designing new drugs.

3.5 Neurogenic Inflammatory Response to RSV

The interaction of RSV with the nervous system is a sometimes overlooked but important component of the inflammatory scenario orchestrated by the body to ward off invading virus. RSV especially affects the airways by making them more susceptible to neurogenic influence through upregulation of the receptor for substance P and increased production of nerve growth factor and its receptor, p75 [42]. Experiments with young animals show important distinctions in the neurological response to RSV infection compared to adult animals. Persistent effects of RSV-induced inflammation on the developing lung and its innervations may increase the tendency towards hyperreactivity and airway dysfunction upon reinfection with RSV [43]. Remodeling of the sensorineural network or changes in neurotrophin expression during the neonatal period could have long-lasting effects. This aspect of the neuroinflammatory process may play a role in the proposed association between repeated RSV infection in infancy and later development of asthma.

3.6 Persistent RSV Infections

RSV was discovered as a pathogen that targets the lungs and it was assumed that following clearance by the immune system, there would be no more virus present. This dogma was recently challenged by findings that RSV could infect and persist in human bone marrow stromal cells long after the virus was cleared from the lungs [44]. Evidence was presented that the presence of the virus altered the bone marrow compartment in ways that might compromise hematopoietic cell differentiation, proliferation, and activity. The existence of extrapulmonary sites of RSV infection and the possibility of reinfection from immunologically privileged stem cells migrating to the lungs are intriguing avenues for new research. Indeed, investigation of patients who have stable COPD has detected RSV transcripts in the absence of disease exacerbation suggesting that the virus may persist in some form in these patients [45].

4 Pathology of RSV Infection

4.1 Pathogenesis

Around 90 % of children have been infected by the age of 2 years [2] and reinfection throughout life is common because complete immunity to RSV is never developed. The first infection is usually the most severe and infants with a history of premature birth, bronchopulmonary dysplasia, congenital heart disease, cystic fibrosis, or immunosuppression are the ones most likely to develop the severest cases of

bronchiolitis and pneumonia, which carry the highest risk of death [11]. However, a comprehensive analysis of RSV-associated deaths between 1979 and 1997 in US children suggested that the majority of deaths do not occur among children who are presumed to be at high risk for severe RSV LRTIs [46]. Although the major clinical manifestation of RSV in older children and adults is upper respiratory tract illness such as rhinitis and acute otitis media, it may also be the main etiological agent in up to 2.4 % of community-acquired pneumonia in these groups [47].

4.2 Clinical Manifestations

4.2.1 Infants and Young Children

Lower respiratory tract involvement and bronchiolitis are the most common types of RSV infection in infants, but in many cases the first symptoms may only be diminished activity, loss of appetite, and shortness of breath. Children may show cold-like symptoms and fever, and only after a couple of days develop a cough or wheezing.

4.2.2 Adults and Elderly Individuals

Healthy adults infected with RSV may be asymptomatic or suffer primarily upper respiratory tract RSV infections with fever, cough, runny nose, and fatigue. The disease is normally self-limiting and lasts for about 5 days. In older adults, RSV was identified as responsible for 10 % of winter hospital admissions, and it has a case-fatality rate that approaches 10 %. In addition, 78 % of RSV-associated deaths occurred in individuals aged 65 years or older with underlying cardiac and pulmonary pathology [48]. Patients taking immunosuppressive drugs are at risk for RSV pneumonia and should be especially careful during peak seasons to avoid contact with infected persons.

While RSV infection used to be looked upon as a serious threat to infants but only a nuisance to adults, the burgeoning populations of the elderly and immunocompromised patients have changed this outlook radically. Hospitalizations for pneumonia, especially among the elderly in community care facilities, have been on the upswing along with a significant increase in the duration of stay with concomitant rise in cost and reduced quality of life. Rates of RSV illness in nursing homes are 5–10 % per year with 10 % of these resulting in pneumonia and 2–5 % ending in death. Individuals with cardiovascular disease or those taking immunosuppressive medications are at greatest risk of severe infection, pneumonia, and death. An estimated 10,000 deaths occur annually among those over 65 years of age because of RSV infection. A 4-year study published in 2005 [49] reported that nearly 80 % of deaths associated with RSV occurred in the elderly who also had respiratory and circulatory problems.

4.3 Differential Pathology in Infants and the Elderly

The increased susceptibility to RTIs and to complications can be traced, at least in part, to a weakened immune system in the elderly that prevents them from mounting an effective defense against microbial attack. Immune dysregulation can occur at the innate and adaptive levels and the age-related inability to mount an ineffective immune response to microbial invasion is often referred to as immunosenescence [49]. A significant decrease in the number and proliferative capacity of lymphoid precursors is a key feature in immunosenescence and declining T-cell function is one of the best-studied characteristics of the aging immune system. Defects can be found along the functional pathway from initial activation of naïve T-cells through to the stage of expansion, effector proliferation, and memory cell production.

In addition to cellular deficiencies, the elderly lungs show substantially reduced rates of mucociliary clearance, loss of elasticity, and a tendency to chronic inflammation. Comorbid conditions such as emphysema, chronic obstructive pulmonary disease, and congestive heart failure all may contribute to a greater susceptibility to severe RSV infection when compared to younger adults. Because of these important differences between the immune responses of young and aged individuals, there is a distinct need for new diagnostic, prophylactic, and therapeutic strategies tailored specifically for combating viral infections such as RSV in the geriatric population [51].

5 Diagnosis, Prophylaxis, and Treatment of RSV Infection

In spite of decades of effort, an effective and safe vaccine for RSV continues to elude biomedical researchers. The antiviral drug, ribavirin, is only moderately effective against RSV in children and the humanized monoclonal antibody against the RSV fusion protein F (palivizumab) is very expensive and restricted to prophylaxis of at-risk neonates. The need for safer, less expensive, more effective alternatives for prophylaxis and treatment of viral respiratory infections, especially with the increasing population of elderly individuals, is obvious and needs to be taken seriously by the pharmaceutical and government funding agencies.

Assays that identify RSV as the causative agent in RTIs are often not performed or else the time required to obtain the results is so long that the illness has already progressed beyond the point where any antiviral treatment would be effective. The currently most effective method for rapid identification of respiratory viruses is the real-time multiplex PCR assay [52]. The cost of instrumentation, staffing, and reagents, however, is beyond reach of many resource-limited clinics. An inexpensive, rapid, point-of-care device capable of distinguishing RSV from influenza, rhinovirus, or bacterial infections would be a tremendous boon for providing global care to the elderly and immunocompromised. If disease could be identified early then ribavirin or intravenous polyvalent (hyper)-immunoglobulins (IVIG) could be administered with some hope of terminating the infection.

Table 5.1 A list of anti-RSV therapy or vaccine candidates in clinical trials

Product	Sponsor	Class	Status
ALN-RSV01	Alnylam	siRNA	Phase IIb (completed)
MEDI-557	MedImmune	Humanized mAb	Phase I (ongoing)
RSV604	Arrow/Novartis	Nucleoprotein inhibitor	Phase II (pending)
ALS-8176	Alios	Nucleoside analogue	Phase I (ongoing)
MDT-637	Teva Pharmaceuticals	Fusion inhibitor	Phase I (completed)
RSV ΔNS2 Δ1313 11314L	NIAID	Vaccine	Phase I (recruiting)
MEDI-559	MedImmune	Vaccine (rA2cp248/404/1030ΔSH)	Phase I–IIa (completed)
RSV-F Nanoparticle	Novavax	RSV-F vaccine with adjuvant	Phase I (ongoing, elderly)

5.1 *New Targets for RSV Prophylaxis*

In the past decade, research has led to the identification of a number of targets, both cellular and viral, that may be useful for the prevention of RSV infection and its accompanying pathology. Differential microarray analysis has been employed to pinpoint gene expression changes in RSV-infected cells and to identify candidate therapeutic genes [53]. Expression plasmids have been constructed for these genes and they have been tested in cultured lung epithelial cells in vitro and in animal models in vivo. In order to deliver these plasmids in the most effective way to target cells, novel carrier systems have been produced based on modified polysaccharide nanoparticles which protect the DNA and facilitate its introduction into the lungs. Characterization of these gene expression changes includes immune modulation, signal transduction, and apoptosis. A summary of lead vaccine and therapy candidates in each of the drug classes ranging from small molecules to DNA-based drugs that are in phase I or II clinical trials is summarized in Table 5.1. However, given the historic failures, it is difficult to speculate if any one of them will translate to anti-RSV product. Some of the recent advances are briefly discussed below.

5.2 *Use of Animal and Three-Dimensional Cellular Models to Develop Anti-RSV Compounds*

Developing antivirals requires a comprehensive molecular understanding of the early events of virus-host interaction necessary for virus fusion and entry into cells and virus replication. To study the virus interaction, researchers have established human monolayer and three-dimensional epithelial cell cultures, human dendritic

cell isolation and culture, and mouse models of RSV infection. RSV affects pulmonary function in BALB/c mice [54] and a number of investigators have utilized this mouse model for the study of asthma and RSV infection [55–59].

As in humans, mouse pulmonary T-cells induce both Th1 and Th2 responses in the lung in response to RSV infection [58–61]. RSV infection induces the expression of intracellular adhesion molecule-1 (ICAM-1) on host cells. The co-localization of RSV and ICAM-1 suggested that ICAM-1 binds to RSV, most likely by interacting with the RSV fusion protein. Treatment of cells with antibodies to ICAM-1 or targeting ICAM-1 in mice significantly inhibited RSV infection and the production of inflammatory mediators, suggesting a potential therapeutic use for anti-ICAM-1 antibodies.

Intranasal administration in mice of a plasmid encoding IFN-γ significantly decreased viral replication and inflammation in mouse lung. From DNA microarray analysis and other molecular and cellular techniques, the 2-5AS-oligoadenylate synthetase (2-5AS) has been identified as playing a major role in the IFN-γ-mediated inhibition of RSV replication. Mice given adenovirus-expressing 2-5AS showed a significant inhibition of RSV replication. From microarray studies aimed at dissecting the early events of RSV infection it was determined that multiple signaling pathways involving STAT1 and STAT3, ERK-1 and ERK-2, and PKC-α are involved in RSV-induced early gene expression and inflammation. PKC-α is a critical target upstream of these signaling pathways, and inhibitors of PKC-α specifically block RSV fusion and cause abortion of infection in normal human bronchial epithelial cells. To elucidate the mechanism of RSV infection, RSV-induced signal transduction pathways involving STAT and PKC were investigated. In an attempt to develop a vaccine, prophylaxis, or treatment based on RSV genes, a multigene DNA vaccine and siRNA-based strategy was explored.

5.3 *Immunoprophylaxis*

Prophylactic IFN-γ gene transfer in BALB/c mice decreased viral replication and induced a Th1-like (increased production of IFN-γ and IL-12), instead of a Th2-like (decreased IL-5), immune response against RSV infection [62–64]. Viral infections induce IFN-γ, which in turn facilitates the resolution of viral infection [63]. IFN-γ levels have been compared in bronchoalveolar lavage (BAL) fluids following infection with RSV in control and pIFN-γ-treated mice. A three- to sixfold increase in IFN-γ production was found in RSV-infected mice compared to uninfected mice; such increases have been considered relatively low compared to other viral infections [62–64]. The finding that a natural, live-virus infection is cleared by elevated IFN-γ production, a response similar to that seen after live-viral infection in mice, suggests that the results from an animal model will be applicable to human RSV disease.

5.4 siRNA-Based Prophylaxis

A new prophylactic approach consists of taking advantage of the RNA interference mechanism initially discovered in plant cells and that also has been found in all species including mammals. RNA interference is triggered by double-stranded RNA that is cleaved by an RNAse-III-like enzyme, Dicer, into 21–25-nucleotide fragments (siRNAs) with characteristic 5′ and 3′ termini [65, 66]. These siRNAs act as guides for a multi-protein complex, including a PAZ/PIWI domain containing the protein Argonaute2 that cleaves the target mRNA [67]. These gene silencing mechanisms are highly specific and induce inhibition of gene expression throughout an organism. RNA interference is a well-characterized phenomenon that has proven effective in silencing a number of genes of different viruses [68–70]. siRNA to viral P and NS1 mRNAs have been shown to be efficient as prophylactics for RSV infection in cellular and animal model studies [21, 71]. The prophylactic intranasal administration of a siRNA formulation specific for RSV-P mRNA was able to significantly reduce the viral load and the disease parameters on RSV-infected mice [71]. A carrier in the formulation was not required. In addition, a very low dose was effective in showing a protective effect. Moreover, siRNA-resistant virus did not appear after using this formulation [71]. While intranasal naked siRNA to humans was found to be safe in a phase I study, other studies have shown toxicity effects.

Since the synthesis of RNA oligonucleotide-based siRNA is expensive, DNA vectors have been engineered to introduce siRNA into RSV-infected human cells and animals. This is based on the principle of the intracellular transcription of small RNA molecules that are synthesized from a DNA template under the control of RNA polymerase III promoters, such as U6. NS1 was selected as the target because NS1 protein interferes with the type-1 IFN-mediated host antiviral response [72]. Silencing of the NS1 gene attenuated RSV replication and boosted the immune response through an increase in IFN-β production [21]. The prophylactic intranasal administration of siNS1 formulated with chitosan nanoparticles significantly reduced the viral load and pulmonary pathology in RSV-infected mice [21]. In addition, mice treated with these prophylactic nanoparticles develop protection from reinfection [21]. The nanoparticle-mediated siRNA silencing of NS1 allowed the normal expression of host-antiviral genes that suppress RSV replication. Nanoparticle-siNS1 represents a novel prophylactic that could be at low cost prepared and distributed globally to save millions of infants and children from RSV disease and death.

Alnylam Pharmaceuticals [http://www.alnylam.com/] has developed and is testing an siRNA (ALN-RSV01) that degrades the viral nucleocapsid protein N mRNA and thus prevents its translation for construction of the RSV nucleocapsid. Without being able to form the nucleocapsid, the virus cannot replicate itself and dies. The safety of ALN-RSV01 has been tested in a phase 1 clinical trial and the formulation proved to be harmless and biocompatible [http://www.alnylam.com/Programs-and-Pipeline/Partner-Programs/index.php].

5.5 *Modified Peptides for Blocking RSV Fusion and Entry*

RSV enters host cells through the fusion protein RSV F, which forms a six-helix fusogenic bundle. Therefore, approaches to RSV prophylaxis target the highly conserved heptad-repeat domains of the RSV fusion protein F, which is an absolute requirement for infection to take place. An approach involving small interfering peptides that prevent bundle formation and limit RSV infection in vitro has been suggested, which is schematically shown in Fig. 5.2. However, these peptides are highly susceptible to degradation in vivo, which has been a major stumbling block for all peptidic drugs.

Recently however in an elegant study, Bird et al. [73] took advantage of the alpha-helical structure of this peptide and colleagues applied hydrocarbon stapling to stabilize the α-helical structure of an RSV F peptide (SAH-RSV). Pretreatment with SAH-RSV prevented infection in both cell culture and murine models of RSV. A series of modified peptides were synthesized that bind to the C-terminal region of the F protein and prevent the conformational change that results in the formation of six-helix bundles.

Intranasal delivery prevented viral infection within the nares, while intratracheal delivery of a nanoparticle preparation of SAH-RSV prevented RSV infection in the lung. Viruses lacking this capability remain extracellular and cannot replicate or

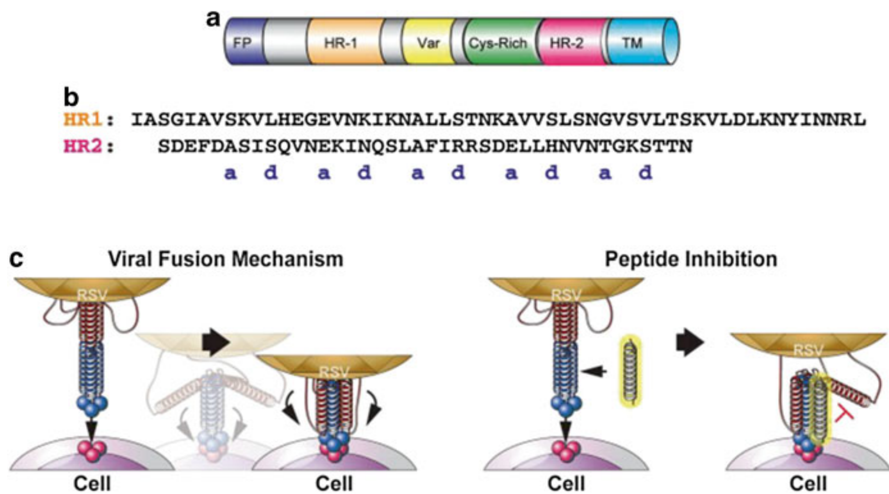

Fig. 5.2 Illustration of the therapeutic strategy for using decoy HR peptides to inhibit the RSV-cell fusion. The fusion protein F of RSV has specific domains known as heptad repeats (HRs) that enable it to merge its envelope with the membrane of a target, usually an epithelial cell, and deposit its contents within the cytosol. A schematic of the HR1 and HR2 domains (**a**) and the amino acid sequence of primary structure of the F1 subunit of RSV-F (**b**) are shown. A strategy deploying decoy peptides to block fusion is also shown (**c**)

infect host epithelial cells. The engineered antiviral peptides are resistant to proteolysis and can be stably delivered to the airways in the form of inhalable biocompatible nanoparticles. Movement of this formulation into phase I clinical trials is essential for continued development of promising approaches to preventing RSV infection in at-risk populations.

6 Conclusions and Future of Anti-RSV Strategies

RSV remains a dangerous pathogen for infants less than 12 months of age and an effective vaccine for that population is unlikely to be developed; thus prophylactic measures are still the mainstay for protecting at-risk infants. The high cost of the currently approved anti-RSV treatment, however, means that many of the world's infants will not have access to it and points to the continuing need for a safe, cost-effective RSV prophylactic. Infants are not the only ones at risk of hospitalization and death from RSV infection. Although not usually recognized as such, RSV is every bit as serious a pathogen as influenza in the elderly and the lack of a vaccine makes RSV pneumonia less preventable than the flu. Given the promising research on methods of blocking RSV attachment to and entry into airway epithelial cells plus the great advances in the use of nanoparticles for drug delivery, it gives cause for hope that a routinely available, safe, and effective RSV preventive is not too far in the future.

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Chapter 6
Surveillance for Hepatitis C

Kathleen N. Ly, Elizabeth M. Hughes, Ruth B. Jiles, R. Monina Klevens, Henry Roberts, and Eyasu Teshale

Abbreviations

CDC	United States Centers for Disease Control and Prevention
CLD	Chronic liver disease
EHR	Electronic health record
ELR	Electronic laboratory reporting
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HIEs	Health information exchanges
HIT	Health information technology
IDU	Injection drug use
MSM	Men who have sex with men
NHANES	National Health and Nutrition Examination Survey
RIBA	Recombinant immunoblot assay
SVR	Sustained virologic response
U.S.	United States

Core Message This chapter provides an overview of hepatitis C surveillance methods.

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1 Importance and Aims of Hepatitis C Surveillance

Hepatitis C virus (HCV) infection is a common type of chronic blood-borne infection [1]. The World Health Organization estimates that 170 million persons are HCV infected globally [2]. In countries that conduct hepatitis C surveillance, hepatitis C may be required to be reported by laboratories and healthcare providers to health departments.

Hepatitis C surveillance includes acute and chronic hepatitis C cases; however, some countries or local areas may not have the necessary confirmatory laboratory tests to distinguish current (or present) from resolved (or past) HCV infection. Additionally, because the chronic hepatitis C burden is large and conducting follow-up investigation can be labor intensive, some countries and local areas might lack the capacity to investigate and confirm cases. Despite the challenges of conducting hepatitis C surveillance, there is hope for hepatitis C eradication because primary prevention is effective, and secondary transmission and complications are preventable through case management, new effective treatments, and alcohol counseling. Surveillance data are essential to the planning, implementation, and evaluation of public health programs and policies [3].

2 Epidemiology

2.1 Characteristics

Hepatitis C traces back to the mid-1970s, though at the time, the virus was broadly termed "non-A, non-B hepatitis" when serologic tests ruled out hepatitis A or B as the cause of acute hepatitis following a blood transfusion [4]. In 1989, hepatitis C was fully distinguished from non-A, non-B hepatitis [4, 5].

HCV infection may be difficult to measure because 70–85 % of HCV-infected persons are asymptomatic. When symptoms are present, they can include jaundice, fever, abdominal pain or discomfort, nausea, vomiting, dark urine, fatigue, joint pain, loss of appetite, and clay-colored stools [6]. For symptomatic HCV-infected individuals, the onset of symptoms usually occurs 6–7 weeks after exposure [7, 8]. For asymptomatic HCV-infected individuals, diagnosis usually occurs incidentally during blood donation screenings and other medical screenings. In the USA, federally supported surveillance captures only a fraction of all acute HCV infections because the identification of acute HCV infection requires the presence of symptoms [9], and the proportion of those with symptoms is relatively small (20–30 %) [6]. However, procedures have been developed that account for asymptomatic HCV infections in estimating the total number of reported acute cases (*estimation procedures are discussed in* Sect. 5.5). Table 6.1 describes the general characteristics of acute and chronic HCV infection.

Table 6.1 Characteristics of acute and chronic hepatitis C virus (HCV) infection

Characteristic	Acute hepatitis C	Chronic hepatitis C
Definition	The first 6 months of a new HCV infection	HCV infection persisting past 6 months
Burden of disease	No global estimate available	170 million persons worldwide
Persons at risk	Persons who have percutaneous exposure to HCV-infected blood, e.g., persons who share needles and persons who seek healthcare services in settings where standard precautions and infection control measures are not strictly implemented	About 80 % of persons who are acutely infected with hepatitis C
Symptoms (if present)	Jaundice, fever, abdominal pain or discomfort, nausea, vomiting, dark urine, fatigue, joint pain, loss of appetite, and clay-colored stools	Symptoms are usually present in advanced stages: cirrhosis, jaundice, liver failure
Rate of spontaneous recovery	20 %	Chronic HCV-infected persons will not recover spontaneously but can achieve a sustained virologic response with treatment
Rate of asymptomatic	70–85 %	Symptoms are usually not present until the advanced stages of liver disease
Laboratory diagnosis	(1) Antibody to hepatitis C-positive followed by HCV RNA-positive result or genotype result and report of recent risk behavior/factor; (2) positive HCV RNA and documented HCV antibody seroconversion within the past 6 months	(1) Two positive HCV RNA or genotype results performed 6 months apart; (2) antibody to hepatitis C-positive followed by HCV RNA-positive result or genotype result and report of risk behavior/factor occurring more than 6 months prior
Mortality	16,000 deaths worldwide in 2010	499,000 deaths worldwide in 2010
Progression to chronic infection	Approximately 80 % of acute HCV infections will progress to chronic HCV infection	Not applicable
Progression to liver cancer	No	Yes, the rate of progression is approximately 4–5 % among chronically HCV-infected persons
Treatment	High rate of sustained virologic response among those treated with ribavirin and/or peginterferon	There are multiple national and regional guidelines for treatment of hepatitis C. The following drugs are currently approved for treatment of hepatitis C: peginterferon and ribavirin, boceprevir, telaprevir, simeprevir, sofosbuvir. Treatment depends on stage of disease and genotype
Primary prevention	Needle exchange programs, standard precaution measures and infection control in healthcare settings, increase awareness of disease	Prevention of acute hepatitis C
Secondary prevention	Screening for HCV infection	Hepatitis A and B vaccination
Tertiary prevention	Some studies have shown that treatment of acute hepatitis C could prevent the progression to chronic disease and provide a cure	Case management, routine medical care, alcohol counseling, treatment

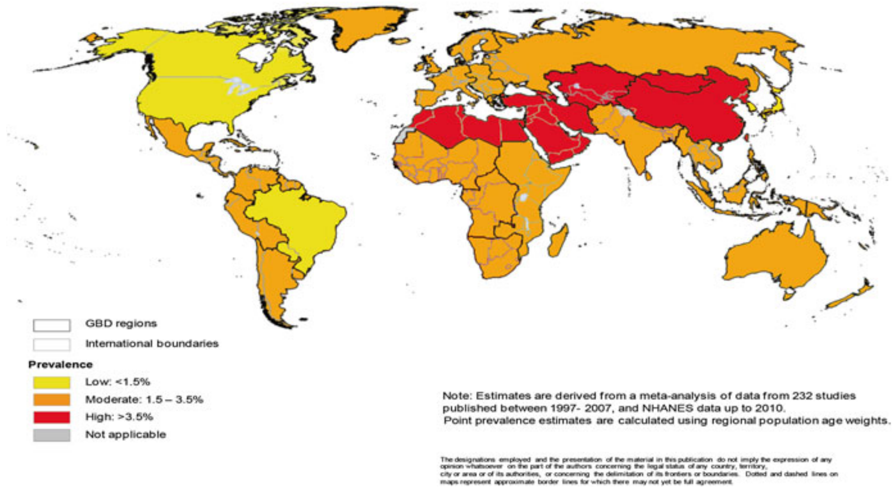

Fig. 6.1 Seroprevalence of hepatitis C antibody by global burden of disease region, 2005. Adapted from Mohd HK, Groeger J, Flaxman AD, Wiersma ST. Global epidemiology of hepatitis C virus infection: new estimates of age-specific antibody to HCV seroprevalence. *Hepatology*. 2013; 57(4):1333–42. Permission to use this figure was obtained from *Hepatology*

2.2 *Geographic Distribution*

Although the overall estimated global prevalence of past/present HCV infection is high (3 %; 170 million persons) [2], there is wide variability in prevalence between geographic regions [10]. Typically, developing countries, such as parts of Africa and Asia, have the highest reported prevalence of >3.5 % (Fig. 6.1). In Egypt, for example, 15 % of persons aged 15–59 years had evidence of past/present HCV infection [11]. In comparison, more developed countries, such as those in North America, northern and western Europe, and Australia, have a low to moderate reported prevalence (<3.5 %) (Fig. 6.1). In the USA, for example, the estimated prevalence of HCV infection is approximately 1.0 %, or 2.7 million persons [12]—disproportionately affecting persons who are middle aged [1, 12, 13]. Hepatitis C has at least six distinct genotypes widely distributed across the globe. In the USA, Europe, and Japan, genotypes 1a and 1b are most predominant although genotypes 2a and 2b are also common [14]. Genotype 2c is prevalent in northern Italy while genotype 3a is prevalent among intravenous drug users in Europe and the USA [14]. In North Africa and the Middle East, genotype 4 is predominant while genotypes 5 and 6 are confined to South Africa and Hong Kong [14].

2.3 Mode of Transmission

HCV is primarily transmitted through percutaneous exposure to infected blood [15]. One of the most common ways by which HCV is transmitted is injection drug use (IDU) [1, 2]. In the USA, among persons with acute hepatitis C who responded to questions about IDU, approximately 60 % reported injection of street drugs [16]. From 2007 to 2011, US surveillance data detected a 44 % increase in the hepatitis C incidence [16], which may be due to a rise in injection drug users among young persons [17–19].

In healthcare settings where standard precautions and infection control measures are less strictly implemented, needle stick injuries and unsafe medical practices are common causes of HCV transmission. In developed countries like the USA, the risk of HCV transmission in healthcare settings has dramatically declined due to the implementation of safe injection and universal infection control practices [20]. Despite the decline, from 2008 to 2012, 16 healthcare-associated hepatitis C outbreaks that resulted in 160 cases of HCV infection were reported to the United States Centers for Disease Control and Prevention (CDC) [21]. Healthcare-associated hepatitis C outbreaks are indicators of failure to implement and strictly adhere to standard precautions and infection control measures. Since the development of the hepatitis C antibody screening test in 1990 and screening of the blood supply for hepatitis C in 1992, HCV transmission from blood transfusions has been greatly reduced in developed countries [22]. In developing countries, this mode of transmission remains significant [22, 23].

HCV also can be transmitted from HIV-coinfected mothers to their infants, and HIV-infected men who have sex with men (MSM) have an increased likelihood of acquiring HCV infection [24]. The risk of HCV transmission among HIV-infected MSM is also increased in the presence of genital ulcerative disease and sexual practices that lead to mucosal trauma [25]. Heterosexual contact among monogamous partners is an unlikely route of transmission [24].

Other demographic groups also are disproportionately affected by hepatitis C, evident by a higher prevalence among those groups. For example, in the USA, the overall hepatitis C antibody prevalence is estimated to be 1.6 %; however, it is 3 % among persons born during 1945–1965 [13]; 5 % among military veterans [26, 27]; 6 % among blacks aged 30–49 years [28]; 14 % among HIV-infected persons [1]; and 23–39 % among the incarcerated [26]. Additionally, some hepatitis C epidemics are fueled by contaminated injection equipment used in mass treatment campaigns, such as schistosomiasis treatment in Egypt during 1960–1980; HCV transmission is still ongoing today [29].

2.4 Complications of Chronic Infection

The burden of hepatitis C, mostly among persons who are undiagnosed and not in care, is evidenced by increasing complications. One such complication is the development and progression of chronic liver disease (CLD). In addition, alcohol use is

independently associated with liver disease progression [13]. Based on representative samples of published reports from at least 1990 and 11 World Health Organization regions, hepatitis C was identified as one of the most common etiologies of CLD throughout most of the world [30] and was associated with 27 % of liver cirrhosis and 25 % of hepatocellular carcinoma (HCC) [30]. From the same study, death due to HCV infection was identified in approximately 211,000 persons with liver cirrhosis and 155,000 persons with liver cancer [30]. Among US adult residents in two sentinel surveillance sites and one healthcare network site, 64 % of newly diagnosed CLD had underlying hepatitis C during 1999–2001 [31]. In the USA, over 15,000 hepatitis C-related deaths occurred in 2007, of which 57 % had CLD, including HCC [32].

Although hepatitis C is widely known to increase the risk of dying from liver-related diseases, recent studies have found that HCV infection also increased the risk of dying from non-liver-related diseases [33–35]. In one study, patients with chronic HCV infection had a non-liver-related mortality risk nearly two times higher than uninfected patients [33]. Similarly, in another study, persons who were HCV antibody positive had significantly higher mortality than persons who were HCV antibody negative. Additionally, persons with detectable HCV RNA levels had significantly higher mortality than persons with undetectable RNA levels [34].

2.5 Laboratory Testing

The traditional approach for detecting HCV infection is to screen persons for a history of risk factors and to test those with any identifiable risk factor [36]. While IDU is the most common mode of transmission in developed countries, additional risk factors, including exposure to unsafe blood products and injection practices, are highly prevalent and contribute to significant HCV transmission in developing countries [37]. There are many international recommendations for hepatitis C testing and all have consistency in their recommendation for testing of persons who inject illicit drugs, prior recipients of transfusions or organ transplants, persons with persistently elevated liver enzymes, children born to HCV-infected mothers, and persons exposed to HCV-positive blood in healthcare [38].

Table 6.2 describes the interpretation of hepatitis C test results and corresponding further actions. The initial test is for HCV antibodies, which are detectable approximately 4–10 weeks after exposure [39]. In symptomatic cases, this time period usually occurs at or before the onset of clinical symptoms. The HCV antibody test is positive in acute, chronic, and resolved infections (Table 6.2). Consequently, HCV antibody tests do not have the capacity to distinguish current infection from past, resolved infection [40]. In addition to standard serologic assays, there are also rapid tests to detect HCV antibodies [41]. The availability of standard and rapid assays varies significantly and is dependent on availability of resources [40]. Over the past decade, new generations of standard tests with high sensitivity and specificity have been developed [40, 42]. However, the proportion of

Table 6.2 Interpretation of results of tests for hepatitis C virus (HCV) infection and further actions

Test outcome	Interpretation	Further actions
HCV antibody nonreactive	No HCV antibody detected	Sample can be reported as nonreactive for HCV antibody. No further action required. If recent exposure in person tested is suspected, test for HCV RNA[a]
HCV antibody reactive	Presumptive HCV infection	A repeatedly reactive result is consistent with current HCV infection, or past HCV infection that has resolved, or biologic false positivity for HCV antibody. Test for HCV RNA to identify current infection
HCV antibody reactive, HCV RNA detected	Current HCV infection	Provide person tested with appropriate counseling and link person tested to care and treatment[b]
HCV antibody reactive, HCV RNA not detected	No current HCV infection	No further action required in most cases. If distinction between true positivity and biologic false positivity for HCV antibody is desired, and if sample is repeatedly reactive in the initial test, test with another HCV antibody assay. In certain situations,[c] follow up with HCV RNA testing and appropriate counseling

Adapted from CDC. Testing for HCV infection: An update of guidance for clinicians and laboratorians. MMWR 2013;62(18)

[a]If HCV RNA testing is not feasible and person tested is not immunocompromised, do follow-up testing for HCV antibody to demonstrate seroconversion. If the person tested is immunocompromised, consider testing for HCV RNA

[b]It is recommended before initiating antiviral therapy to retest for HCV RNA in a subsequent blood sample to confirm HCV RNA positivity

[c]If the person tested is suspected of having HCV exposure within the past 6 months, or has clinical evidence of HCV disease, or if there is concern regarding the handling or storage of the test specimen

false-positive HCV antibody results is inversely related to the HCV prevalence in that setting [40, 43]. False-negative HCV antibody results also occur, particularly in individuals with severe immunodeficiency [39, 44], but rarely among the general population.

In contrast to HCV antibodies, HCV RNA can detect current infection and is detectable in serum as early as 1–2 weeks after exposure [41]. There are a number of qualitative and quantitative HCV RNA assays [43]. However, these tests are expensive and not widely available. Further, because these tests detect HCV RNA, they are important for differentiating current infection from past, resolved infection [45]. Therefore, both in the clinical practice and in epidemiologic studies, it is important to follow up on every HCV antibody-positive result with an RNA assay [43]. In conditions where the HCV antibody test is likely a false negative, RNA testing may provide the correct diagnosis [43]. For these reasons, quantitative HCV RNA assays and genotype studies are important in the clinical management of chronic HCV infection.

Other tests available for hepatitis C detection and diagnosis include the HCV core antigen test [46] and core antigen/antibody tests [47]. These tests are available only in some countries. While the HCV core antigen test is sensitive, will yield results early in the course of infection, and detects active infection [46, 48], the HCV core antigen/antibody test is positive in the presence of either one or both antigen and antibody [47] making it difficult to differentiate between active and resolved infection. Blood donations are screened by testing for HCV RNA and core antigen [49].

Dried blood spot assays are well developed and validated for HIV detection [50]. HCV antibody and RNA testing on a dried blood spot sample are used in research activities whereby a blood draw is not feasible [51, 52]. However, these tests are not well validated and require highly qualified personnel. Therefore, its use is restricted to research use only.

In many sub-Saharan African countries, a high rate of false-positive HCV antibody test results have been reported, even when the latest generation of serologic assays are used [53]. In the majority of HCV antibody-positive samples, the recombinant immunoblot assay (RIBA) yields either a negative or indeterminate result [53]. RIBA is a more specific blood test for detecting HCV antibodies and is sometimes used as a confirmatory test to less specific antibody tests [43]. In the USA, RIBA was phased out in 2013 [41]. Even among RIBA-confirmed HCV antibody-positive samples, the large majority are HCV RNA negative. Such findings have been a challenge for clinical diagnosis, epidemiological studies, and screening of blood products for transfusion. While the cause of the high false positivity is still unknown [54], it raises questions about the best strategy to test for HCV infection in some countries.

2.6 Treatment

The goal of treatment for chronic HCV infection is to achieve sustained virologic response (SVR), or cure, currently defined as having an undetectable viral load 24 weeks after the end of treatment [55]. Achieving SVR, in turn, is associated with long-term clearance of the virus and reduced long-term health complications such as cirrhosis, HCC, liver failure, and all-cause mortality [56, 57]. Novel therapies with direct-acting antivirals have demonstrated high virus eradication rates. Persons diagnosed as HCV positive should be medically evaluated and entered into routine care, as appropriate. The evaluation should include confirmation of chronic infection by viral testing including genotype and viral load, an assessment of liver function, stage of liver fibrosis, evidence of liver cancer, and eligibility for treatment [58].

The traditional treatment is pegylated interferon with ribavirin [7]. The duration of treatment is determined by the virologic response, which in turn is associated with the person's genotype. With pegylated interferon and ribavirin therapy, a 24-week treatment course is recommended for genotypes 2 and 3 and a 48-week

treatment course for other genotypes [7]. Persons with genotypes 2 and 3 who followed this regimen have ≥80 % SVR rate compared with a 40–50 % SVR rate for persons with genotype 1 [7].

Pegylated interferon and ribavirin is associated with many side effects, requires frequent injections, and has shown low success rates with hepatitis C genotype 1 [59]. In 2011, the United States (US) Food and Drug Administration approved the use of two new protease inhibitors, boceprevir and telaprevir, for the treatment of hepatitis C genotype 1 [7, 59]—the most common genotype reported in the USA. When used in combination with pegylated interferon and ribavirin, boceprevir and telaprevir demonstrated SVR rates of 63–66 % and 69–75 %, respectively [7], a marked increase from traditional standard of care therapy alone.

In November and December 2013, the US Food and Drug Administration approved the use of two new drugs for the treatment of chronic HCV infection, simeprevir and sofosbuvir. These drugs are approved for use in HCV-infected patients with genotypes 1 and 4, which showed >90 % SVR rates in clinical trials [60]. Although treatment is very costly, these are among the new drugs that offer promising hope towards the global eradication of hepatitis C. As new therapies continue to be developed, evidence-based hepatitis C management recommendations are continuously updated to address issues ranging from testing and linkage to care to the optimal treatment regimen in specific patient situations [36].

3 Assessment of Priorities

The success of a surveillance system for hepatitis C is dependent on the dedicated resources and established priorities for the surveillance system. For example, if the priority is to measure the overall burden of disease, the design of the system might be to conduct a seroprevalence survey. However, if the objective is to obtain data for case management and evaluation of local area prevention programs, then individual cases should be monitored and records updated over time [61]. Furthermore, the population for which information is needed is an important determinant of surveillance methods. For example, in an enhanced surveillance pilot study in select neighborhoods in England, there were concerns about increases in HCV infection among MSM. Health officials quickly recruited and collected information from certain drug treatment facilities and implemented a surveillance project that provided results that were applied and published in less than a year [62]. In another study, researchers sought to determine which hepatitis C genotypes were circulating among injection drug users in Hungary [63]. They approached needle exchange programs and drug treatment facilities in all health districts and found that HCV strains among injection drug users were very different compared to HCV-infected persons who did not acquire their infections from injecting drugs [63]. The objectives and expected use of the surveillance data also should be tempered with the resources available to conduct the activities.

4 United States Surveillance Methods

4.1 Passive Surveillance

In a population-based passive surveillance system, sources of hepatitis C reports (e.g., hospitals, clinics, laboratories) routinely report cases to health departments [64]. However, in this type of surveillance system, notification may not be timely enough to alert health officials of a potential outbreak. In addition, the data reported are often incomplete because few, if any, incentives are given to the laboratories and healthcare providers to report information [64].

In the USA, as of 2013, acute hepatitis C is reportable in all states and the District of Columbia, and past/present hepatitis C is reportable in 43 states and the District of Columbia. Due to resource constraints, it is oftentimes difficult for health departments to obtain the necessary confirmatory laboratory tests from laboratories or healthcare providers to distinguish current from resolved or past HCV infection. Therefore, these cases are labeled as "past/present hepatitis C." Under the current national surveillance system, acute and past/present hepatitis C are passively and voluntarily reported on a weekly basis by health departments to a national surveillance network at CDC. The system relies on laboratories and healthcare providers to submit case reports to health departments, as mandated by states. Health departments process case reports to determine that they represent new, unique cases and store data with personal identifiers.

Most laboratory reports and some physician reports are submitted electronically to health departments. However, reporting can be accomplished by fax or telephone, even using toll-free numbers or automated recording devices available at all hours. Time and lack of resources greatly limit such a system to a small percentage of most reportable diseases, but as long as the reporting system and requirements remain unchanged, the changes in incidence may reflect meaningful patterns of disease.

The advantage of a case reporting system is that there is an organized system of reporting and tabulating cases at both the local and national level. Also, at the local level, individuals are identified for intervention. However, case-reporting systems also have a number of disadvantages, including the following: (1) not all cases are reported despite legal requirements, primarily because of the lack of both symptoms and resources; (2) the variability in reporting from one jurisdiction to another; and (3) the lack of hepatitis C laboratory tests that distinguish between acute and chronic HCV infection.

Due to the large volume of past/present hepatitis C case reports and the resource-intensive process of identifying and classifying a case, chronic hepatitis C is grossly underreported in the USA. Current estimates indicate 2.2–3.2 million persons chronically infected with HCV [12].

4.2 Active Surveillance

Active surveillance requires health departments to contact sources of hepatitis C reports at regular intervals and request specific information for case reports [64]. Reporting frequency is monitored and data on epidemiologic features, such as complications of infections, which would not otherwise be collected through passive surveillance, are obtained. In addition, the data are reported in a more timely fashion than in a passive system. However, unlike passive surveillance, active surveillance is expensive and resource intensive.

4.3 Enhanced Surveillance

CDC provides additional funding to support enhanced surveillance programs. These programs conduct follow-up investigation on cases to obtain additional information, including information about risk behaviors and/or exposures. As a result, data are more complete than passive surveillance. These additional data allow the surveillance infrastructure to answer discrete surveillance and research questions. From 1982 through 2006, the Sentinel Counties Study of Acute Viral Hepatitis enrolled all acute viral hepatitis patients in six county/city health departments in the USA [65]. From this project, funded sites collected data about cases of acute viral hepatitis from hospitals, healthcare providers, and other agencies and patient care sources. These data were used to describe the incidence of acute viral hepatitis [66], characterize individual cases, and identify and describe risk behaviors/exposures. From 2005 through 2011, CDC funded seven sites to conduct enhanced viral hepatitis surveillance throughout major US cities and states. Because of additional resources, completeness of reporting significantly improved in the enhanced surveillance sites [67].

4.4 Analysis of Specimens/Supplementary Data Sources

In the USA, there are existing data from other sources to augment hepatitis C surveillance data. For example, cancer registries have information on HCC [68]. Vital statistics generally include information on the number of deaths for which hepatitis C was listed among causes of death and calculating trends in HCV infection as a cause of death relative to other causes is useful [32]. Healthcare administrative data are available electronically and may be a useful source of data as well. Events available from administrative data include diagnosis, procedure codes, and cost information to examine the economic impact of hepatitis C [69].

Currently, CDC uses data from a variety of sources to further understand the burden of chronic HCV infection; characterize persons who receive treatment;

describe treatments, results of treatment, and sequelae of disease; and characterize those who die with and as a consequence of hepatitis C:

(a) *Vital Statistics*: The oldest form of surveillance in the USA is mortality registration. Registration of death, using a death certificate, is legally required in the USA. As a result, virtually all deaths are included in the registries. Cause of death listed on the death certificate is dependent on the presence/absence of a physician or family member who is knowledgeable about the health of the deceased, severity of disease, complexity of the disease, associated illnesses, and whether or not an autopsy or diagnostic laboratory testing was performed.

    Death certificates are completed by funeral directors based on information from attending physicians, medical examiners, coroners, and family members. Death certificates are filed in vital statistics offices within each state and the District of Columbia. States share information from death certificates with CDC through the National Vital Statistics System, which then produces public-use mortality files containing death information with cause of death coded in accordance with the International Classification of Disease, Tenth Revision [70]. These data are used to determine the national burden of mortality associated with specific diseases, including viral hepatitis. A recent analyses of these mortality data from 1999 through 2007 indicated that the hepatitis C mortality rate exceeded the HIV mortality rate in 2007 [32].

(b) *Surveys*: Health surveys are used for a variety of reasons, including augmenting our understanding of viral hepatitis from surveillance. Currently, the CDC uses several national surveys, which may include seroprevalence data, to describe and understand hepatitis C-related prevalence, hospitalizations, treatments, and development of CLD. For example, the National Health and Nutrition Examination Survey (NHANES) has provided valuable seroprevalence data on hepatitis A [71], hepatitis B [72], and hepatitis C [12] that are representative of the US noninstitutionalized civilian population [73]. The National Ambulatory Medical Care Survey and the National Hospital Ambulatory Medical Care Survey use a national sample of visits from nonfederal employed office-based physicians who provide direct patient care and from emergency departments and outpatient department of noninstitutional general and short-stay hospitals, respectively, to obtain information about the use and provision of ambulatory medical care services, including viral hepatitis-related visits [74], in these settings. The National Hospital Discharge Survey was a nationally representative survey conducted from 1965-2010 that provided information from patients of non-federal, short-stay hospitals in the USA about the characteristics of these patients, conditions for which they were treated, cost of treatment, and a number of other public health topics of interest. The National Hospital Care Survey is a new survey which links the inpatient data that was collected by the National Hospital Discharge Survey with the emergency department, outpatient department, and ambulatory surgery center data collected by the National Hospital Ambulatory Medical Care Survey. Together, these population-based surveys provide a wealth of readily available data that are already in electronic format. As a result, they can be relatively inexpensive sources of useful information in addition to that obtained from surveillance.

5 Sequence in Case-Based Surveillance Processes

5.1 Sequential Flow of Case Data for Hepatitis C Surveillance to CDC

CDC uses message mapping guides to standardize the data transmitted for all notifiable diseases monitored by health departments transmitted to the national surveillance network. States report basic demographic, clinical, and risk information on cases of notifiable conditions to the national surveillance network. The CDC Division of Viral Hepatitis retrieves data from servers once a week. These data are used to detect outbreaks, flag cases requiring immediate public health follow-up, and perform data quality checks. Viral hepatitis data are summarized in annual surveillance reports and are made available to the public on the CDC Division of Viral Hepatitis website [75].

5.2 Case Definitions in the USA, Europe, and Australia

Cases of notifiable conditions must meet standard case definitions. In the USA, these case definitions are developed and updated in collaboration with the Council of State and Territorial Epidemiologists and CDC. The 2012 hepatitis C surveillance case definitions require a combination of symptoms and laboratory findings for acute disease and laboratory findings only for past/present hepatitis C [9]. Because the clinical characteristics are the same for acute hepatitis A, B, and C, laboratory testing is needed to identify the specific viral cause of illness. For both acute and past/present hepatitis C cases, laboratory findings include a positive antibody to hepatitis C virus screening test, nucleic acid test for HCV RNA, and genotype testing. A special definition is applied to identify new seroconversions that require only one positive test and a previous negative test within the past 6 months [9].

In 2012, the European Union decided not to require clinical signs/symptoms for a confirmed case. Instead, at least one laboratory finding (RNA, core antigen, or antibody) in a person aged >18 months represents a confirmed case. The definition classifies an acute hepatitis C case as one that has a seroconversion within 12 months or has detected either RNA or core antigen but is antibody negative. A chronic case is defined as two samples positive for RNA or core antigen detected at least 12 months apart [76]. In Australia, case reports supplement other sources of information on HCV infection. A confirmed case in Australia requires laboratory evidence of either an antibody or nucleic acid test (either genotype or RNA) in a person aged at least 24 months, and who does not meet the criteria for a newly acquired case; that is, there is no evidence that the infection was acquired in the 24 months before diagnosis [77].

5.3 Follow-Up Investigation and Case Management

A hepatitis C case report is usually initiated with a positive antibody test, which can indicate either acute or chronic infection. After checking the surveillance database to determine whether the potential case was previously reported and had other epidemiologic or laboratory information, health departments can either attempt follow-up investigation or wait for future laboratory information to be received. Follow-up might require contacting the case patient's provider to determine whether symptoms of hepatitis were present. Together with information that the case was not previously reported suggests a newly reported acute hepatitis C case. Follow-up with the case patient and/or the healthcare provider is required to obtain additional epidemiologic data. Cases might be divided into groups of interest, such as persons aged <30 years that may indicate IDU [78] or adults aged >65 years that may indicate transmission in healthcare and extended care facilities. Prioritizing groups of interest reduces the number of cases to be investigated, which makes the task more achievable. Basic demographic, clinical, and risk information are collected using a standard case report form (Fig. 6.2). This information is needed to confirm the classification, determine the most likely source of infection, and limit further transmission [79].

5.4 Uses of Surveillance Data in the USA

The uses of surveillance data vary depending on the public health agency's need for the data. In general, at the national level, surveillance data are used to understand the burden of disease, inform local partners of disease clusters or outbreaks within and across jurisdictions, identify high-risk populations, and inform, prioritize, and evaluate prevention activities. At the local level, surveillance data are used to identify the most likely mode of transmission in the community to limit further transmission, detect and control local outbreaks, improve outreach services, and provide appropriate case management including screening and linking infected persons into care and counseling. Additionally, hepatitis C surveillance data can be matched with other disease registries, such as HIV, in order to integrate medical services for each individual and further understand disease burden. Surveillance data can also be used to evaluate the quality of care, including implementation of hepatitis A and B vaccine recommendations, among HCV-infected patients. Hepatitis A and B vaccine history can be obtained through follow-up investigation of cases and can be used to improve vaccine coverage rates.

The following questions should be asked for every case of viral hepatitis

Form Approved OMB No. 0920-0728 Exp. Date 01/31/2014

Prefix: (Mr. Mrs. Miss Ms. etc) ____ Last: ____ First: ____ Middle: ____
Preferred Name (nickname): ____ Maiden: ____
Address: Street: ____
City: ____ Phone: (_ _ _) _ _ _ - _ _ _ _ Zip Code: _ _ _ _ _ - _ _ _ _
SSN # (optional) _ _ _ - _ _ _ - _ _ _ _

— Only data from lower portion of form will be transmitted to CDC —

State: ____ County: ____ Date of Public Health Report MM / DD / YYYY

Case ID: ____
Legacy Case ID: ____

DEMOGRAPHIC INFORMATION

RACE: (check all that apply)
❑ Amer Indian or Alaska Native ❑ Black or African American ❑ White
❑ Asian ❑ Native Hawaiian or Pacific Islander ❑ Other Race, specify ____

ETHNICITY:
Hispanic ❑
Non-hispanic ❑
Other/Unknown ❑

SEX: Male ❑ Female ❑ Unk ❑ **PLACE OF BIRTH:** ❑ USA ❑ Other: ____
DATE OF BIRTH: MM / DD / YYYY **AGE:** _ _ _ (years) (00 = <1yr , 999 = Unk)

CLINICAL & DIAGNOSTIC DATA

REASON FOR TESTING: (check all that apply)
❑ Year of birth (1945-1965) ❑ Symptoms of acute hepatitis ❑ Prenatal screening
❑ Screening of asymptomatic patient with reported risk factors ❑ Blood/organ donor screening ❑ Unknown
❑ Screening of asymptomatic patient with no risk factors (e.g., patient requested) ❑ Evaluation of elevated liver enzymes
❑ Follow-up testing for previous marker of viral hepatitis ❑ Other: specify: ____

CLINICAL DATA:

Diagnosis date: MM / DD / YYYY	Yes	No	Unk
Is patient symptomatic?	❑	❑	❑
if yes, onset date: MM / DD / YYYY			
At diagnosis, was the patient			
• Jaundiced?	❑	❑	❑
• Hospitalized for hepatitis?	❑	❑	❑
Was the patient pregnant?	❑	❑	❑
Due date: MM / DD / YYYY			
Did the patient die from hepatitis?	❑	❑	❑
• Date of death: MM / DD / YYYY			
Was the patient aware they had viral hepatitis prior to lab testing?	❑	❑	❑
Does the patient have a provider of care for hepatitis?	❑	❑	❑
Does the patient have diabetes?	❑	❑	❑
Diabetes diagnosis date: MM / DD / YYYY			

DIAGNOSTIC TESTS: (CHECK ALL THAT APPLY)

	Pos	Neg	Unk
• Total antibody to hepatitis A virus [total anti-HAV]	❑	❑	❑
• IgM antibody to hepatitis A virus [IgM anti-HAV]	❑	❑	❑
• Hepatitis B surface antigen [HBsAg]	❑	❑	❑
• Total antibody to hepatitis B core antigen [total anti-HBc]	❑	❑	❑
• Hepatitis B "e" antigen [HBeAg]	❑	❑	❑
• IgM antibody to hepatitis B core antigen [IgM anti-HBc]	❑	❑	❑
• Nucleic Acid Testing for hepatitis B [Hep B NAT]	❑	❑	❑
• Antibody to hepatitis C virus [anti-HCV]	❑	❑	❑
– anti-HCV signal to cut-off ratio ____			
• Supplemental anti-HCV assay [e.g., RIBA]	❑	❑	❑
• Antibody to hepatitis D virus [anti-HDV]	❑	❑	❑
• Antibody to hepatitis E virus [IgM anti-HEV]	❑	❑	❑

LIVER ENZYME LEVELS AT TIME OF DIAGNOSIS
• ALT [SGPT] Result ____ Upper limit normal ____
• Date of ALT result MM / DD / YYYY
• AST [SGOT] Result ____ Upper limit normal ____
• Date of AST result MM / DD / YYYY

	Yes	No	Unk
If this case has a diagnosis of hepatitis A that has not been serologically confirmed, is there an epidemiologic link between this patient and a laboratory-confirmed hepatitis A case?	❑	❑	❑

DIAGNOSIS: (check all that apply)
❑ Acute hepatitis A ❑ Acute hepatitis C ❑ Chronic HBV infection ❑ Perinatal HBV infection
❑ Acute hepatitis B ❑ Acute hepatitis E ❑ HCV infection (Past or Present)

Fig. 6.2 Viral hepatitis case report form

Patient History — Acute Hepatitis A

Case ID: ____

During the **2-6 weeks** prior to onset of symptoms-	**Yes**	**No**	**Unk**
Was the patient a contact of a person with confirmed or suspected hepatitis A virus infection?	❑	❑	❑
If yes, was the contact (check one)			
• household member (non-sexual)	❑	❑	❑
• sex partner	❑	❑	❑
• child cared for by this patient	❑	❑	❑
• babysitter of this patient	❑	❑	❑
• playmate	❑	❑	❑
• other ______________________			
Was the patient			
• a child or employee in a day care center, nursery, or preschool?	❑	❑	❑
• a household contact of a child or employee in a day care center, nursery or preschool?	❑	❑	❑
If yes for either of these, was there an identified hepatitis A case in the child care facility?	❑	❑	❑

What is the sexual preference of the patient?

❑ Heterosexual ❑ Homosexual ❑ Bisexual ❑ Unknown

Please ask both of the following questions regardless of the patient's gender.

In the **2–6 weeks** before symptom onset how many	**0**	**1**	**2–5**	**>5**	**Unk**
male sex partners did the patient have?	❑	❑	❑	❑	❑
female sex partners did the patient have?	❑	❑	❑	❑	❑

In the **2–6 weeks** before symptom onset	**Yes**	**No**	**Unk**
Did the patient inject drugs not prescribed by a doctor?	❑	❑	❑
Did the patient use street drugs but not inject?	❑	❑	❑
Did the patient **travel or live** outside of the U.S.A. or Canada?	❑	❑	❑

If yes, where? 1) ______________________ 2) ______________________

(Country) 3) ______________________

What was the principle reason for travel? ❑ Business ❑ New Immigrant ❑ Other

❑ Tourism ❑ Visiting relatives/friends ❑ Adoption ❑ Unknown

	Yes	**No**	**Unk**
In the **3 months** prior to symptom onset did anyone in the patient's household travel outside of the U.S.A. or Canada?	❑	❑	❑

If yes, where? 1) ______________________ 2) ______________________

(Country) 3) ______________________

	Yes	**No**	**Unk**
Is the patient suspected as being part of a common-source outbreak?	❑	❑	❑
If yes, was the outbreak			
Foodborne — associated with an infected food handler	❑	❑	❑
Foodborne — **NOT** associated with an infected food handler	❑	❑	❑
Specify food item ______________________			
Waterborne	❑	❑	❑
Source not identified	❑	❑	❑
Was the patient employed as a food handler during the **TWO WEEKS** prior to onset of symptoms or while ill?	❑	❑	❑

VACCINATION HISTORY

	Yes	**No**	**Unk**	
• Has the patient ever received the hepatitis A vaccine?	❑	❑	❑	
	1	**>2**		
If yes, how many doses?	❑	❑		• In what year was the last dose received? _ _ _ _ (year)
	Yes	**No**	**Unk**	
• Has the patient ever received immune globulin?	❑	❑	❑	• If yes, when was the last dose received? _ _ / _ _ _ _ (mo/year)

Fig. 6.2 (continued)

Patient History — Acute Hepatitis B

Case ID: ______________________

	Yes	No	Unk
During the **6 weeks – 6 months** prior to onset of symptoms was the patient a contact of a person with confirmed or suspected acute or chronic hepatitis B virus infection?	❑	❑	❑
If yes, type of contact			
Sexual	❑	❑	❑
Household (non-sexual)	❑	❑	❑
Other: ______________			

During the **6 weeks – 6 months** prior to onset of symptoms **Did the patient:**	Yes	No	Unk
• undergo hemodialysis?	❑	❑	❑
• have an accidental stick or puncture with a needle or other object contaminated with blood?	❑	❑	❑
• receive blood or blood products [transfusion]	❑	❑	❑
If yes, when? MM/DD/YYYY			
• receive any IV infusions and/or injections in the outpatient setting	❑	❑	❑
• have other exposure to someone else's blood	❑	❑	❑
specify: ______________			
During the **6 weeks – 6 months** prior to onset of symptoms			
• Was the patient employed in a medical or dental field involving direct contact with human blood?	❑	❑	❑
If yes, frequency of direct blood contact? ❑ Frequent (several times weekly) ❑ Infrequent			
• Was the patient employed as a public safety worker (fire fighter, law enforcement or correctional officer) having direct contact with human blood?	❑	❑	❑
If yes, frequency of direct blood contact? ❑ Frequent (several times weekly) ❑ Infrequent			
• Did the patient receive a tattoo?	❑	❑	❑
Where was the tattooing performed? (select all that apply) ❑ commercial parlor/shop ❑ correctional facility ❑ other ______			

	Yes	No	Unk
Did the patient ever receive hepatitis B vaccine?	❑	❑	❑
	1	2	3+
If yes, how many shots?	❑	❑	❑
• In what year was the last shot received? ____			
	Yes	No	Unk
Was the patient tested for antibody to HBsAg (anti-HBs) within 1-2 months after the last dose	❑	❑	❑
• If yes, was the serum anti-HBs ≥ 10mIU/ml?	❑	❑	❑
(answer 'yes' if the laboratory result was reported as 'positive' or 'reactive')			

What is the sexual preference of the patient?
❑ Heterosexual ❑ Homosexual
❑ Bisexual ❑ Unknown

Ask both of the following questions regardless of the patient's gender.

In the **6 months** before symptom onset, how many	0	1	2–5	>5	Unk
• male sex partners did the patient have?	❑	❑	❑	❑	❑
• female sex partners did the patient have?	❑	❑	❑	❑	❑

	Yes	No	Unk
Was the patient **EVER** treated for a sexually-transmitted disease?	❑	❑	❑
• If yes, in what year was the most recent treatment? YYYY			

During the **6 weeks – 6 months** prior to onset of symptoms	Yes	No	Unk
• inject drugs not prescribed by a doctor?	❑	❑	❑
• use street drugs but not inject?	❑	❑	❑
• Did the patient have any part of their body pierced (other than ear)?	❑	❑	❑
Where was the piercing performed? (select all that apply) ❑ commercial parlor/shop ❑ correctional facility ❑ other ______			
• Did the patient have dental work or oral surgery?	❑	❑	❑
• Did the patient have surgery ? (other than oral surgery)	❑	❑	❑
Was the patient: (check all that apply)			
• hospitalized?	❑	❑	❑
• a resident of a long term care facility?	❑	❑	❑
• incarcerated for longer than 24 hours	❑	❑	❑
if yes, what type of facility (check all that apply)			
prison	❑	❑	❑
jail	❑	❑	❑
juvenile facility	❑	❑	❑

	Yes	No	Unk
During his/her lifetime, was the patient **EVER** incarcerated for longer than 6 months?	❑	❑	❑
• If yes, what year was the most recent incarceration? YYYY			
for how long? MMM (mos)			
Did patient have a negative HBsAg test within 6 months prior to positive test?	❑	❑	❑
Verified test date: MM/DD/YYYY			
Was the patient tested for hepatitis D?	❑	❑	❑
Did patient have a co-infection with hepatitis D?	❑	❑	❑

Fig. 6.2 (continued)

Perinatal Hepatitis B Virus Infection

Case ID: ____________________

RACE OF MOTHER:

❑ Amer Ind or Alaska Native ❑ Black or African American ❑ White ❑ Unknown

❑ Asian ❑ Native Hawaiian or Pacific Islander

❑ Other Race, specify: ____________________

ETHNICITY OF MOTHER:

Hispanic................................❑

Non-hispanic ....❑

Other/Unknown...................❑

	Yes	No	Unk
Was **Mother** born outside of United States?..	❑	❑	❑
Was the **Mother** confirmed HBsAg positive prior to or at time of delivery?..	❑	❑	❑
• If no, was the mother confirmed HBsAg positive after delivery?	❑	❑	❑
Date of earliest HBsAg positive test result..	M M / D D / Y Y Y Y		

If yes, what country? ____________________

	0	1	2	3+
How many doses of hepatitis B vaccine did the child receive ?......................	❑	❑	❑	❑

• When?
  - Dose 1 M M / D D / Y Y Y Y
  - Dose 2 M M / D D / Y Y Y Y
  - Dose 3 M M / D D / Y Y Y Y

	Yes	No	Unk
Did the child receive hepatitis B immune globulin (HBIG)?.........................	❑	❑	❑
• If yes, on what date did the child receive HBIG?	M M / D D / Y Y Y Y		

Fig. 6.2 (continued)

Patient History — Acute Hepatitis C

Case ID: ____________________

	Yes	No	Unk
During the **2 weeks – 6 months** prior to onset of symptoms was the patient a contact of a person with confirmed or suspected acute or chronic hepatitis C virus infection?........	❑	❑	❑
If yes, type of contact			
Sexual..	❑	❑	❑
Household (non-sexual)...	❑	❑	❑
Other: ____________________	❑	❑	❑

	Yes	No	Unk
During the **2 weeks – 6 months** prior to onset of symptoms **Did the patient:**			
• undergo hemodialysis?..	❑	❑	❑
• have an accidental stick or puncture with a needle or other object contaminated with blood?.........................	❑	❑	❑
• receive blood or blood products [transfusion]..............	❑	❑	❑
If yes, when?			
• receive any IV infusions and/or injections in the outpatient setting..	❑	❑	❑
• have other exposure to someone else's blood	❑	❑	❑
specify: ____________________			
During the **2 weeks – 6 months** prior to onset of symptoms			
• Was the patient employed in a medical or dental field involving direct contact with human blood ?	❑	❑	❑
If yes, frequency of direct blood contact? ❑ Frequent (several times weekly) ❑ Infrequent			
• Was the patient employed as a public safety worker (fire fighter, law enforcement or correctional officer) having direct contact with human blood?......................	❑	❑	❑
If yes, frequency of direct blood contact? ❑ Frequent (several times weekly) ❑ Infrequent			
• Did the patient receive a tattoo?	❑	❑	❑
Where was the tattooing performed? (select all that apply) ❑ commercial parlor/shop ❑ correctional facility ❑ other __________			

What is the sexual preference of the patient?
❑ Heterosexual ❑ Homosexual
❑ Bisexual ❑ Unknown

Ask both of the following questions regardless of the patient's gender.

In the **6 months** before symptom onset, how many	0	1	2–5	>5	Unk
• male sex partners did the patient have?...............	❑	❑	❑	❑	❑
• female sex partners did the patient have?............	❑	❑	❑	❑	❑

	Yes	No	Unk
Was the patient EVER treated for a sexually-transmitted disease?...	❑	❑	❑
• If yes, in what year was the most recent treatment? Y Y Y Y			
During the **2 weeks – 6 months** prior to onset of symptoms			
• inject drugs not prescribed by a doctor?	❑	❑	❑
• use street drugs but not inject?............................	❑	❑	❑
Did the patient have a negative HCV antibody test within 6 months to a positive test?	❑	❑	❑
Vertified test date M M / D D / Y Y Y Y			
During the **2 weeks – 6 months** prior to onset of symptoms			
• Did the patient have any part of their body pierced (other than ear)?......................................	❑	❑	❑
Where was the piercing performed? (select all that apply) ☐ commercial parlor/shop ☐ correctional facility ☐ other __________			
• Did the patient have dental work or oral surgery?	❑	❑	❑
• Did the patient have surgery ? (other than oral surgery)...	❑	❑	❑
Was the patient – (check all that apply)			
• hospitalized?..	❑	❑	❑
• a resident of a long term care facility?	❑	❑	❑
• incarcerated for longer than 24 hours	❑	❑	❑
If yes, what type of facility (check all that apply)			
prison..	❑	❑	❑
jail	❑	❑	❑
juvenile facility	❑	❑	❑

	Yes	No	Unk
During his/her lifetime, was the patient **EVER** incarcerated for longer than 6 months?.....................	❑	❑	❑
• If yes, what year was the most recent incarceration? Y Y Y Y; for how long? M M M (mos)			
Has the patient recieved medication for the type of hepatitis being reported?..	❑	❑	❑

Fig. 6.2 (continued)

Patient History — Chronic Hepatitis B Infection

Case ID: ______________________________

The following questions are provided as a guide for the investigation of lifetime risk factors for HBV infection. Routine collection of risk factor information for persons who test HBV positive is not required. However, collection of risk factor information for such persons may provide useful information for the development and evaluation of programs to identify and counsel HBV-infected persons.

	Yes	No	Unk
Did the patient receive clotting factor concentrates produced prior to 1987?	❑	❑	❑
Was the patient ever on long-term hemodialysis?	❑	❑	❑
Has the patient ever injected drugs not prescribed by a doctor even if only once or a few times?	❑	❑	❑
How many sex partners has the patient had (approximate lifetime)? ________			
Was the patient ever incarcerated?	❑	❑	❑
Was the patient ever treated for a sexually transmitted disease?	❑	❑	❑
Was the patient ever a contact of a person who had hepatitis?	❑	❑	❑
If yes, type of contact			
• Sexual	❑	❑	❑
• Household [Non-sexual]	❑	❑	❑
• Other	❑	❑	❑

	Yes	No	Unk
Was the patient ever employed in a medical or dental field involving direct contact with human blood?	❑	❑	❑
What is the birth country of the mother ? ______________________			
Has the patient recieved medication for the type of hepatitis being reported?	❑	❑	❑

Patient History — Hepatitis C Infection (past or present)

Case ID: ______________________________

The following questions are provided as a guide for the investigation of lifetime risk factors for HCV infection. Routine collection of risk factor information for persons who test HCV positive is not required. However, collection of risk factor information for such persons may provide useful information for the development and evaluation of programs to identify and counsel HCV-infected persons.

	Yes	No	Unk
Did the patient receive a blood transfusion prior to 1992?	❑	❑	❑
Did the patient receive an organ transplant prior to 1992?	❑	❑	❑
Did the patient receive clotting factor concentrates produced prior to 1987?	❑	❑	❑
Was the patient ever on long-term hemodialysis?	❑	❑	❑
Has the patient ever injected drugs not prescribed by a doctor even if only once or a few times?	❑	❑	❑
How many sex partners has the patient had (approximate lifetime)? ________			
Was the patient ever incarcerated?	❑	❑	❑
Was the patient ever treated for a sexually transmitted disease?	❑	❑	❑
Was the patient ever a contact of a person who had hepatitis?	❑	❑	❑
If yes, type of contact			
• Sexual	❑	❑	❑
• Household [Non-sexual]	❑	❑	❑
• Other	❑	❑	❑

	Yes	No	Unk
Was the patient ever employed in a medical or dental field involving direct contact with human blood?	❑	❑	❑
Has the patient recieved medication for the type of hepatitis being reported?	❑	❑	❑

Fig. 6.2 (continued)

5.5 *Estimation of Hepatitis C Incidence in the USA*

Most HCV infections are not captured through surveillance because many of these infections are asymptomatic. Estimation methodologies are used to account for underreporting of asymptomatic hepatitis C infections. The methodology employed by the CDC to estimate the incidence of HCV infections in the USA was revised in 2011. This methodology uses a simple probability model to estimate all new hepatitis C infections that occur in the USA during a calendar year [80].

Under this model, the estimated number of acute HCV infections in the USA is the number of reported confirmed acute hepatitis C cases multiplied by the joint probability that acute HCV-infected persons who would have developed symptoms, sought healthcare tests, and been reported to health officials [80]. CDC conducted a meta-analysis of peer-reviewed studies to inform the three parameters [80]. However, more sophisticated models informed by representative and comprehensive studies are needed to best estimate acute HCV infections in the USA.

5.6 Security and Confidentiality

While hepatitis C surveillance data can serve many useful public health purposes, these data must be collected, stored, shared, and used in a way that protects the identity of infected individuals [81]. Countries with a well-established hepatitis C surveillance system may have data security and confidentiality policies and procedures in place while countries that are still developing their surveillance system may not. In general, there are guiding principles [82] that can be followed by all countries in order to guarantee security and confidentiality of public health data. These principles are summarized below:

- Data collection and use policies should respect the rights of individuals and community groups and minimize undue burden.
- Program officials should be active, responsible stewards of public health data.
- Programs should:
  - Require that public health data be acquired, used, disclosed, and stored for legitimate public health purposes
  - Collect the minimum amount of personally identifiable information necessary to conduct public health activities
  - Have strong policies to protect the privacy and security of personally identifiable data
  - Have policies and procedures to ensure the quality of any data they collect or use
  - Have the obligation to use and disseminate summary data to relevant stakeholders in a timely manner
  - Have public health data maintained in a secure environment and transmitted through secure methods
  - Share data for legitimate public health purposes and establish data-use agreements to facilitate sharing data in a timely manner
  - Minimize the number of persons and entities granted access to identifiable data

6 Limitations and Challenges of Surveillance

6.1 Distinguishing Acute and Chronic HCV Infection

Many countries do not distinguish between acute and chronic HCV infection and use a case definition based on HCV antibody results alone [76]. For example, in a survey of countries, the European Centre for Disease Prevention and Control found that Germany, the Netherlands, Sweden, England and Wales, and Scotland were enumerating hepatitis C cases based on an antibody test alone [76].

Jaundice may be a practical method for conducting viral hepatitis surveillance in areas where laboratory testing is not routinely included in healthcare visits. For example, in a surveillance system developed to measure the frequency of hepatitis E in northern Uganda, epidemiologists implemented a system originally designed for malaria surveillance [83]. Upon presentation of a person with jaundice to one of the facilities where providers were trained, providers completed a brief case report form, and collected a specimen that was then sent to a central laboratory [83]. At a centralized location, the information from the case report forms and the laboratory results were processed and analyzed to determine the frequency of the different etiologies of jaundice [83].

6.2 Underreporting

Even with the technological advances made in the areas of health information in the USA, the surveillance of hepatitis C continues to be hampered by underreporting, misclassification of cases, and need for more complete data since hepatitis C reporting to the national surveillance network is voluntary. In the USA, CDC estimates that for every reported case of acute hepatitis C, another 12 infections go unreported [80]. There are many reasons why hepatitis C is underreported in the USA. First, HCV infections can only be considered acute if symptoms are present or there is a documented seroconversion. However, 70–85 % of HCV-infected individuals are asymptomatic; about one-half are unaware of their infection [84]. Second, a large percentage of HCV-infected persons may lack access to healthcare services and are thus not reported to health departments. In addition, many states lack the funding needed to conduct enhanced surveillance and do not have the capacity to develop a surveillance system capable of receiving and processing the large number of positive HCV antibody laboratory reports. The number of hepatitis C cases annually reported to health departments often outpaces the amount of resources on hand to fully conduct follow-up investigations to determine if the newly reported case is acute. For these reasons, identifying hepatitis C cases is challenging.

6.3 Resources

Case-based hepatitis C surveillance is a resource-intensive process. Because of many low-resource settings and competing priorities to dedicate the majority of resources to surveillance for acute and effectively preventable conditions, hepatitis C surveillance is often not well developed. As a result, many health departments do not have the capacity to fully investigate every newly reported hepatitis C case. The inability to depend solely on serologic testing to identify acute hepatitis C cases combined with the inability to fully investigate all newly reported cases often leads to the inability to determine if hepatitis C cases are confirmed; these cases instead have a case status label of "probable," "suspect," or "unknown." Unconfirmed cases may never be tested or investigated to determine if they are currently infected [85]. An important decision should be made about the specific objectives and needs for hepatitis C surveillance data such that resources can be used most efficiently and effectively.

6.4 Laboratory Issues

Although a number of highly sensitive and specific rapid tests are available in order to accurately and quickly identify HCV-infected persons and link them to the appropriate care, these tests are often not available in resource-limited settings. The barriers for correctly identifying HCV-infected persons include the lack of simple laboratory assays, need for additional confirmatory testing, and lack of a test for delineating acute from chronic infection.

7 Future Directions

7.1 Health Information Technology

Health information technology (HIT) provides the tools necessary for healthcare providers to better manage patient care through the secure electronic exchange of health information [86]. In a fragmented healthcare system such as that in the USA where multiple healthcare providers are making individual healthcare decisions on the same patient, benefits of the widespread use of HIT include improved quality of healthcare, significantly reduced medical errors, decreased healthcare costs, increased administrative efficiencies, decreased paperwork, and expanded access to affordable healthcare [86, 87]. For example, in 2004, the Massachusetts eHealth Collaborative was formed to establish an electronic health record (EHR) system that would improve the quality, efficiency, and safety of patient care in Massachusetts [88]. By August 2007, nearly 600 physicians participating in the initiative were using EHRs [89].

Historically, a series of critical events which occurred during the late 1990s and early 2000s drew concerns regarding the ability of the USA to respond effectively to acts of bioterrorism and natural epidemics while continuing to protect the health of the nation. These events included the anthrax attacks; destruction of the World Trade Center and the attack on the Pentagon; and emerging disease epidemics such as SARS, avian influenza, and West Nile virus [90]. Having a national system with medical and health information on its citizens would be critical. In response, on April 27, 2004, the president of the USA signed Executive Order 12225, which created the Office of the National Coordinator of Health Information Technology, the principal federal entity charged with supporting the widespread meaningful use of HIT and coordinating efforts to implement and use a nationwide interoperable and secure health information exchange system [90].

7.1.1 Electronic Integration

Ideally, electronic sources of data on HCV infections would have some standardization allowing easy aggregation, supplementation, and analyses. In the USA, with the exception of 16 states, viral hepatitis surveillance systems are neither integrated nor interoperable to produce a singular national electronic surveillance system. Such a system would help to prevent the spread of viral hepatitis and help understand the relationship between viral hepatitis and comorbidities [91]. Hepatitis C surveillance can be greatly improved by expanding health information exchanges (HIEs) and electronic laboratory reporting (ELR).

Where electronic medical records are not integrated, separate data streams can be used to identify persons with HCV infection, for example, using pharmacy records that list antiviral medications specific for hepatitis C [60].

7.1.2 Health Information Exchanges

The framework for a nationwide health information network that connects independent but interoperable public health data systems dates back to 2004 [92]. A key goal of a nationwide health information network is to create an electronic system that can accurately and in a timely fashion exchange patient health information while following security and other protection protocols [93]. HIEs facilitate information flow across various healthcare delivery systems including hospitals, healthcare provider groups, insurers, and government agencies, and are characterized by formal agreements and technologies that facilitate the electronic movement of health-related information [94]. In the USA, funding by the CDC and other public health agencies have supported the development of HIEs and a nationwide health information network [95, 96].

The ability of HIEs to strengthen patient safety through improving laboratory result processing, diagnoses, treatment modalities, and communication between pro-

viders and patients has magnified the potential uses for HIEs. Despite the potential uses of HIEs and the great amount of progress that has occurred over the past 10 years, resource constraints prevent widespread implementation of HIEs [97, 98].

7.1.3 Electronic Laboratory Reporting

In the USA, electronic laboratory reporting is conducted by the automated transmittal of laboratory test results of notifiable diseases from commercial, public health, and hospital laboratories to health departments through a laboratory information management system. The goal of ELR for reporting of hepatitis C is to improve the accuracy, timeliness, and completeness by reducing the number of laboratory reports that are manually entered by health departments. When using ELR, laboratories export data from their information systems in a standard file format and electronically transmit it to their health departments through the laboratory information management system.

Prior to the advances in ELR technology in the USA, manual data entry of paper laboratory reports was the standard procedure for collecting data on viral hepatitis infections. However, manual data entry of paper laboratory reports is both labor intensive and costly. ELR has been shown to identify almost three times as many hepatitis C cases as the traditional paper-based method, and, on average, identified those cases nearly 5.5 days earlier than the conventional method [99].

Although ELR shows promising hope for timely and accurate laboratory reporting, there are challenges. First, these systems report only data listed on laboratory reports and do not contain the clinical information required to confirm a hepatitis C case. Secondly, these systems do not report any enhanced epidemiologic data including risk behaviors/exposures, hepatitis A and B vaccination history, and pregnancy status. These additional components are obtained through enhanced follow-up investigation with the provider and patient. Because of the overwhelming burden of past/present hepatitis C laboratory reports that are submitted to health departments, follow-up investigations are often an enormous endeavor, and for highly populated areas such as New York State, only a sample of total past/present hepatitis C reports can be followed. Additionally, complex ELR algorithms that are either inept or inefficient often lead to incorrect detection of new viral hepatitis cases [99].

7.2 Lessons Learned from Enhanced Surveillance in the USA

From 2005 through 2011, the CDC funded seven health departments to conduct enhanced hepatitis C surveillance throughout the USA. Experiences from this collaboration suggest that certain elements are critical to the success of conducting complete, useful surveillance:

(a) *Electronic infrastructure to receive and process hepatitis C laboratory reports*. Most clinical laboratories have the capacity to report tests associated with all

notifiable diseases in an electronic format to the health departments. However, because laboratories lack a standardized system and health departments vary in their capacity to receive and process electronic information, the large number of hepatitis C reports easily overwhelmed their systems. The lesson learned was that health departments needed to invest in electronic data systems that allowed significant numbers of test results to be received, de-duplicated to result in patient-level information, and then processed to determine electronically whether the patient had been reported previously or was a new case [100].

(b) *Funding for staff at health departments to conduct follow-up investigation*. Because of limited human resources, it became clear that attempting to follow-up on all de-duplicated cases was not feasible. The solution to this problem was to conduct follow-up investigation on a random sample of cases. In most sites, sampling was conducted prospectively on recently reported cases, allowing a 3-month waiting period to ensure that providers had notified patients of their test results. The goal in most sites was to sample ≥10 % of reported cases and to obtain supplemental information from the healthcare provider associated with the positive test result.

(c) *Flexibility of data collection instruments and data entry and storage systems*. Previously, information on mode of transmission was considered desirable, but more helpful to prevention was the identification of which individuals were linked to care. Several health departments had the flexibility to pilot new information items including whether the individual had seen a healthcare provider for hepatitis-related care, and whether they had ever been treated for hepatitis C.

(d) *Secure and standardized transmission of data to a central office*. Difficulties with the larger electronic system for notifiable diseases resulted in the use of an independent, secure transfer protocol mechanism to receive electronic data from sites.

(e) *Capacity to conduct analyses at the central office*. The application of standardized case definitions was complicated at the local level by subjective interpretations and applications of the definition. Data collected on all positive HCV antibody tests allows surveillance programs to understand the population testing positive, and not only those who have the additional confirmatory testing requirements to meet the case definition (e.g., RIBA, RNA). Therefore, a best practice is to receive all data elements and observations health departments are able to collect and send them to a central office. Then, standardized selection criteria can be applied prior to data analyses. For example, the current US hepatitis C case definition requires a confirmatory antibody test; however, understanding the frequency with which persons test positive and are then not reported to have a follow-up test is useful for prevention [85].

7.3 Conclusions

Hepatitis C surveillance can yield useful information for understanding burden of disease, preventing outbreaks, identifying high-risk populations, and planning and evaluating prevention activities. However, careful consideration of objectives should

be balanced with available resources. The current US hepatitis C surveillance system forms the backbone of surveillance and provides incidence data. Enhanced surveillance activities provide additional risk and exposure information on cases. To describe the complete spectrum of HCV disease, HCV-related information from additional sources of data, including population-based surveys, is used. As health-care services evolve in their application of informatics, surveillance of HCV infection can take advantage of the events generated from HCV-related medical encounters in electronic medical records. Testing data from laboratories could be used to monitor the implementation of screening recommendations, and results from nucleic acid tests could be useful to distinguish between current present and resolved or past HCV infection.

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Chapter 7
Nipah Virus Emergence, Transmission, and Pathogenesis

Emmie de Wit and Vincent J. Munster

Core Message Nipah virus is an important emerging virus with potentially global impact. In this chapter, we discuss the emergence of Nipah virus and the current state of knowledge on Nipah virus pathogenesis and countermeasures.

1 Nipah Virus Emergence

1.1 Nipah Virus Outbreaks

1.1.1 Malaysia and Singapore, 1998–1999

In September of 1998, an outbreak of febrile illness with encephalitis was reported in Malaysia [1]. Besides the disease observed in humans, disease in the local pig population was noticed simultaneously. These pigs were suffering from pronounced respiratory and neurological disease, also named "barking pig syndrome," albeit not with a high morbidity and mortality [2]. Human cases occurred primarily in adult men who had been in close contact with pigs, indicating that pigs acted as an amplifying, intermediate host enabling the transmission of the virus from bats to humans (Fig. 7.1) [1, 3–5]. By February 1999, the outbreak spread to humans and pigs in other regions of Malaysia; this spread was associated with the movement of pigs [1, 2]. This movement of pigs also resulted in the spread of the outbreak to abattoir workers in Singapore in March 1999 [6].

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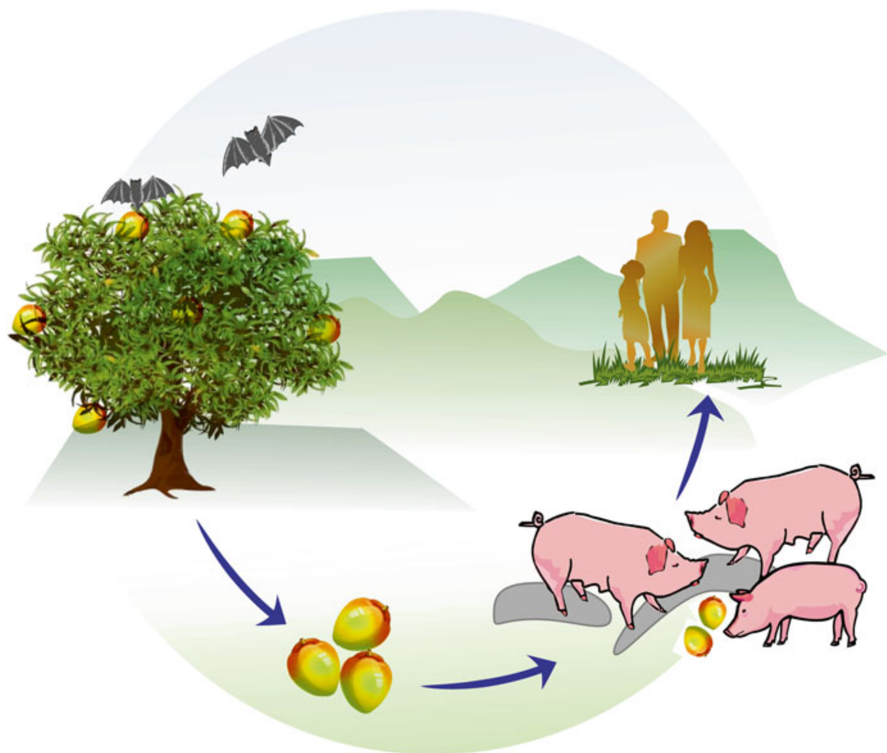

Fig. 7.1 Nipah virus transmission cycle in Malaysia. Pteropid fruit bats are the natural reservoir of Nipah virus. Bats roosting in fruit trees on pig farms transmitted the virus to pigs. Pigs transmitted Nipah virus to people in close contact with them

Initially, the outbreak in Malaysia and subsequently Singapore was suspected to be caused by Japanese encephalitis virus, but this was dismissed as the causative agent when it became clear that most cases were adult men rather than young children and when mosquito control measures and vaccination programs were shown to be ineffective in controlling the outbreak. A previously unknown paramyxovirus was subsequently isolated from the cerebrospinal fluid (CSF) of several fatal encephalitis cases. This virus was named Nipah virus and was later classified in the genus Henipavirus together with Hendra virus [7]. Like Hendra virus, Nipah virus was categorized as a WHO Risk Group 4 Pathogen and thus can only be handled in laboratories of the highest containment level (biosafety level 4).

In total, 276 human cases of encephalitis occurred in the 1998–1999 outbreak (265 cases in Malaysia; 11 cases in Singapore), of which 106 were fatal; more than 1,000,000 pigs were killed from close to 900 farms to control the outbreak [2, 7]. Human-to-human transmission of Nipah virus in Malaysia or Singapore was not described other than four potential cases of nosocomial transmission to health care workers [8, 9]. Human cases of Nipah virus have not been detected in Malaysia since the end of the original outbreak in 1999.

1.1.2 India, 2001, 2007

An outbreak with 66 human cases of encephalitis in India in January and February of 2001 was retrospectively determined to have been caused by Nipah virus [10]. The outbreak occurred in Siliguri, a large city in north eastern India. The outbreak centered around four hospitals in the area; Nipah virus cases were hospitalized patients, their family contacts, and medical staffs. Seventy-five percent of patients were exposed to Nipah virus in a hospital setting [10]; however, there is no indication of how or where the zoonotic transmission occurred that started the outbreak. The area around Siliguri overlaps with the geographic spread of fruit bats belonging to the *Pteropus* genus, but studies to investigate whether an intermediate host was involved in this outbreak or whether Nipah virus was transmitted directly from bats to humans were not conducted. The disease outcome was unknown for some of the cases during this outbreak, but the case-fatality rate was estimated to be around 74 % [10]. In 2007, five cases of Nipah virus disease occurred in a village in West Bengal, India. Four of these five cases were likely the result of human-to-human transmission from the index case; all five cases were fatal [11].

1.1.3 Bangladesh, 2001–Present

In Bangladesh, outbreaks of Nipah virus disease have occurred repeatedly since 2001. Two outbreaks of encephalitis with respiratory disease in Bangladesh, one in 2001 and the other in 2003, were retrospectively diagnosed as having been caused by Nipah virus [12, 13]. In 2001, 13 cases were identified with 9 fatalities; in 2003 12 cases resulted in 8 deaths. In 2004, two outbreaks occurred in Bangladesh, one in January involving 29 cases (22 fatalities) [14] and the other in April involving 36 cases (27 fatalities) [15]. Then there was another outbreak from December 2004 to January 2005 [16]. No outbreaks were identified in 2006. Intensified surveillance has resulted in the identification of Nipah virus outbreaks and sporadic cases in Bangladesh every year since then [17–23]. Although early studies identified contact with sick livestock [12], contact with a herd of pigs [13], and climbing trees [16] as risk factors for acquiring Nipah virus infection, zoonotic transmission of Nipah virus in Bangladesh is most commonly associated with the consumption of raw date palm sap [16, 22]. Date palm sap is collected in Bangladesh in the winter months; bats have been observed to drink from the date palm collection pots [24]. Saliva, feces, or urine of these bats containing Nipah virus could contaminate the collection pots and be transmitted to people through drinking of the sap (Fig. 7.2). Another important means of acquiring Nipah virus infection in Bangladesh is through human-to-human transmission. Respiratory symptoms including coughing are more prevalent in Nipah virus patients in Bangladesh than in Malaysia, a factor that is statistically associated with human-to-human transmission [17, 25]. In some of the Nipah virus outbreaks in Bangladesh, the majority of cases are the result of human-to-human transmission. A striking example is a Nipah virus outbreak in Faridpur district in 2004, where 92 % of cases were the result of human-to-human transmission; a religious leader who

Fig. 7.2 Proposed Nipah virus transmission cycle in Bangladesh. Pteropid fruit bats are the natural reservoir of Nipah virus. During the collection of date palm sap, fruit bats drink from the sap and contaminate the sap with Nipah virus through saliva, urine, or feces. People drinking the date palm sap become infected with Nipah virus and transmit the virus to close contacts

acquired Nipah virus from his aunt subsequently transmitted the virus to 22 family members and followers [26]. Although the outbreaks in Bangladesh are generally smaller in size than the outbreak in Malaysia in 1998–1999, the case-fatality rate tends to be higher in Bangladesh [27]. Whether the increased human-to-human transmission and case-fatality rate in Bangladesh as compared to Malaysia are due to intrinsic differences in transmissibility and pathogenicity between the virus isolate that caused the Malaysian outbreak and the Nipah virus isolates in Bangladesh or whether this is due to differences in health care practices, route of zoonotic transmission or other factors is currently unclear. Experimental infection of hamsters suggests that a Nipah virus isolate from Bangladesh is not more pathogenic or more transmissible than an isolate from Malaysia [28–30].

1.2 The Natural Reservoir of Nipah Virus

1.2.1 Fruit Bats of the *Pteropus* Genus Are the Natural Reservoir of Nipah Virus

The genus *Henipavirus* in the family *Paramyxoviridae* contains the species Hendra virus, Nipah virus, and a tentative third species, Cedar virus [31]. The first indication of the potential involvement of bats in the circulation of henipaviruses came shortly after the discovery of Hendra virus as the causative agent of an outbreak of acute respiratory disease in horses and humans in 1994 in Australia. To explore the potential of a wildlife reservoir from which Hendra virus was transmitted to horses a large serosurvey was initiated. More than 5,000 sera were collected from 46 different species; none of these sera contained antibodies against Hendra virus [32]. Comparison of viral sequences from the two Hendra virus outbreak areas in Brisbane and Mackay suggested a common source of the outbreak and the potential involvement of specific fruit bats, the flying foxes (*Pteropus* spp.; Fig. 7.3) based on their presence and spatial connectivity at both outbreak sites. Serologic investigations subsequently revealed the presence of neutralizing antibodies against Hendra virus in these flying foxes [32].

Because of the close genetic relationship between Nipah virus and Hendra virus, the search for the natural reservoir of Nipah virus focused directly on bats [33].

Fig. 7.3 Flying fox, the natural reservoir of Nipah virus (photo: Vincent Munster)

A serosurvey among bats belonging to species in Malaysia revealed the presence of neutralizing antibodies in four species of fruit bats and one species of insectivorous bats [33]. Especially flying foxes have been identified to play a role in the circulation of Nipah virus; evidence of Nipah virus infection has been detected in the island flying fox (*Pteropus hypomelanus*), Malayan flying fox (*Pteropus vampyrus*), the Indian flying fox (*Pteropus giganteus*), and Lyle's flying fox (*Pteropus lylei*), confirming the role of flying foxes as natural reservoir for Nipah virus [18, 33–38]. Compared to Hendra virus, evidence for Nipah virus circulation in fruit bats has been detected over a wider geographical range including Malaysia, Bangladesh, India, Papua New Guinea, Cambodia, Indonesia, East Timor, Vietnam, and Thailand [33, 35–46]. Flying foxes are nomadic fruit bats capable of long-distance travel [47, 48], thereby connecting populations spread out over large parts of southeastern Asia. The short- and long-distance movements by these fruit bats have been implicated in the spread of pathogens and might facilitate intra- and cross-species transmission. The distribution of Nipah and Hendra virus appears to be predominantly determined by the range of their host species rather than geographical features such as the Wallace line [45].

1.2.2 Factors Affecting Zoonotic Transmission of Henipaviruses

Human activities, such as deforestation, have likely contributed to the emergence of Hendra and Nipah virus [49, 50]. Fruit bat populations are highly mobile and seasonally nomadic in response to local food abundance. Changes in migratory behavior due to resource supplementation by alternative food sources such as mangos on plantations and habitat alteration facilitated close contact with agricultural amplifying hosts (horses for Hendra virus and pigs for Nipah virus) and thereby the risk of zoonotic transmission [51]. Additional stress factors, such as nutritional stress, may also impact disease dynamics [52]. Hendra virus seroprevalence was highest in little red flying foxes (*Pteropus scapulatus*) under nutritional stress [52], suggesting that alterations in food abundance, due to habitat fragmentation and climate change, could increase the risk for Hendra virus zoonotic transmission. In Australia, the number of flying foxes in contact with human and domestic animal populations has increased as a result of urban habituation and decreased migration after anthropogenic transformation of bat habitat [53]. Ten of the 14 known Hendra virus outbreaks occurred near urbanized or sedentary flying fox populations, indicating the potential risk associated with anthropogenic habitat transformation. Similar processes could potentially affect Nipah virus prevalence and zoonotic transmission as well.

1.2.3 Nipah Virus Infection of Bats

Nipah virus is considered to be nonpathogenic in its natural hosts. Experimental infection of Australian grey-headed flying foxes (*Pteropus poliocephalus*) with Nipah virus did not result in clinical disease, but subclinical infection occurred as

indicated by viral shedding, viral isolation, and seroconversion [54]. These findings were confirmed upon experimental inoculation of Malayan flying foxes and black flying foxes (*Pteropus alecto*) with Nipah virus, with no disease signs and virus shedding predominantly in urine [18, 39, 54–56]. The isolation of Nipah virus from the urine of fruit bats, both in the wild and experimentally, and the isolation of Nipah virus from partially eaten fruits suggest that direct contact as well as fomite transmission are important routes of transmission within the natural host species.

1.2.4 Geographic Spread of Henipaviruses in Bats

Recently, serological and molecular surveys identified the circulation of henipaviruses in fruit bats in Africa and South and Central America, suggesting an even larger geographical circulation of henipaviruses than previously recognized [57–61]. A wide variety of different bat species, fruit bats, as well as insectivorous bats appear to harbor henipaviruses. However, the pathogenic and zoonotic potential of these henipaviruses is currently unknown, nor have these newly discovered viruses been associated with any outbreaks in humans or domestic animals. For instance, the recently isolated Cedar virus, a close relative of Nipah and Hendra virus circulating in the black flying fox population of Australia, did not cause any disease in experimentally infected ferrets and guinea pigs, suggesting limited pathogenic potential. The question whether these newly detected henipaviruses have zoonotic potential remains to be addressed.

2 Nipah Virus Pathogenesis

2.1 Nipah Virus Disease in Humans

Nipah virus mainly causes encephalitis in infected individuals; a subset of patients also suffer from virus-induced respiratory disease. The incubation time for Nipah virus disease in Malaysia was estimated to be 10 days; time from onset of symptoms to death in fatal cases was approximately 16 days [62]. During the Nipah virus outbreak in Malaysia and Singapore patients generally presented with fever and altered mental status or decreased consciousness [6, 62, 63]. Neurological signs of disease progressed over time and resulted in coma, and ultimately death in severe cases. Mechanical ventilation was required in most of the severe cases [6, 62–64]. Magnetic resonance imaging during the acute as well as during later phases of illness revealed focal lesions disseminated throughout the brain, mainly in the subcortical and deep white matter of the cerebral hemispheres [6, 63, 65, 66]. Nipah virus was isolated from throat swabs, nose swabs, urine, tracheal secretions, and CSF collected from patients in Malaysia [63, 67]. From day 7 after onset of symptoms onwards, infectious virus could no longer be detected in swabs or urine. There was no correlation between shedding of virus and disease outcome [67]; however, the presence of Nipah virus in CSF was correlated with a poor prognosis [68].

Clinical disease in patients in Bangladesh was similar to that described in Malaysian and Singaporean patients with a few differences. First of all, up to 69 % of patients in Bangladesh had respiratory symptoms, whereas only about 25 % of patients in Malaysia had these symptoms. Secondly, the time from onset to symptoms to death was shorter in Bangladesh (approximately 7 days). Finally, the case-fatality ratio was much higher in Bangladesh at about 70 % calculated over all cases, with the case-fatality rate reaching 100 % in certain outbreaks, compared of about 40 % in the outbreak in Malaysia and Singapore [27, 69].

A follow-up study that included 107 patients from the Malaysian outbreak showed that 19 % of surviving Nipah virus patients had long-term neurological deficits lasting for more than 5 months [62]. After the outbreaks in Malaysia and Bangladesh, late-onset or relapse encephalitis has been described, that was fatal in some cases, anywhere between 4 months and 11 years after the original exposure to Nipah virus [70–73].

Postmortem autopsies were only performed on cases from the 1998–1999 outbreak in Malaysia. Histologic changes were mainly observed in the central nervous system (CNS) of patients, followed by lungs and spleen, and incidentally heart or kidneys [64, 74]. The main observed lesion was vasculitis of small blood vessels and capillaries. This vasculitis was characterized by segmental endothelial destruction, mural necrosis, and karyorrhexis. In the CNS, lesions were observed in blood vessels of the grey and white matter. Viral inclusions were detected in neurons. In the lungs, besides vasculitis, alveolar hemorrhage, pulmonary edema, and aspiration pneumonia were often observed. There was no vasculitis in the spleen; rather, white pulp depletion and acute necrotizing inflammation were observed [74].

2.2 Nipah Virus Disease in Pigs

The Nipah virus outbreak in Malaysia in 1998–1999 was the first time this disease was observed in pigs. Due to the fact that the Nipah virus lethality in pigs was 1–5 % and disease signs are similar to those caused by other pig diseases, Nipah virus infection in pigs may have gone undetected if it had not occurred simultaneously with a disease outbreak in humans [2]. Indeed, modeling has shown that the outbreak in 1998–1999 was unlikely to be the first introduction of Nipah virus in pigs on the index farm [51]. In pigs, Nipah virus disease presentation depended on age. Adult boars and sows presented mainly with neurological disease, whereas pigs under 6 months of age mainly suffered from respiratory disease. Histologically, pigs developed moderate to severe interstitial pneumonia. Like in human Nipah virus infections, pigs developed widespread vasculitis in lungs, brain, and kidneys. In the brain, nonsuppurative meningitis with gliosis was observed [2]. Experimentally, pigs have been inoculated subcutaneously, orally [75], or a combination of intranasally, orally, and ocularly [76]. Although oral inoculation alone did not result in disease, subcutaneous as well as combined intranasal, oral, and ocular inoculation

resulted in disease that recapitulated the disease observed in naturally infected pigs in Malaysia. There was uniform development of disease in all animals; only a subset of animals developed neurological signs. Virus was detected in the respiratory tract and the CNS [75, 76]. Nipah virus reached the CNS via transport along cranial nerves as well as by crossing the blood–brain barrier [76].

2.3 *Animal Models*

Unlike most paramyxoviruses, henipaviruses have a very broad host range. Nipah virus infection has been identified in humans, bats, pigs, cats, and dogs; experimentally, bats, pigs, cats, Syrian hamsters, ferrets, guinea pigs, squirrel monkeys, and African green monkeys have been infected. The use of animal models is essential for understanding Nipah virus transmission and pathogenesis and to test potential intervention strategies.

2.3.1 The Syrian Hamster Model: Elucidating Nipah Virus Pathogenesis and Transmission

Syrian hamsters are currently the most commonly used Nipah virus animal model. The outcome of disease in the hamster model generally depends on the route of inoculation and inoculum dose: intraperitoneally inoculated hamsters develop disease faster than intranasally inoculated animals [77] and a low-dose inoculum results in neurological disease whereas a high dose results in respiratory disease [78]. Evidence for virus replication was detected in lungs, trachea, CNS, spleen, liver, kidneys, and heart. Histologically, focal vasculitis and endothelial syncytia were observed in blood vessels of the lung, brain, liver, kidney, and heart; bronchointerstitial pneumonia was observed in the lungs; animals had meningitis; and viral inclusions could be detected in neurons [77–79]. The Nipah virus Syrian hamster model has been used to study Nipah virus pathogenesis and transmission and to test the efficacy of antiviral treatments and vaccines. Using the Syrian hamster model, it was shown that Nipah virus can be transported by leukocytes, without these cells becoming infected, providing an explanation for the systemic dissemination of Nipah virus during infection [80]. Furthermore, Nipah virus was transported rapidly into the CNS via olfactory neurons in the nasal cavity, providing a route of entry into the CNS that does not require breakdown of the blood–brain barrier [79].

By using reverse genetics techniques, recombinant Nipah viruses were generated that lacked the open reading frame for the nonstructural proteins V, C, or W. Inoculation of Syrian hamsters with these recombinant viruses showed a reduced virulence of viruses lacking proteins V or C, but not W [81]. The mechanism through which nonstructural proteins V and C affect Nipah virus pathogenicity is still unclear;

however, in vitro experiments indicated that their effect on pathogenicity is likely not related to their interferon-antagonist function [81].

A virus isolate from a fatal human case in Malaysia was compared to one from Bangladesh in the Syrian hamster model; although virus replication and disease progressed slower in hamsters inoculated with the Bangladeshi isolate, the clinical signs, tissue tropism, histopathology, and virus titers were comparable in the end stage of disease [29]. These results suggest that clinical differences observed in humans may not be a result of intrinsic differences in viral pathogenicity, but could rather be a result of differences in health care practices and route or dose of acquiring the Nipah virus infection.

The Syrian hamster was also used to model virus transmission, showing that Nipah virus is most likely transmitted between humans through direct contact rather than via fomites or aerosols [28]. Moreover, it was confirmed that, as suggested by epidemiological investigations of Nipah virus outbreaks in Bangladesh, foodborne transmission of Nipah virus via drinking of palm sap containing the virus resulted in virus replication in the respiratory tract and neurological disease [30]. Surprisingly, a Nipah virus isolate from Malaysia transmitted as well as an isolate from Bangladesh in the Syrian hamster model of human-to-human transmission [28, 30].

2.3.2 The Ferret Model

The ferret model of Nipah virus disease has mainly been used to study the efficacy of antivirals and vaccines. In ferrets, Nipah virus caused respiratory and neurological disease upon oronasal inoculation. Histologically, ferrets developed systemic vasculitis, meningitis, and bronchointerstitial pneumonia [82]. Using the Nipah virus ferret model, the pathogenicity of a virus isolate from a fatal human case in Malaysia was compared to a virus isolate from a fatal human case in Bangladesh. No obvious differences in clinical signs caused by the two different isolates were observed; the main difference was the amount of virus shed via the throat: more virus was detected in oral swabs of ferrets inoculated with a Nipah virus strain from Bangladesh [83]. Although increased shedding of the Nipah virus isolate from Bangladesh could be related to the human-to-human transmission observed in Bangladesh, the ferret model has so far not been used to model human-to-human transmission.

2.3.3 The African Green Monkey Model

African green monkeys (*Chlorocebus aethiops*) mainly suffered from severe respiratory disease after inoculation with Nipah virus, with neurological signs occurring in some infected animals [84]. Viral RNA was detected in blood, with the peak viremia occurring at the time of euthanasia. Viral RNA could also be detected in throat and nose swabs. In line with the viremia detected in blood and a resulting systemic infection, viral RNA could be isolated from many different organs, including lungs and brain [84].

2.3.4 Other Animal Models

Experimental inoculation of common squirrel monkeys (*Saimiri sciureus*), guinea pigs, cats, and mice lacking the IFN-α/β receptor (IFNAR-KO mice) were performed. Intravenous inoculation of squirrel monkeys resulted in respiratory and neurological disease; however, disease severity and outcome were not uniform [85]. Guinea pigs inoculated intraperitoneally with a high dose of Nipah virus only showed transient fever and weight loss from which they recovered [77].

Experimental inoculation of cats was performed because of a report of a Nipah virus-infected cat during the Malaysian outbreak of Nipah virus. In this naturally infected cat, generalized vasculitis in multiple organs was the main histological finding [86]. Cats inoculated with Nipah virus oronasally or subcutaneously developed a febrile illness, with respiratory signs developing 24 h after fever [75, 87]. Vertical transmission was observed in a pregnant cat; placental fluid contained high quantities of infectious virus and viral genome copies could be detected in all fetal tissues tested [88].

Whereas regular laboratory mouse strains are not susceptible to Nipah virus disease, intracerebral or intraperitoneal inoculation of IFNAR-KO resulted in uniformly lethal disease; intranasal inoculation with the same dose resulted in lethal infection of 60 % of animals, indicating an important role of interferon signaling in Nipah virus pathogenesis [89]. Histologically, IFNAR-KO mice had widespread vasculitis, meningitis, and lung inflammation [89].

3 Outbreak Intervention Strategies and Treatment Options

3.1 Outbreak Intervention Strategies

3.1.1 Successful Implementation of Outbreak Intervention Strategies in Malaysia

Once it became clear that pigs were the intermediate, amplifying host during the Nipah virus outbreak in Malaysia, a stamping out policy was put into effect. Initially, more than 900,000 pigs in the outbreak area were culled; when serologic tests became available screening of pig farms for the presence of Nipah virus was initiated, resulting in the targeted culling of more than 170,000 pigs [2]. Moreover, the planting of fruit trees on or in the vicinity of pig farms is actively discouraged in an attempt to prevent Nipah virus transmission from bats to pigs [27].

No Nipah virus cases have been detected in Malaysia since 1999, indicating the effectiveness of the implemented outbreak intervention measures.

3.1.2 Development of Outbreak Intervention Strategies in Bangladesh

Developing outbreak intervention strategies in Bangladesh was not as straightforward as in Malaysia. Several outbreaks were not diagnosed until long after they occurred and outbreaks were much smaller. Epidemiologically, there is an association between

drinking of raw date palm sap and Nipah virus infection [16, 22]. Although Nipah virus has so far not been detected in date palm sap, outbreak intervention strategies mainly focus on preventing fruit bats from having access to date palm collection pots. By working with the date palm sap harvesters, methods to prevent bats from having access to the sap were devised [90]. Two methods were assessed for their usefulness in preventing bats drinking from date palm sap by date palm sap harvesters: application of lime to the tree and the collection pots and a bamboo skirt covering the collection pot and sap stream. Only the bamboo skirts were deemed practical and effective by the palm sap harvesters [91]. A randomized control trial to study the effectiveness of bamboo skirts in preventing bats' access to date palm sap collection pots showed that these bamboo skirts, when placed correctly over the tree bark and collection pots, dramatically reduced the number of bat-sap contacts, proving the effectiveness of this method [92].

An assessment of the cultural health practices in Bangladesh revealed that cultural practices in Bangladesh include extensive physical contact with sick patients; moreover, disease transmission is poorly understood by the general public as well as health care workers [93]. Thus, education of the general population and health care workers on prevention of Nipah virus transmission could potentially aid in the prevention of human-to-human transmission and thereby reduce the number of Nipah virus cases.

3.2 The Search for Effective Antiviral Treatments

Due to the severity of Nipah virus outbreaks and their sporadic nature, antiviral treatment options would be very valuable. Currently, the only treatment available to Nipah virus patients is supportive care in hospital settings. Attempts to develop antivirals against Nipah virus are under way. An overview of antiviral treatments tested in one or more of the Nipah virus animal models is given in Table 7.1.

Table 7.1 The efficacy of antiviral treatments tested in Nipah virus animal models

Antiviral	Species	Efficacy	References
Ribavirin	Human (Malaysia outbreak)	36 % reduction in mortality	[95]
	Syrian hamster	No beneficial effect	[96, 97]
Chloroquine	Syrian hamster	No beneficial effect	[96]
	Ferret	No beneficial effect	[104]
Neutralizing antibodies	Syrian hamster	100 % survival with pretreatment; partial protection with posttreatment	[100]
m102.4 antibody	Ferret	100 % survival when treated 24 h after inoculation	[82]
poly(I)-poly(C_{12}U)	Syrian hamster	80 % survival when treated 2 h after inoculation and 9 additional days	[97]
VIKI-PEG4-chol	Syrian hamster	40 % survival when treated 2 days after inoculation	[105]

3.2.1 Ribavirin

During the Nipah virus outbreak in Malaysia, patients were treated with oral or intravenous ribavirin when it became available in sufficient quantities. Ribavirin is a broad-spectrum antiviral that inhibits replication of many DNA and RNA viruses, including the paramyxovirus human respiratory syncytial virus (reviewed in [94]). Comparison of treated vs. non-treated patients indicated a 36 % reduction in lethality [95]. However, all treated cases occurred later in the outbreak whereas most non-treated patients were from the early stage of the outbreak before ribavirin became available. Thus, other confounding factors such as a generally better case management later in the outbreak cannot be excluded to have resulted in the reduced lethality in ribavirin-treated patients [95]. In Syrian hamsters, treatment with ribavirin did not reduce Nipah virus lethality [96, 97]. Although the efficacy of ribavirin against Nipah virus has not been tested in African green monkeys, ribavirin was not effective in changing disease outcome in the African green monkey model for the closely related Hendra virus [98].

3.2.2 Antibody Treatment

Passive transfer studies using sera from Syrian hamsters vaccinated with recombinant vaccinia virus expressing Nipah virus glycoprotein F and/or G resulted in protection of animals from lethal Nipah virus infection [99]. Based on the successful treatment of animals with Nipah virus antibodies, this therapeutic option was further explored. Treatment of Syrian hamsters with different mouse monoclonal antibodies selected to neutralize the Nipah virus glycoproteins G or F before virus inoculation with a lethal dose of Nipah virus resulted in 100 % survival. Unfortunately, administration of antibodies at 24 h or more after inoculation with Nipah virus only partially protected animals from lethal disease [100]. A human monoclonal antibody, m102.4, that neutralizes the Hendra virus as well as Nipah virus glycoprotein G was selected from a naïve human phage-displayed antibody library [101]. Treatment of ferrets with this m102.4 antibody 10 h after inoculation with Nipah virus protected ferrets from lethal Nipah virus infection [82]. Treatment of African green monkeys with m102.4 up to 72 h after challenge with a lethal dose of Hendra virus resulted in survival, although the animals were not protected from disease [102]. Moreover, the m102.4 antibody has been used as prophylactic treatment in humans exposed to Hendra virus in Australia. Although adverse effects of treatment were not noted, it is unknown whether the treatment was efficacious as it was not clear if these people were actually infected with Hendra virus (reviewed in [103]).

3.2.3 Other Antiviral Treatment Options

The efficacy of chloroquine, an antimalarial agent, was tested in the Syrian hamster and ferret models; no effect of treatment on the outcome of disease was detected in either of these models [96, 104].

Treatment of Syrian hamsters with poly(I)-poly(C_{12}U), a potent inducer of type I interferon, for 10 days, starting at 2 h after inoculation with a lethal dose of Nipah virus, resulted in 80 % of animals surviving until 30 days after inoculation [97]. Although poly(I)-poly(C_{12}U) has been tested in human clinical trials, it is currently not approved for use in humans.

A C-terminus heptad repeat peptide, VIKI-PEG4-chol, that binds an intermediate form of the Nipah virus glycoprotein F during fusion of the virus membrane with the host cell membrane, was designed to inhibit fusion and subsequent entry of the virus into the cell. Administration of this peptide at 2 days after inoculation to Syrian hamsters with a lethal dose of Nipah virus increased survival to 40 % [105]. Further clinical testing and approval for use in humans would be required before this peptide could be used in Nipah virus-infected patients.

Several other compounds have been shown to inhibit Nipah virus replication in vitro [106–109]. However, since these compounds are not approved for use in humans and/or have not been tested in at least one of the available Nipah virus animal models, their application as therapeutic agents in human Nipah virus patients is currently unknown.

3.3 Vaccine Development

Since 2012, an equine Hendra virus vaccine is available in Australia. This vaccine, Equivac HeV, consisting of soluble glycoprotein G, aims to protect horses from acquiring Hendra virus. Since humans are largely exposed to Hendra virus through infected horses, the vaccine at the same time aims to prevent transmission of Hendra virus to humans (reviewed in [103]). Since there is no intermediate reservoir involved in the transmission of Nipah virus to humans in the Bangladeshi outbreaks, a similar vaccination strategy cannot be adopted there. Moreover, Nipah virus outbreaks in Bangladesh are small and sporadic, thereby making it unlikely that large-scale vaccination campaigns will ever be used in the human population. However, a Nipah virus vaccine may be useful to employ a ring vaccination strategy during Nipah virus outbreaks. For such a strategy to be successful, fast-acting vaccines need to be developed. An overview of vaccine candidates tested in one or more of the Nipah virus animal models is presented in Table 7.2.

3.3.1 Viral Vector-Based Vaccine Candidates

The first proof-of-principle vaccine consisted of a recombinant vaccinia virus expressing Nipah virus glycoprotein F or G. Syrian hamsters vaccinated subcutaneously with VV-NiV.F or VV-NiV.G or a combination of the two were completely protected from a lethal Nipah virus infection at 3 months after the last vaccination [99]. Moreover, passive transfer of sera from vaccinated hamsters to naïve animals at 1 h before and 24 h after lethal Nipah virus challenge also resulted in complete

Table 7.2 The efficacy of vaccine candidates tested in Nipah virus animal disease models

Vaccine	Species	Efficacy	References
Vaccinia virus vector	Syrian hamster	Protection from lethal challenge after a single vaccination	[99]
Canarypox virus vector	Pig	Protection from clinical disease after two vaccinations	[110]
VSVΔG	Ferret	Protection from heterologous lethal challenge after a single vaccination	[113]
	Syrian hamster	Protection from lethal challenge after a single vaccination	[112]
Adeno-associated virus vector	Syrian hamster	Protection from lethal challenge after a single vaccination	[114]
Measles virus	Syrian hamster	Protection from lethal challenge after two vaccinations	[115]
	African green monkey	Protection from clinical disease after two vaccinations	[115]
sG_{NiV}	Cat	Protection from clinical disease after three vaccinations	[87]
sG_{HeV}	Cat	Protection from clinical disease after two vaccinations	[118]
	Ferret	Protection from lethal challenge up to 1 year after two vaccinations	[120]
	African green monkey	Protection from lethal challenge after two vaccinations	[119]

protection of these animals [99]. Based on the results from this study, subsequent Nipah virus vaccines have focused on producing neutralizing antibodies against glycoproteins F and/or G.

A canarypox virus vector (ALVAC) expressing glycoprotein F or G was tested for use as a vaccine in pigs, as several veterinary vaccines based on the canarypox virus vector are already licensed. Pigs received two vaccine doses with a 2-week interval of ALVAC-F, ALVAC-G, or a combination of both. All animals developed neutralizing antibodies; a combination of ALVAC-F and -G induced the highest neutralizing antibody titer. When animals were challenged 2 weeks after the second vaccination with a nonlethal dose of Nipah virus, none of the vaccinated pigs developed clinical signs of disease, whereas unvaccinated control animals did [110].

Several vaccine studies using recombinant vesicular stomatitis Indiana virus (VSV) have been performed. Deletion of the VSV glycoprotein and insertion of the Nipah virus glycoprotein F or G result in a virus that is replication incompetent, but that does induce neutralizing antibodies in mice [111]. VSVΔG-NiVG/F vaccines have been tested in ferrets as well as Syrian hamsters [112, 113]. Ferrets vaccinated once with an intramuscular injection of VSVΔG expressing G or F from a Nipah virus isolate from a patient in Bangladesh were completely protected from a challenge with a lethal dose of a heterologous Nipah virus isolate from Malaysia at 28 days after vaccination [113]. Syrian hamsters were completely protected from a

homologous lethal infection at 32 days after intramuscular injection with VSVΔG-NiVG/F [112].

Expression of the Nipah virus glycoprotein G from an adeno-associated virus also resulted in complete protection of hamsters with a single intramuscular vaccination [114].

A recombinant measles virus expressing Nipah virus G was first tested in Syrian hamsters; two intraperitoneal vaccinations 3 weeks apart resulted in 100 % survival from a lethal Nipah virus challenge 1 week after the second vaccination [115]. Subsequently, the vaccine was tested in two African green monkeys. Two subcutaneous vaccinations with a 4-week interval resulted in complete protection from clinical disease in a nonlethal Nipah virus challenge model. Although concerns likely exist over preexisting measles virus antibodies in the human population, measles virus-seropositive African green monkeys developed high antibody titers to Nipah virus after two vaccinations [115].

Venezuelan equine encephalitis virus and Newcastle disease virus vectors expressing Nipah virus F or G induced neutralizing antibodies in mice, but these vaccine candidates were not tested further in a Nipah virus challenge model [116, 117].

3.3.2 Soluble Glycoprotein G-Based Vaccine Candidate

A vaccine consisting of recombinant expressed, soluble Nipah virus glycoprotein G (sG_{NiV}) plus adjuvant was shown to protect cats from clinical Nipah virus disease after three vaccinations at 2-week intervals; cats similarly vaccinated with soluble glycoprotein G from Hendra virus were also protected from clinical disease after Nipah virus challenge [87]. As a result of this cross-protection, subsequent experiments used the soluble glycoprotein G from Hendra virus that is part of the licensed Equivac HeV vaccine. When this sG_{HeV} was tested in cats, it protected cats from clinical Nipah virus disease after two vaccinations with the sG_{HeV} at a dose as low as 5 μg protein with adjuvant [118]. Subsequent testing of sG_{HeV} in African green monkeys resulted in complete protection from lethal Nipah virus challenge after two vaccinations with doses of sG_{HeV} as low as 10 μg plus adjuvant [119]. Long-lasting protection after vaccination with sG_{HeV} was shown in the ferret model, when ferrets were completely protected from lethal Nipah virus challenge more than a year after receiving two doses of sG_{HeV} with adjuvant [120].

3.3.3 Other Vaccine Candidates

DNA vaccination with a pCAGGS vector expressing Nipah virus glycoproteins F or G and viruslike particles consisting of Nipah virus matrix protein and glycoproteins F and G were shown to induce neutralizing antibodies in mice; however, the efficacy of these vaccination strategies was not tested in a Nipah virus challenge model [121, 122].

4 Concluding Remarks

It has been almost two decades since the first emergence of Nipah virus. Nipah virus continues to cause annual outbreaks in Bangladesh, with low case numbers but high case fatality rates. Although human-to-human transmission has so far been relatively limited, adaptation of the virus could result in more efficient human-to-human transmission, potentially resulting in large-scale human outbreaks. Effective therapeutic or prophylactic treatment options are currently still lacking; however, even once these become available their implementation in the resource-poor outbreak areas in Bangladesh may be difficult. Therefore, efforts should be focused on the design of low-cost intervention strategies aimed at blocking zoonotic and human-to-human transmission.

The detection of Nipah virus in flying foxes, animals with a wide geographical range partially overlapping with areas of very high human population density, suggests a potential of Nipah virus to cause outbreaks over a large part of southeastern Asia, potentially affecting much larger populations than it has done to date. The discovery of henipaviruses closely related to Nipah virus in bats in Africa and South and Central America further suggests that with contact between bats and humans increasing as a result of habitat destruction and climate change, we could be facing more henipavirus zoonotic transmission events in the future.

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Chapter 8
A Decade of Giant Virus Genomics: Surprising Discoveries Opening New Questions

Hiroyuki Ogata and Masaharu Takemura

Core Message After the discovery of giant viruses at the beginning of this century, viral research started to exert important influences in ever-broader areas of biology. This chapter presents a review of the discoveries of giant viruses such as mimiviruses and pandoraviruses, their spectacular biology, and revolutionary ideas proposed for their origin and evolution, by particularly addressing the implications that have been brought to reassess our classical perception of a virus.

1 The Nature of Viruses: A Traditional View

Viruses are traditionally regarded as small biological entities, which were once termed *filterable agents*. Since their discovery in the late nineteenth century, and particularly after the observation of crystallized tobacco mosaic virus in 1935, they have rarely been regarded as living organisms. They have no cellular structure, the unit and a common trait of living organisms reproducing by binary division. Viruses do not produce energy (i.e., ATPs) required for their reproduction. In contrast, viruses first replicate their genomic material in large numbers, and then package the genomes into capsids. They cannot replicate autonomously outside their hosts but instead hijack host molecular machinery for their replication.

Viral particles contain nucleic acids (either DNA or RNA [1]) as well as proteins in most cases, which are enclosed in a capsid. The capsid might in turn be covered by an envelope of lipid bilayer membranes for certain viruses. Viruses are classified

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into DNA viruses or RNA viruses according to the type of nucleic acids they carry in their particles. Viruses are too small to be visualized easily using optical microscopy. Even the observation of poxviruses (approximately 0.25 μm in length), the largest known viruses until the beginning of the twenty-first century, requires electron microscopy. Most viruses possess a gene (or genes) for genome replication (i.e., DNA polymerase or RNA polymerase), but they often lack genes for transcription and they never encode genes for translation machinery. Viruses depend on their host proteins for these latter steps of the central dogma (i.e., transcription and translation). Therefore, their metabolic capacity is crucially insufficient for autonomous self-reproduction.

A tremendously large body of research has been devoted to understand viruses from medical, agricultural, biochemical, and genetics perspectives. There had always been a clear-cut boundary between viruses and living organisms (i.e., life). However, an extremely large virus now called Acanthamoeba polyphaga mimivirus discovered in 2003 triggered a remarkable change in the perception of viruses, at least among certain microbiologists [2].

2 Discovery of Giant Viruses

Acanthamoeba polyphaga mimivirus (APMV) is an amoeba-infecting large DNA virus, with virus particles reaching 0.75 μm in diameter, including the glycosylated fibrous structure on the surface [3]. In microbiology laboratories, amoebas are used as tools to isolate bacterial pathogens such as *Legionella*. APMV was captured in this type of effort to isolate human pathogens in water samples from a cooling tower of a hospital in England by Timothy Rowbotham [4]. Its particle propagating in the amoeba culture was initially assumed to be an intracellular bacterium because of its large size comparable to small bacterial cells and for its Gram-positive staining property. The particle was therefore given the tentative name of “Bradford coccus,” reflecting the name of the city of its isolation. However, efforts to amplify rRNA gene fragments were unsuccessful, leaving the characterization of the bacterium-like particles pending for years.

In 2003, the sample was brought to the group of Didier Raoult in France (Aix-Marseille University), who painstakingly examined the bacterium-like particles using electron microcopy. Unexpectedly, the particles had a regular, icosahedral form that was typical for a virion. Their reproduction cycle had an eclipse period, which was followed by the sudden emergence of hundreds of particles inside their host amoeba cells. The viral characteristics of the large particles were therefore revealed, and the virus was formally designated as “Mimivirus” to emphasize its size (i.e., a bacterium-“mimicking” virus) before its current name, APMV, was assigned.

In 2004, the complete genome sequence of APMV was determined [2]. The linear dsDNA genome, which turned out to be 1.18 Mbp in length, was found to encode more than 1,000 genes, most of which are transcribed during its infection cycle [5, 6]. The viral nature of APMV was also evident from its gene composition (e.g., capsid genes),

and gene phylogenies firmly placed APMV within the nucleocytoplasmic large DNA virus (NCLDV) group [7], which has been proposed, but not yet approved, as a new order, "*Megavirales*" [8]. Certain members of the NCLDV group, such as poxviruses, infecting vertebrates or insects, as well as chloroviruses, infecting unicellular algae such as chlorella, are large dsDNA viruses, already recognized as "giant viruses" even before the discovery of APMV [9] (Table 8.1). However, these classical giants of viruses measure only 0.18–0.25 μm and possess a genome of ca. 300 kbp. Therefore, APMV was truly exceptional in terms of its particle size and genome size among viruses known at that time.

Is APMV an intriguing, but unique, exception of the virosphere, standing at the extremity in the size spectrum of viruses? Alternatively, have researchers somehow missed opportunities to see and capture such giant viruses (now colloquially called as "giruses" [10])? Soon after the genome sequencing of APMV, comparative sequence studies of genetic data from environmental microbial samples suggested the existence of viruses related to APMV in marine ecosystems [11, 12]. Environmental samplings thus started with the aim of hunting the next giant viruses in different environments including marine ecosystems, and led to the isolation of mimivirus strains from diverse environments [13] and to the discoveries of new giant viruses in the sea, including a large virus infecting bacteriovorus marine nanoflagellate *Cafeteria roenbergensis* (CroV, 750 kbp), which confirmed the predicted presence of mimivirus relatives in the sea [14]. In 2011, again using amoeba cultures, another virus tentatively named "*Megavirus chilensis*" with a genome (1.26 Mbp) slightly larger than that of APMV was isolated from marine sediment sampled at a Chilean coast [15]. In 2013, Philippe et al. reported the discovery of pandoraviruses [16], the largest viruses ever found, with many features that had not been found in the giant viruses reported earlier.

3 Pandoraviruses

Pandoraviruses are atypical among large viruses in their virion morphology. Their virions are not icosahedral, but instead display an irregular ovoid form measuring 1 μm by 0.5 μm with a little apical pore, which makes it reminiscent of Pandora's jar in Greek mythology. They were identified as lytic agents of amoeba cultures, as in the case of mimiviruses and "*Megavirus chilensis*." Pandoravirions are visible by optical microscopy, and were initially given a nickname of "New Life Form (NLF)." Two similar particles were isolated: one from a sediment sample taken at the mouth of the Tunquen River, Chile, and the other from the bottom of a freshwater pond near Melbourne, Australia. Genome sequence analyses revealed that these two parasitic particles (respectively tentatively named "*Pandoravirus salinus*" and "*Pandoravirus dulcis*") represent related but distinct members of a newly proposed genus of giant virus. Except for regions with repetitive sequences at one extremity, their linear dsDNA genomes were sequenced completely. The size of the whole genome was estimated as 2.77 Mb (2,556 predicted genes) for "*P. salinus*" and as 1.91 Mbp (1,502 predicted genes) for "*P. dulcis*."

Table 8.1 Viral genomes larger than 300 kbp recorded in the NCBI/RefSeq database

Virus name	Genome size (bp)	Number of genes	Genome type	NCBI classification	Accession
"*Pandoravirus salinus*"	2473870[a]	2,541[a]	Linear	Viruses; dsDNA viruses, no RNA stage; unclassified dsDNA viruses[b]	NC_022098
"*Pandoravirus dulcis*"	1908524[a]	1,487[a]	Linear	Viruses; dsDNA viruses, no RNA stage; unclassified dsDNA viruses[b]	NC_021858
"*Megavirus chiliensis*"	1259197	1,120	Linear	Viruses; dsDNA viruses, no RNA stage; *Mimiviridae*; unclassified *Mimiviridae*[b]	NC_016072
"*Megavirus lba*"	1230522	1,176	Linear	Viruses; unclassified viruses[b]	NC_020232
Acanthamoeba polyphaga mimivirus (APMV)	1181549	979[a]	Linear	Viruses; dsDNA viruses, no RNA stage; *Mimiviridae*; *Mimivirus*[b]	NC_014649
"Acanthamoeba polyphaga moumouvirus"	1021348	894	Linear	Viruses; dsDNA viruses, no RNA stage; *Mimiviridae*; unclassified *Mimiviridae*[b]	NC_020104
Cafeteria roenbergensis virus BV-PW1 (CroV)	617453	544	Linear	Viruses; dsDNA viruses, no RNA stage; unclassified dsDNA viruses[b]	NC_014637
Phaeocystis globosa virus	459984	434	Linear	Viruses; dsDNA viruses, no RNA stage; *Phycodnaviridae*; *Prymnesiovirus*; unclassified prymnesiovirus[b]	NC_021312
Emiliania huxleyi virus 86	407339	472	Circular	Viruses; dsDNA viruses, no RNA stage; *Phycodnaviridae*; *Coccolithovirus*[b]	NC_007346
Paramecium bursaria Chlorella virus NY2A	368683	886	Linear	Viruses; dsDNA viruses, no RNA stage; *Phycodnaviridae*; *Chlorovirus*[b]	NC_009898
Acanthamoeba polyphaga marseillevirus (APMaV)	368454	428	Circular	Viruses; dsDNA viruses, no RNA stage; *Marseilleviridae*; *Marseillevirus*[b]	NC_013756
Canarypox virus	359853	328	Linear	Viruses; dsDNA viruses, no RNA stage; *Poxviridae*; *Chordopoxvirinae*; *Avipoxvirus*[b]	NC_005309
Cronobacter phage vB_CsaM_GAP32	358663	545	Linear	Viruses; dsDNA viruses, no RNA stage; *Caudovirales*; *Myoviridae*	NC_019401
"Lausannevirus"	346754	444	Circular	Viruses; dsDNA viruses, no RNA stage; *Marseilleviridae*; *Marseillevirus*[b]	NC_015326

Enterobacteria phage vB_KleM-RaK2	345809	534	Linear	Viruses; dsDNA viruses, no RNA stage; *Caudovirales*; *Myoviridae*	NC_019526
Paramecium bursaria chlorella virus AR158	344691	814	Linear	Viruses; dsDNA viruses, no RNA stage; *Phycodnaviridae*; *Chlorovirus*; unclassified chlorovirus[b]	NC_009899
Ectocarpus siliculosus virus 1	335593	240	Circular	Viruses; dsDNA viruses, no RNA stage; *Phycodnaviridae*; *Phaeovirus*[b]	NC_002687
Aramecium bursaria chlorella virus 1 (PBCV-1)	330611	802	Linear	Viruses; dsDNA viruses, no RNA stage; *Phycodnaviridae*; *Chlorovirus*[b]	NC_000852
Paramecium bursaria chlorella virus FR483	321240	849	Linear	Viruses; dsDNA viruses, no RNA stage; *Phycodnaviridae*; *Chlorovirus*[b]	NC_008603
Pseudomonas phage 201phi2-1	316674	461	Linear	Viruses; dsDNA viruses, no RNA stage; *Caudovirales*; *Myoviridae*; *Phikzlikevirus*; unclassified phiKZ-like viruses	NC_010821
Choristoneura biennis entomopoxvirus 'L'	307691	334	Linear	Viruses; dsDNA viruses, no RNA stage; *Poxviridae*; *Entomopoxvirinae*; *Betaentomopoxvirus*[b]	NC_021248
Shrimp white spot syndrome virus (white spot bacilliform virus)	305108	532	Circular	Viruses; dsDNA viruses, no RNA stage; *Nimaviridae*; *Whispovirus*	NC_003225

[a]Genome size and the number of predicted genes are based on the records in the NCBI/RefSeq database, and they slightly differ from the numbers from original publications cited in the main text

[b]Viruses classified in the NCLDV group

Pandoraviruses are also unique in terms of their gene contents. Only 401 predicted genes from "*P. salinus*" have significant sequence similarity to other sequences in the current sequence databases, whereas the remaining 2,155 predicted genes (84 %) lack detectable known homologs (i.e., "orphan genes"). A proteomic analysis of "*P. salinus*" virions identified 210 proteins originating from its genome, of which 80 % had no detectable sequence similarity to any other sequence in the public databases. The proteomic identification of gene products from "*P. salinus*" orphan genes suggests the *bona fide* gene status for the many of the predicted orphan genes in its genome.

Electron microscopic analysis revealed the following infection/replication cycle in *Acanthamoeba* cultures. Pandoravirions enter the cytoplasm of amoeba host cells by phagocytosis, as in the case of mimiviruses. The particle empties its contents (genomic DNA and probably associated proteins) into the cytoplasm through its apical pore. The injection process involves the fusion of the internal lipid membrane of the viral particle and the phagocytic vacuolar membrane. This genome delivery step is followed by an eclipse period during which the contents of the particles become invisible, as in other viruses. No binary division was observed. Later, the nuclear membranes disappear gradually and numerous newly assembled virion particles emerge at the periphery of the region formerly occupied by the nucleus. The location of the emergent pandoravirions is therefore different from those produced by mimiviruses and "*Megavirus chilensis*," which create an electron-dense intracellular compartment called a "virion factory," a viral replication and assembly center, in the cytoplasm of the infected cell. Therefore, pandoraviruses are assumed to use the host nucleus for their replication. The replication cycle lasts for 10–15 h.

Another interesting feature of pandoravirus genomes is the presence of spliceosomal type introns in many genes. Although precise delineation of exon/intron structure requires deep sequencing of transcripts, it is estimated that ca. 10 % of "*P. salinus*" genes contain spliceosomal introns. Spliceosomal introns differ from self-splicing introns (found in many viruses) and have been found only rarely in viral genomes. The presence of spliceosomal introns in pandoravirus genes is also indicative of the use of the host nucleus for their transcription.

Pandoraviruses share only a handful of genes with other previously characterized large DNA viruses. Therefore, their evolutionary relation with other viruses might not be readily apparent. Detailed phylogenetic analyses of a few genes common to pandoraviruses and other viruses suggest that they are distantly related to phycodnaviruses in the NCLDV group [17].

4 Are Viruses Alive?

Are viruses really non-organisms? The discoveries of these giant viruses strongly shook some of the beliefs of microbiologists and evolutionary biologists, and either reactivated or initiated old and new issues related to the concept of viruses. Succinctly put, a clear boundary that had been perceived between organisms (cellular life forms)

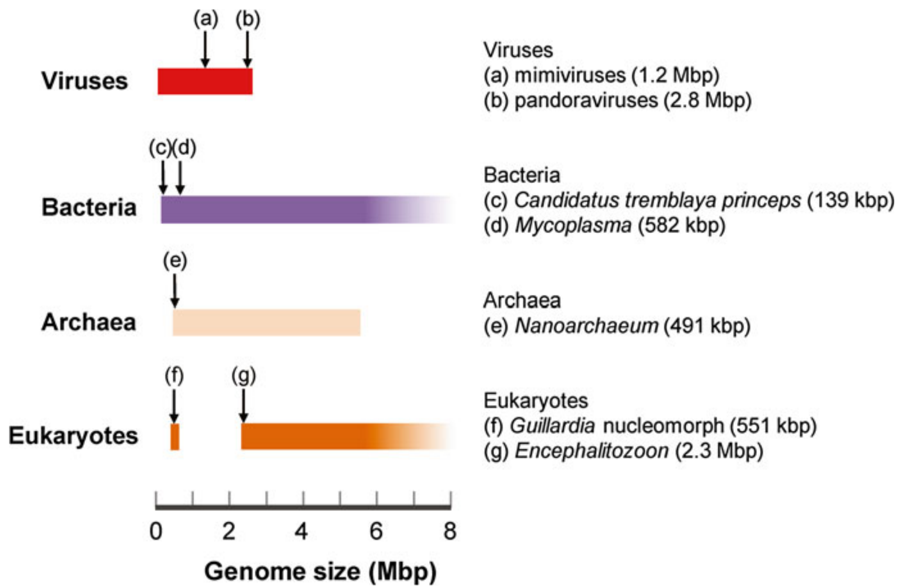

Fig. 8.1 Genome size of viruses and cellular organisms

and viruses became blurred considerably for the first time in the history of virology. Viruses had been thought to represent an ultimate form of parasite that carries a minimal set of genes that are necessary for nucleic acid replication and packaging. However, the size ranges of viral genomes and cellular genomes now mutually overlap. Mimivirus genome sizes exceed those of parasitic bacterial and archaeal genomes, whereas the size of the "*P. salinus*" genome falls in the size range of standard bacterial genomes and exceeds the size of parasitic eukaryotic genomes (Fig. 8.1).

Given the diversity of genes encoded in giant virus genomes, their reproduction strategies would not be expected to be simpler than those of cellular organisms. Furthermore, the APMV genome was found to encode genes for part of the translation system, which is regarded as a hallmark molecular apparatus distinguishing cellular organisms from viruses. APMV has four aminoacyl-tRNA synthetase genes and three genes for translation initiation, elongation, and termination, in addition to six tRNA genes. To date, no virus has been found that encodes genes for the ribosome, but the presence of these translation-related genes in APMV suggests that giant viruses actively participate in the translation process. They are not completely dependent on their host at every phase of the central dogma during their reproduction. CroV and "*Megavirus chilensis*" have several translational genes, although no such gene was identified in the genomes of pandoraviruses. In addition, APMV particles were found to contain both RNA (mRNAs) and DNA (genomic DNA), which further blurred the conceptual barrier between organisms and viruses.

The mimivirus DNA delivery system also illustrates how the molecular machineries of giant viruses are sophisticated [18]. Upon infection of particle contents into

the host cell cytoplasm, APMV opens five triangular faces around a vertex of its icosahedral capsid. The machinery, called "stargate," shows no structural similarity to the DNA delivery systems in other viruses such as the tails of bacteriophages.

The discovery of virophages supports the self-contained characteristics of the reproduction machinery of giant viruses, at least to a certain degree. Virophages are small viruses with genomes ca. 20 kbp dsDNA in length [19–22]. They are incapable of infecting cellular organisms independently, but they start reproduction when they are co-infected with a giant virus such as APMV. In fact, virophages infect the virion factory that giant viruses build inside the cytoplasm of the host. Infection of virophages can lead to abortive forms and abnormal capsid assembly of giant viruses. Therefore, virophages are small viruses (with their own DNA replication genes) that infect other larger viruses. The existence of virophages now appears to be a common phenomenon associated with giant viruses of the family *Mimiviridae* [23]. These observations indicate a high level of integrity and flexibility of the virion factory, and revived the old contention, that is, "are viruses not alive?" [24].

These discoveries during the last decade provided opportunities to reexamine the concept of viruses and their placement in the evolutionary history of life.

5 Fourth Domain Hypothesis

Mimiviruses possess several genes that are widely conserved in cellular organisms, such as RNA polymerase genes and aminoacyl-tRNA synthetase genes. Molecular-phylogenetic analyses suggest deep evolutionary origins for those genes, which might predate the radiation of the eukaryotic kingdom. Based on this observation, Raoult et al. reported that mimiviruses and related giant viruses might constitute a fourth domain of life [2], in addition to the other three established domains of life composed of eukaryotes, bacteria, and archaea (a domain is the highest taxonomic rank of organisms) (Fig. 8.2). This initial proposal was followed by others that supported the same idea or that extended the hypothesis by providing different evidence and arguments [25–29]. However, as expected, several lines of counter-argument have been raised by others [30–32].

In fact, some mimivirus gene phylogenies that were used to support the viruses' deep evolutionary origins in early studies later supported more recent origins after additional data for eukaryotic genomes were added [31]. Nevertheless, the phylogenetic analyses of different genes (including RNA polymerase and DNA polymerase gene) still support early branching positions for these genes near the roots of trees of cellular homologs.

In support of recent origins of giant virus genes, Moreira and Brochier-Armanet used phylogenetic analyses to prove that many mimivirus genes were acquired from cellular organisms by horizontal gene transfer in the course of evolution, and suggested "giant chimeric" characteristics of mimivirus genomes [33]. This type of result, implying that large virus genomes were derived from smaller virus genomes through the accretion of genes, enjoys certain popularity. However, the evidence of

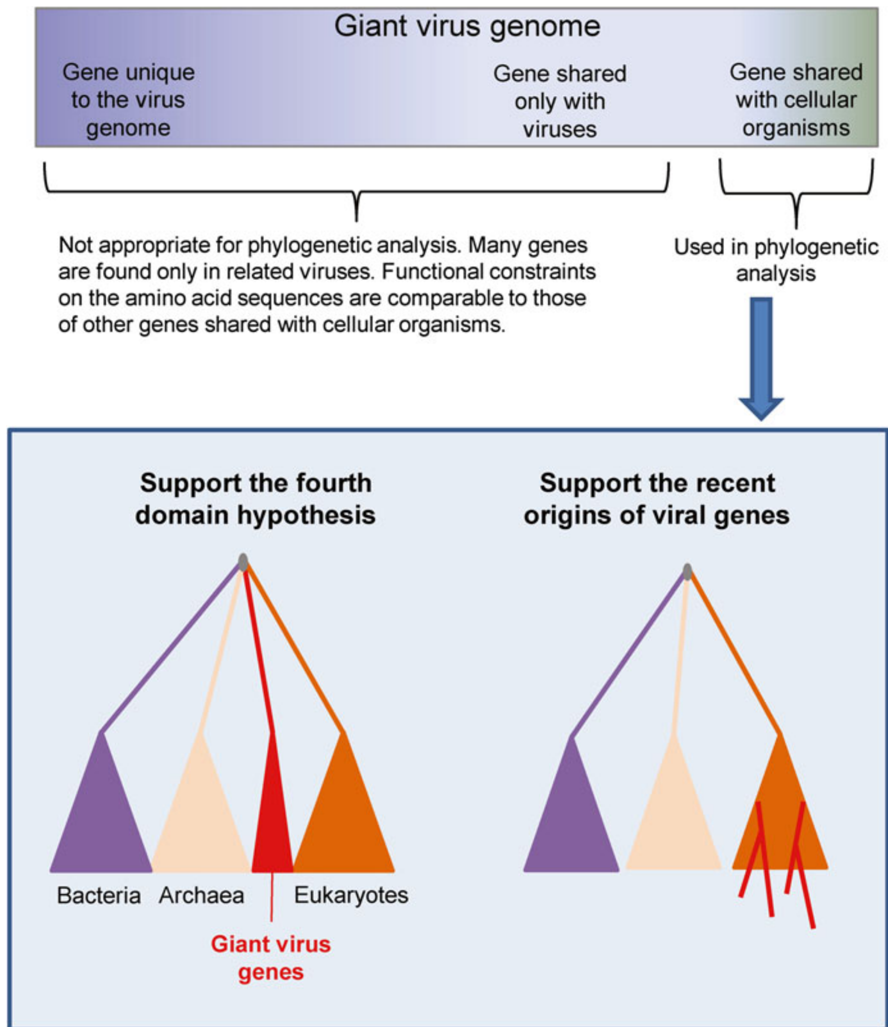

Fig. 8.2 Schematic drawing for the gene composition of typical giant virus genome and evolutionary analysis results

lateral gene acquisitions is in fact limited to a rather small subset (i.e., <10 %) of the entire gene set encoded in giant virus genomes [24, 34]. Such a level of detection of gene transfer is at the same level as that of bacterial genomes. For instance, "*P. salinus*" has 92 genes that might be of host amoeba origin, but this corresponds to only 3.6 % of the 2,556 genes encoded in its genome [16].

Giant viruses possess numerous genes with no detectable homologs in any cellular organism. Several authors have inferred that this fact might result from deep evolutionary origins of giant viruses [35]. More specifically, the existence of those

genes with obscure origins is not compatible with the classical idea that viral genomes are (mainly) derived from cellular genomes. A typical counter-argument to this invokes the high evolutionary rate of viral genomes that can erase the trace of homology between viral and cellular gene homologs. Ogata and Claverie refuted this counter-argument by demonstrating that no significant difference exists in the relative rates of evolution (more specifically, in the levels of functional constraints on sequences) between genes found only in closely related large DNA viruses and those with cellular homologs [36]. RNA viruses and ssDNA viruses (albeit at a lesser extent than RNA viruses) are known to evolve rapidly, but currently no reliable estimate exists for the evolutionary rate of giant virus genomes that can be compared directly with those of cellular genomes [37, 38].

The fourth domain issue would be revolutionary if the hypothesis is true, but the issue might be more complicated than the third (Archaea) domain proposition by Carl Woese in 1977 [39], which has now become widely established after a long debate (but see [40] for a recent discussion). Several important but different points might be readily apparent in the debates on the fourth domain hypothesis. They are discussed at different levels: some scientific and others epistemological. Crucial questions include the following: Are viruses organisms? Are viruses as old as cellular organisms? Even if we accept their deep ancestry, does the evidence from gene sequences support their old origins? How are they connected with the early history of the evolution of cells? Can we regard all viruses, from small RNA viruses to large DNA viruses, as a single biological group? Further characterization of giant viruses is expected to contribute to the resolution of these entangled issues.

6 Viral Origin of the Nucleus

Presumably, an important issue in the biology of giant viruses is the elucidation of the virion factory, an intracellular compartment for viral replication, and assembly that large viruses create inside their host cells. Nearly nothing is known about the virion factory, which can be as large as the nucleus and which would involve hundreds of viral proteins and other host factors. Investigation of the composition, structure, and function of the virion factory will definitely engender a better understanding of the nature of giant viruses. Here we briefly revisit a hypothesis that links the ancestor of large DNA viruses (and virion factory) with the origin of the nucleus.

Before the discovery of APMV, it had been proposed that eukaryotic DNA polymerase genes originated from ancient large DNA viruses based on the deep phylogenetic positions suggested for DNA polymerase genes of large DNA viruses [41, 42]. Takemura [42] and Bell [43] independently proposed further that an ancient large dsDNA virus infecting an archaeal ancestor of eukaryotes might be the origin of the eukaryotic nucleus (i.e., viral eukaryogenesis hypothesis). These authors identified several intriguing functional similarities between the nucleus and large DNA viruses such as poxviruses. Both poxviruses and the nucleus replicate only inside the

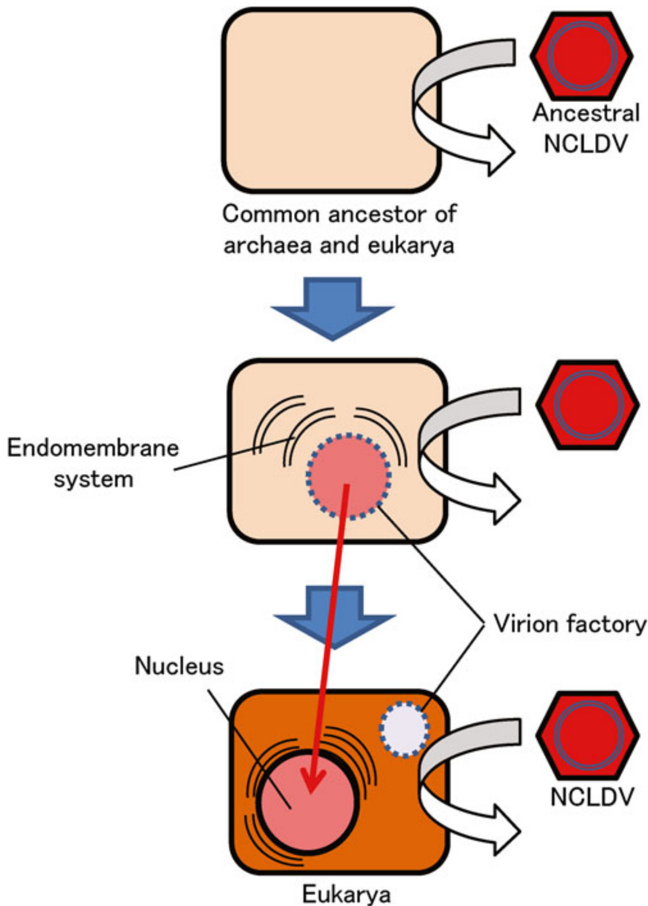

Fig. 8.3 Virion factory and the origin of the eukaryotic nucleus

cytoplasm of a eukaryotic cell. The translation process requires the translation system (ribosomes) located in the cytoplasmic region of the cell in both cases, although the spatial arrangement of cytoplasmic ribosomes and the virion factory are not known. Both poxviruses and the nucleus possess mechanisms to export mRNAs. The presence of repetitive sequences at the extremities of their linear dsDNA genomes is common between large DNA viruses and the nucleus. The virion factory of poxviruses arranges endoplasmic reticulum (ER) membranes at its periphery [44], reminiscent of the membrane surrounding the nucleus.

The viral eukaryogenesis hypothesis is an endosymbiotic hypothesis for the nucleus. It has been revisited and extended since the discovery of APMV and its large virion factory [45, 46] (Fig. 8.3). The endosymbiotic origin of the mitochondrion and the chloroplast is now widely accepted among biologists. In contrast, various theories have been proposed for the origin of the nucleus by researchers. These are

divisible into two categories, symbiotic or non-symbiotic theories, but none has yet been widely accepted. Symbiotic hypotheses are based on the symbiosis of organisms belonging to two species, such as archaeal and bacterial cells. The syntrophic eukaryogenetic theory, proposed by Moreira and López-García, invokes a syntrophic association of a sulfate-reducing δ-proteobacterium and a methanogenic archaeon [47]. However, the non-syntrophic eukaryogenetic theory, proposed by Cavalier-Smith, emphasizes the co-evolution of organelles including the nucleus, and postulates the fusion of ER membranes as the origin of the nuclear membranes [48]. Martin and Koonin hypothesized that nucleus–cytosol compartmentalization occurred to separate an mRNA splicing reaction, which proceeds more slowly, from a translation reaction, which proceeds more rapidly [49].

In spite of the proposal of these hypotheses corroborated by updated biological knowledge, an enigma remains. When and how did these events start? Was there a critical event that started everything, or did they occur gradually? The viral eukaryogenesis hypothesis has acquired more attention because of the discoveries of giant viruses and their properties consistent with the hypothesis, as described above. In a recent work, Takemura suggests that the infection of an ancestral NCLDV to the common ancestor of archaea and eukaryotes was a critical evolutionary event that spurred the emergence of the cell nucleus (Takemura, submitted).

Another effort to establish evolutionary and conceptual links between viruses with cellular organisms is the examination of the definition of viruses. For instance, Raoult and Forterre suggested the definition of viruses as "capsid-encoding organisms" and cellular organisms as "ribosome-encoding organisms" [50]. Traditionally, the term virus refers to a viral particle (i.e., virion) [1]. Claverie and Forterre proposed that the crucially important characteristics of "metabolically active state" of a virus reside in the virion factory [46] or in the whole infected cell (i.e., the "virocell" concept) [35, 51]. It is noteworthy that viral research is changing after the discoveries of giant viruses as well as other previously unrecognized viruses such as archaea viruses and symbiotic viruses, and now has marked influence in ever-broader areas of biology [52]. It is likely that further studies of giant viruses will continue to reveal their fascinating biology and will engender a unified evolutionary picture of the viral and cellular worlds.

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Chapter 9
Expanded Host Diversity and Global Distribution of Hantaviruses: Implications for Identifying and Investigating Previously Unrecognized Hantaviral Diseases

Richard Yanagihara, Se Hun Gu, and Jin-Won Song

Core Message

- Discovery of genetically distinct hantaviruses in multiple species of shrews and moles (order Eulipotyphla) and insectivorous bats (order Chiroptera) heralds a new frontier in hantavirology.
- Acquisition of new knowledge about the spatial and temporal distribution, host range and genetic diversity of newfound hantaviruses harbored by shrews, moles, and bats was accelerated by having access to archival tissue collections.
- Newfound hantaviruses in shrews, moles, and bats are genetically more diverse than those hosted by rodents (order Rodentia), suggesting that the evolutionary origins of hantaviruses are more ancient and complex than previously contemplated.
- Phylogenetic analyses indicate four distinct hantavirus clades, with evidence of both co-divergence and host switching, and suggest that shrews, moles, and/or bats may have predated rodents as the early reservoir hosts of primordial hantaviruses.
- Detection of hantavirus RNA in ethanol-fixed tissues greatly expands the pool of specimens for future hantavirus-discovery efforts, particularly in other insectivorous small mammals, such as hedgehogs and tenrecs.
- The lack of cell culture isolates of the newly detected hantaviruses hosted by shrews, moles, and bats has hampered the identification and investigation of novel hantaviral diseases.

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1 Introduction

In the spring of 1993, four decades after their forefathers in Korea were faced with an epidemic febrile illness with renal failure, a disease then unknown to American medicine called Korean hemorrhagic fever [1–3], emergency room physicians and health-care workers in the Four Corners region of the southwestern USA were confronted with a terrifying outbreak of a rapidly progressive, frequently fatal respiratory disease, now known as hantavirus cardiopulmonary syndrome (HCPS) [4]. No one had the prescience to predict that this previously unrecognized disease would be caused by a once-exotic group of rodent-borne viruses, belonging to the *Hantavirus* genus of the *Bunyaviridae* family.

Present-day hantavirology dates to the seminal discovery of Hantaan virus (HTNV) as the prototype virus of hemorrhagic fever with renal syndrome (HFRS) in the striped field mouse (*Apodemus agrarius*) [5]. This milestone made possible the identification of other HFRS-causing hantaviruses, such as Puumala virus (PUUV) in the bank vole (*Myodes glareolus*) [6], Seoul virus (SEOV) in the brown rat (*Rattus norvegicus*) [7], and Dobrava virus (DOBV) in the yellow-necked field mouse (*Apodemus flavicollis*) [8]. Similarly, the identification of Sin Nombre virus (SNV) in the deer mouse (*Peromyscus maniculatus*) [9, 10] and Andes virus (ANDV) [11, 12] in the long-tailed colilargo (*Oligoryzomys longicaudatus*), as the causative agents of HCPS, marked the next major benchmark in hantavirology. Several other genetically distinct hantaviruses harbored by neotomine and sigmodontine rodents in the USA, such as New York virus (NYV) in the white-footed mouse (*Peromyscus leucopus*) [13–15], Bayou virus (BAYV) in the marsh rice rat (*Oryzomys palustris*) [16–18], and Black Creek Canal virus (BCCV) in the hispid cotton rat (*Sigmodon hispidus*) [19, 20], have been associated with HCPS.

Recently, a new frontier in hantavirology has been forged with the discovery of highly divergent lineages of hantaviruses in multiple species of shrews and moles (order Eulipotyphla) and insectivorous bats (order Chiroptera) from widely separated geographic regions. Phylogenetic analyses suggest that ancestral shrews and moles and/or bats may have predated rodents as the early reservoir hosts of primordial hantaviruses [21, 22]. However, to what extent one or more of these newfound non-rodent-borne hantaviruses might cause infection and disease in humans is unknown.

Nevertheless, both HFRS and HCPS are excellent examples of how the initial identification and subsequent investigation of previously unrecognized emerging infectious diseases are dependent on the coordinated efforts of collaborative teams, comprising clinicians, epidemiologists, microbiologists, mammalogists and field ecologists, and pathologists. In such outbreaks, the initial observational acumen and clinical experience of medical and paramedical personnel—whether they be in the best-equipped tertiary-care referral hospitals or in resource-constrained rural clinics or field settings in low-income countries—are critical to suspect that something out of the ordinary might be occurring. Moreover, the persistence or stubbornness and strong conviction of health-care practitioners, who refuse to readily accept negative laboratory tests, is an important prerequisite for identifying new, emerging and reemerging infectious diseases. Thus, effective early-warning systems are heavily

dependent on individual people, and the importance of this first step in recognition of new diseases cannot be over emphasized. Also vital is the unwavering support of human resources and public health infrastructure, which are increasingly aided by powerful social media applications and sophisticated data-sharing communications and information technology platforms.

In this chapter, we will not attempt to review the rich diversity of hantaviruses and their genotypes in myriad neotomine and sigmodontine rodents of various species in the Americas, largely because this has been elegantly summarized, with the clear demonstration that the majority of South American hantaviruses segregate into three phylogenetic clades, comprising ANDV and ANDV-like viruses, Laguna Negra virus (LANV) and LANV-like viruses, and Rio Mamore virus (RIOMV) and RIOMV-like viruses [23]. Instead, we focus mainly on reviewing the host diversity and geographic distribution of the newfound non-rodent-borne hantaviruses and summarize efforts to identify human infection and to investigate diseases that may be caused by these still-orphan hantaviruses. We draw from the detailed studies on the first rodent-borne hantavirus from sub-Saharan Africa, namely Sangassou virus (SANGV) harbored by the African wood mouse (*Hylomyscus simus*) [24], and the first shrew-borne hantavirus to be isolated in nearly four decades, namely Imjin virus (MJNV) hosted by the Ussuri white-toothed shrew (*Crocidura lasiura*) [25]. We also discuss some of the challenges associated with definitively linking newly described orphan viruses to previously unrecognized infectious diseases in humans.

2 Reservoir Host Diversity

Like all other members of the *Bunyaviridae* family, viruses in the *Hantavirus* genus possess a negative-sense, single-stranded RNA genome consisting of three segments, designated large (L), medium (M), and small (S), which encode an RNA-dependent RNA polymerase, envelope glycoproteins (Gn, Gc) and a nucleocapsid (N) protein, respectively [26, 27]. However, unlike the more than 400 other members in this virus family, hantaviruses are unique in that they are harbored by small mammals. Whether or not arthropod vectors, such as mites, are involved in the transmission dynamics and maintenance of the enzootic cycle have again been raised recently [28], and renewed investigations are now underway.

Initially, rodents were believed to serve as the exclusive reservoir hosts of hantaviruses [29]. Moreover, the conventional view held that each genetically distinct hantavirus is carried by a rodent of a single species, with which it coevolved. This now appears to be an overly simplistic paradigm, particularly in light of the expanded host range and genetic diversity of hantaviruses [21, 22]. Mounting evidence supports the concepts of host sharing and host switching. That is, as shown in Table 9.1, the same hantavirus may be harbored by more than one reservoir rodent, such as Tula virus (TULV) in the common vole (*Microtus arvalis*), Russian common vole (*Microtus rossiaemeridionalis*), field vole (*Microtus agrestis*), and European pine vole (*Pitymys subterraneus*) [30–34]. TULV has also been reported in the Eurasian

Table 9.1 Hantaviruses and rodent-host and disease associations[a]

Family	Subfamily	Reservoir host species	Virus name	Disease
Muridae	Murinae	*Apodemus agrarius*	Hantaan	HFRS
		Apodemus agrarius	Dobrava (Kurkino)	HFRS
		Apodemus agrarius	Dobrava (Saaremaa)	HFRS?
		Apodemus flavicollis	Dobrava (Dobrava)	HFRS
		Apodemus ponticus	Dobrava (Sochi)	HFRS
		Apodemus peninsulae	Amur	HFRS
		Apodemus peninsulae	Soochong	HFRS
		Hylomyscus simus	Sangassou	Unknown
		Niviventer confucianus	Da Bie Shan	Unknown
		Rattus losea	Seoul	HFRS?
		Rattus norvegicus	Seoul	HFRS
		Rattus rattus	Seoul	HFRS
		Bandicota indica	Thailand	HFRS
		Bandicota savilei	Thailand-like	Unknown
		Rattus rattus	Thailand (Anjozorobe)	Unknown
		Rattus tanezumi	Thailand (Serang)	Unknown
		Rattus tanezumi	Thailand (Jurong)	Unknown
		Stenocephalemys albipes	Tigray	Unknown
Cricetidae	Arvicolinae	*Eothenomys miletus*	Luxi	Unknown
		Microtus agrestis	Tatenale	Unknown
		Microtus agrestis	Tula	Unknown
		Microtus arvalis	Tula	Unknown
		Microtus rossiaemeridionalis	Tula	Unknown
		Pitymys subterraneus	Tula	Unknown
		Arvicola amphibius	Tula	Unknown
		Microtus californicus	Isla Vista	Unknown
		Microtus ochrogaster	Bloodland Lake	Unknown
		Microtus fortis	Khabarovsk	Unknown
		Microtus maximowiczii	Khabarovsk	Unknown
		Microtus fortis	Vladivostok	Unknown
		Microtus fortis	Yuanjiang	Unknown
		Microtus pennsylvanicus	Prospect Hill	Unknown
		Myodes glareolus	Puumala	HFRS
		Myodes rufocanus	Puumala	HFRS
		Myodes rufocanus	Hokkaido	Unknown
		Myodes regulus	Muju	HFRS?
		Lemmus sibiricus	Topografov	Unknown
	Neotominae	*Peromyscus boylii*	Limestone Canyon	Unknown
		Peromyscus beatae	Montano	Unknown
		Peromyscus leucopus	Blue River	Unknown
		Peromyscus leucopus	New York	HCPS
		Peromyscus maniculatus	Sin Nombre	HCPS
		Reithrodontomys megalotis	El Moro Canyon	Unknown
		Reithrodontomys sumichrasti	El Moro Canyon	Unknown

(continued)

Table 9.1 (continued)

Family	Subfamily	Reservoir host species	Virus name	Disease
		Reithrodontomys mexicanus	Rio Segundo	Unknown
	Sigmodontinae	*Akodon azarae*	Pergamino	HCPS
		Akodon montensis	Ape Aime	Unknown
		Akodon montensis	Jaborá	Unknown
		Akodon paranaensis	Jabora	Unknown
		Akodon serrensis	Jabora	Unknown
		Bolomys lasiurus	Araraquara	HCPS
		Bolomys obscurus	Maciel	HCPS
		Calomys laucha	Laguna Negra	HCPS
		Calomys callosus	Laguna Negra	HCPS
		Holochilus chacoensis	Alto Paraguay	Unknown
		Oligoryzomys chacoensis	Bermejo	HCPS
		Oligoryzomys fornesi	Anajatuba	HCPS
		Oligoryzomys longicaudatus	Oran	HCPS
		Oligoryzomys longicaudatus	Andes	HCPS
		Necromys benefactus	Andes	HCPS
		Oligoryzomys nigripes	Araucária	HCPS
		Oxymycterus judex	Araucária	HCPS
		Oligoryzomys flavescens	Lechiguanas	HCPS
		Oligoryzomys delicatus	Maporal	Unknown
		Oligoryzomys fulvescens	Maporal	Unknown
		Oligoryzomys fulvescens	Choclo	HCPS
		Oligoryzomys costaricensis	Choclo	HCPS
		Oligoryzomys microtis	Rio Mamore	HCPS
		Oligoryzomys nigripes	Itapúa	Unknown
		Oligoryzomys nigripes	Juquitiba	HCPS
		Oligoryzomys fornesi	Juquitiba	HCPS
		Oligoryzomys utiaritensis	Castelo dos Sonhos	HCPS
		Oryzomys couesi	Catacamas	Unknown
		Oryzomys couesi	Playa de Oro	Unknown
		Oryzomys palustris	Bayou	HCPS
		Sigmodon alstoni	Cano Delgadito	Unknown
		Sigmodon hispidus	Muleshoe	Unknown
		Sigmodon hispidus	Black Creek Canal	HCPS
		Zygodontomys brevicauda	Calabazo	Unknown

[a]This table is not meant to be exhaustive or comprehensive. Rather its intent is to display the vast diversity of hantaviruses harbored by rodents in the Muridae and Cricetidae families. In particular, the large number of hantaviruses hosted by multiple sigmodontine rodent hosts in South America is emphasized. However, many of these viruses probably do not represent distinct species but fall into one of three phylogenetic clades: ANDV, LANV, and RIOMV. The rodent reservoirs of some HCPS-causing hantaviruses, such as Tunari virus, Maripa virus, and Paranoá virus, have not been identified. Disease associations, such as HFRS or HCPS, are shown, when known. Otherwise, the "Unknown" descriptor is used

ANDV Andes virus, *HCPS* hantavirus cardiopulmonary syndrome, *HFRS* hemorrhagic fever with renal syndrome, *LANV* Laguna Negra virus, *RIOMV* Rio Mamore virus

water vole (*Arvicola amphibius*) [35]. It is unclear if this represents spillover from common voles or a host switch. Host sharing and/or host switching seems to apply also to other rodent-borne hantaviruses, such as Thailand virus (THAIV) in the greater bandicoot rat (*Bandicota indica*) [36, 37] and Savile's bandicoot rat (*Bandicota savilei*) [38], as well as THAIV-like hantaviruses in the black rat (*Rattus rattus*) and tanezumi rat (*Rattus tanezumi*) [39, 40]. Moreover, genetic variants of PUUV, designated Hokkaido virus (HOKV) and Muju virus (MUJV), have been reported in the gray red-backed vole (*Myodes rufocanus*) in Japan [41] and the royal vole (*Myodes regulus*) in Korea [42, 43], respectively. In addition, as discussed in greater detail later, some hantaviruses harbored by soricine shrews and insectivorous bats have been detected in hosts belonging to more than one species, but further research is necessary to better understand these host–virus relationships.

Spillover of hantaviruses to syntopic rodents and host-switching events, on the one hand, are contrasted by the same rodents also hosting more than one hantaviruses. For example, the field vole hosts TULV in Europe and a newly discovered hantavirus, named Tatenale virus (TATV), in the UK [44]; and the striped field mouse, which serves as the reservoir of HTNV in Asia, also hosts the Kurkino and Saaremaa genotypes of DOBV in Europe [45]. It is noteworthy that the least virulent genotypes of DOBV are those harbored by the striped field mouse in Europe, whereas in Asia, the striped field mouse harbors the prototypic virulent hantavirus, known as HTNV. On the other hand, DOBV genotypes Dobrava and Sochi, which are hosted by the yellow-necked field mouse and the Caucasus field mouse (*Apodemus ponticus*), respectively, are more pathogenic and account for the majority of HFRS fatalities in Europe [45]. The molecular basis for this differential virulence is unknown.

Whereas HFRS- and HCPS-causing hantaviruses are only known to be harbored by rodents thus far, the global landscape of hantaviruses has been forever altered by the discovery of highly divergent lineages of hantaviruses in shrews, moles, and insectivorous bats [21, 22]. As such, the evolutionary origins and phylogeography are clearly ancient and far more complex than previously contemplated [21, 22, 46]. Although unimaginable a few years ago, the entire host diversity has presumably not been attained and many more genetically distinct hantaviruses, particularly those hosted by shrews, moles, and bats, still await discovery.

2.1 Hantaviruses in Rodents

A rich literature exists on hantaviruses harbored by rodents of the Muridae and Cricetidae families. Since most of the attention has understandably been paid to hantaviruses that cause HFRS and HCPS, the reader is often left with the mistaken impression that all hantaviruses are pathogenic. In fact, the majority of rodent-borne hantaviruses has not been associated with human infection and disease. This is particularly true for hantaviruses carried by arvicoline rodents, and in particular those harbored by members of the *Microtus* genus, the prototype being

Prospect Hill virus (PHV), the first hantavirus isolated from an indigenous wild rodent, the meadow vole (*Microtus pennsylvanicus*), in North America [47]. Other prominent examples include Khabarovsk virus (KHAV) and Vladivostok virus (VLAV), hosted by the Maximowicz's vole (*Microtus maximowiczii*) and reed vole (*Microtus fortis*), respectively, which do not appear to cause infection or disease in humans [48, 49]. Also, not all genetic variants or genotypes of the same hantavirus appear to have the identical degree of pathogenicity. For example, no human disease has been associated with HOKV, harbored by the gray red-backed vole in Japan, despite its close genetic and phylogenetic relationship with PUUV [41]. Also, the Saaremaa genotype of DOBV, carried by the striped field mouse in Estonia, seems non-pathogenic [45].

Table 9.1 lists the hantaviruses detected in rodents and indicates which hantaviruses are known to be pathogenic. As previously mentioned, extensive host sharing, in which the same hantavirus is harbored by rodents belonging to more than one species, is evident. It is not clear in every instance whether this has resulted from spillover or host-switching events and subsequent species-specific adaptation. Examples can be found in rodent-borne hantaviruses of the same rodent host family and subfamily. The bewildering constellation of rodents of divergent species and designations of hantaviruses, particularly in South America, have recently been simplified by in-depth analysis of hantavirus isolates from HCPS patients and rodents. As mentioned earlier, the majority of South American hantaviruses, and in particular ANDV, LANV, and RIOMV, belong to three distinct hantavirus species [23]. However, not all strains of ANDV, LANV, and RIOMV appear to cause HCPS. Also, hantaviruses carried by closely related rodent hosts, such as Choclo virus (CHOV) and Maporal virus (MAPV) in the Costa Rican pygmy rice rat (*Oligoryzomys costaricensis*) and the delicate pygmy rice rat (*Oligoryzomys delicatus*), respectively, exhibit vastly different pathogenic potential, with CHOV causing a full spectrum from subclinical infection to severe HCPS [50, 51], and MAPV showing no disease in humans [52]. Both CHOV and MAPV were previously thought to be hosted by the fulvous colilargo (*Oligoryzomys fulvescens*) [53–55].

Hantavirus infection in the rodent host is subclinical, generally with short-lived viremia but with dissemination of virus in multiple tissues, including lung, salivary gland and kidney [56–59]. The demonstration of virus antigen in brown fat of overwintering live-caught bank voles in the former Soviet Union suggests a possible mechanism of virus maintenance [60]. Virus excretion in urine and feces persists for months or possibly lifelong in infected rodents, despite high-titered serum neutralizing antibodies. There is no evidence of vertical transmission of hantaviruses in rodents [29, 61, 62]. Arthropod vectors do not appear to be involved in hantavirus infection among humans [29, 63], but questions have again been raised about the role of mites in the maintenance of the hantavirus enzootic cycle [28].

Hantavirus-infected reservoir rodents tend to be localized in small, circumscribed foci, rather than being uniformly distributed in any given geographical area [29]. As such, transmission and prevalence rates of rodent-borne hantavirus infections are regulated within reservoir host populations and typically vary in time and space [64].

Since the recognition of HCPS in the Americas, the epizootiology of SNV infection in deer mouse populations has been intensively studied. Among the more consistent findings have been the widespread nature of the SNV enzootic in the reservoir rodent species, the greater preponderance of infection in adult male deer mice, the decreasing antibody prevalence with age (suggesting passively acquired immunity in pups), the higher SNV antibody prevalence in peri-domestic compared to sylvan settings, and the correlation between population size and hantavirus-antibody prevalence [61, 65–71]. In addition, SNV RNA was repeatedly detected in serially collected blood samples, particularly in antibody-positive male deer mice, suggesting their role in virus shedding for prolonged periods [72].

2.2 Hantaviruses in Shrews

Shrews have been generally ignored in the transmission dynamics and evolutionary origins of hantaviruses, despite the fact that Thottapalayam virus (TPMV), a previously unclassified virus isolated from the Asian house shrew (*Suncus murinus*), captured near Vellore in Tamil Nadu, India [73, 74], predated the isolation of HTNV. Also, the early reports of the detection of HFRS antigens in tissues of the Eurasian common shrew (*Sorex araneus*), alpine shrew (*Sorex alpinus*), and Eurasian water shrew (*Neomys fodiens*) in Russia and the former Yugoslavia [60, 75, 76] went largely unnoticed.

The antigenic relationship between TPMV and 31 other hantavirus isolates has been investigated by cross-enzyme immunoassay (ELISA) and cross-plaque-reduction neutralization tests (PRNT) using antisera from experimentally infected animals [77]. Antisera prepared against strains of HTNV, PUUV, SEOV, THAIV, and PHV, exhibited 16-fold or lower ELISA titers to cell culture-derived TPMV antigen than to the homotypic hantaviral antigen [77]. Of the 32 hantaviruses examined by PRNT, TPMV was the only one that displayed no cross-neutralization with any other hantavirus; that is, none of the heterologous antisera neutralized TPMV and the antiserum to TPMV did not neutralize any other hantavirus [77].

Recently, TPMV strains have been detected in Asian house shrews captured in Nepal [78] and China [79]. Phylogenetic analysis of the partial and full genome sequences of prototype TPMV and other newfound TPMV strains demonstrate that they form a separate phylogenetic clade, suggesting an early evolutionary divergence from other hantaviruses [80–82]. Using oligonucleotide primers based on TPMV, a novel hantavirus, named MJNV, was detected in Ussuri white-toothed shrews (*Crocidura lasiura*) captured along the Imjin River, near the demilitarized zone in the Republic of Korea [25]. High prevalence of MJNV infection has been demonstrated within discrete foci during the autumn months, with evidence of marked male predominance [25]. The absence of cross neutralization between MJNV and rodent-borne hantaviruses indicates that it is antigenically distinct.

Empowered by the full genomes of TPMV and MJNV, we launched an opportunistic search for hantavirus RNA using reverse transcription polymerase chain reaction

(RT-PCR). Initially, we envisioned that the genomes of TPMV and MJNV would make finding new hantaviruses a trivial exercise. Instead, the unexpectedly vast genetic diversity of the shrew-borne hantaviruses posed considerable challenges in designing suitable primers for the amplification of their genes. Also, in the belief that the probability of success for finding novel hantaviruses would be highest in frozen tissues, we initially limited our search to such specimens. However, we soon learned that this approach placed unnecessary restrictions on our virus-discovery attempts, so we expanded our search to include tissues which were either preserved in RNAlater® RNA Stabilization Reagent or fixed in 90 % ethanol.

The generosity of museum curators and field mammalogists, who provided access to their valuable archival tissue collections, accelerated the acquisition of new knowledge about the host range and spatial and temporal distribution of hantaviruses. In analyzing RNA, extracted from more than 1,500 tissues from nearly 50 shrew species collected throughout Europe, Asia, North America, and Africa, between 1980 and 2012, we have discovered multiple genetically distinct hantaviruses, including Seewis virus (SWSV) in the Eurasian common shrew [83–86], Ash River virus (ARRV) in the masked shrew (*Sorex cinereus*) [87], Jemez Springs virus (JMSV) in the dusky shrew (*Sorex monticolus*) [87], Kenkeme virus (KKMV) in the flat-skulled shrew (*Sorex roboratus*) [88], Amga virus (MGAV) in the Laxmann's shrew (*Sorex caecutiens*) [89], Sarufutsu virus (SRFV) in the long-clawed shrew (*Sorex unguiculatus*) [90], Cao Bang virus (CBNV) in the Chinese mole shrew (*Anourosorex squamipes*) [91], Xinyi virus (XYIV) in the Taiwanese mole shrew (*Anourosorex yamanashi*) [92], Camp Ripley virus (RPLV) in the northern short-tailed shrew (*Blarina brevicauda*) [93], Iamonia virus (AMNV) in the southern short-tailed shrew (*Blarina carolinensis*) (unpublished), Boginia virus (BOGV) in the Eurasian water shrew [94], Azagny virus (AZGV) in the West African pygmy shrew (*Crocidura obscurior*) [95], Jeju virus (JJUV) in the Asian lesser white-toothed shrew (*Crocidura shantungensis*) [96], Bowé virus (BOWV) in the Doucet's musk shrew (*Crocidura douceti*) [97], Uluguru virus (ULUV) in the geata mouse shrew (*Myosorex geata*) [98], and Kilimanjaro virus (KMJV) in the Kilimanjaro mouse shrew (*Myosorex zinki*) [98] (Table 9.2).

As for rodent-borne hantaviruses, examples of host sharing or spillover have been found for SWSV in the Eurasian pygmy shrew [86, 99], tundra shrew

Table 9.2 Genetically distinct Hantaviruses detected in shrews (order Eulipotyphla, family Soricidae)

Virus name	Virus abbreviation	Reservoir host species	Country	Year of capture	References
Azagny	AZGV	*Crocidura obscurior*	Côte d'Ivoire	2009	[95]
Bowé	BOWV	*Crocidura douceti*	Guinea	2012	[97]
Imjin	MJNV	*Crocidura lasiura*	Korea	2004	[25]
Jeju	JJUV	*Crocidura shantungensis*	Korea	2007	[96]
Tanganya	TGNV	*Crocidura theresae*	Guinea	2004	[102]

(continued)

Table 9.2 (continued)

Virus name	Virus abbreviation	Reservoir host species	Country	Year of capture	References
Thottapalayam	TPMV	*Suncus murinus*	India	1964	[73, 81, 82]
			Nepal	1996	[78]
			China	2009	[79]
Kilimanjaro	KMJV	*Myosorex zinki*	Tanzania	2002	[98]
Uluguru	ULUV	*Myosorex geata*	Tanzania	1996	[98]
Cao Bang	CBNV	*Anourosorex squamipes*	Vietnam	2006	[91]
			China	2006	Unpublished
Xinyi	XYIV	*Anourosorex yamashinai*	Taiwan	1989	[92]
Camp Ripley	RPLV	*Blarina brevicauda*	USA	1998	[93]
			Canada	1983	Unpublished
Iamonia	AMNV	*Blarina carolinensis*	USA	1983	Unpublished
Amga	MGAV	*Sorex caecutiens*	Russia	2006	[89]
			Japan	2010	[89]
Ash River	ARRV	*Sorex cinereus*	USA	1994	[87]
Asikkala	ASIV	*Sorex minutus*	Czech Republic	2010	[104]
Boginia	BOGV	*Neomys fodiens*	Poland	2011	[94]
Jemez Springs	JMSV	*Sorex monticolus*	USA	1996	[87]
		Sorex palustris	Canada	2005	Unpublished
		Sorex trowbridgii	USA	1996	Unpublished
		Sorex vagrans	USA	1996	Unpublished
Kenkeme	KKMV	*Sorex roboratus*	Russia	2006	[88]
Sarufutsu	SRFV	*Sorex unguiculatus*	Japan	2006	[90]
Seewis	SWSV	*Sorex araneus*	Switzerland	2006	[83]
			Hungary	1997	[84]
			Finland	1982	[84]
			Germany	2007	[99]
			Czech Republic	2010	[99]
			Poland	2010	[86, 94]
			Slovakia	2008	[99]
			Slovenia	1990	[100, 101]
			Russia	2006	[85]
		Sorex daphaenodon	Russia	2006	[85]
		Sorex minutus	Germany	2005	[84]
			Poland	2012	[86]
		Sorex tundrensis	Russia	2006	[85]
			Mongolia	2010	Unpublished
		Neomys anomalus	Austria	2007	Unpublished
			Poland	2011	[86]
Qian Hu Shan	QHSV	*Sorex cylindricauda*	China	2005	[105]
Yakeshi	YAKV	*Sorex isodon*	China	2006	[103]

(*Sorex tundrensis*) [85], large-toothed Siberian shrew (*Sorex daphaenodon*) [85], and Mediterranean water shrew (*Neomys anomalus*) [86]. Also, JMSV, which is harbored by the dusky shrew, has been found in the vagrant shrew (*Sorex vagrans*), Trowbridge's shrew (*Sorex trowbridgii*), and American water shrew (*Sorex palustris*) in North America (unpublished). In addition, other investigators have independently reported SWSV among Eurasian common shrews in central Europe [99–101], well as additional shrew-borne hantaviruses, including Tanganya virus in the Therese's shrew (*Crocidura theresae*) [102], Yakeshi virus in the taiga shrew (*Sorex isodon*) [103], Asikkala virus (ASIV) in the Eurasian pygmy shrew (*Sorex minutus*) [104], and Qian Hu Shan virus in the stripe-backed shrew (*Sorex cylindricauda*) [105].

2.3 Hantaviruses in Moles

Tissues from moles belonging to 10 of the 40 extant species, tested to date, have yielded five genetically distinct hantaviruses, including Asama virus (ASAV) in the Japanese shrew mole (*Urotrichus talpoides*) [106], Oxbow virus (OXBV) in the shrew mole (*Neurotrichus gibbsii*) [107], Nova virus (NVAV) in the European mole (*Talpa europaea*) [108], Rockport virus (RKPV) in the eastern mole (*Scalopus aquaticus*) [109], and Dahonggou Creek virus (DHCV) in the long-tailed mole (*Scaptonyx fusicaudus*) (unpublished) (Table 9.3). Undoubtedly, this represents a gross underestimation of the number of talpid-borne hantaviruses, because many more moles belonging to other species were unavailable for testing and for the ten species tested, the sample sizes were small, numbering fewer than ten individuals. More targeted searches for hantavirus RNA in moles that share common ancestries with the known talpid reservoirs will likely lead to the discovery of additional hantaviruses and/or clarify whether or not host sharing occurs among moles. In addition, studies of moles, which are sympatric and syntopic with shrews and rodents, are warranted to ascertain host-switching events.

The most highly divergent lineage of hantaviruses is represented by NVAV [108]. Recent studies indicate high prevalences of NVAV infection exceeding 50 % in

Table 9.3 Genetically distinct Hantaviruses detected in moles (order Eulipotyphla, family Talpidae)

Virus name	Virus abbreviation	Reservoir host species	Country	Year	Reference
Asama	ASAV	*Urotrichus talpoides*	Japan	2008	[106]
Dahonggou Creek	DHCV	*Scaptonyx fusicaudus*	China	1989	Unpublished
Nova	NVAV	*Talpa europaea*	Hungary	1999	[108]
			France	1912	[110]
			Poland	2010	[86]
Oxbow	OXBV	*Neurotrichus gibbsii*	USA	2003	[107]
Rockport	RKPV	*Scalopus aquaticus*	USA	1986	[109]

European moles from France and Poland, suggesting efficient enzootic virus transmission and a well-established, long-standing reservoir host–hantavirus relationship [86, 110]. Much like SWSV is widespread in the Eurasian common shrew throughout Europe, NVAV probably occurs throughout the vast distribution of the European mole. The rodent-borne hantavirus counterparts are PUUV in the bank vole in Europe and PUUV-like hantaviruses, such as HOKV and MUJV, in other arvicoline rodent species in Far East Asia, as well as SNV in the deer mouse and SNV-like hantaviruses, such as NYV, in other neotomine rodents in North America.

2.4 Hantaviruses in Bats

Attempts by our group and others to find hantavirus RNA by RT-PCR in more than 1,500 tissue samples from insectivorous and frugivorous bats belonging to approximately 100 species have resulted in the identification of six hantaviruses (Table 9.4). These include Mouyassué virus (MOYV) in the banana pipistrelle from Côte d'Ivoire [111, 112], Magboi virus (MGBV) in the hairy slit-faced bat (*Nycteris hispida*) from Sierra Leone [113], Makokou virus (MAKV) in the Noack's round-leaf bat (*Hipposideros ruber*) from Gabon [114], Xuan Son virus (XSV) in the Pomona round-leaf bat (*Hipposideros pomona*) from Vietnam [112, 115], Huangpi virus (HUPV) in the Japanese pipistrelle (*Pipistrellus abramus*), and Longquan virus (LQUV) in the Chinese rufous horseshoe bat (*Rhinolophus sinicus*), Formosan lesser horseshoe bat (*Rhinolophus monoceros*), and intermediate horseshoe bat (*Rhinolophus affinis*) from China [103]. Thus far, hantaviruses have not been detected in fruit bats (flying foxes).

Compared to the much higher success rates of detecting hantavirus RNA in shrews and moles, the very low success rate of similar efforts in bat tissues may be attributed to several factors. For one, the genomes of bat-borne hantaviruses may be too different to be readily amenable to the current primer-based screening methodologies, and primer mismatches and suboptimal PCR cycling conditions need to be overcome [111, 112, 115]. Also, the very focal nature of hantavirus infection, small sample sizes from any given bat species and poorly preserved or degraded RNA may be contributory.

Table 9.4 Genetically distinct Hantaviruses detected in insectivorous bats (order Chiroptera)

Virus name	Virus abbreviation	Reservoir host species	Country	Year	References
Huangpi	HUPV	*Pipistrellus abramus*	China	2011	[103]
Longquan	LQUV	*Rhinolophus sinica*	China	2011	[103]
		Rhinolophus affinis	China	2011	[103]
		Rhinolophus monoceros	China	2011	[103]
Magboi	MGBV	*Nycteris hispida*	Sierra Leone	2010	[113]
Makokou	MAKV	*Hipposideros ruber*	Gabon	2012	[114]
Mouyassué	MOYV	*Neoromicia nanus*	Côte d'Ivoire	2011	[111, 112]
Xuan Son	XSV	*Hipposideros pomona*	Vietnam	2012	[112, 115]

Alternatively, bats may be less susceptible to hantavirus infection or may have developed immune mechanisms to curtail viral replication and/or viral persistence. While bats of fewer species might serve as reservoirs, the hantaviruses they harbor are among the most genetically diverse described to date [103, 111–115]. As such, intensified studies on the phylogeography and transmission dynamics of hantaviruses in bats may provide additional insights into their evolutionary origins.

Although frozen tissues are intuitively preferred in virus-discovery efforts, the successful detection of hantavirus RNA in ethanol-fixed tissue from bat tissues [111] should substantially expand the pool of specimens for hantavirus hunting, especially in tissues from bats and other small mammals, such as hedgehogs and tenrecs, which may also carry hantaviruses. Such studies, currently underway, will further explore the host range of hantaviruses.

3 Geographic Distribution

Hantaviruses have now been identified in rodents, shrews, moles, and bats from widely separated geographic regions. For rodents and shrews, hantaviruses have been found in members of multiple species in four continents. Although far from comprehensive, the geographic distribution of hantaviruses is shown in Table 9.5, and the geographic origins of hantaviruses detected in shrews, moles, and bats are shown in Figs. 9.2, 9.3, and 9.4. The hantaviruses in South America have been

Table 9.5 Geographic distribution of rodent-, shrew-, mole-, and bat-borne hantaviruses[a]

		Hantaviruses in			
Continent	Country	Rodent	Shrew	Mole	Bat
Asia	Cambodia	SEOV, THAIV			
	China	AMRV, DBSV, HTNV, KHAV, LUXV, PUUV, SEOV	CBNV, MJNV, QHSV, TPMV, YAKV	DHCV	HUPV, LQUV
	India	SEOV	TPMV		
	Indonesia	SEOV, THAIV	TPMV		
	Japan	HOKV, SEOV	SRFV	ASAV	
	Korea	HTNV, MUJV, SEOV, SOOV	JJUV, MJNV		
	Mongolia		SWSV		
	Nepal		TPMV		
	Russia	AMRV, DOBV, KHAV, PUUV, SEOV, TULV, VLAV	KKMV, MGAV, SWSV		
	Singapore	SEOV, THAIV			
	Taiwan	SEOV	XYIV		
	Thailand	SEOV, THAIV			
	Vietnam	SEOV	CBNV, TPMV		XSV

(continued)

Table 9.5 (continued)

		Hantaviruses in			
Continent	Country	Rodent	Shrew	Mole	Bat
Europe	Austria	PUUV, TULV	SWSV		
	Belgium	PUUV, SEOV			
	Czech Republic	DOBV, PUUV, TULV	ASIV, SWSV		
	Finland	PUUV	ASIV, SWSV		
	France	PUUV, SEOV, TULV		NVAV	
	Germany	DOBV, PUUV, TULV	ASIV, SWSV		
	Hungary	DOBV, PUUV, TULV	SWSV	NVAV	
	Poland	DOBV, PUUV, TULV	BOGV, SWSV	NVAV	
	Serbia	DOBV, PUUV, SEOV, TULV			
	Slovakia	DOBV, PUUV, TULV	SWSV		
	Slovenia	DOBV, PUUV, SEOV, TULV	SWSV		
	Switzerland	TULV	SWSV		
	UK	SEOV, TATV			
Africa	Cote d'Ivoire		AZGV		MOYV
	Ethiopia	TIGV			
	Gabon				MAKV
	Guinea	SANGV	BOWV, TGNV		
	Madagascar	THAIV			
	Sierra Leone				MGBV
	Tanzania		ULUV, KMJV		
North America	USA	BAYV, BCCV, BLLV, EMCV, ISLAV, MULV, NYV, PHV, SEOV, SNV	AMNV, ARRV, JMSV, RPLV	OXBV, RKPV	
	Canada	SNV	JMSV		

[a]This table is not meant to be exhaustive. For example, the hantaviruses in South America are not listed because reservoir hosts other than rodents are not known

Rodent-borne hantaviruses: *AMRV*, Amur virus; *BAYV*, Bayou virus; *BCCV*, Black Creek Canal virus; *BLLV*, Bloodland Lake virus; *DBSV*, Da Bie Shan virus; *DOBV*, Dobrava virus; *EMCV*, El Moro Canyon virus; *HTNV*, Hantaan virus; *HOKV*, Hokkaido virus; *ISLAV*, Isla Vista virus; *KHAV*, Khabarovsk virus; *LUXV*, Luxi virus; *MULV*, Muleshoe virus; *MUJV*, Muju virus; *NYV*, New York virus; *PHV*, Prospect Hill virus; *PUUV*, Puumala virus; *SANGV*, Sangassou virus; *SEOV*, Seoul virus; *SNV*, Sin Nombre virus; *SOOV*, Soochong virus; *TATV*, Tatenale virus; *THAIV*, Thailand virus; *TIGV*, Tigray virus; *TULV*, Tula virus; *VLAV*, Vladivostok virus. Several rodent-borne hantaviruses in North America, such as Blue River virus and Limestone Canyon virus, detected in *Peromyscus leucopus* and *Peromyscus boylii*, respectively, are not listed

Shrew-borne hantaviruses: *AMNV*, Iamonia virus; *ARRV*, Ash River virus; *ASIV*, Asikkala virus; *AZGV*, Azagny virus; *BOGV*, Boginia virus; *BOWV*, Bowé virus; *CBNV*, Cao Bang virus; *JJUV*, Jeju virus; *JMSV*, Jemez Springs virus; *KMJV*, Kilimanjaro virus; *MGAV*, Amga virus; *MJNV*, Imjin virus; *QHSV*, Qian Hu Shan virus; *RPLV*, Camp Ripley virus; *SRFV*, Sarufutsu virus; *SWSV*, Seewis virus; *TGNV*, Tanganya virus; *TPMV*, Thottapalayam virus; *ULUV*, Uluguru virus; *YAKV*, Yakeshi virus

Mole-borne hantaviruses: *ASAV*, Asama virus; *DHCV*, Dahonggou Creek virus; *NVAV*, Nova virus; *OXBV*, Oxbow virus; *RKPV*, Rockport virus

Bat-borne hantaviruses: *HUPV*, Huangpi virus; *LQUV*, Longquan virus; *MAKV*, Makokou virus; *MGBV*, Magboi virus; *MOYV*, Mouyassué virus; *XSV*, Xuan Son virus

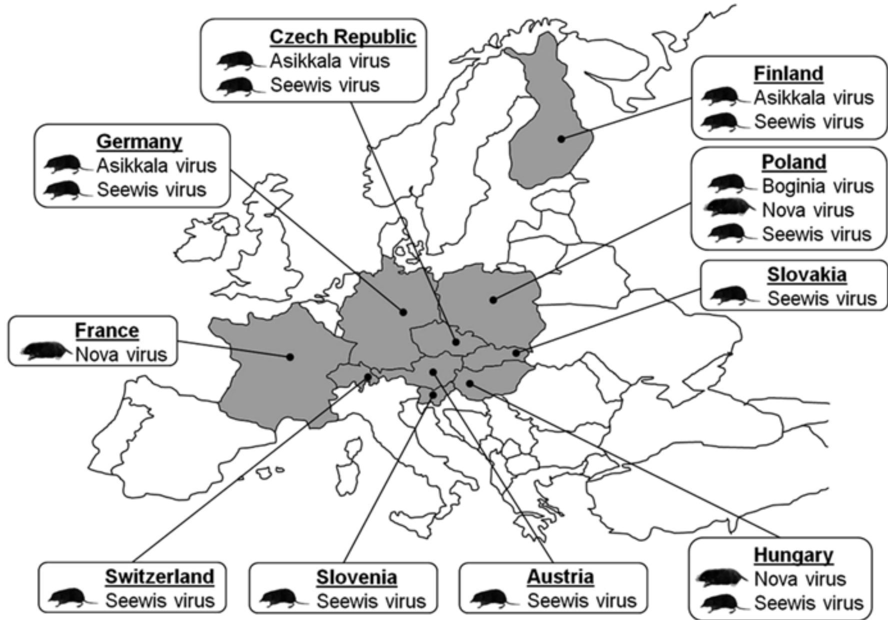

Fig. 9.1 Map of Europe, showing the countries where shrew- and mole-borne hantaviruses have been found. Table 9.5 provides a list of the hantaviruses harbored by rodents, shrews and moles in Europe

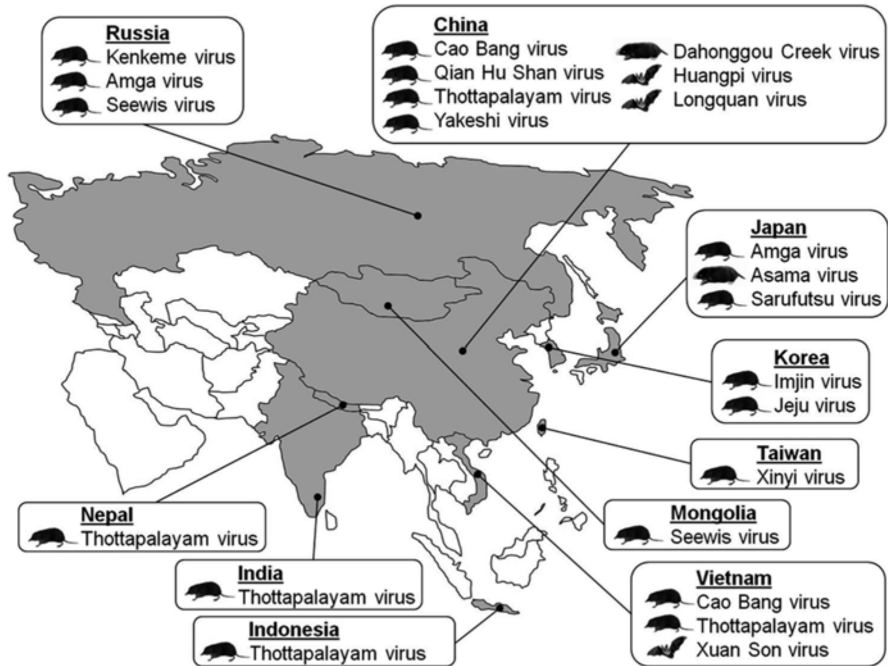

Fig. 9.2 Map of Asia, showing the countries where shrew-, mole-, and bat-borne hantaviruses have been found. Table 9.5 provides a list of the hantaviruses harbored by rodents, shrews, moles, and bats in Asia

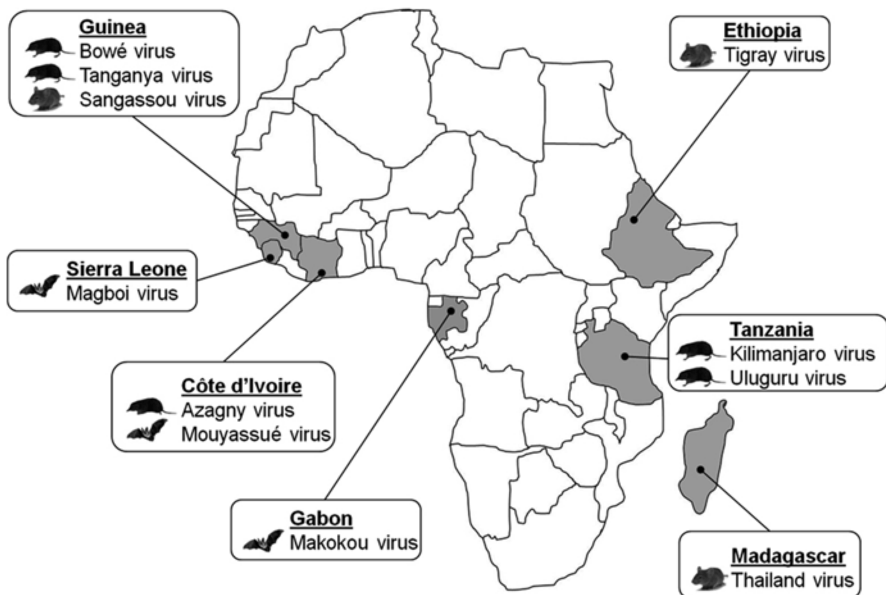

Fig. 9.3 Map of Africa, showing the countries where shrew- and bat-borne hantaviruses have been found. Table 9.5 provides a list of the hantaviruses harbored by rodents, shrews and bats in Africa

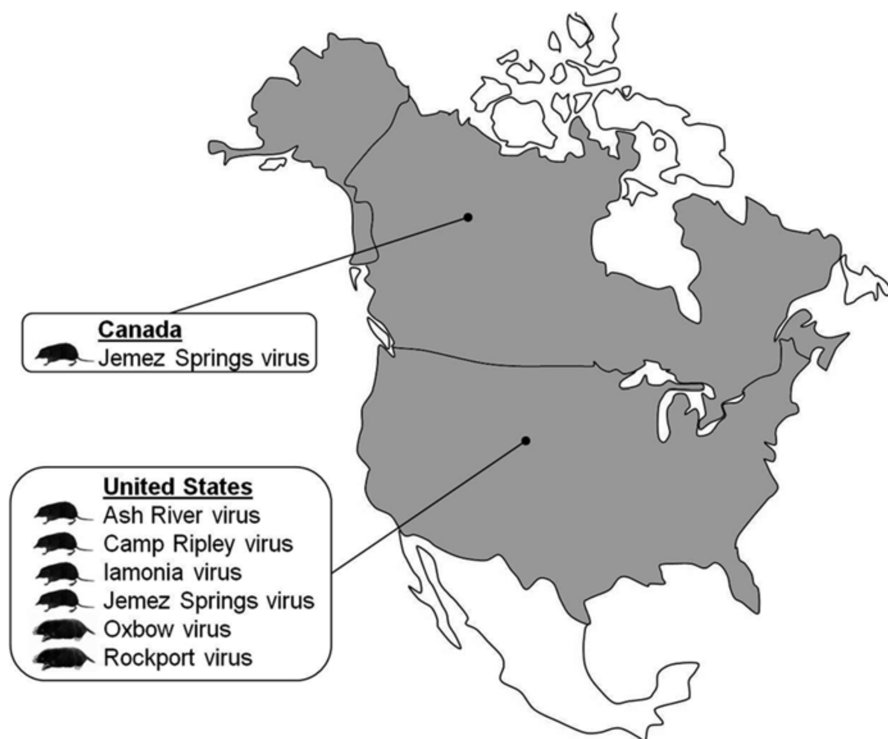

Fig. 9.4 Map of North America, showing the countries where shrew- and mole-borne hantaviruses have been found. Table 9.5 provides a list of the hantaviruses harbored by rodents, shrews and moles in North America

excluded intentionally because hosts other than rodents have not been identified. Similarly, countries in which only SEOV has been detected in rats are not included, in part because of their nearly global distribution, possibly accounted for by international shipping. The distribution of the reservoir host may also result from intentional anthropogenic activities. For example, it is highly likely that the present-day distribution of the Asian house shrew is due to human migration (S.D. Ohdachi, personal communication).

Of the 33 genetically distinct hantaviruses identified in shrews, moles, and bats (Tables 9.2, 9.3, and 9.4), each differs from known hantaviruses by more than 7 % in the amino acid sequence of the S segment-encoded nucleocapsid protein, suggesting that they may all represent new hantavirus species. However, in the absence of virus isolates in tissue culture, all of the current criteria mandated by the International Committee on Taxonomy of Viruses (ICTV) [116] cannot be met. Nevertheless, assuming for the moment that the 22 hantaviruses in shrews (Table 9.2), five in moles (Table 9.3), and six in bats (Table 9.4) represent distinct species, we can make the following observations: the preponderance of 15 hantaviruses in eulipotyphlya and chiropterans from Asia [25, 73, 88–92, 96, 103, 105, 106, 112] (Fig. 9.2), compared to the comparatively lower number of four from Europe [83, 94, 104, 108] (Fig. 9.1), eight from Africa [95, 97, 98, 102, 111, 113, 114] (Fig. 9.3), and six from North America [87, 93, 107, 109] (Fig. 9.4), and the far greater genetic diversity of hantaviruses hosted by Asian eulipotyphla and chiropterans and their basal positions in phylogenetic trees suggest that hantaviruses originated in Asia [22, 95]. An Asian origin was similarly concluded following an analysis of 190 S-segment sequences of rodent-borne hantaviruses, found in 30 countries during 1985–2010, retrieved from GenBank [117].

Previously, geographic-specific genetic variation has been demonstrated for HTNV in the striped field mouse [118], Soochong virus (SOOV) in the Korean field mouse (*Apodemus peninsulae*) [119], PUUV in the bank vole [120–124], MUJV in the royal vole [42, 43], TULV in the European common vole [32, 125], and ANDV in the long-tailed colilargo [23, 126]. Similarly, phylogenetic analyses show that hantaviruses harbored by shrews [84, 85, 99] and moles [86, 110] segregate along geographically specific lineages, suggesting long-standing associations between hantaviruses and their reservoir eulipotyphlan hosts.

While long suspected, novel hantaviruses have only recently been detected in rodents [24, 127] and shrews [95, 97, 98, 102], as well as insectivorous bats [111, 113, 114], in sub-Saharan Africa (Table 9.5 and Fig. 9.3). Notably, the five hantaviruses detected in African shrews and three detected in African bats, compared to only two hantaviruses reported from African rodents, despite the testing of tissues from many more rodents than shrews or bats, suggest that rodents may not have been the primordial mammalian hosts of ancestral hantaviruses [21, 22]. It is very probable that many more hantaviruses are extant in Africa, where unique lineages of shrews have diversified and evolved [95, 97, 98, 102]. Thus, more intensified investigations are warranted, not only in well-recognized biodiversity hotspots in West Africa but also in less-studied savannah and desert biomes.

4 Hantavirus Evolution

Before discussing the evolutionary dynamics of hantaviruses, it needs to be made clear that, while the newfound hantaviruses in shrews, moles, and bats are undoubtedly viruses, this does not infer that they have been adopted by the ICTV as hantavirus species (116). In fact, almost none of these viruses have been isolated in cell culture and their existence is inferred from partial or whole genome sequences. However, as evidenced by the extent of amino acid sequence differences observed compared to ICTV-classified hantaviruses and their unique ecological niches, it is likely that most of these newly reported hantaviruses will prove to be distinct hantavirus species.

Currently, the genomic database comprises sequences for 33 genetically distinct hantaviruses hosted by shrews, moles, and bats (Tables 9.2, 9.3, and 9.4). Whole genomes are available for only seven (BOWV, CBNV, JJUV, MJNV, RKPV, TPMV, YAKV), and full-length S-segment sequences have been completed for 20. None of the bat-borne hantaviruses have been fully sequenced, and full-length M-segment sequences are generally lacking. The paucity of whole-genome sequences of the newfound eulipotyphla- and chiroptera-borne hantaviruses has greatly hampered attempts at clarifying their evolutionary origins and phylogeography [21, 22]. And thus far, efforts at employing next-generation sequencing technology have been largely unsuccessful, primarily because of the limited availability of tissues and poor-quality of tissue RNA.

Phylogenetic analysis, based on partial or full genome sequences of all three segments, results in trees consisting of four distinct clades (Fig. 9.5). One clade comprises hantaviruses harbored by rodents of the Muridae family; a second by hantaviruses hosted by rodents of the Cricetidae family; a third by hantaviruses in eulipotyphlans of the Soricidae family; and a fourth by hantaviruses harbored by talpid moles (Talpinae subfamily) and insectivorous bats, which represent the most divergent hantaviruses found to date (Fig. 9.5). Eulipotyphla-borne hantaviruses are divided into two phylogenetic lineages: one that is paraphyletic with murid rodent-borne hantaviruses, includes soricine and crocidurine shrew-borne hantaviruses, and two hantaviruses carried by shrew moles (ASAV and OXBV); the other lineage includes TPMV and MJNV, two crocidurine shrew-associated hantaviruses that are phylogenetically more closely related to bat-borne hantaviruses (HUPV, LQUV, MGBV, MOYV, XSV).

Previously, the segregation of hantaviruses into clades that paralleled the molecular phylogeny of their rodent hosts in the Murinae, Arvicolinae, Neotominae, and Sigmodontinae subfamilies suggested the concept of co-divergence [128]. Recently, this concept has been challenged on the basis of the disjunction between the evolutionary rates of the hosts and viruses. Preferential host switching and local host-specific adaptation have been proposed to account for the largely congruent phylogenies [129]. However, host-switching events alone do not completely explain the coexistence and distribution of genetically distinct hantaviruses among hosts of different species in three divergent taxonomic orders of small mammals spanning across four continents [108]. Moreover, phylogenetic trees reconstructed for co-phylogeny mapping, using consensus topologies based on amino acid sequences

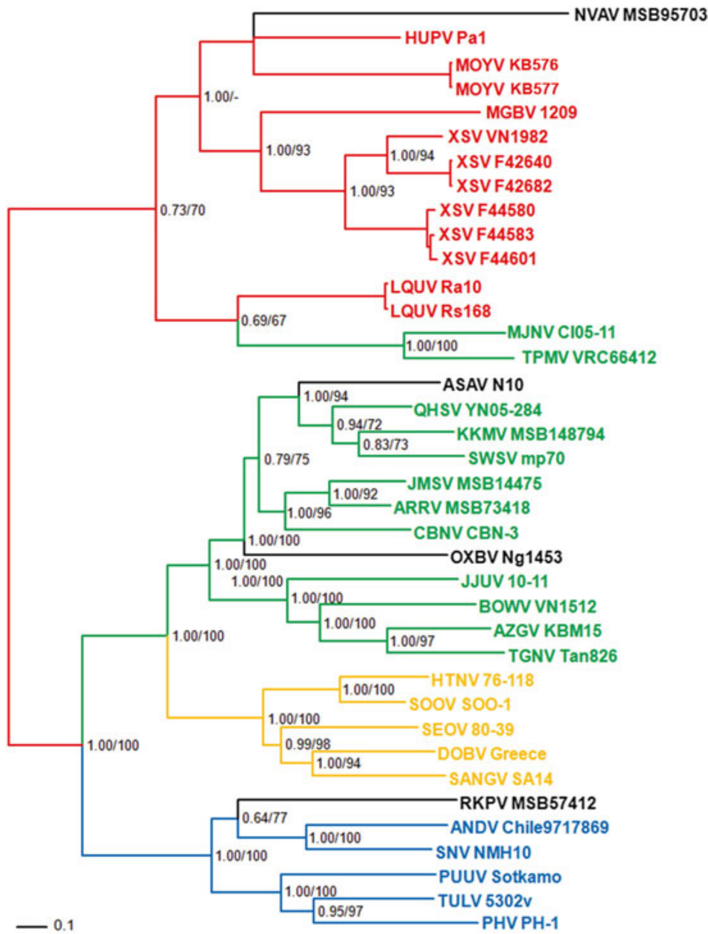

Fig. 9.5 Phylogenetic tree generated by maximum-likelihood and Bayesian methods, based on the alignment of the L-segment sequences of hantaviruses. The phylogenetic positions of Xuan Son virus (XSV) and Mouyassué virus (MOYV) are shown in relationship to other bat-borne hantaviruses (shown in *red*), including Magboi virus (MGBV), Longquan virus (LQUV) and Huangpi virus (HUPV), and representative shrew-borne hantaviruses (shown in *green*), including Thottapalayam virus (TPMV VRC66412), Imjin virus (MJNV Cl05-11), Seewis virus (SWSV mp70), Kenkeme virus (KKMV MSB148794), Cao Bang virus (CBNV CBN-3), Ash River virus (ARRV MSB 73418), Jemez Springs virus (JMSV MSB144475), Qian Hu Shan virus (QHSV YN05-284), Tanganya virus (TGNV Tan826), Azagny virus (AZGV KBM15), Jeju virus (JJUV 10-11), Bowé virus (BOWV VN1512); mole-borne hantaviruses (shown in *black*), including Asama virus (ASAV N10), Oxbow virus (OXBV Ng1453), Nova virus (NVAV MSB95703), and Rockport virus (RKPV MSB57412). Also shown are representative Murinae rodent-borne hantaviruses (shown in *orange*), including Hantaan virus (HTNV 76-118), Soochong virus (SOOV SOO-1), Dobrava virus (DOBV Greece), Seoul virus (SEOV 80-39), and Sangassou virus (SANG SA14); Arvicolinae rodent-borne hantaviruses (shown in *blue*), including Tula virus (TULV M5302v), Puumala virus (PUUV Sotkamo), and Prospect Hill virus (PHV PH-1); and Neotominae and Sigmodontinae rodent-borne hantaviruses (shown in *blue*), Sin Nombre virus (SNV NMH10) and Andes virus (ANDV Chile9717869). The numbers at each node are posterior node probabilities (*left*) based on 150,000 trees and bootstrap values (*right*) based 1,000 replicates executed on the RAxML BlackBox web server, respectively. The scale bar indicates nucleotide substitutions per site

of the nucleocapsid protein, Gn and Gc glycoproteins and RNA-dependent RNA-polymerase, exhibited congruent segregation of hantaviruses according to the subfamily of their eulipotyphlan reservoir hosts, with no evidence of host switching except for two hantaviruses carried by shrew moles [107].

Host-switching events in hantavirus evolution have been documented between hosts of the same family (Soricidae and Soricidae), of different families (Soricidae and Talpidae) and of separate orders (Eulipotyphla and Rodentia) [103, 106, 107]. The importance of such virus-host switching lies in the possible emergence of disease-causing hantaviruses. The close association between distinct hantaviruses and specific rodents, shrews, and moles probably resulted from alternating and periodic episodes of host/pathogen co-divergence through deep evolutionary time [95]. That is, as evidenced by the overall congruence between the phylogenies of hantavirus genes and their rodent and eulipotyphlan hosts, hantaviruses have likely co-diverged with specific reservoir hosts during part of their evolutionary history [108, 109].

5 Hantaviral Diseases

In a now classic volume, published in 1953, Gajdusek conjectured that Korean hemorrhagic fever in Asia and nephropathia epidemica in Scandinavia, while occurring in different geographic locations and exhibiting differential clinical severity, were manifestations of the same disease and were caused by the same virus or closely related viruses [1]. This conjecture, made more than a decade before the discovery of HTNV, was verified shortly after the isolation of HTNV in cell culture [130–133]. And while the literature contains more than 150 synonyms for this clinical syndrome, the designation of HFRS has been dominant since the isolation of HTNV. With the advent of HCPS, as a disease with predominantly cardiac and pulmonary involvement, the conventional view was that of two clinically distinguishable syndromes caused by hantaviruses harbored by rodents belonging to different rodent subfamilies in the Old and New Worlds. That is, HFRS was caused by hantaviruses carried by rodents of the Murinae and Arvicolinae subfamilies, while hantaviruses hosted by rodents in the Neotominae and Sigmondontinae subfamilies caused HCPS.

This tidy trans-Atlantic classification may have outlived its usefulness and is being subjected increasingly to intense scrutiny, particularly as clinicians in both the Old and New Worlds encounter cases of HFRS which lack renal involvement but exhibit prominent cardiopulmonary features, and conversely as cases of HCPS with renal insufficiency but without pulmonary involvement are documented [51, 134–138]. Once downplayed or sometimes intentionally ignored, the considerable overlap between HFRS and HCPS is challenging the long-accepted distinction of two separate clinical syndromes. A proposed nosology would entail the moniker “hantavirus fever” [51, 139]. Much more discussion is obviously needed for ultimate consensus and adoption, but this particular name might not necessarily solve the current conundrum. For instance, some diseases, caused by arboviruses, such as dengue

fever and West Nile fever, typically refer to the milder, non-life-threatening clinical forms of infections with dengue and West Nile viruses. For patients with clinically severe diseases with either flavivirus, different names are typically used, such as dengue hemorrhagic fever and dengue shock syndrome, or West Nile virus meningoencephalitis, respectively. For dengue, the World Health Organization (WHO) has recently issued revised guidelines for classifying dengue virus-infected patients, based on clinical severity and laboratory tests [140], into three levels: dengue; dengue with warning signs; and severe dengue.

Although there is no unanimity of opinion in accepting the new WHO guidelines for dengue [141, 142], a similar nosological approach may be contemplated for hantavirus-infected patients: namely, hantavirus fever; hantavirus fever with warning signs; and severe hantavirus fever. Irrespective of the resultant new classification, however, it is imperative that the guidelines are concise, clearly stated, easily implemented and relevant to the diagnosis and clinical management of patients with hantavirus disease. As with dengue, a list of warning signs to alert physicians to better identify severe cases, or potentially severe cases, and to make appropriate changes in clinical care, especially in resource-poor settings, would be valuable.

5.1 HFRS and HCPS

Outbreaks of HFRS usually follow encroachment of rodent habitats or irruptions of reservoir rodent populations with subsequent invasion of human dwellings. The respiratory droplet route of aerosolized rodent excreta constitutes the principal mode of viral transmission to humans [3, 29]. Humans infected with pathogenic hantaviruses usually develop mild to severe clinical disease, but subclinical infection also occurs to varying degrees depending on the hantavirus. In Scandinavia, HFRS is often still referred to as nephropathia epidemica, which, while usually mild, may run a more fulminant course [3]. Inapparent or subclinical hantavirus infection is not uncommon, depending on the particular virus, as with Choclo and Calabazo viruses on the Azuero peninsula of Panama [143]. Human population-based serosurveys in HFRS- and HCPS-endemic geographic areas indicate low (<1–5 %) to very high (>30 %) prevalences of anti-hantavirus antibodies [143–145]. Infections among children are uncommon, and seroprevalence tends to increase with age.

Vascular leak, or increased endothelial permeability, is the principal pathophysiological feature of severe HFRS and HCPS. The principal symptoms and clinical features of both syndromes include high fever, chills, headache, generalized myalgia, abdominal pain, and nausea and vomiting. In the classical descriptions of HFRS, five distinct phases were described [3, 29, 63, 146]. Febrile phase, which begins abruptly; hypotensive phase, on the fifth day of illness; oliguric phase, on the ninth day of illness, with associated thrombocytopenia, proteinuria, hemorrhage and plasma leakage; diuretic phase, usually between days 12 and 14; and convalescent phase, which is gradual over several months. Depending on the severity of disease, not all HFRS patients exhibit all phases, or the phases may overlap [147]. The early

stage of HCPS, which resembles the febrile phase of HFRS, is somewhat nondescript and can be easily confused with other, more common, acute-onset febrile infectious diseases. But at 4–10 days after the onset of illness, HCPS patients experience rapidly progressive respiratory distress, characterized by dry cough and extreme shortness of breath, or dyspnea [4, 148–150]. Multivariate analysis showed that the clinical features of dizziness, nausea and vomiting and absence of cough at the time of hospital admission, and the initial laboratory abnormalities of thrombocytopenia, low serum bicarbonate level and elevated hematocrit served to identify HCPS patients [151].

The clinical management of HFRS and HCPS is largely supportive, with careful fluid management and monitoring of cardiopulmonary and/or renal function, administered in an intensive care hospital setting. Dialysis may be required for some patients with severe HFRS. For HCPS patients, mechanical ventilation is frequently required, and other life-saving measures, such as extracorporeal membrane oxygenation, may be necessary [4, 152]. The use of antiviral drugs is uncommon, despite the significant benefit from intravenous ribavirin, as demonstrated in a prospective, randomized, double-blind, placebo-controlled clinical trial involving 242 patients with serologically confirmed HFRS in China [153]. In a subsequent study, intravenous ribavirin significantly reduced the occurrence of oliguria and the severity of renal insufficiency in HFRS patients [154]. Similarly well-controlled trials of intravenous ribavirin in HCPS have not been conducted. However, because of the lack of clinical benefit in an open-label trial of ribavirin, conducted during the 1993 HCPS outbreak, a trial which was not designed to assess efficacy [155], and the partial results from a placebo-controlled, double-blind trial that was prematurely terminated because of inadequate patient accrual [156], ribavirin is currently not recommended in the treatment of HCPS or available for this use under any existing research protocol. Recent findings from *in vivo* studies in the Syrian hamster HCPS model, indicating that ribavirin provides effective post-exposure prophylaxis against HCPS-causing ANDV infection [157, 158], should prompt serious reconsideration of the current, possibly unjustified verdict against the use of ribavirin in HCPS. This is more than an academic issue, for while the lethality of HFRS ranges from <1 % to more than 20 % [3, 63], the lethality of HCPS is much higher, ranging from 30 to 50 % or more in the Americas [148–150]. As such, adjunct therapy with ribavirin, or other newly developed antiviral compound, could potentially reduce the number of HCPS-related deaths. A well-designed, properly controlled and sufficiently powered clinical trial of intravenous ribavirin for HCPS should be conducted in South America, where more than 4,000 HCPS cases have been diagnosed up until 2013 [150].

A fundamental epidemiological factor in HFRS and HCPS is exposure to rodent-infested habitats. Seemingly trivial exposure to environments contaminated with rodent excretions can lead to infection and disease. On the other hand, the intimate handling of rodents does not necessarily constitute sufficient exposure. Thus, although individuals, such as mammalogists, who have frequent occupational contact with rodents, are presumed at increased risk to rodent-borne pathogens, several studies have indicated insignificant prevalence of hantavirus infections [159–162]. This has been corroborated in a recent study, in which only four of 757 persons who had handled neotomine or sigmodontine rodents in North America exhibited serum

IgG antibodies against SNV [163]. Also, during the height of the HCPS outbreak in the Four Corners region, forest and park service personnel showed no evidence of SNV infection [164]. By contrast, studies in Eurasia show clear associations between hantavirus infection and exposure to rodent excreta among certain high-risk occupation groups, such as animal trappers, forestry workers and farmers [165–167], and individuals, such as hunters, whose recreational activities encroach on wildlife habitats [168].

No evidence of SNV or ANDV transmission was found among health-care workers exposed to patients with confirmed HCPS [169, 170]. Similarly, there are no reports of hantavirus transmission from HFRS patients to physicians or medical personnel or to family members. On the other hand, there are well-substantiated reports of person-to-person transmission of ANDV in Argentina and Chile [171–174]. In a study of household contacts of persons with HCPS in Chile, the risk was highest among sex partners [174]. Also, epidemiological data suggest that prolonged and close contact with HCPS patients during the prodromal phase of disease, before patients seek medical attention, may constitute the period of increased risk [173].

5.2 *Identifying and Investigating Previously Unrecognized Hantaviral Diseases*

Not all orphan viruses, or viruses in search of diseases, warrant investigations to ascertain their pathogenic potential at the time of discovery. However, selected viruses, particularly those related to viruses known to cause severe and life-threatening diseases, such as HFRS and HCPS, are worthy of high research priority. No one would have predicted that rodent-borne viruses, previously known to cause acute renal insufficiency with varying degrees of hemorrhage and shock, would also cause an acute respiratory disease. The realization that rodent-borne hantaviruses are capable of causing HFRS and HCPS raises the possibility that soricid-associated hantaviruses may similarly cause a wide spectrum of febrile illnesses. In this regard, prospective studies of neotomine and sigmodontine rodent-borne hantaviruses in the early 1980s might have provided important clues about their pathogenicity long before the recognition of HCPS in 1993. In much the same way, one or more of the newly identified soricid-borne hantaviruses may cause outbreaks of human disease and/or serve as surrogate antigens for the diagnosis of previously unrecognized hantaviral diseases. Robust serological assays and other sensitive technologies, now under development, will assist in establishing if these newest members of the *Hantavirus* genus are pathogenic for humans. Also, studies on the genetics, transmission dynamics and disease-causing potential of one or more of the newly identified hantaviruses in shrews, moles, and insectivorous bats, as well as African rodents, may better prepare the next generation of health-care workers before the next newly recognized hantaviral disease.

By focusing too heavily on the syndromic features of renal and/or cardiopulmonary dysfunction, the full spectrum of hantavirus disease may be obscured or missed. Possibly, a detailed examination of atypical cases of HFRS and HCPS may provide

clues about other previously unrecognized diseases caused by hantaviruses, particularly those newly discovered in shrews, moles, and bats. In this regard, before the recognition of HCPS, serological surveys were conducted for evidence of hantavirus infection among patients with fever of unknown etiology in the USA, including individuals with pneumonia, rickettsial-like illnesses and leptospirosis-negative tests [175]. However, as in any serosurvey, one can be misled into thinking that an orphan virus is nonpathogenic if the 'wrong' patient groups are studied. In the case of HCPS, only HCPS patients had evidence of SNV infection.

5.2.1 In Search of SANGV Infection and Disease

As summarized recently, many thousands of sera from randomly selected human populations in Algeria, Benin, Burkino Faso, Cameroon, Central African Republic, Chad, Djibouti, Egypt, Gabon, Nigeria, Senegal, and countries in South Africa have been tested for evidence of hantavirus infection [176]. In all such studies across the African continent, IgG antibodies against HTNV, and occasionally SEOV, PUUV, or PHV, were sought, using either enzyme-linked immunosorbent assay (ELISA) or immunofluorescent antibody test (IFA). Because confirmatory tests were not employed in nearly all of these studies, the reported seroprevalences, which ranged from 0.2 to 17 %, cannot be interpreted [162]. With the recent detection of rodent- and shrew-borne hantaviruses in both West and East Africa, and with improvements in serological testing, more accurate information about the true burden of hantavirus infection and disease in humans may be within reach.

In large part, this is being made possible by SANGV, which is the first hantavirus discovered in the African wood mouse in sub-Saharan Africa [24] and the only African hantavirus isolated in cell culture [177]. The whole genome of SANGV has been sequenced and studies indicate that SANGV uses β(1) integrin as a cell-entry receptor [177]. Previously, pathogenic hantaviruses, which cause HFRS (HTNV, SEOV, PUUV, DOBV) and HCPS (SNV, NYV), have been shown to utilize αvβ3 integrin for cell entry, compared to nonpathogenic hantaviruses (PHV) which use β1 integrin [178–181]. β1 integrin usage would suggest that SANGV is nonpathogenic. Nevertheless, detailed serological surveys have been conducted to ascertain if SANGV causes human infection and disease.

In analyzing 717 serum specimens from inhabitants of 29 villages in Forest Guinea (including 68 samples from residents of Sangassou village) by ELISA, with confirmation by IFA, western blot (WB), and focus-reduction neutralization test (FRNT), Klempa and colleagues found approximately 1 % of tested individuals to be antibody positive [182]. Also, in a separate study of 253 sera from residents of Upper Guinea [183] and in a survey of 1,442 samples from the Republic of South Africa [176], the seroprevalence was 1 %. However, the prevalence was much higher (4.4 %) among 68 patients from Sangassou village, who had fever of unknown origin [183]. Two of the three seropositive children had neutralizing antibodies against SANGV and had an illness compatible with HFRS [183]. Although HFRS is usually uncommon in children [184–187], SANGV may differ in this regard from other HFRS-causing

hantaviruses. Alternatively, the selection of febrile study participants in Sangassou village might have skewed the findings.

5.2.2 In Search of MJNV Infection and Disease

The isolation of MJNV from the Ussuri white-toothed shrew also raised questions about its pathogenic potential. From one standpoint, however, the objective of demonstrating MJNV infection in humans might be considered ill conceived for the simple reason that shrew populations are generally much smaller than rodent populations, making the probability of contact between humans and shrews (and their excretions) extremely low. Also, the Ussuri white-toothed shrew is not found in peri-domestic habitats, unlike the Asian house shrew, which carries a closely related hantavirus known as TPMV, making even less likely exposure to MJNV-infected fomites. While this line of thinking is logical, zoonotic microbes, in general, tend to rarely infect humans, but they are nevertheless of significant medical importance. In this regard, HCPS itself is a rare disease. And quite likely, in the absence of an outbreak of human disease caused by MJNV, one would be looking for such a rare event. Placed in proper perspective, therefore, HCPS would have probably gone unnoticed, had cases not clustered in time and space and had a closely knit group of dedicated and astute health-care workers not recognized that something very unusual was happening.

Our search for evidence of MJNV infection was focused almost entirely on patients with acute febrile illnesses, and in whom other zoonotic infectious diseases (such as leptospirosis, scrub typhus, murine typhus and HFRS caused by HTNV and SEOV) had been ruled out. A summary of the study populations, comprising 2,800 participants, is shown in Table 9.6. Acute-phase sera from clinic and hospitalized patients, as well as sera from individuals with HFRS-like symptoms, were screened

Table 9.6 Serological survey of MJNV infection

	Serum	ELISA MJNV		IFA				
Study Population	Tested	IgM+	IgG+	MJNV+	TPMV+	RT-PCR +	WB +	PRNT +
Paju Adult and Pediatric Clinic	52	0	ND	0	0	ND	ND	ND
Guro Hospital	327	1	ND	3	2	0	ND	ND
HFRS-like disease 2003	593	2	ND	2	0	ND	ND	ND
HFRS-like disease 2004	1074	0	ND	7	7	0	ND	ND
HFRS-like disease 2006	656	5	2	6	3	0	3	0
HFRS-like disease 2011	30	0	ND	0	0	ND	ND	ND

Abbreviations: *HFRS*, hemorrhagic fever with renal syndrome; *IFA*, indirect immunofluorescence antibody test; *IgG*, immunoglobulin G; *IgM*, immunoglobulin M; *MJNV*, Imjin virus; *ND* = test not done; *PRNT*, plaque-reduction neutralization test; *RT-PCR*, reverse transcription polymerase chain reaction; *TPMV*, Thottapalayam virus; *WB*, western blot

Table 9.7 Serological testing of individuals with suspected MJNV infection

Study group	Patient	Age	ELISA MJNV		IFA		RT-PCR				
		Sex	IgM	IgG	MJNV	TPMV	L	M	S	WB	PRNT
Guro Hospital	1	37 M	–	–	64	–	ND	–	ND	ND	ND
	2	24 M	200	–	–	–	ND	–	ND	ND	ND
	3	49 F	–	–	256	–	–	–	–	ND	ND
	4	58 F	–	–	32	–	–	–	–	ND	ND
HFRS-like disease 2003	5	79 F	–	ND	32	–	–	–	–	ND	ND
	6	69 F	–	ND	32	–	–	–	–	ND	ND
	7	40 M	400	ND	–	–	–	–	–	ND	ND
	8	56 M	400	ND	–	–	–	–	–	ND	ND
HFRS-like disease 2004	9	34 M	–	–	64	–	–	–	–	ND	ND
	10	35 M	–	–	128	–	ND	–	ND	ND	ND
	11	22 M	–	–	256	–	–	–	–	ND	ND
	12	35 M	–	–	128	–	–	–	–	ND	ND
	13	UNK	–	–	256	–	–	–	–	ND	ND
	14	80 M	–	–	128	–	–	–	–	ND	ND
	15	UNK	–	–	64	–	–	–	–	ND	ND
HFRS-like disease 2006	16	33 M	200	–	64	32	ND	–	ND	–	–
	17	36 M	400	–	–	32	ND	–	ND	–	–
	18	53 F	400	–	64	–	ND	–	ND	+	–
	19	26 M	800	–	256	–	ND	–	ND	–	–
	20	65 F	800	–	32	128	ND	–	ND	–	–
	21	UNK	–	400	128	–	ND	–	ND	+	–
	22	45 M	–	400	1024	–	ND	–	ND	+	–

Definitions: ELISA IgM and IgG: defined as <200; IFA MJNV and TPMV: defined as <32; PCR: defined as undetectable hantavirus RNA; WB: defined as <40; PRNT: defined as <40. ND = test not done

Abbreviations: *ELISA*, enzyme-linked immunosorbent assay; *IFA*, indirect immunofluorescence antibody test; *IgG*, immunoglobulin G; *IgM*, immunoglobulin M; *L*, L segment; *M,* M segment; *MJNV*, Imjin virus; *PRNT*, plaque-reduction neutralization test; *RT-PCR*, reverse transcription polymerase chain reaction; *S*, S segment; *TPMV*, Thottapalayam virus; *WB*, western blot

for IgM and IgG antibodies against MJNV by ELISA and IFA. Confirmatory tests included WB and PRNT, and sera from some suspect cases were tested by RT-PCR for MJNV RNA (Table 9.7). The test results of 22 study subjects with suggestive evidence of MJNV infection are shown in Table 9.7. Three patients with HFRS-like diseases had detectable antibodies to MJNV, as determined by ELISA, IFA and WB, but confirmation by PRNT was lacking. Overall, no serological evidence of MJNV infection was found.

An important shortcoming of any serological survey in search of a rare infectious event is the failure to recruit individuals who are affected by that rare event. On the one hand, the inability to find individuals with antibodies against MJNV may indicate that MJNV does not cause infection in humans. On the other hand, this same (negative)

result could mean that the study population simply failed to enroll subjects with MJNV infection. In other words, if MJNV infection is associated with a rare or uncommon disease, we would be unable to show infectivity in humans. In this regard, even at the height of the 1993 HCPS outbreak in the Four Corners region, no serological evidence of SNV infection could be found in patients with a variety of diseases or in health-care workers, parks service personnel and mammalogists. Only patients with HCPS had evidence of SNV infection. Thus, even with the most lethal of infectious agents, one would erroneously conclude that the microbe is nonpathogenic or noninfectious, if the "right" patients are not tested.

6 Concluding Remarks

With the expanded host diversity and geographic distribution of hantaviruses has come a reexamination of previously long-held dogma about the host range, evolutionary origins and phylogeography of hantaviruses. Many more hantaviruses, possibly some in hosts belonging to other taxonomic orders and in unanticipated geographic regions, await discovery. Textbook chapters on hantaviruses will also need to be rewritten, as more information becomes known about the emergence and pathogenic potential of newfound hantaviruses. In this regard, some of the uncertainties and conundrums in hantavirus research is a direct consequence of the dearth of full-length genomes and hantavirus isolates. In particular, nearly all of the newly identified hantaviruses in shrews, moles, and bats have yet to be isolated. In fact, to date, there are only two non-rodent-borne hantavirus isolates in cell culture. One is TPMV, the prototype shrew-borne hantavirus, isolated from the Asian house shrew [73, 74], and the other is MJNV, isolated from the Ussuri white-toothed shrew [25]. There are no hantavirus isolates from moles or bats (or other shrews). Virus isolates would dramatically accelerate the acquisition of whole genome sequences of recently discovered hantaviruses.

The isolation of hantaviruses, however, is fraught with difficulty, with numerous failed attempts. Recently, the isolation of HOKV was achieved only after establishing a cell line from the rodent reservoir, the gray red-backed vole [188]. Whether such strategies will prove helpful or become necessary for other hantaviruses hosted by shrews, moles, and bats is worthy of serious consideration. In any case, until such time that multiple non-rodent-borne hantaviruses are isolated in cell culture, the biology, taxonomy and pathogenicity of these newly identified hantaviruses will remain speculative at best. Thus, the road ahead, at the dawn of a new era in hantavirology, is laden with challenges, but also innumerable opportunities and unlimited possibilities. Many discoveries and giant leaps in newfound knowledge can be anticipated. Above all, strong partnerships between health-care providers, public health workers, veterinarians, mammalogists, ecologists, and pathologists will be vital for the identification and rapid diagnosis of previously unrecognized infectious diseases, caused by newfound hantaviruses and other vector-borne and zoonotic microbial agents [189].

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Chapter 10
Family *Bunyaviridae*

Chris A. Whitehouse, Jens H. Kuhn, Jiro Wada, and Koray Ergunay

Core Message The family *Bunyaviridae* is one of the largest and most diverse of the established viral families. Viruses within this family infect a wide range of organisms including invertebrates, vertebrates, and plants. Bunyaviruses are transmitted by mammals or arthropods, including ticks, mosquitoes, biting midges, sandflies, and thrips. Some viruses within this family are important pathogens causing encephalitis or hemorrhagic fever in humans, abortions in pregnant animals, or devastating disease in economically important plants.

1 Introduction

The large family *Bunyaviridae* includes more than 400 distinct members (bunyaviruses) that are grouped into five genera—*Hantavirus*, *Nairovirus*, *Orthobunyavirus*, *Phlebovirus*, and *Tospovirus*. Bunyaviruses possess a tripartite, single-stranded RNA genome that encodes four structural and, in some cases, one or two additional

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nonstructural proteins in a mostly negative-sense (i.e., anti-message) manner. Exemplifying the great diversity of the family *Bunyaviridae*, viruses in this family are known to infect invertebrates, vertebrates, and plants. Indeed, most viruses of the family are arthropod-borne, and various mosquitoes, ticks, biting flies, and thrips are vectors for transmission of orthobunyaviruses, nairoviruses, phleboviruses, and tospoviruses, respectively. Small mammals serve as amplifying hosts for most bunyaviruses, although some members (hantaviruses) use small mammal reservoirs and are transmitted via aerosolized excreta. Bunyaviruses have a worldwide geographic distribution, and some of these viruses are associated with significant human illnesses, including encephalitides and viral hemorrhagic fevers. Other bunyaviruses cause diseases in domestic animals and plants that are associated with significant economic impact.

2 History and Classification

Undoubtedly, many bunyaviral diseases were known long before viruses were discovered in the late nineteenth century. As far back as 960 A.D., the Chinese described illnesses closely resembling hemorrhagic fever with renal syndrome (HFRS) [1, 2], which is now known to be caused by certain hantaviruses. During the Napoleonic Wars (1803–1815), soldiers suffered from an epidemic of febrile illness, named Mediterranean fever that had symptoms similar to sandfly fever [3] that is caused by a phlebovirus. Sandfly fever was first clinically described by Alois Pick in 1886 in the Balkans region where the disease was prevalent in an endemic form [4].

In 1943, Smithburn and colleagues initially isolated Bunyamwera virus from *Aedes* mosquitoes during studies of yellow fever in Uganda [5] that is now classified in the genus *Orthobunyavirus*. Early classification of arboviruses (arthropod-borne viruses) relied mostly on serological relatedness, using methods such as complement fixation and neutralization tests. By 1960, eastern equine encephalitis virus, western equine encephalitis virus, and certain other viruses were known to be related to each other and were referred to as group A arboviruses (which are now classified in the genus *Alphavirus* in the family *Togaviridae*). Japanese encephalitis virus, yellow fever virus, St. Louis encephalitis virus, West Nile virus, and certain other viruses were also known to be related to each other and were referred to as group B arboviruses (which are now classified in the family *Flaviviridae*). Following the isolation of Bunyamwera virus, several other arboviruses were isolated that clearly did not fit into these two antigenic groups. These viruses were subsequently assigned to what became known as the group C arboviruses [6]. The family *Bunyaviridae* was formally established in 1975 to incorporate this group [7], and its members are now grouped into five genera—*Hantavirus*, *Nairovirus*, *Orthobunyavirus*, *Phlebovirus*, and *Tospovirus*. Viruses in all genera infect vertebrates, except for those in the genus *Tospovirus*, which infect plants [8].

3 Physical Properties

Bunyavirion morphology varies among viruses of each of the five genera; however, virions are generally spherical, 80–120 nm in diameter, and possess surface glycoprotein projections of 5–10 nm. These peplomers are embedded in a lipid bilayered envelope approximately 5 nm thick and are thought to mostly consist of heterodimers of the two viral glycoproteins (Fig. 10.1). The previous designations of these glycoproteins, G1 and G2, were based on their relative migration in polyacrylamide gels. Today, these proteins are referred to as Gn and Gc, referring to the amino-terminal and carboxy-terminal coding of the proteins [9]. Orthobunyavirions have surfaces covered with closely packed, knob-like morphologic units with no detectable order. Similarly, no obvious order is found for the small surface structures with central cavities observed on nairovirions [10]. In contrast, hantavirions have a surface structure that is distinctly ordered in a square grid-like pattern [10]. Phlebovirions have round, closely packed subunits approximately 10–11 nm in diameter with central cavities approximately 5 nm in diameter [10]. The appearances of tospovirions are similar to those of nairovirions in which, other than the presence of glycoprotein spikes, distinctive surface structures have not been observed.

Bunyavirions consist of 2 % RNA, 58 % protein, 33 % lipid, and 7 % carbohydrate (estimated for Uukuniemi virus of the genus *Phlebovirus*) and are sensitive to heat, lipid solvents, detergents, and formaldehyde [11]. Treatment with lipid solvents or detergents removes the viral envelope, resulting in loss of infectivity in arthropods and mammals [11]. Interestingly, however, the envelope is not required for viral entry or replication in plant cells of the plant-infecting tomato spotted wilt virus [12].

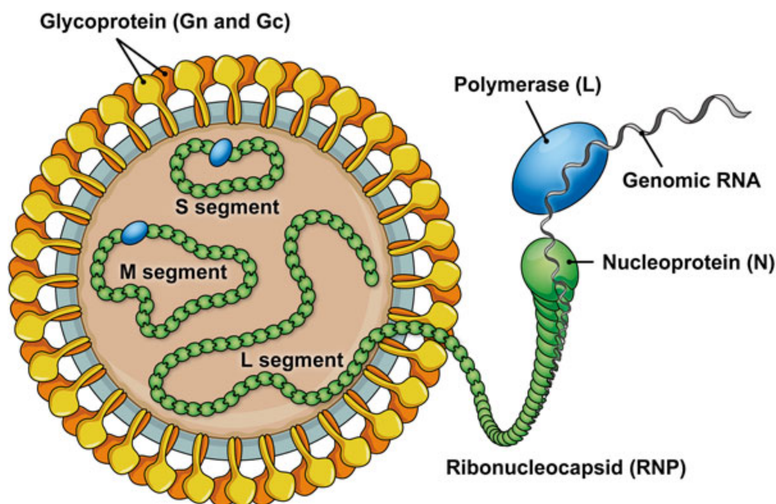

Fig. 10.1 Schematic enveloped, spherical bunyavirion, 80–120 nm diameter. Image courtesy of ViralZone, SIB Swiss Institute of Bioinformatics, with permission

4 Genome Structure

The bunyaviral genome comprises three segments of negative or ambisense single-stranded RNA designated as small (S), medium (M), and large (L). The lengths of the genomic segments vary among the genera, with the total genome lengths of approximately 11–19 kb (Table 10.1). The 3′ and 5′ terminal nucleotides of each genomic segment are highly conserved among viruses of a given genus, but differ among viruses of different genera (Table 10.2). Stretches of the 3′ and 5′ termini are complementary. Thus, the termini of each segment base-pair, forming noncovalently closed, panhandle-like RNAs. Direct support for base-pairing comes from electron microscopy studies of extracted RNA from Uukuniemi virions, in which three sizes of circular RNAs are evident [13].

5 Protein Coding and Viral Replication Strategies

The S, M, and L genome segments of all bunyaviruses encode a nucleocapsid protein (N), two envelope glycoproteins (Gn and Gc), and an RNA-dependent RNA polymerase (L), respectively (Fig. 10.2).

The S segment is approximately 1.0–3.0 kb and has one open reading frame (ORF) in the negative-sense orientation that codes for N. N is the most abundant component of virions and viral product in infected cells. N plays several important roles in viral replication, including protecting the RNA from degradation. The S segment of the orthobunyaviruses encodes for both the N protein and a nonstructural (NS)s protein in overlapping reading frames. Likewise, some hantavirus

Table 10.1 Approximate length (kb) of the genomic RNA segments of viruses belonging to the five genera included in the family *Bunyaviridae*

	Genus				
RNA segment	*Orthobunyavirus*	*Hantavirus*	*Nairovirus*	*Phlebovirus*	*Tospovirus*
S	1.0	1.7	1.7	1.7	2.9
M	4.5	3.9	4.9	3.2	4.8
L	6.9	6.5	12.2	6.4	8.9
Total	12.4	12.1	18.8	11.3	16.6

Table 10.2 Bunyavirus genus-specific consensus 3′ and 5′ terminal nucleotide sequences of the viral genomic RNAs

Orthobunyavirus	3′-UCAUCACAUGA……………………..UCGUGUGAUGA-5′
Hantavirus	3′-AUCAUCAUCUG…………………………AUGAUGAU-5′
Nairovirus	3′-AGAGUUUCU…………………………..AGAAACUCU-5′
Phlebovirus	3′-UGUGUUUC………………………………GAAACACA-5′
Tospovirus	3′-UCUCGUUA……………………………CUAACGAGA-5′

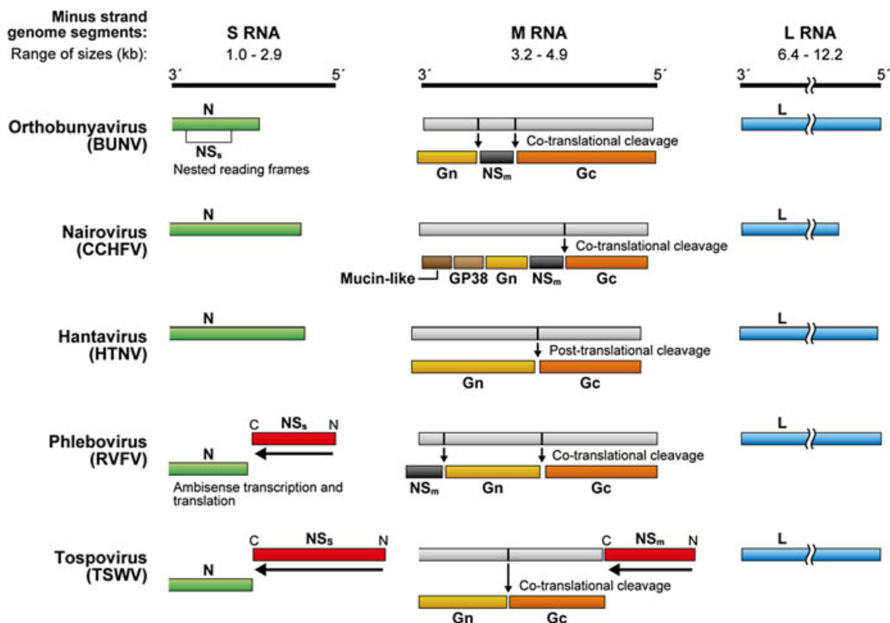

Fig. 10.2 Genome organization of viruses of the five bunyavirus genera. Structural proteins are N (nucleocapsid) and the two viral glycoproteins, Gn and Gc, named according to their proximity to the N or C termini of the precursor polyprotein, respectively. The L protein possesses RNA-dependent RNA polymerase activity. Virus abbreviations: *BUNV* Bunyamwera virus, *CCHFV* Crimean-Congo hemorrhagic fever virus, *HTNV* Hantaan virus, *RVFV* Rift Valley fever virus, *TSWV* tomato spotted wilt virus

genomes have an ORF within the N ORF, and an NSs protein has been detected in cells infected with some hantaviruses [14, 15].

A single, continuous ORF in the M RNA segment encodes the polyprotein precursor of the glycoproteins, which is co-translationally cleaved by a cellular protease into mature Gn and Gc. The M segment of nairoviruses is 30–50 % larger than M segments of viruses of the other genera and has the coding potential of up to 240 kDa of protein [16]. The M segment of Crimean-Congo hemorrhagic fever virus (CCHFV) encodes a precursor Gn (preGn) that undergoes posttranslational cleavage to two proteins of unknown functions, a mucin-rich protein, and glycoprotein GP38 [17]. The M segments of some bunyaviruses (but not those of hantaviruses) also encode a NSm protein. NSm is encoded in negative-sense orientation in the case of orthobunyaviruses and phleboviruses, but in positive-sense orientation in the case of tospoviruses. The nairovirus CCHFV also encodes NSm [18]. This integral membrane protein is cleaved off from the C-terminal region of preGn and is detected in cells infected with CCHFV, but not in virion pellets [18]. The function of this protein is currently unknown. In fact, the only M segment NS protein to have a defined role is the NSm of tospoviruses, which aids viral cell-to-cell movement

Table 10.3 Approximate mass (kDa) of structural proteins of viruses belonging to the five bunyavirus genera

Protein	Genus				
	Orthobunyavirus	*Hantavirus*	*Nairovirus*	*Phlebovirus*	*Tospovirus*
N	25	50	50	30	30
Gn	35	70	35	55–70	45
Gc	110	55	75	65	75
L	250	250	450	250	330

(see below). The L segment uses a conventional negative-sense (i.e., complementary to mRNA) coding strategy.

The bunyaviral L segments encode the RNA-dependent RNA polymerase (L). Hantaviral, orthobunyaviral, and phleboviral L is of similar mass ≈250 kDa, whereas tospoviral and nairoviral L is considerably larger (330 kDa and 450 kDa, respectively) (Table 10.3).

Bunyavirions gain entry into host cells by mechanisms similar to many other enveloped viruses. The viral glycoproteins, Gn and Gc, are primarily responsible for attachment to host cells and fusion with cellular membranes. Attachment to the host cell is followed by virion endocytosis. Most bunyaviruses assemble and bud into the Golgi apparatus (visualized by electron microscopy in the case of Bunyamwera virus) [19]. After budding into the Golgi cisternae, maturing virions are transported to the plasma membrane in small vesicles. By a process that resembles normal exocytosis, virion release occurs after fusion of the vesicles with the plasma membrane [20].

6 Ecology and Epidemiology

6.1 Orthobunyaviruses

The genus *Orthobunyavirus* includes ≈53 species and contains more than 193 viruses. Most of these viruses are grouped into ≈20 serogroups based on antigenic relationships (Table 10.4) [8, 21]. The vast majority of the viruses are vectored by mosquitoes; however, some orthobunyaviruses have culicoid flies (i.e., biting midges of the genus *Culicoides*) or ticks as vectors. These viruses are distributed worldwide; however, the majority of these viruses are not well studied. The exceptions are those viruses that have medical (e.g., La Crosse and Oropouche viruses) or veterinary importance (e.g., the newly emerged Schmallenberg virus).

La Crosse virus (LACV) is a member of the California serogroup and one of the most significant bunyaviruses in terms of causing human encephalitis. LACV is transmitted by its primary vector, the forest-dwelling, tree-hole-breeding mosquito *Aedes triseriatus* [22] (Fig. 10.3). This mosquito is found throughout the northern, midwestern, and northeastern USA. LACV is maintained in these mosquitoes by

Table 10.4 Viruses in the genus *Orthobunyavirus*

Virus species (in italics) and their member viruses	Vector	Distribution	Disease host
Anopheles A serogroup			
Anopheles A virus			
Anopheles A virus (ANAV)	Mosquitoes	South America	–
Arumateua virus (ARTV)	Mosquitoes	South America	–
Caraipé virus (CPEV)	Mosquitoes	South America	–
Las Maloyas virus (LMV)	Mosquitoes	South America	–
Lukuni virus (LUKV)	Mosquitoes	South America	–
Trombetas virus (TRMV)	Mosquitoes	South America	–
Tucuruí virus (TUCV)	Mosquitoes	South America	–
Tacaiuma virus			
Tacaiuma virus (TCMV)	Mosquitoes	South America	Human
CoAr 1071 virus	Mosquitoes	South America	–
CoAr 3627 virus	Mosquitoes	South America	–
Virgin River virus (VRV)	Mosquitoes	North America	–
Anopheles B serogroup			
Anopheles B virus			
Anopheles B virus (ANBV)	Mosquitoes	South America	–
Boracéia virus (BORV)	Mosquitoes	South America	–
Bakau serogroup			
Bakau virus			
Bakau virus (BAKV)	Mosquitoes	Asia	–
Ketapang virus (KETV)	Mosquitoes	Asia	–
Nola virus (NOLAV)	Mosquitoes		–
Tanjong Rabok virus (TRV)	ND	Asia	–
Telok Forest virus (TFV)	ND	Asia	–
Bunyamwera serogroup			
Bunyamwera virus			
Batai virus (BATV)	Mosquitoes	Asia	Human
Birao virus (BIRV)	Mosquitoes	Africa	–
Bozo virus (BOZOV)	Mosquitoes	Africa	–
Bunyamwera virus (BUNV)	Mosquitoes	Africa	Human
Cache Valley virus (CVV)	Mosquitoes	North America	Sheep, cattle, human
Fort Sherman virus (FSV)	Mosquitoes	South America	Human
Germiston virus (GERV)	Mosquitoes	Africa	Human
Iaco virus (IACOV)	Mosquitoes	South America	–
Ilesha virus (ILEV)	Mosquitoes	Africa	Human
Lokern virus (LOKV)	Mosquitoes/culicoid flies	North America	–
Maguari virus (MAGV)	Mosquitoes	South America	–
Mboke virus (MBOV)	Mosquitoes	Africa	–
Ngari virus (NRIV)[a]	Mosquitoes	Africa	Human

(continued)

Table 10.4 (continued)

Virus species (in italics) and their member viruses	Vector	Distribution	Disease host
Northway virus (NORV)	Mosquitoes	North America	–
Playas virus (PLAV)	Mosquitoes	South America	–
Potosi virus (POTV)	Mosquitoes	North America	–
Santa Rosa virus (SARV)	Mosquitoes	North America	–
Shokwe virus (SHOW)	Mosquitoes	Africa	Human
Stanfield virus	Mosquitoes	North America	–
Tensaw virus (TENV)	Mosquitoes	North America	–
Tlacotalpan virus (TLAV)	Mosquitoes	North America	–
Tucunduba virus (TUCV)	Mosquitoes	South America	–
Xingu virus (XINV)	Mosquitoes	South America	Human
Guaroa virus			
Guaroa virus (GROV)	Mosquitoes	North America, South America	Human
Kairi virus			
Kairi virus (KRIV)	Mosquitoes	South America	Horse
Main Drain virus			
Main Drain virus (MDV)	Mosquitoes/culicoid flies	North America	Horse
Bwamba serogroup			
Bwamba virus			
Bwamba virus (BWAV)	Mosquitoes	Africa	Human
Pongola virus (PGAV)	Mosquitoes	Africa	Human
California serogroup			
California encephalitis virus			
California encephalitis virus (CEV)	Mosquitoes	North America	Human
Chatanga virus	Mosquitoes	Asia, Europe	–
Inkoo virus (INKV)	Mosquitoes	Europe	Human
Jamestown Canyon virus (JCV)	Mosquitoes	North America	Human
Jerry Slough virus			
Keystone virus (KEYV)	Mosquitoes	North America	–
La Crosse virus (LACV)	Mosquitoes	North America	Human
Lumbo virus (LUMV)	Mosquitoes	Africa	Human
Melao virus (MELV)	Mosquitoes	South America	–
Morro Bay virus (MBV)			
San Angelo virus (SAV)	Mosquitoes	North America	–
Serra do Navio virus (SDNV)	Mosquitoes	South America	–
Snowshoe hare virus (SSHV)	Mosquitoes	North America	Human
South River virus (SORV)	Mosquitoes	North America	–
Tahyña virus (TAHV)	Mosquitoes	Europe	Human
Trivittatus virus (TVTV)	Mosquitoes	North America	–
Capim serogroup			
Acara virus			
Acara virus (ACAV)	Mosquitoes	North America, South America	–
Moriche virus (MORV)	Mosquitoes	South America	–

(continued)

Table 10.4 (continued)

Virus species (in italics) and their member viruses	Vector	Distribution	Disease host
Benevides virus			
Benevides virus (BVSV)	Mosquitoes	South America	–
Capim virus			
Capim virus (CAPV)	Mosquitoes	South America	–
Gamboa serogroup			
Alajuela virus			
Alajuela virus (ALJV)	Mosquitoes	North America	–
San Juan virus (SJV)	Mosquitoes	South America	–
Gamboa virus			
Gamboa virus (GAMV)	Mosquitoes	North America	–
Pueblo Viejo virus (PVV)	Mosquitoes	South America	–
Group C serogroup			
Caraparú virus			
Apeú virus (APEUV)	Mosquitoes	South America	Human
Bruconha virus (BRUV)	Mosquitoes	South America	–
Caraparú virus (CARV)	Mosquitoes	North America, South America	Human
Ossa virus (OSSAV)	Mosquitoes	North America	Human
Vinces virus (VINV)	Mosquitoes	South America	–
Madrid virus			
Madrid virus (MADV)	Mosquitoes	North America	Human
Marituba virus			
Gumbo Limbo virus (GLV)	Mosquitoes	North America	–
Marituba virus (MTBV)	Mosquitoes	South America	Human
Murutucú virus (MURV)	Mosquitoes	South America	Human
Nepuyo virus (NEPV)	Mosquitoes	North America, South America	Human
Restan virus (RESV)	Mosquitoes	South America	Human
Zungarococha virus (ZUNV)	ND	South America	Human
Oriboca virus			
Itaquí virus (ITQV)	Mosquitoes	South America	Human
Oriboca virus (ORIV)	Mosquitoes	South America	Human
Guama serogroup			
Bertioga virus			
Bertioga virus (BERV)	ND	South America	–
Cananeia virus (CNAV)	Mosquitoes	South America	–
Guaratuba virus (GTBV)	Mosquitoes	South America	–
Itimirim virus (ITIV)	ND	South America	–
Mirim virus (MIRV)	Mosquitoes	South America	–
Bimiti virus			
Bimiti virus (BIMV)	Mosquitoes	South America	–
Guama virus			
Ananindeua virus (ANUV)	Mosquitoes	South America	–

(continued)

Table 10.4 (continued)

Virus species (in italics) and their member viruses	Vector	Distribution	Disease host
Guama virus (GMAV)	Mosquitoes	North America, South America	Human
Mahogany Hammock virus (MHV)	ND	North America	–
Moju virus (MOJUV)	Mosquitoes	South America	–
Kongool serogroup			
Koongol virus			
Koongol virus (KOOV)	Mosquitoes	Australia	–
Wongal virus (WONV)	Mosquitoes	Australia	–
Minatitlan serogroup			
Minatitlan virus			
Minatitlan virus (MNTV)	ND	North America	–
Palestina virus (PLSV)	Mosquitoes	South America	–
Nyando serogroup			
Nyando virus			
Nyando virus (NDV)	Mosquitoes	Africa	Human
Eretmapodites virus (ERETV)	Mosquitoes	Africa	–
Olifantsvlei serogroup			
Bomtambi virus			
Bomtambi virus (BOTV)	Mosquitoes	Africa	–
Olifantsvlei virus			
Bobia virus (BIAV)	Mosquitoes	Africa	–
Dabakala virus (DABV)	Mosquitoes	Africa	–
Olifantsvlei virus (OLIV)	Mosquitoes		–
Oubi virus (OUBIV)	Mosquitoes	Africa	–
Patois serogroup			
Patois virus			
Abras virus (ABRV)	Mosquitoes	South America	–
Babahoya virus (BABV)	Mosquitoes	South America	–
Pahayokee virus (PAHV)	Mosquitoes	North America	–
Patois virus (PATV)	Mosquitoes	North America	–
Shark River virus (SRV)	Mosquitoes	North America	–
"Sedlec serogroup"			
Sedlec virus			
I612045 virus	ND	Asia	–
Oyo virus	ND	Africa	–
Sedlec virus (SEDV)	ND	Europe	–
Simbu serogroup			
Akabane virus			
Akabane virus (AKAV)	Mosquitoes/culicoid flies	Africa, Asia, Australia	Cattle
Sabo virus (SABOV)	Culicoid flies	Africa	–

(continued)

Table 10.4 (continued)

Virus species (in italics) and their member viruses	Vector	Distribution	Disease host
Tinaroo virus (TINV)	Culicoid flies	Australia	–
Yaba-7 virus (Y7V)	ND	Africa	–
"*Leanyer virus*"			
Leanyer virus (LEAV)	Mosquitoes	Australia	–
Oropouche virus			
Facey's Paddock virus (FPV)	ND	Australia	–
Madre de Dios virus	ND	South America	–
Oropouche virus (OROV)	Mosquitoes/culicoid flies	South America	Human
Pintupo virus	ND	North America	–
Utinga virus (UTIV)	ND	South America	–
Utive virus (UVV)	ND	North America	–
Sathuperi virus			
Douglas virus (DOUV)		Australia	–
Sathuperi virus (SATV)	Mosquitoes/culicoid flies	Africa, Asia	–
Simbu virus			
Simbu virus (SIMV)	Mosquitoes/culicoid flies	Africa	–
Shamonda virus			
Peaton virus (PEAV)	Culicoid flies	Australia	–
Sango virus (SANV)	Mosquitoes/culicoid flies	Africa	–
Shamonda virus (SHAV)	Culicoid flies	Africa	–
Shuni virus			
Aino virus (ANOV)	Mosquitoes/culicoid flies	Asia, Australia	–
Kaikalur virus (KAIV)	Mosquitoes	Asia	–
Shuni virus (SHUV)	Mosquitoes/culicoid flies	Africa	–
Thimiri virus			
Thimiri virus (THIV)	ND	Africa, Asia	–
Iquitos virus (IQTV)[b]	ND	South America	Human
Jatobal virus (JATV)[b]	ND	South America	–
Schmallenberg virus (SBV)[b]	Culicoid flies	Europe	Cattle
Tete serogroup			
Batama virus			
Batama virus (BMAV)	ND	Africa	–
Tete virus			
Bahig virus (BAHV)	Ticks	Asia, Europe	–
Matruh virus (MTRV)	Ticks	Africa, Asia	–
Tete virus (TETEV)	ND	Africa	–
Tsuruse virus (TSUV)	ND	Asia	–
Weldona virus (WELV)	Culicoid flies	North America	–
Turlock serogroup			
M'Poko vius			
M'Poko virus (MPOV)	Mosquitoes	Africa	–
Yaba-1 virus (Y1V)	Mosquitoes	Africa	–

(continued)

Table 10.4 (continued)

Virus species (in italics) and their member viruses	Vector	Distribution	Disease host
Turlock virus			
Lednice virus (LEDV)	Mosquitoes	Europe	–
Turlock virus (TURV)	Mosquitoes	North America, South America	–
Umbre virus (UMBV)	Mosquitoes	Asia	–
Wyemoyia serogroup			
Wyeomyia virus			
Anhembi virus (AMBV)	Mosquitoes	South America	–
BeAr 328208 virus (BAV)	Mosquitoes	South America	–
Cachoeira Porteira virus (CPOV)			
Iaco virus (IACOV)			
Macaua virus (MCAV)	Mosquitoes	South America	–
Sororoca virus (SORV)	Mosquitoes	South America	–
Taiassui virus (TAIAV)	Mosquitoes	South America	–
Tucunduba virus (TUCV)			
Wyeomyia virus (WYOV)	Mosquitoes	South America	Human
Ungrouped			
Bushbush virus			
Benfica virus	Mosquitoes	South America	–
Bushbush virus (BSBV)	Mosquitoes	South America	–
Juan Diaz virus (JDV)	ND	North America	–
Catu virus			
Catu virus (CATUV)	Mosquitoes	South America	Human
Estero Real virus			
Estero Real virus (ERV)	Ticks	North America	–
Guajara virus			
Guajara virus (GJAV)	Mosquitoes	North America, South America	–
Kaeng Khoi virus			
Kaeng Khoi virus (KKV)	Nest bugs	Asia	–
Manzanilla virus			
Buttonwillow virus (BUTV)	Culicoid flies	North America	–
Cat Que virus	Mosquitoes	Asia	–
Ingwavuma virus (INGV)	Mosquitoes	Africa, Asia	Pig
Inini virus (INIV)	ND	South America	–
Manzanilla virus (MANV)	ND	South America	–
Mermet virus (MERV)	Mosquitoes	North America	–
Timboteua virus			
Timboteua virus (TBTV)	Mosquitoes	South America	–
Zegla virus			
Zegla virus (ZEGV)	ND	North America	–

(continued)

Table 10.4 (continued)

Virus species (in italics) and their member viruses	Vector	Distribution	Disease host
Unclassified			
Abbey Lake bunyavirus (Ab-BUNV)	Mosquitoes	Asia	–
Brazoran virus	Mosquitoes	North America	–
Enseada virus (ENSV)	Mosquitoes	South America	–
Khurdun virus (KHURV)	ND	Europe	–
Kowanyama virus (KOWV)	Mosquitoes	Australia	–
Mojuí dos Campos virus (MDCV)	ND	South America	–
Murrumbidgee virus (MURBV)	Mosquitoes	Australia	–
Salt Ash virus (SASHV)	Mosquitoes	Australia	–
Termeil virus (TERV)	Mosquitoes	Australia	–

ND not determined
[a]Includes Garissa virus
[b]It is currently unclear to which species these reassortant Simbu serogroup viruses belong

Fig. 10.3 *Aedes triseriatus*, commonly known as the "treehole mosquito," obtaining a blood meal from a human hand (courtesy of James Gathany, obtained from the Centers for Disease Control and Prevention (CDC) Public Health Image Library at http://phil.cdc.gov/phil/home.asp [accessed Feb 25, 2014])

transovarial transmission, which allows overwintering of the virus in mosquito eggs [23]. Squirrels, chipmunks, foxes, and woodchucks often serve as amplifying reservoir hosts [24]. Most cases of La Crosse encephalitis occur in the summer and early fall when risk of being bitten by infected mosquitoes is highest. The majority of cases occur in the Mississippi and Ohio River basins with over 90 % of cases reported from Wisconsin, Minnesota, Iowa, Indiana, Ohio, and Illinois; however, cases also occur throughout most of the eastern half of the USA (Fig. 10.4).

Oropouche virus (OROV) was first identified from Trinidad in 1955 [25] and was later isolated from the blood of a pale-throated sloth (*Bradypus tridactylus*) in 1960 [26]. Recently, OROV has become the second most frequent cause of arboviral infection in Brazil, surpassed only by dengue viruses [26]. OROV has been associated with large and explosive outbreaks of febrile disease in South and Central

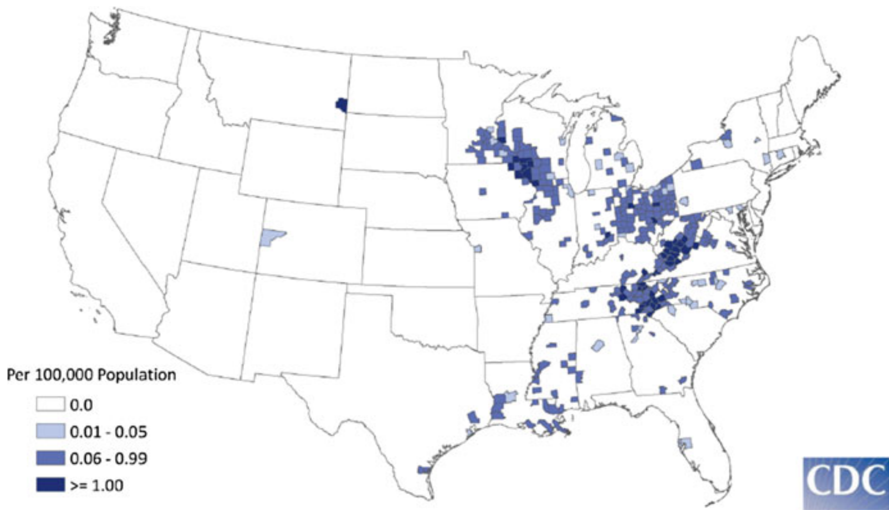

Fig. 10.4 Most reported cases of California serogroup virus neuroinvasive disease (encephalitides, meningoencephalitides, or meningitides) are due to La Crosse virus (LACV). Counties are *shaded* according to incidence ranging from less than 0.06, 0.06–0.99, and greater than 1.00 per 100,000. Most of the counties with the highest incidence are located along the Appalachian range and the upper Mississippi River basin (Obtained from the CDC website at http://www.cdc.gov/lac/tech/epi.html [accessed Feb 25, 2014])

America, especially in the Amazon Basin. More than half a million cases have been reported in the Americas [26]. In addition to outbreaks, OROV can also cause sporadic human infections [27]. OROV is transmitted to sloths, marsupials, primates, and birds by *Aedes serratus* and *Culex quinquefasciatus* mosquitoes. Notably, OROV has adapted to an urban cycle involving man, with biting midges (*Culicoides paraensis*) as the primary vector [28, 29].

In addition to human pathogens, the genus *Orthobunyavirus* also contains pathogens of significant veterinary importance. One example is the recently emerged Schmallenberg virus (SBV). In late 2011, a nonspecific febrile syndrome occurred in dairy cattle that was characterized by decreased milk production and watery diarrhea [30]. The affected farms were located along the German-Dutch boarder in North Rhine-Westphalia, near the city of Schmallenberg, Germany. Next-generation sequencing and metagenomic analysis was used to identify the novel orthobunyavirus from blood samples of deceased cows from a farm in Schmallenberg [30]. Since the first detection in Germany, SBV has spread rapidly over large parts of northern and western Europe (Fig. 10.5). In addition to the febrile syndrome initially described for the virus, transplacental infection often results in the birth of malformed calves, lambs, and goat kids [31, 32]. Based on similarities to other related viruses affecting livestock, researchers suspected that Schmallenberg virus was transmitted by biting midges (*Culicoides*). In fact, several studies have detected the virus in field-collected midges [33–36], and viral replication and dissemination in *C. sonorensis* midges

Fig. 10.5 Distribution of Schmallenberg virus by country in Europe as of February 2013 (courtesy of O. Smooth, obtained at http://en.wikipedia.org/wiki/Schmallenberg_virus [accessed Feb 27, 2014])

have been observed under laboratory conditions [37]. Interestingly, SBV appears to be a reassortant virus, with the M segment from Sathuperi virus and the S and L segments from Shamonda virus, both members of the Simbu serogroup of orthobunyaviruses [38].

6.2 Nairoviruses

The genus *Nairovirus* includes ≈50 predominantly tick-borne viruses in eight established and four proposed species [39, 40] (Table 10.5). The pathogenicity of many of these viruses is not known. However, two nairoviruses are known to be serious pathogens: Crimean-Congo hemorrhagic fever virus (CCHFV) and Nairobi sheep disease virus (NSDV). CCHFV causes severe hemorrhagic fever in humans, and NSDV causes severe gastroenteritis in sheep and goats. Case-fatality rates of infections with either virus can reach 90 % [41, 42]. CCHF is the most widespread tick-borne viral infection of humans, occurring across a vast area including western China through Southern Asia, the Middle East to southeastern Europe, and most of Africa [43]. CCHFV is

Table 10.5 Viruses in the genus *Nairovirus*

Virus species (in italics) and their member viruses	Original source	Year of description
"*Ahun virus*"[a]		
Ahun virus	Common pipistrelle (*Pipistrellus pipistrellus*), whiskered myotis (*Myotis mystacinus*)	2014
"*Artashat virus*"		
Artashat virus (ARTSV)	Ticks: *Ornithodoros alactagalis*	1972
Crimean-Congo hemorrhagic fever virus		
Crimean-Congo hemorrhagic fever virus (CCHFV)	Humans	1967
Hazara virus (HAZV)	Ticks: *Ixodes redikorzevi*	1970
Khasan virus (KHAV)	Ticks: *Haemaphysalis longicornis*	1978
Dera Ghazi Khan virus		
Abu Hammad virus (AHV)	Ticks: *Argas hermanni*	1971
Abu Mina virus (AMV)	Ticks: *Argas streptopelia*	1963
Dera Ghazi Khan virus (DGKV)	Ticks: *Hyalomma dromedarii*	1970
Kao Shuan virus (KSV)	Ticks: *Argas robertsi*	1970
Pathum Thani virus (PTHV)	Ticks: *Argas robertsi*	1970
Pretoria virus (PREV)	Ticks: *Argas africolumbae*	1973
Dugbe virus		
Dugbe virus (DUGV)	Ticks: *Amblyomma variegatum*	1970
Ganjam virus (GANV)[b]	Ticks: *Haemaphysalis intermedia*	1969
Kupe virus	Ticks: *Amblyomma gemma*, *Rhipicephalus pulchellus*	2009
Nairobi sheep disease virus (NSDV)	Domestic sheep	1910
Hughes virus		
Caspiy virus (CASV)[c]	Ticks: *Ornithodoros maritimus*	1970
Farallon virus (FARV)	Ticks: *Carios capensis*	1964
Fraser Point virus (FPV)	ND	ND
Great Saltee virus (GRSV)	Ticks: *Ornithodoros* maritimus	1976
Hughes virus (HUGV)	Ticks: *Ornithodoros capensis*	1962
Puffin Island virus (PIV)	Ticks: *Ornithodoros maritimus*	1979
Punta Salinas virus (PSV)	Ticks: *Ornithodoros* sp.	1967
Raza virus (RAZAV)	Ticks: *Carios denmarki*	1962
Sapphire II virus (SAPV)	Ticks: *Argas cooley*	ND
Soldado virus (SOLV)	Ticks: *Ornithodoros* sp.	1963
Zirqa virus (ZIRV)	Ticks: *Ornithodoros* sp.	1969
"*Issyk-kul virus*"		
Issyk-kul virus (ISKV)	Bats: noctule (*Nyctalus noctula*); ticks: *Argas* sp.	1973
Kasokero virus (KASV)	Bats: Egyptian rousettes (*Rousettus aegyptiacus*)	1986
Keterah virus (KTRV)	Bats: lesser Asiatic yellow house bat (*Scotophilus kuhli temminckii*)	1976

(continued)

Table 10.5 (continued)

Virus species (in italics) and their member viruses	Original source	Year of description
Yogue virus (YOGV)	Bats: Egyptian rousettes (*Rousettus aegyptiacus*)	1986
Qalyub virus		
Bakel virus (BAKV)	ND	ND
Bandia virus (BDAV)	Multimammate rat (*Mastomys* sp.)	1965
Chim virus (CHIMV)	Ticks: *Ornithodoros tartakovskyi*	1971
Omo virus (OMOV)	Multimammate rat (*Mastomys* sp.)	1971
Qalyub virus (QYBV)	Ticks: *Ornithodoros erraticus*	1952
Sakhalin virus		
Avalon virus (AVAV)[d]	Ticks: *Ixodes* uriae	1972
Clo Mor virus (CMV)	Ticks: *Ixodes* uriae	1973
Finch Creek virus	Ticks: *Ixodes uriae*	2009
Kachemak Bay virus (KBV)	Ticks: *Ixodes signatus*	1974
Sakhalin virus (SAKV)	Ticks: *Ixodes putus*	1970
Taggert virus (TAGB)	Ticks: *Ixodes* uriae	1972
Tillamook virus (TILLV)	Ticks: *Ixodes uriae*	1970
"*South Bay virus*"		
South Bay virus (SBV)	Ticks: *Ixodes scapularis*	2014
Tamdy virus		
Tamdy virus (TDY)	Ticks: *Hyalomma* sp.	1976
Thiafora virus		
Erve virus (ERVEV)	Greater white-toothed shrew (*Crocidura russula*)	1982
Thiafora virus (TFAV)	White-toothed shrew (*Crocidura* sp.)	1971

ND not determined
[a]Viruses in quotation marks represent proposed species
[b]Some consider GANV to be an Asian variant of NSDV
[c]Exact taxonomic position is currently unclear
[d]Also known as Paramushir virus (PRMV)

maintained through vertical and horizontal transmission in ixodid (hard) ticks of several genera, which spread the virus to a variety of wild and domestic mammals. Affected mammals develop transient viremia without signs of illness. Human infections occur through tick bite or exposure to the blood or other body fluids from infected animals or humans. Ticks of the genus *Hyalomma* (Fig. 10.6) are the principal source of human infection, probably because both the immature and the adult forms of the tick actively seek hosts for their obligate blood meals. Of note, during a recent viral metagenomics study, novel nairovirus sequences were detected in France in bats belonging to two different species [44]. These sequences diverge significantly from all known nairovirus genomes and thus represent a new nairovirus, Ahun virus, which will probably to be assigned to a novel species.

Fig. 10.6 *Hyalomma marginatum rufipes* tick, a known vector of Crimean-Congo hemorrhagic fever. This specimen was collected from Kenya and has the characteristic banded coloration pattern on the legs, typical of *Hyalomma* ticks (courtesy of Alan R. Walker, obtained at http://en.wikipedia.org/wiki/Ticks_of_domestic_animals [accessed Feb 26, 2014])

6.3 Hantaviruses

The *Hantavirus* genus contains 36 viruses classified into 24 species and an additional ≈67 unclassified viruses (Table 10.6). Unlike all other members of the family *Bunyaviridae*, hantaviruses are not arboviruses, but infect rodents, eulipotyphla (shrews and moles), and bats [45]. Human hantavirus infection is thought to occur following exposure to excretions from infected mammalian hosts. Researchers generally agree that hantaviruses have co-evolved with their hosts over the course of several hundreds of thousands to millions of years [46]. Phylogenetic trees visualize that most hantaviruses clearly group according to their mammalian hosts (Fig. 10.7) [47]. Rodent-borne hantaviruses form two basic lineages, Old World and New World, which primarily reflect the geographic distribution of their rodent hosts. Significant human pathogens among the Old World viruses include Hantaan, Seoul, Puumala, and Dobrava-Belgrade viruses, which cause HFRS. Hantaan virus, named after the Hantaan River in Korea, is the prototype hantavirus and was first isolated from the striped field mouse (*Apodemus agrarius*) in 1976 [48]. After this discovery, other related viruses were characterized and classified including Puumala virus from the bank vole (*Myodes glareolus*) [49], Seoul virus from brown and roof rats (*Rattus norvegicus* and *R. rattus*) [50], and Dobrava-Belgrade virus from the yellow-necked field mouse (*Apodemus flavicollis*) [51].

Hantaviruses were first recognized in the New World in 1982 (Prospect Hill virus) and then again in 1993 when Sin Nombre virus was identified as the causative agent of an outbreak of acute respiratory distress, today called hantavirus (cardio-) pulmonary syndrome (HCPS), in the Four Corners area of the US Southwest [52]. Sin Nombre virus (Fig. 10.8) was ultimately isolated from North American deer-mice (*Peromyscus maniculatus*), which were confirmed as the primary rodent reservoirs for this hantavirus [53]. Since the first detection of this Sin Nombre virus in

Table 10.6 Viruses in the genus *Hantavirus*

Virus species (in italics) and their member viruses	Host	Distribution	Disease in humans
Andes virus			
Andes virus (ANDV)	Argentine akodont (*Necromys benefactus*), long-tailed colilargo (*Oligoryzomys longicaudatus*)	South America	HCPS
Bermejo virus (BMJV)	Chacoan colilargo (*Oligoryzomys chacoensis*)	South America	HCPS
Lechiguanas virus (LECV)	Flavescent colilargo (*Oligoryzomys flavescens*)	South America	HCPS
Maciel virus (MCLV)	Dark-furred akodont (*Necromys obscurus*)	South America	HCPS
Orán virus (ORNV)	Long-tailed colilargo (*Oligoryzomys longicaudatus*)	South America	HCPS
Pergamino virus (PRGV)	Azara's akodont (*Akodon azarae*)	South America	HCPS
Tunari virus (TUNV)	Unknown	South America	HCPS
Bayou virus			
Bayou virus (BAYV)	Marsh oryzomys (*Oryzomys palustris*)	North America	HCPS
Black Creek Canal virus			
Black Creek Canal virus (BCCV)	Hispid cotton rat (*Sigmodon hispidus*)	North America	HCPS
Caño Delgadito virus			
Caño Delgadito virus (CADV)	Alston's cotton rat (*Sigmodon alstoni*)	South America	Unknown
Dobrava-Belgrade virus			
Dobrava-Belgrade virus (DOBV)	Yellow-necked field mouse (*Apodemus flavicollis*)	Europe	HFRS
Kurkino virus	Striped field mouse (*Apodemus agrarius*)	Europe	HFRS
Sochi virus	Caucasus field mouse (*Apodemus ponticus*)	Europe	HFRS
El Moro Canyon virus			
El Moro Canyon virus (ELMCV)	Sumichrast's harvest mouse (*Reithrodontomys sumichrasti*), western harvest mouse (*Reithrodontomys megalotis*)	North America	HCPS
Hantaan virus			
Amur/Soochong virus (AMRV/SOOV)	Korean field mouse (*Apodemus peninsulae*)	Asia	HFRS
Hantaan virus (HTNV)	Striped field mouse (*Apodemus agrarius*)	Asia	HFRS

(continued)

Table 10.6 (continued)

Virus species (in italics) and their member viruses	Host	Distribution	Disease in humans
Isla Vista virus			
Isla Vista virus (ISLAV)	Californian vole (*Microtus californicus*)	North America	Unknown
Khabarovsk virus			
Khabarovsk virus (KHAV)	Reed vole (*Microtus fortis*), Maximowicz's vole (*Microtus maximowiczii*)	Asia	Unknown
Laguna Negra virus			
Laguna Negra virus (LANV)	Big laucha (*Calomys callosus*), little laucha (*Calomys laucha*)	South America	HCPS
Muleshoe virus			
Muleshoe virus (MULV)	Hispid cotton rat (*Sigmodon hispidus*)	North America	HCPS
New York virus			
New York virus (NYV)	White-footed deermouse (*Peromyscus leucopus*)	North America	HCPS
Prospect Hill virus			
Bloodland Lake virus (BLLV)	Prairie vole (*Microtus ochrogaster*)	North America	Unknown
Prospect Hill virus (PHV)	Meadow vole (*Microtus pennsylvanicus*)	North America	Nonpathogenic[a]
Puumala virus			
Hokkaido virus (HOKV)	Gray red-backed vole (*Myodes rufocanus*)	Asia	Unknown
Muju virus (MUJV)	Korean red-backed vole (*Myodes regulus*)	Asia	HFRS
Puumala virus (PUUV)	Bank vole (*Myodes glareolus*), gray red-backed vole (*Myodes rufocanus*)	Europe	HFRS/HCPS
Río Mamoré virus			
Maripa virus	Unknown	South America	HCPS
Río Mamoré virus (RIOMV)	Small-eared colilargo (*Oligoryzomys microtis*)	South America	HCPS
Río Segundo virus			
Río Segundo virus (RIOSV)	Mexican harvest mouse (*Reithrodontomys mexicanus*)	North America	Unknown
Saaremaa virus			
Saaremaa virus (SSAV)	Striped field mouse (*Apodemus agrarius*)	Europe	HFRS

Seoul virus			
Seoul virus (SEOV)	Brown rat (*Rattus norvegicus*), Losea rat (*Rattus losea*), roof rat (*Rattus rattus*)	Worldwide[b]	HFRS
Sin Nombre virus			
Blue River virus (BRV)	White-footed deermouse (*Peromyscus leucopus*)	North America	HCPS
Monongahela virus (MGLV)	North American deermouse (*Peromyscus maniculatus*)	North America	HCPS
Sin Nombre virus (SNV)	North American deermouse (*Peromyscus maniculatus*)	North America	HCPS
Thailand virus			
Anjozorobe virus	Major's tufted-tailed rat (*Eliurus majori*), roof rat (*Rattus rattus*)	Africa	Unknown
Jurong virus	Oriental house rat (*Rattus tanezumi*)	Asia	Unknown
Serang virus (SERV)	Oriental house rat (*Rattus tanezumi*)	Asia	Unknown
Thailand virus (THAIV)	Greater bandicoot rat (*Bandicota indica*), Savile's bandicoot rat (*Bandicota savilei*)	Asia	Unknown
Thottapalayam virus			
Thottapalayam virus (TPMV)	Asian house shrew (*Suncus murinus*)	Asia	Nonpathogenic[c]
Topografov virus			
Topografov virus (TOPV)	Siberian brown lemming (*Lemmus sibiricus*)	Asia	Unknown
Tula virus			
Tula virus (TULV)	Common vole (*Microtus arvalis*), East European vole (*Microtus levis*), Eurasian water vole (*Arvicola amphibius*), field vole (*Microtus agrestis*)	Europe	HFRS/HCPS
Unclassified			
Altai virus (ALTV)	Common shrew (*Sorex araneus*)	Asia	Unknown
Alto Paraguay virus (ALPAV)	Chacoan marsh rat (*Holochilus chacarius*)	South America	Unknown
Anajatuba virus (ANJV)	Fornes' colilargo (*Oligoryzomys fornesi*)	South America	HCPS
Ape Aime virus (AAIV)	Montane akodont (*Akodon montensis*)	South America	Unknown
Araraquara virus (ARAV)	Hairy-tailed akodont (*Necromys lasiurus*)	South America	HCPS
Araucária virus (ARAUV)	Black-footed colilargo (*Oligoryzomys nigripes*)	South America	HCPS
Artybash/Amga virus (ARTV/MGAV)	Laxmann's shrew (*Sorex caecutiens*)	Asia, Europe	Unknown
Asama virus (ASAV)	Japanese shrew mole (*Urotrichus talpoides*)	Asia	Unknown

(continued)

Table 10.6 (continued)

Virus species (in italics) and their member viruses	Host	Distribution	Disease in humans
Ash River virus (ARRV)	Cinereus shrew (*Sorex cinereus*)	North America	Unknown
Asikkala virus (ASIV)	Eurasian pygmy shrew (*Sorex minutus*)	Europe	Unknown
Azagny virus (AZGV)	West African pygmy shrew (*Crocidura obscurior*)	Africa	Unknown
Boginia virus (BOGV)[d]	Eurasian water shrew (*Neomys fodiens*)	Europe	Unknown
Bowé virus (BOWV)	Doucet's musk shrew (*Crocidura douceti*)	Africa	Unknown
Calabazo virus	Short-tailed zygodont (*Zygodontomys brevicauda*)	North America	Unknown
Camp Riley virus (RPLV)	Northern short-tailed shrew (*Blarina brevicauda*)	North America	Unknown
Cao Bang virus (CBNV)[e]	Chinese mole shrew (*Anourosorex squamipes*)	Asia	Unknown
Carrizal virus (CARV)	Sumichrast's harvest mouse (*Reithrodontomys sumichrasti*)	North America	Unknown
Castelo dos Sonhos virus (CASV)	Brazilian colilargo (*Oligoryzomys eliurus*)	South America	HCPS
Catacamas virus (CATV)	Coues' oryzomys (*Oryzomys couesi*)	North America	Unknown
CGRn9415 virus[f]	Brown rat (*Rattus norvegicus*)	Asia	Unknown
Choclo virus (CHOV)	Fulvous colilargo (*Oligoryzomys fulvescens*)	North America	HCPS
Da Bie Shan virus (DBSV)	Confucian niviventer (*Niviventer confucianus*)	Asia	Unknown
Dahonggou Creek virus (DHCV)	Long-tailed mole (*Scaptonyx fusicaudus*)	Asia	Unknown
Gou virus (GOUV)	Brown rat (*Rattus norvegicus*), roof rat (*Rattus rattus*), Oriental house rat (*Rattus tanezumi*)	Asia	HFRS
Huangpi virus (HUPV)	Japanese pipistrelle (*Pipistrellus abramus*)	Asia	Unknown
Huitzilac virus (HUIV)	Western harvest mouse (*Reithrodontomys megalotis*)	North America	Unknown
Iamonia virus (AMNV)	Southern short-tailed shrew (*Blarina carolinensis*)	North America	Unknown
Imjin virus (MJNV)	Ussuri white-toothed shrew (*Crocidura lasiura*)	Asia	Unknown
Itapúa virus	Black-footed colilargo (*Oligoryzomys nigripes*)	South America	Unknown
Jaborá virus (JABV)	Montane akodont (*Akodon montensis*), Paraná akodont (*Akodon paranaensis*), Serra do Mar akodont (*Akodon serrensis*)	South America	Unknown
Jeju virus (JJUV)	Asian lesser white-toothed shrew (*Crocidura shantungensis*)	Asia	Unknown
Jemez Springs virus (JMSV)[g]	Dusky shrew (*Sorex monticolus*)	North America	Unknown

Juquitiba virus (JUQV)	Black-footed colilargo (*Oligoryzomys nigripes*), Fornes' colilargo (*Oligoryzomys fornesi*)	South America	HCPS
Kenkeme virus (KKMV)	Flat-skulled shrew (*Sorex roboratus*)	Asia	Unknown
Kilimanjaro virus (KMJV)	Kilimanjaro mouse shrew (*Myosorex zinki*)	Africa	Unknown
Lena River virus (LNAV)?	Laxmann's shrew (*Sorex caecutiens*)	Europe	Unknown
Limestone Canyon virus (LSCV)	Brush deermouse (*Peromyscus boylii*)	North America	Unknown
Longquan virus (LQUV)	Chinese Rufous horseshoe bat (*Rhinolophus sinicus*), Formosan lesser horseshoe bat (*Rhinolophus monoceros*), intermediate horseshoe bat (*Rhinolophus affinis*)	Asia	Unknown
Luxi virus (LUXV)	Yunnan red-backed vole (*Eothenomys miletus*)	Asia	Unknown
Magboi virus (MGBV)	Hairy slit-faced bat (*Nycteris hispida*)	Africa	Unknown
Makokou virus (MAKV)	Noack's roundleaf bat (*Hipposideros ruber*)	Africa	Unknown
Maporal virus (MAPV)	Fulvous colilargo (*Oligoryzomys fulvescens*)	South America	Unknown
Montano virus (MTNV)	Orizaba deermouse (*Peromyscus beatae*)	North America	Unknown
Mouyassué virus (MOYV)	Banana pipistrelle (*Neoromicia nanus*)	Africa	Unknown
Nova virus (NVAV)	European mole (*Talpa europaea*)	Europe	Unknown
Oxbow virus (OXBV)	American shrew mole (*Neurotrichus gibbsii*)	North America	Unknown
Paranoá virus	Unknown	South America	HCPS
Playa de Oro virus (OROV)	Coues' oryzomys (*Oryzomys couesi*)	North America	Unknown
Qian Hu Shan virus (QHSV)[h]	Stripe-backed shrew (*Sorex cylindricauda*)	Asia	Unknown
Rockport virus (RKPV)	Eastern mole (*Scalopus aquaticus*)	North America	Unknown
Sangassou virus (SANGV)	Allen's hylomyscus (*Hylomyscus alleni*)	Africa	Unknown
Sarufutsu virus (SFSV)	Long-clawed shrew (*Sorex unguiculatus*)	Asia	Unknown
Seewis virus (SWSV)	Common shrew (*Sorex araneus*), Eurasian pygmy shrew (*Sorex minutus*), Siberian large-toothed shrew (*Sorex daphaenodon*), tundra shrew (*Sorex tundrensis*)	Europe, Asia	Unknown
Tanganya virus (TGNV)	Therese's shrew (*Crocidura theresae*)	Africa	Unknown
Tatenale virus (TATV)	Field vole (*Microtus agrestis*)	Europe	Unknown

(continued)

Table 10.6 (continued)

Virus species (in italics) and their member viruses	Host	Distribution	Disease in humans
Tigray virus (TIGV)	White-footed stenocephalemys (*Stenocephalemys albipes*)	Africa	Unknown
Uluguru virus (ULUV)	Geata mouse shrew (*Myosorex geata*)	Africa	Unknown
Vladivostok virus (VLAV)	Reed vole (*Microtus fortis*)	Asia	Unknown
Xinyi virus (XYIV)	Taiwanese mole shrew (*Anourosorex yamashinai*)	Asia	Unknown
Xuan Son virus (XSV)	Pomona leaf-nosed bat (*Hipposideros pomona*)	Asia	Unknown
Yakeshi virus (YKSV)	Taiga shrew (*Sorex isodon*)	Asia	Unknown
Yuanjiang virus (YUJV)	Reed vole (*Microtus fortis*)	Asia	Unknown

HCPS hantavirus (cardio-)pulmonary syndrome, *HFRS* hemorrhagic fever with renal syndrome
[a]PHV is considered nonpathogenic to humans based on the lack of reported human cases, despite widespread distribution of the virus in indigenous rodents [135]
[b]SEOV has a worldwide distribution likely due to the spread of brown rats from Asia that follow humans [136]
[c]Data on specific cellular receptor usage of TPMV suggest that the virus is not pathogenic to humans [137]
[d]Includes strain “Laihi virus (LAIV)” found in Eurasian water shrews (*Neomys fodiens*)
[e]Also known as Lianghe virus (LHEV)
[f]Natural reassortant of HTNV and SEOV
[g]Includes the genotypes “Fox Creek virus (FXCV)” found in American water shrews (*Sorex palustris*), “Powell Butte virus (PWBV)” found in vagrant shrews (*Sorex vagrans*), and “Tualatin River virus (TLNV)” found in Trowbridge’s shrews (*Sorex trowbridgii*)
[h]Also known as Qiandao Lake (QDLV)

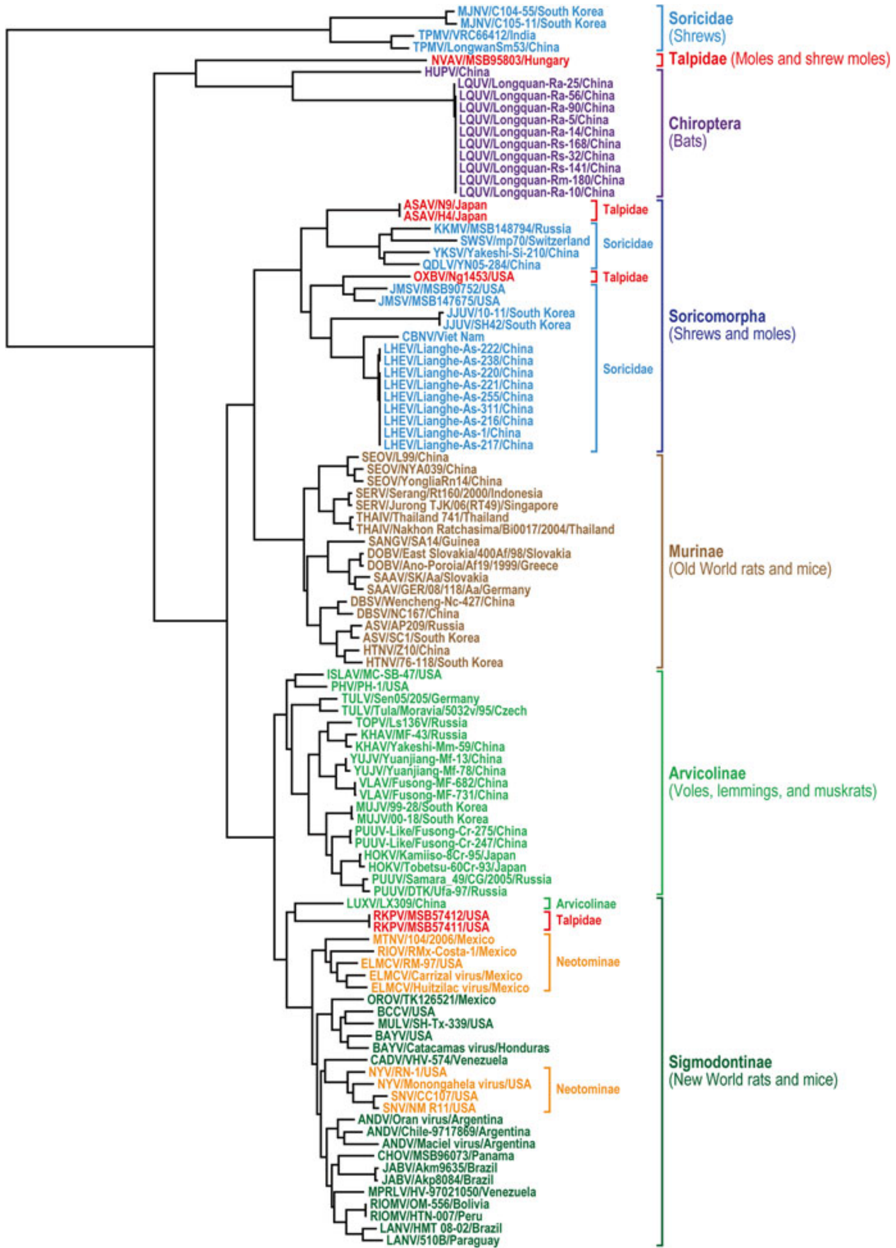

Fig. 10.7 Phylogenetic tree based on the coding sequence of the S segments of hantavirus genomes showing coevolution with their mammalian reservoir hosts. Image modified from Guo et al. [47]

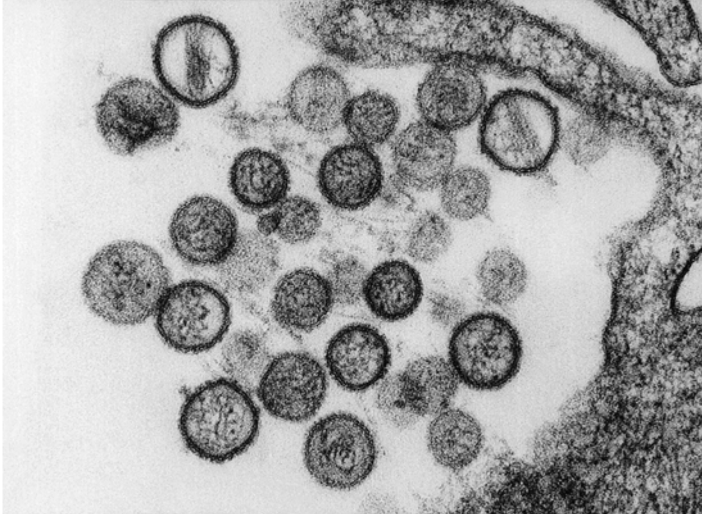

Fig. 10.8 Transmission electron micrograph showing the ultrastructure of virions of the hantavirus Sin Nombre virus (courtesy of CDC/Brian W.J. Mahy and Luanne H. Elliott, obtained from CDC's Public Health Image Library at http://phil.cdc.gov/phil/home.asp [accessed Feb 11, 2014])

the USA, numerous additional hantaviruses have been discovered throughout the Americas, some of which (e.g., Andes virus) are clearly pathogenic to humans and cause HCPS [54].

Thottapalayam virus was isolated from an Asian house shrew in India in 1964 [55], but was not classified as a hantavirus until 1989 [56]. Since 2006, at least 26 new hantaviruses have been identified from eulipotyphla [8, 57]. In addition, more recently, hantaviruses have been identified in bats from diverse geographic locations, including Africa [58, 59] and Asia [47, 60]. Until now, none of the non-rodent-borne hantaviruses has been associated with disease.

6.4 *Phleboviruses*

The genus *Phlebovirus* includes ≈24 species and ≈115 viruses (Table 10.7) that can be classified based on their antigenic similarities, arthropod vectors, and the presence of an open reading frame in M segments coding for a non-structural protein. Phleboviruses are transmitted by arthropod vectors, namely, sandflies, mosquitoes, biting midges of the *Culicoides* genus, or ticks [8]. Infection of the vector is frequently persistent and lifelong. Within the vectors, the viruses multiply and spread transovarially (i.e., vertical transmission) and venereally (i.e., horizontal transmission) [61–63]. Generally, phleboviruses are specific with regard to arthropod vectors and vertebrate hosts. However, some phleboviruses infect multiple vectors, with

Table 10.7 Viruses in the genus *Phlebovirus*

Virus species (in italics) and their member viruses	Vector	Distribution	Disease in humans
"*Aguacate virus*"[a]			
Aguacate virus (AGUV)	Sandflies	South America	–
Armero virus (ARMV)	Sandflies	South America	–
Durania virus (DURV)	Sandflies	South America	–
Ixcanal virus (IXCV)	Sandflies	South America	–
"*Bhanja virus*"			
Bhanja virus (BHAV)	Ticks	Eurasia, Africa	Encephalitis, febrile illness
Forécariah virus (FORV)	Ticks	Africa	–
Heartland virus (HRTV)	Ticks	North America	Febrile illness
Hunter Island group virus (HIGV)	Ticks	Australia	–
Kisemayo virus (KISV)	Ticks	Africa	–
Lone Star virus (LSV)	Ticks	North America	–
Malsoor virus	ND	Asia	–
Palma virus (PALV)	Ticks	Europe	–
Razdan virus (RAZV)	Ticks	Asia	–
Severe fever with thrombocytopenia virus (SFTSV)	Ticks	Asia	Febrile illness, hemorrhagic fever
Bujaru virus			
Bujaru virus (BUJV)	ND	South America	–
Munguba virus (MUNV)	Sandflies	South America	–
Candiru virus			
Alenquer virus (ALEV)	ND	South America	Febrile illness
Ariquemes virus (ARQV)	Sandflies	South America	–
Candiru virus (CDUV)	ND	South America	Febrile illness
E(s)charate virus (ESVC)	ND	South America	Febrile illness
Itaituba virus (ITAV)	ND	South America	–
Jacunda virus (JCNV)	ND	South America	–
Maldonado virus (MLOV)	ND	South America	Febrile illness
Morumbi virus (MRMBV)	ND	South America	Febrile illness
Mucura virus (MCRV)	Mosquitoes	South America	–
Nique virus (NIQV)	Sandflies	South America	–
Oriximiná virus (ORXV)	Sandflies	South America	–
Serra Norte virus (SRNV)	ND	South America	Febrile illness
Turuna virus (TUAV)	Sandflies	South America	–
Chilibre virus			
Cacao virus (CACV)	Sandflies	South America	–
Chilibre (CHIV)	Sandflies	South America	–
Frijoles virus			
Frijoles virus (FRIV)	Sandflies	South America	–
Joa virus (JOAV)	Sandflies	South America	–

(continued)

Table 10.7 (continued)

Virus species (in italics) and their member viruses	Vector	Distribution	Disease in humans
"*Grand Arbaud virus*"			
Grand Arbaud virus (GAV)	Ticks	Europe	–
"*Icoaraci virus*"			
Belterra virus (BELTV)	ND	South America	–
Icoaraci virus (ICOV)	Mosquitoes, sandflies	South America	–
"*Karimabad virus*"			
Gabek Forest virus (GFV)	Sandflies	Africa	–
Karimabad virus	Sandflies	Asia	–
"*Manawa virus*"			
Manawa virus (MWAV)	Ticks	Asia	–
"*Murre virus*"			
Murre virus (MURV)	ND	North America	–
RML-105-105455 virus (RMLV)	Ticks	North America	–
Sunday Canyon virus	Ticks	North America	–
"*Precarious Point virus*"			
Catch-me-cave virus	Ticks	Australia	–
Precarious Point virus (PPV)	Ticks	Australia	–
"*Provencia virus*"			
Provencia virus	Sandflies	Europe	–
Punta Toro virus			
Buenaventura virus (BUEV)	Sandflies	South America	–
Punta Toro virus (PTV)	Sandflies	South America	Febrile illness
Rift Valley fever virus			
Rift Valley fever virus (RVFV)	Mosquitoes	Africa, Asia	Encephalitis, febrile illness, hemorrhagic fever
Salehabad virus			
Adria virus (ADRV)	Sandflies	Europe	–
Arbia virus (ARBV)	Sandflies	Europe	–
Arumowot virus (AMTV)	Mosquitoes	Africa	–
Odrenisrou virus (ODRV)	Mosquitoes	Africa	–
Olbia virus	Sandflies	Europe	–
Salehabad virus (SALV)	Sandflies	Asia	–
Sandfly fever Naples virus			
Fermo virus	Sandflies	Europe	–
Gordil virus	ND	Africa	–
Granada virus (GRV)	ND	Europe	–
Massila virus	Sandflies	Europe	–
Punique virus (PUNV)	Sandflies	Africa	–
Saint-Floris virus (SAFV)	ND	Africa	–
Sandfly fever Naples virus (SFNV)	Sandflies	Asia	Febrile illness

(continued)

Table 10.7 (continued)

Virus species (in italics) and their member viruses	Vector	Distribution	Disease in humans
Tehran virus (THEV)	Sandflies	Asia	–
Toscana virus (TOSV)	Sandflies	Eurasia	Febrile illness, meningoencephalitis
“*Sandfly fever Sicilian virus*”			
Chagres virus (CHGV)	Sandflies	South America	Febrile illness
Chios virus	ND	Europe	Encephalitis
Corfou virus (CFUV)	Sandflies	Europe	–
Sandfly fever Cyprus virus	Sandflies	Eurasia	Febrile illness
Sandfly fever Sicilian virus (SFSV)	Sandflies	Eurasia	Febrile illness
Sandfly fever Turkey virus	Sandflies	Asia	Febrile illness
Uukuniemi virus			
Chizé virus (CHZV)	Ticks	Europe	–
EgAN 1825-61 virus	ND	Africa	–
Fin V 707 virus	Ticks	Europe	–
Khasan virus (KHAV)	Ticks	Asia	–
Oceanside virus (OCV)	Ticks	North America	–
Ponteves virus (PTVV)	Ticks	Europe	–
Rukutama virus (RUKV)	Ticks	Europe	–
St. Abbs Head virus (SAHV)	Ticks	Europe	–
Tunis virus (TUNV)	Ticks	Africa	–
Uukuniemi virus (UUKV)	Ticks	Eurasia	Febrile illness
Zaliv Terpenyia virus (ZTV)	Ticks	Eurasia	–
Unclassified			
Ambe virus (AMBEV)	Sandflies	South America	–
American dog tick phlebovirus (ADTPV)	Ticks	North America	–
Anhanga virus (ANHV)	ND	South America	–
Arboledas virus (ADSV)	Sandflies	South America	–
Blacklegged tick phlebovirus (BTPV)	Ticks	North America	–
Caimito virus (CAIV)	Sandflies	South America	–
Itaporanga virus (ITPV)	Mosquitoes	South America	–
Komandory virus (KOMV)	Ticks	Asia	–
Leticia virus (LTCV)	Sandflies	South America	–
Mariquita virus (MRQV)	Sandflies	South America	–
Morolillo virus (MOLV)	ND	South America	–
Otter fecal phlebovirus	ND	Eurasia	–
Pacui virus (PACV)	Sandflies	South America	–
Phasi Chaeron-like virus (PCLV)	Flies	Asia	–
Phasi Chaeron virus (PhaV)	Flies	Asia	–
Red fox fecal phlebovirus	ND	Eurasia	–
Rio Grande virus (RGV)	ND	South America	–

(continued)

Table 10.7 (continued)

Virus species (in italics) and their member viruses	Vector	Distribution	Disease in humans
Salanga virus	ND	Africa	–
Salobo virus (SBOV)	ND	South America	–
Tapara virus (TAPV)	Sandflies	South America	–
Uriurana virus (URIV)	Sandflies	South America	–
Urucuri virus (URUV)	ND	South America	–

ND not determined
[a]Viruses in quotation marks represent proposed species

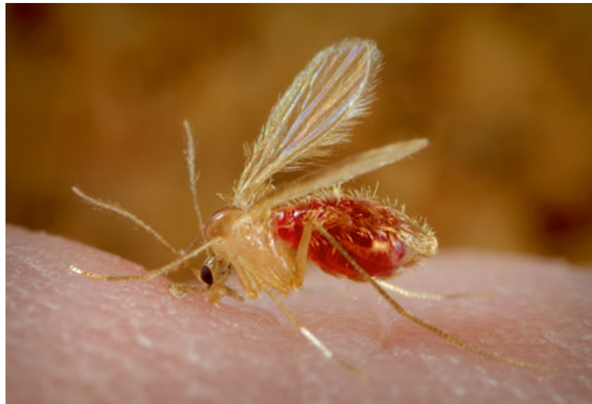

Fig. 10.9 *Phlebotomus papatasi* sandfly, a known vector of sandfly fever Naples and sandfly fever Sicilian viruses, taking a blood meal (photo courtesy of CDC/James Gathany, obtained from CDC's Public Health Image Library at http://phil.cdc.gov/phil/home.asp [accessed March 18, 2014])

several distinct phleboviruses using the same vectors. This multivirus infection provides considerable opportunity for reassortment of the genome segments and therefore results in the evolution of new viruses. The vertebrate hosts or reservoirs of most phleboviruses have not been characterized to date. However, the role of the amplifying hosts might be minimal or secondary in regard to the horizontal amplification of the viruses in vectors [64].

Phleboviruses occupy broad geographic and ecologic niches, defined by the distribution, host competency, and biological behavior of their vectors. Phleboviruses are endemic in Europe, Africa, Central Asia, and the Americas, and have even been found on a subantarctic island [65, 66]. There is considerable evidence that phlebovirus habitats, especially for sandfly-borne viruses and Rift Valley fever virus, are expanding, possibly due to climate change and thereby changing ranges of vectors [64, 67].

Sandfly-borne phleboviruses are transmitted by the sandflies in the family *Psychodidae*, which are present in the warm zones of Asia, Africa, Australia, Southern Europe, and the Americas [68]. In the Old World (Figs. 10.9 and 10.10), the most important human pathogens are sandfly fever Sicilian virus (SFSV), sandfly fever Naples virus (SFNV), and Toscana virus (TOSV), all of which are

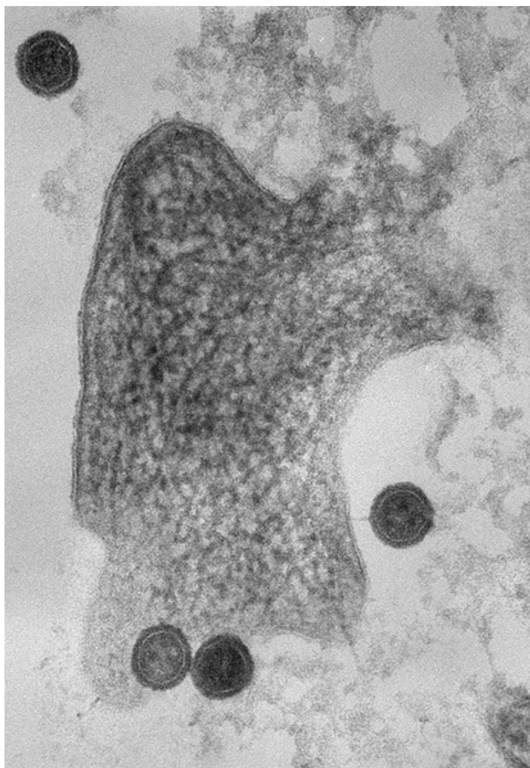

Fig. 10.10 Transmission electron micrograph of virions of the phlebovirus sandfly fever Turkey virus (50,000× magnification, courtesy of Dr. Stefan Frey, Bundeswehr Institute of Microbiology, Munich, Germany)

transmitted by *Phlebotomus* sandflies and cause febrile disease [64]. The risk for phlebovirus exposure via sandflies is high in populations residing in regions south and east of the Mediterranean Sea [64]. Little is known about the epidemiology of sandfly-borne phleboviruses of the New World that are transmitted by *Lutzomyia* sandflies. Several viruses, such as Alenquer, Candiru, Chagres, E(s)charate, Morumbi, Punta Toro, and Serra Norte viruses, were isolated from humans with clinical febrile disease in Brazil, Panama, Columbia, Peru, and regions of northern South America. The phleboviral diseases of tropical America are probably associated with rodents living in forests that may act as amplifying hosts [69, 70].

Transmission of sandfly-borne phleboviruses to susceptible humans and animals occurs during the blood meal of female sandflies, which are the primary reservoirs for these viruses [64, 65]. Vertical and horizontal transmissions, demonstrated experimentally and in natural habitats, are contributing mechanisms that assure long-term maintenance of phleboviruses in these vectors [71]; however, the exact role and efficiency of these mechanisms in the transmission cycles of various phleboviruses have not been fully explored. Researchers generally agree that humans or other large vertebrates are dead-end hosts that do not participate in the natural virus transmission cycle. Horizontal transmission among infected vertebrates does not occur. Toscana virus was isolated from a Kuhl's pipistrelle (*Pipistrellus kuhli*) in Italy [72], but the

role of bats in virus circulation remains clear. Direct aerosol or parenteral transmission of sandfly-borne phleboviruses has not been demonstrated.

Rift Valley fever virus (RVFV) is the best characterized mosquito-borne phlebovirus. RVFV is the causative agent of Rift Valley fever (RVF), an acute disease of domestic ruminants characterized by deaths of newborn animals and abortion in pregnant sheep, goats, and cattle. RVF was initially described in British Kenya in 1931 [73]. The disease is endemic in sub-Saharan Africa and has caused major outbreaks in several African countries including Egypt, Kenya, Madagascar, Mauritania, Tanzania, Senegal, Somalia, South Africa, and Sudan [74, 75]. The largest human epidemic occurred in Egypt in 1977–1978, when there were an estimated 200,000 human infections, with some 18,000 cases of illness and 600 deaths. In 2000, an outbreak occurred in Saudi Arabia and Yemen, the first outbreak occurring outside of Africa. This outbreak raises concern that the disease could spread further into Asia or Europe, or even to the Western Hemisphere where susceptible mosquitoes may be present [76, 77]. In its enzootic regions, RVFV persists in the environment through vertical transmission in mosquitoes and horizontal transmission by mosquitoes among domestic and wild herbivores, including cattle, buffalo, sheep, and goats. The principal hosts for RVFV have not been identified but presumably involve native ungulates and rodents [76, 78]. Bats can also be infected experimentally [79]. RVFV may replicate in a number of potential vectors, including ticks and a variety of flies [80–82]. However, various mosquitoes, including *Aedes* and *Culex* mosquitoes, are the main arthropod vectors in the natural environment [77]. RVFV is transmitted to vertebrates by the bites of infected mosquitoes or by direct contact with infected tissues, blood, body fluids, and fomites, particularly if associated with abortions. Aborted materials and placental membranes contain high numbers of virions, which can either contaminate the local environment or infect animals or humans in close contact. In vitro experiments have described the relatively long-term persistence of RVFV in the environment [77, 83]. Because of this persistence and the potential for aerosol transmission, biosafety level 3/4 laboratory conditions are recommended for handling the virus. Due to habitat expansion of competent vectors, increases in live animal trade, and impact of climatic and environmental changes, the risk of an introduction of RVFV into naive zones and preparedness for a probable emergence have been considered by several national and international agencies [77, 84].

Tick-borne phleboviruses [8] are transmitted via competent hematophagous hard (ixodid) or soft (argasid) ticks to various warm-blooded vertebrates [51]. Uukuniemi virus (UUKV) is the prototype tick-borne phlebovirus in the Old World and was originally isolated from a pool of *Ixodes ricinus* ticks collected in southern Finland [85]. Subsequently, UUKV was detected in Scandinavia and central and Eastern Europe and from Azerbaijan in Central Asia. Vertebrate hosts for UUKV are forest rodents (e.g., bank voles) and ground-feeding passerine birds [86]. Serological surveys suggest that humans have been exposed to UUKV without serious consequences. In fact, other than UUKV, which has been associated with a self-limiting

acute febrile disease in individuals from southern Russia, no human illness has been reported with the other Uukuniemi group viruses [86, 87].

Bhanja virus (BHAV) is another tick-borne phlebovirus that was initially isolated from *Haemaphysalis intermedia* ticks collected from a paralyzed goat in India. BHAV has subsequently been isolated in Europe and Africa from ticks of several other species, domestic animals, and humans, and has been associated with febrile disease in domestic animals (sheep, goats, and cows) and meningoencephalitis in humans [86].

Novel tick-borne phleboviruses causing disease in humans have recently emerged in China and the USA. These include severe fever with thrombocytopenia syndrome virus (SFTSV; also known as Huaiyangshan virus or Henan fever virus) and Heartland virus [88–91]. SFTSV causes a severe and potentially fatal febrile illness with thrombocytopenia and hemorrhagic manifestations. SFTSV mainly occurs in the rural areas of eastern, central, and northeastern China, but SFTSV has also been reported from South Korea and Japan [92]. SFTSV has been detected in *Haemaphysalis longicornis* and *Rhipicephalus microplus* ticks collected in the endemic region of China [93]. A wide range of animals, including sheep, goats, cattle, pigs, dogs, chickens, and rodents have tested positive for antibodies against SFTSV [94]. Incidence of livestock infection was significantly higher than the incidence in poultry, humans, and rats, suggesting livestock might serve as amplifying hosts in human transmission. Heartland virus (HRTV) has been associated with human cases of severe febrile illness with thrombocytopenia after tick exposure in the central USA [88]. Ticks are implicated as potential vectors, as the virus was recently detected in field-collected *Amblyomma americanum* ticks [95]. Preliminary investigations also suggest exposure to HRTV or an antigenically similar virus in cattle, sheep, goats, deer, and elk [96]. Genetically, Bhanja group virus and its close relatives form a clade distinct from those of SFTSV/HRTV and UUKV.

7 Clinical Features of Human and Animal *Bunyaviridae* Infections

7.1 Orthobunyaviruses

At least 36 orthobunyaviruses have been associated with human disease (Table 10.4), causing a range of syndromes such as febrile illnesses, encephalitides, or hemorrhagic fevers. California serogroup viruses are important causes of disease among humans in the USA. For example, La Crosse virus (LACV) is a common cause of arboviral encephalitis in children in the midwestern USA, whereas Jamestown Canyon virus commonly causes encephalitis in adults [97]. The onset of illness is usually sudden and characterized by fever, headache, malaise, nausea, and vomiting. Within a few days, these signs and symptoms are accompanied by meningeal signs and lethargy. In severe forms, the disease may progress to seizures (approximately 50 %) and coma

(about 10 %) [97]. The case-fatality rate is approximately 1 % or less, and the total duration of the illness rarely exceeds 2 weeks [97]. Most infected individuals do not experience neurologic sequelae. Other orthobunyaviruses (e.g., Cache Valley virus) cause abortion or teratogenic effects in pregnant livestock.

7.2 *Nairoviruses*

Crimean-Congo hemorrhagic fever virus (CCHFV) is the most important of the nairoviruses in terms of human disease [42, 43]. CCHFV infection can result in a severe hemorrhagic syndrome with a 5–30 % case-fatality rate. The course of the disease is divided into four phases: incubation period, prehemorrhagic phase, hemorrhagic phase, and convalescent phase. The length of the incubation period appears to depend in part on the mode of acquisition of virus. Following a tick bite, the incubation period ranges from 1 to 5 days, whereas it is usually 5–7 days (maximum 13 days) following contact with infected blood or tissues [98]. The prehemorrhagic phase begins as a sudden and nonspecific prodrome that is characterized by fever, myalgia, arthralgia, and lower back and abdominal pain [98]. In patients who progress to hemorrhagic signs, the start of the hemorrhagic phase is abrupt and begins approximately 3–6 days following the onset of signs and symptoms. Bleeding occurs at various sites, including the brain and respiratory, gastrointestinal, and urogenital tracts. At this phase, the most common manifestation is a petechial rash of the skin, conjunctivae, and other mucous membranes, which progresses to large cutaneous ecchymoses. In those patients who succumb to infection, death occurs approximately 6–10 days after the first signs and symptoms from irreversible shock (e.g., loss of blood pressure, elevated levels of proinflammatory cytokines, and disseminated intravascular coagulation) [98, 99]. In patients who survive CCHF, full recovery may take up to a year [98].

7.3 *Hantaviruses*

Hantavirus infections are associated with two clinical disorders in humans: HFRS and HCPS. HFRS is associated with Old World viruses (e.g., Hantaan, Seoul, Dobrava, and Puumala viruses), whereas, HCPS is associated with New World viruses (e.g., Sin Nombre and Andes viruses) (Table 10.6). In general, the severity of HFRS varies with the causative agent. HFRS caused by Hantaan and Dobrava viruses are more severe, while disease caused by Seoul virus is more moderate and disease caused by Puumala virus is mild ("nephropathia epidemica"). Hantaan virus-associated HFRS, which first came to the attention of western physicians during the Korean War, is among the most severe forms of HFRS, resulting in a case-fatality rate of 5–15 % [100]. Classically, the clinical course of HFRS occurs in five distinct phases. First, a febrile phase consists of headache, high fever, and chills. A hypotensive phase then follows during which blood platelet numbers drop

and petechial hemorrhage can be observed. Abdominal pain and tachycardia are also observed during this stage. An oliguric phase may occur next in which decreased urine production, proteinuria and may progress to kidney failure. Urine production is then increased in the diuretic phase, which can last for months before the patient enters the convalescent phase and recovery [101].

In contrast, HCPS is a severe acute disease associated with a rapid onset of respiratory failure and cardiogenic shock [102]. HCPS bears some resemblance to HFRS except that the lungs are targeted for capillary leakage, instead of the kidneys [101]. As with HFRS, the clinical presentation and case-fatality rate depends on the etiological hantavirus [101]. The onset of HCPS in the Americas is generally characterized by nonspecific symptoms such as fever, myalgia, cough, gastrointestinal symptoms, chills, and headache. HCPS evolves rapidly (1–3 days) to a lung capillary leak syndrome, resulting in respiratory distress, followed by respiratory failure and then cardiogenic shock. These clinical signs are responsible for the majority of deaths (case-fatality rate up to 50 %) [100].

Although in general the Old World hantaviruses target the kidneys (resulting in HFRS) and New World hantaviruses target the lungs and heart (resulting in HCPS), this distinction is far from absolute. Indeed, controversy exists among hantavirologists whether the terms HFRS and HCPS should be abandoned in favor of the more generic single designation, "hantavirus fever" [103]. In the last few years, cases of hantavirus infection with divergent symptomatology have been reported. For example, cases of Puumala virus infection with pulmonary involvement were observed in Europe, and acute renal failure has been recognized in patients infected with New World hantaviruses [104, 105]. Similarly, in a recent study in China, investigators examined the clinical characteristics and outcome of 48 patients with HFRS who also had acute respiratory distress syndrome [106]. Patients in this study were in critical condition, with 21 succumbing to the disease (43.8 % case-fatality rate).

7.4 Phleboviruses

The best-known clinical condition associated with phleboviruses is a febrile illness known as sandfly fever (also known as phlebotomus, papatacci, or 3-day fever in the Old World). Sandfly fever is prevalent in the countries in the Mediterranean Basin, Northern Africa, and parts of Central, Western, and Southern Asia [65]. Cases and outbreaks of sandfly fever still occur in non-immune individuals in endemic regions [107, 108]. Sandfly fever has also been described as an important travel-related infection, and several cases of imported sandfly fever have been reported in patients following visits to endemic regions [107, 109]. Sandfly fever is characterized by high fever, headache, retroorbital pain, photophobia, generalized aches, malaise, and chills. Abdominal pain, discomfort, diarrhea, or constipation may also occur. The duration of fever is usually 2–4 days, but may be extended in some cases. Following the febrile stage, the affected patients frequently suffer from fatigue and weakness. Convalescence may require a few days to several weeks and can be

incapacitating. The prognosis is favorable without any complications, sequelae, or lethality. In infected individuals, viral replication is controlled by the immune response. The immunity to sandfly fever phleboviruses is specific to the virus, and neutralizing antibodies produced during exposure are sufficient to suppress the occurence of symptoms upon rechallenge with homotypic agent [110, 111]. In the Old World, phleboviruses responsible for sandfly fever are sandfly fever Sicilian virus (SFSV) and sandfly fever Naples Virus (SFNV) [64, 110]. Regional SFSV variants, sandfly fever Cyprus virus (SFCV) and sandfly fever Turkey virus (SFTV), are also associated with the clinical picture of sandfly fever similar to that caused by SFSV and SFNV, but induce more prominent gastrointestinal symptoms, including elevation of hepatic enzymes and thrombocytopenia, with a more emphasized post-infectious asthenic syndrome [112–114]. In the New World, primarily Alenquer, Chagres, Candiru, and Punta Toro viruses have been detected in individuals with febrile diseases [61, 70]. Tick-borne Uukuniemi virus has also been identified in a limited number of cases of acute illness characterized by fever, headache, muscle and joint pain, facial hyperemia, and body rash [86, 87].

Toscana virus (TOSV), another sandfly-borne phlebovirus, transmitted by the bites of *Phlebotomus perniciosus* and *P. perfiliewi* sandflies, is a significant human pathogen due to its distinct neurotropism, causing central nervous system infections in endemic regions around the Mediterranean Sea [115]. TOSV has been pinpointed as a major agent of seasonal aseptic meningitis or meningoencephalitis, especially occurring during spring-summer months when the activity of vectors increase. TOSV infection is also a travel-associated infection in individuals leaving endemic regions [115]. Similar to the other phleboviral infections, the majority of the TOSV exposures result in an asymptomatic seroconversion or subclinical infection. Clinical manifestations in symptomatic TOSV infections are frequently characterized by high fever, severe headache, myalgia, and neurological symptoms of cortical and/or meningeal involvement from which patients generally recover within 7–10 days [64, 110, 115]. Central nervous system involvement in most cases is associated with a favorable outcome, but severe and lethal infections have also been reported [116]. Moreover, a variety of clinical signs and symptoms including hydrocephalus, impaired speech, paresis, hearing loss, diffuse intravascular coagulation, myositis-fasciitis, and testicular manifestations have also been reported [116–120].

RVF is the clinical manifestation of infection with the mosquito-borne RVFV, affecting ruminant animals and humans. Outbreaks of RVF in ruminants are often recognized initially as an abortion storm in herds of pregnant animals. Sheep are the most susceptible mammals, while cattle, goats, and camels demonstrate variations in susceptibility. Forty to 100 % of pregnant RVFV-infected sheep abort, and the fetuses often have malformations [76]. Newborn lambs suffer from an acute disease, characterized by necrotic hepatitis with 95–100 % lethality. Some breeds of adult sheep also exhibit hemorrhagic signs, similar to those seen in humans. In humans, exposure to RVFV frequently remains asymptomatic. In those with clinical signs, the disease presents with an influenza-like febrile disease without complications. However, severe manifestations such as hepatitis, retinitis, encephalitis, and hemorrhagic disease have been observed in a small number of cases with RVF, with the overall case-fatality rate estimated to be between 0.5 and 2 % [77, 121].

Severe fever with thrombocytopenia syndrome (SFTS) is a recently described human clinical disease caused by the emerging tick-borne phlebovirus SFTSV [90]. The disease is characterized by high fever, fatigue, anorexia, vomiting, and diarrhea, as well as laboratory findings of thrombocytopenia, leukocytopenia, and elevation of certain serum enzymes, including alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase, creatine phosphokinase, and creatine kinase. The symptoms of SFTS frequently resolve after a week. However, in some cases, the patient's condition progresses to multiorgan dysfunction, disseminated intravascular coagulation, shock, and/or acute respiratory distress syndrome, with neurologic and hemorrhagic manifestations [122, 123]. Case-fatality rates as high as 30 % have been reported [124]. HRTV infections present similar to SFTS. However, the prognosis is favorable, with no respiratory or kidney involvement or coagulation abnormalities [88].

8 General Features of Plant-Infecting Tospoviruses

Currently, 11 species are included in the genus *Tospovirus*, each of which has one member virus. Many other tospoviruses have yet to be classified into species (Table 10.8) [8]. Thrips of numerous species (Fig. 10.11) of the genera *Frankliniella*, *Thrips*, *Scirtothrips*, and *Ceratothripoides* have been reported to transmit tospoviruses to a wide variety of plant species [8]. Transmission can also occur by mechanical means via infected plant sap. Tomato spotted wilt virus (TSWV) is the prototype tospovirus, and, contrary to its name, has a broad host range with susceptible plants belonging to more than 925 species of 70 botanical families [8]. Spotted wilt disease of tomato was first described in 1915 in Australia. The disease was later shown to be transmitted by thrips and caused by TSWV in 1930 [125]. The worldwide dispersal in the 1980s of the western flower thrip (*Frankliniella occidentalis*), a major vector of TSWV, led to a reemergence of the disease. By 1994, worldwide losses of tomato were estimated to be in excess of US $1 billion annually [126]. In addition to tomatoes, other economically important plants affected by TSWV include peppers, lettuce, peanuts, and chrysanthemums [127]. Tospoviruses cause variable signs, including necrotic or chlorotic rings and flecking on leaves, stems and fruits, with early infections leading to one-sided growth, drooping or deformed leaves, wilting, stunting, and death [127] (Fig. 10.12). Late in TSWV infection, tomatoes produce unmarketable fruit with necrotic ringspots that often appear only when the fruit reaches full color (Fig. 10.13).

Molecular and structural studies, mostly performed on TSWV, have shown many similarities with animal-infecting bunyaviruses, including particle morphology, genomic organization, and replication and transcription cycles [128]. The presence of an envelope membrane is relatively unique among plant viruses, and is only found on tospovirions and plant-adapted rhabdovirions [128]. In contrast to the many similarities tospoviruses have with animal-infecting bunyaviruses, some interesting differences reflect tospovirus adaptation to plants. Unlike all other bunyaviruses, the NSm protein of tospoviruses is translated using a genomic ambi-

Table 10.8 Viruses in the genus *Tospovirus*

Virus species Member viruses	Vector	Distribution
Groundnut bud necrosis virus		
Groundnut bud necrosis virus (GBNV)[a]	Thrips: *Frankliniella schultzei*, *Scirtothrips dorsalis*,*Thrips palmi*	Asia
Groundnut ringspot virus		
Groundnut ringspot virus (GRSV)	Thrips: *Frankliniella gemina*, *Frankliniella intonsa*, *Frankliniella occidentalis*, *Frankliniella schultzei*	Africa, North America, South America
Groundnut yellow spot virus		
Groundnut yellow spot virus (GYSV)[b]	Thrips: *Scirtothrips dorsalis*	Asia
Impatiens necrotic spot virus		
Impatiens necrotic spot virus (INSV)	Thrips: *Frankliniella fusca*, *Frankliniella intonsa*, *Frankliniella occidentalis*, *Frankliniella schultzei*	Africa, Asia, Australia, Europe, North America, South America
Polygonum ringspot virus		
Polygonum ringspot virus (PolRSV)	Thrips: *Dictyothrips betae*	Europe
Tomato chlorotic spot virus		
Tomato chlorotic spot virus (TCSV)	Thrips: *Frankliniella intonsa*, *Frankliniella occidentalis*, *Frankliniella schultzei*	Puerto Rico, South America
Tomato spotted wilt virus		
Tomato spotted wilt virus (TSWV)	Thrips: *Frankliniella bispinosa*, *Frankliniella cephalica*, *Frankliniella fusca*, *Frankliniella gemina*, *Frankliniella intonsa*, *Frankliniella occidentalis*, *Frankliniella schultzei*, *Thrips palmi, Thrips setosus*, *Thrips tabaci*	Africa, Asia, Australia, Europe, North America, South America
Watermelon silver mottle virus		
Watermelon silver mottle virus (WSMoV)	Thrips: *Thrips palmi*	Asia
Zucchini lethal chlorosis virus		
Zucchini lethal chlorosis virus (ZLCV)	Thrips: *Frankliniella zucchini*	South America
Unclassified		
Alstroemeria necrotic streak virus (ANSV)	Thrips: *Frankliniella occidentalis*	South America
Bean necrotic mosaic virus (BeNMV)	ND	Central America, South America
Calla lily chlorotic spot virus (CCSV)	Thrips: *Thrips palmi*	Asia
Capsicum chlorosis virus (CaCV)[c]	Thrips: *Ceratothripoides claratris*, *Frankliniella schultzei*, *Thrips palmi*	Asia, Australia
Chrysanthemum stem necrosis virus (CSNV)	Thrips: *Frankliniella gemina*, *Frankliniella occidentalis*, *Frankliniella schultzei*	Asia, Europe, South America

(continued)

Table 10.8 (continued)

Virus species Member viruses	Vector	Distribution
Hippeastrum chlorotic ringspot virus (HCRV)	ND	Asia
Iris yellow spot virus (IYSV)	Thrips: *Frankliniella fusca*, *Thrips tabaci*	Africa, Asia, Australia, Europe, Hawaii, North America, South America,
Lisianthus necrotic ringspot virus (LNRV)	ND	Asia
Melon severe mosaic virus (MeSMV)	ND	North America, South America
Melon yellow spot virus (MYSV)[d]	Thrips: *Thrips palmi*	Asia
Mulberry vein banding virus (MuVBV)	ND	Asia
Peanut chlorotic fan-spot virus (PCFV)[e]	Thrips: *Scirtothrips dorsalis*	Asia, South America
Pepper necrotic spot virus (PNSV)[f]	ND	South America
Soybean vein necrosis-associated virus (SVNaV)	ND	North America
Tomato necrotic ringspot virus (TNRV)	Thrips: *Ceratothripoides claratris*, *Thrips palmi*	Thailand
Tomato yellow (fruit) ring virus (TYRV)	Thrips: *Thrips tabaci*	Asia, Africa
Tomato zonate spot virus (TZSV)	Thrips: *Frankliniella occidentalis*, *Thrips palmi*, *Thrips tabaci*	Asia
Watermelon bud necrosis virus (WBNV)	*Thrips palmi*	Asia

ND not determined
[a]Also known as peanut bud necrosis virus (PDNV)
[b]Also known as peanut yellow spot virus (PYSV)
[c]Also known as Gloxinia tospovirus, tomato necrosis virus (TNRV), or Thailand tomato tospovirus
[d]Also known as Physalis severe mottle virus (PhysSMV)
[e]Also known as groundnut chlorotic fan-spot virus (GCFSV)
[f]Also known as pepper Peruvian necrotic virus

sense strategy [129] (Fig. 10.2). Also, the NSm protein of tospoviruses is the only NSm in the family for which a well-defined function is known. Unlike the animal-infecting bunyaviruses, tospovirus particles accumulate in large vesicles within the cell and are not secreted from plant cells. Spread of the viruses to neighboring plants only occurs after larval-stage thrips have acquired the virus after feeding on infected plants [130]. After a latency period, thrips can transmit the tospovirus during the adult stage. Furthermore, due to the presence of a rigid cell wall in plants, plant viruses can only spread to neighboring cells through channels connecting the plant

Fig. 10.11 A thrip on a person's finger. Thrips are small insects with a distinctive cigar-shaped body. They are the primary vectors of plant diseases caused by tospoviruses (courtesy of OpenCage, obtained at http://en.wikipedia.org/wiki/Thrips [accessed March 18, 2014])

Fig. 10.12 Pepper plant infected with tomato spotted wilt virus showing the initial development of necrotic spots on the leaves (courtesy of Carlos Gonzalez, obtained at http://en.wikipedia.org/wiki/Tospovirus [accessed March 18, 2014])

Fig. 10.13 Tomato infected with tomato spotted wilt virus showing the development of necrotic ringspots on the fruit (obtained at http://en.wikipedia.org/wiki/Tospovirus [accessed March 18, 2014])

cells called plasmodesmata. As TSWV and most other plant viruses have a diameter of 80–120 nm, these virus cannot pass through the plasmodesmata that have a diameter in the range of only 5 nm. Thus, to facilitate movement, many plant viruses have adapted and encode cell-to-cell movement proteins (MPs) that modify plasmodesmata to allow passage of macromolecules, including virions [131]. The NSm protein of TSWV has been identified as the cell-to-cell MP that allows virus to move between plant cells [132–134].

9 Unclassified Bunyaviruses

At least 28 viruses, mostly isolated from mosquitoes or ticks and with unknown pathogenic potential for humans, are possible bunyaviruses, but currently remain unclassified (Table 10.9).

Table 10.9 Unclassified Bunyaviruses

Virus	Host	Distribution	Disease in humans
Antequera virus (ANTV)	Mosquitoes	South America	Unknown
Bangui virus (BGIV)	Humans?	Africa	Fever/rash
Barranqueras virus (BQSV)	Mosquitoes	South America	Unknown
Belem virus (BLMV)	Birds	South America	Unknown
Belmont virus (BELV)	Mosquitoes	Australia	Unknown
Bobaya virus (BOBV)	Birds	Africa	Unknown
Caddo Canyon virus (CDCV)	Ticks	North America	Unknown
Chim virus (CHIMV)	Ticks	Asia	Unknown
Cumutu virus (CUMV)	Mosquitoes	North America	Unknown
Gan Gan virus (GGV)	Mosquitoes	Australia	Arthritis/rash
Gouléako virus (GOLV)	Mosquitoes	Africa	Unknown
Herbert virus (HEBV)	Mosquitoes	Africa	Unknown
Hissar virus	Ticks	Asia	Unknown
Kaisodi virus (KSOV)	Ticks	Asia	Unknown
KF298274 virus	Mosquitoes	Europe	Unknown
Kibale virus (KIBV)	Mosquitoes	Africa	Unknown
Kigluaik phantom virus (KIGV)	Biting midges	North America	Unknown
Lanjan virus (LJNV)	Ticks	Asia	Unknown
Mapputta virus (MAPV)	Mosquitoes	Australia	Unknown
Maprik virus (MPKV)	Mosquitoes	Australia	Unknown
Nome phantom virus (NOMV)	Biting midges	North America	Unknown
Okola virus (OKOV)	Mosquitoes	Africa	Unknown
Pacora virus (PCAV)	Mosquitoes	North America	Unknown
Para virus (PARAV)	Mosquitoes	South America	Unknown
Resistencia virus (RTAV)	Mosquitoes	South America	Unknown

(continued)

Table 10.9 (continued)

Virus	Host	Distribution	Disease in humans
Santarem virus (STMV)	Mosquitoes	South America	Unknown
Silverwater virus (SILV)	Ticks	North America	Unknown
Sunday Canyon virus (SCAV)	Ticks	North America	Unknown
Taï virus (TAIV)	Mosquitoes	Africa	Unknown
Tanga virus (TANV)	Mosquitoes	Africa	Unknown
Tataguine virus (TATV)	Mosquitoes, humans	Africa	Febrile illness
Trubanaman virus (TRUV)	Mosquitoes	Australia	Arthritis/rash
Wanowrie virus (WANV)	Ticks, humans	Asia	Unknown
Witwatersrand virus (WITV)	Mosquitoes	Africa	Unknown
Yacaaba virus (YAVC)	Mosquitoes	Australia	Unknown

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Chapter 11
Viral Hemorrhagic Fevers of Animals Caused by Negative-Strand RNA Viruses

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Core Message As ecological pressures force humans to seek new, high-quality protein sources, the role of aquaculture in human society will gain greater importance. Serious diseases of both nonhuman primates and food and fiber animals are outlined here, along with zoonotic viruses that occasionally cause serious disease and death in humans. Finally, honorable mention is given to Rinderpest virus, a success of global eradication efforts in that this virus once caused serious societal effects, but has been relegated to a footnote in textbooks because of a global eradication effort that was declared successful in 2011.

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1 Introduction

The term "viral hemorrhagic fever" (VHFs) is clearly inadequate to fully encapsulate a single spectrum of either ecologically, pathologically, or clinically defined syndromes. The term becomes most inappropriate in a nonhuman context. Some animals are poikilothermic (i.e., cold-blooded) and are incapable of producing a fever. Other animals might only hemorrhage as a minor occurrence in the spectrum of presenting clinical signs. Hemorrhage may occur only after infection with certain virus strains or variants (e.g., bovine viral diarrhea virus 2-associated hemorrhagic disease) or when a particular constellation of genomic mutations is met (e.g., feline calicivirus) [1].

Given that none of the viruses generally considered to be causing VHFs are primarily neurotropic, the fevers they induce are likely secondary to an inflammatory process and not due to an infection of or primary effect on the hypothalamus (i.e., they are not centrally neurogenic fevers). While fever is a common finding in the originally defined group of VHFs (hemorrhagic fever with renal syndrome, Crimean–Congo hemorrhagic fever, and Omsk hemorrhagic fever), it is reasonable to suppose that fever is secondary to the underlying infectious process(es) and not a proximate cause of clinical disease.

Assuming that people generally consider hemorrhage (and not fever) to be the defining characteristic of those serious viral diseases commonly referred to as VHFs, we can continue to use the term for hemorrhagic viruses of poikilotherms, even though it is recognized as inadequate to that situation. Additionally, if we assume that (like humans) overt hemorrhage need not be a major part of the clinical spectrum of disease, we can adequately define a set of serious viral diseases of animals that could be termed "VHFs."

The set of viruses presented here meet these criteria—they cause hemorrhage, or metabolic and/or physiologic deficits that could lead to hemorrhage, as a typical clinical sign. There are many other viruses that can cause severe hemorrhage as an infrequent or minor clinical sign, or cause minor hemorrhage as a significant clinical sign (e.g., petechiae in the brain). These "VHF-like viruses" are mentioned

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briefly in each of the four chapters describing VHFs of animals other than humans. Viruses that induce severe disruptions in fluid balance (e.g., feline infectious peritonitis virus, rotaviruses) or compromise the physical barriers to fluid loss through direct cellular lysis and/or immunological deficits (e.g., in progressive vaccinia or eczema vaccinatum) are not included because the defining characteristic of virally induced hemorrhage is overt blood loss from the vascular system and not simply a tissue-barrier deficit that leads to fluid loss (e.g., skin barrier compromise or edematous conditions).

Whether Čumakov's original, geographic criteria of "natural focus infections" (see [2]) should be reintroduced to the discussion can be debated, but we believe that clinicopathological features of disease should continue to be the defining criteria of the "VHF group." More focused criteria, such as "only endotheliotropic viruses," would exclude viruses that should clearly be considered, like the hepatotropic Rift valley fever virus. Combining a constellation of cellular tropisms may lead to a useful VHF definition, but would not include those viruses that induce deficits in the hemostatic system, including cytokine disturbances and/or thrombocyte function deficits. Examining the embryological origin of the affected tissues also does not properly delineate the group of affected cellular types, as for instance endothelium is of mesodermal origin whereas hepatocytes are derived from endoderm. In the final analysis, every classification system has advantages and deficits, as they are black-and-white distinctions applied to the wonderous and varied greys of Nature.

Having decided that we can use the term VHF to describe some diseases of animals, we must also briefly examine the "animal" part of the phrase "VHFs of animals," especially as it relates to human or ecological health. When considering "public health/one health" issues surrounding animal diseases, many authors focus exclusively on their zoonotic potential, and we followed that paradigm here. However, it is important to remember that a broader definition of both public health and One Health issues may be more appropriate—one that considers the effect of animal disease on human issues other than direct or indirect infection or disease [3]. Issues such as food security, economic harm that can lead to depression and/or suicide [4], or the ecological effects of losing large grazing animals from monoculture systems should be considered in this larger context. For example, a rise in available plant matter can favor an increase in arthropod vector concentrations, which can then precipitate an increase in the transmission of arthropod-borne diseases of importance to humans [5].

One Health issues encompass the assumptions of traditional wisdom. Quite literally, the proverbial "teaching a man to fish" is predicated on the presence of fish, whereas an epizootic depopulation due to infectious hematopoietic necrosis virus infection could negate the value of that knowledge. Similarly, some charitable organizations are predicated upon the idea that a single animal (or small group of animals) can change the economic security of whole families (and perhaps whole villages) in developing countries. However, the social equations of such organizations (e.g., Heifer International) would be radically altered by a Rift Valley fever virus abortion storm that removed the economic benefit of young animals for an entire season.

Animal diseases can take on an importance to a population of humans that exceeds the personal tragedy represented by any single infection (or group of infections) of individual humans. While it may be understandably "specie-ist" to feel the disease and loss of humans more than we feel the disease and loss of animals, we must remember that animals play a vital (and sometimes outsized) importance in human society. A focus on veterinary issues in the context of human issues is an important and necessary allocation of resources that is the basis for the current public health focus on One Health. As diseases of societal importance continue to emerge (or re-emerge) from natural reservoirs, many of which are mammalian species, this focus can and should expand to meet those coming threats.

2 Infectious Hematopoietic Necrosis Virus

2.1 Etiologic Agent and Natural History

2.1.1 Definition

Infectious hematopoietic necrosis virus (IHNV) is a novirhabdovirus that infects and causes hemorrhagic disease in salmon and trout belonging to several species. IHNV targets the hematopoietic tissue of the kidneys and spleen, as well as several other tissues and organs. The virus is endemic to the North American Pacific Northwest and has been introduced outside of this range to various countries in Europe and Asia by transport of infected fish and eggs.

2.1.2 Etiology and Evolution

The disease caused by IHNV was known as Oregon sockeye virus, Sacramento River chinook disease, and Columbia River sockeye disease, among others, before the etiological agent was fully described. The suspected viral disease was first described by Rucker et al. [6] and Watson et al. [7] in cultured sockeye salmon (*Oncorhynchus nerka*) from Washington State, USA. The causative virus was later identified and characterized by isolation in fish cell culture [8–10] and used experimentally to reproduce the disease [11].

IHNV has a linear, single-stranded negative-sense RNA genome of approximately 11,000 nucleotides, containing six genes in the order of 3′-N-P-M-G-Nv-L-5′ that encode the nucleocapsid (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), nonvirion protein (Nv), and RNA-dependent RNA polymerase (L), respectively [12, 13]. The presence of the unique nonvirion protein (Nv) gene and sequence similarity with certain other fish rhabdoviruses such as viral hemorrhagic septicemia virus are characteristics of viruses of the genus *Novirhabdovirus* [14, 15]. IHNV particles are bullet-shaped and enveloped (Fig. 11.1) with mean measurements of 150–190 nm in length and diameters of 65–75 nm. Virions are sensitive to heat, acid, and ether. The

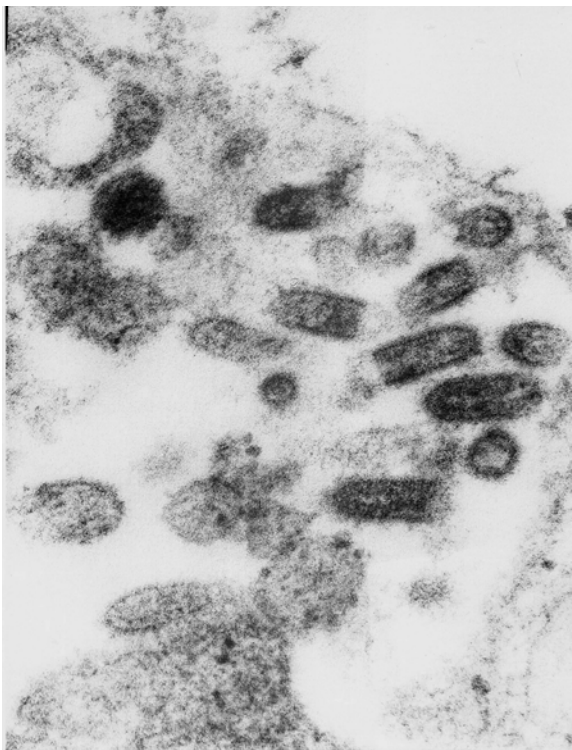

Fig. 11.1 Transmission electron micrograph of cultured epithelioma papulosum cyprini epithelial cells (EPC) infected with infectious hematopoietic necrosis virus (IHNV). IHNV particles (*arrows*) are in cell debris. Uranyl acetate and lead citrate (65,000×)

replicating temperature range of IHNV is 4–20 °C with an optimum of 15 °C [11], consistent with its presence in coldwater fish.

In the 1980s, results of studies using protein electropherotyping [16], monoclonal antibodies, [17–20] and RNase fingerprinting [21] indicated that IHNV genotypes are geographically distinct. This hypothesis was further supported by phylogenetic analysis of the complete G and Nv gene sequences of 12 IHNV isolates [298]. The study of IHNV genetic diversity and evolution was continued by examining the most variable 303 nucleotide region of the G gene of 323 virus isolates obtained between 1966 and 2001 from the USA and Canadian West Coast. The three major genogroups identified occurred in different geographical areas irrespective of the host, with some overlap, and were designated L (lower), M (middle), and U (upper) genogroups [22]. Specifically, the L genogroup was found in California and along the Southern Oregon coast; the M genogroup was seen in Idaho (Hagerman Valley) and sporadically in the US portion of the Columbia River Basin; and the U genogroup was shown to span the national border between the USA and Canada as it was present in Alaska, Washington, and Oregon coasts, and the Columbia River Basin, including most of British Columbia, Canada. The genetic diversity among isolates was relatively low overall (maximum of 3.6 % nucleotide diversity), but was

most conserved in the U clade (no subgroups) and most diverse in the M clade, which has been divided into subgroups A through E [23–25]. Less diversity was found in the L group than in the M group, which was subdivided into subgroups L1 and L2 [26]. Subsequent examination of eight IHNV isolates from sockeye salmon in Russia indicated they were genetically homogeneous and belonged to the U clade [27]. Similar genetic studies investigating several Italian, German, French, and Austrian IHNV isolates indicated they belonged to the M genogroup, suggesting a monophyletic origin for all IHNV isolated in Europe, likely originating from rainbow trout imported from the USA [28, 29]. These viruses have since evolved in rainbow trout farms to form a new European (E) genogroup [30, 31].

Parallel studies with mouse monoclonal antibodies showed there was some variation in nucleoprotein epitopes that did not fully correlate with genotypic classification [29]. Genotyping of Japanese IHNV indicated that five isolates from 1971 to 1982 clustered with the U genogroup, whereas five others formed a new divergent genogroup JRt (Japanese rainbow trout) that shared a common source with the North American U genogroup [24]. The first detection of IHNV in Japan was in kokanee salmon fry (land-locked sockeye salmon) at the Mori Hatchery in Hokkaido in 1971. Mori Hatchery received eggs from the Chitose Hatchery, where sockeye salmon eggs from Alaska had been received in 1969 [32, 33]. Additional genotyping of Chinese and Korean IHNV isolates indicated they were closely related to Japanese JRt and likely introduced from Japan in or after 1985 (Jia et al. 2013) and just prior to 1990 [34, 35], respectively.

Based on recent phylogenetic studies of novirhabdoviruses [36], IHNV and Hirame rhabdovirus shared a common ancestor that was sister to that of viral hemorrhagic septicemia virus and Snakehead rhabdovirus. However, the ancestral origin of all IHNV was likely the U genogroup originating from Alaska and British Columbia, where the virus had a long-term association with sockeye salmon. The virus was inadvertently disseminated to its present U clade range by transplant of salmonids and the early practice of feeding raw, unpasteurized sockeye salmon viscera to salmon fry during the 1950s and 1960s [22]. This diversifying selective pressure caused the virus to jump from sockeye salmon to Chinook salmon and to a lesser extent to steelhead [anadromous rainbow trout (*Oncorhynchus mykiss*)], thus leading to the current range of the U clade. Further evolutionary and geographical divergence occurred, resulting in the L clade that infects mostly Chinook salmon and some steelhead populations. The U and L clades likely remained genetically isolated and distinct through nonoverlapping ocean migration ranges of their associated hosts.

The greater diversity observed in the M clade also evolved from the U clade viruses by adaptation to captive farmed rainbow trout. These adaptive mutations occurred at a higher water temperature, along with several other selective pressures that significantly increased the replication of IHNV. In this captive population, reduced viral competition, reduced transmission, and temporal bottlenecks facilitated greater virus diversification at a more rapid rate than would otherwise occur in other host populations [22]. Thus, M clade viruses have lost pathogenicity for

sockeye salmon [37]. The earliest M clade isolate available for genotyping was from Hagerman Valley, Idaho in 1978. IHNV epizootics in Hagerman Valley were first reported in 1977. By 1980, the virus was endemic throughout this trout farming area [38, 39]. The first extension of M clade IHNV to the remaining Columbia River Basin was reported in anadromous salmonid hatcheries from 1980 to 1982, most likely resulting from virus disease outbreaks upstream in Hagerman Valley. These occurrences have been transient and sporadic, probably because the environmental adaptations of these viruses for farmed trout render them less fit for northern portions of the IHNV range [22]. However, since 2007 the M genogroup viruses have emerged to infect steelhead trout in the Washington coastal rivers of the Olympic Peninsula where salmon comanagers are concerned about further geographic extension [40].

2.1.3 Geographic Distribution and Economic Effects

The endemic range of IHNV is the Pacific Northwest of California, Oregon, Washington, and Columbia River Basin including Hagerman Valley, Idaho, British Columbia, Canada, and Alaska (Fig. 11.2) [11]. In 1969, transport of IHNV-infected fish or eggs to other parts of the USA (Minnesota, then South Dakota, West Virginia, Montana, New York, Colorado, Utah) did not establish the virus [41]. Introduction of the virus beyond North America occurred with more shipments of infected eggs or fish to Japan, Italy, France, Austria, Germany, Belgium, Croatia, Czech Republic, Iran, the Netherlands, Poland, Slovenia, Spain, Switzerland, northeast China, Taiwan, Korea, and Russia [29, 41, 42]. IHNV has become established in most of these countries with a high prevalence in major trout growing regions.

Infectious hematopoietic necrosis (IHN) has resulted in large losses of fish reared in freshwater and in seawater net pens, causing significant negative economic impact to salmon and trout growers in endemic areas of the Pacific Northwest and elsewhere. The disease is listed as reportable to the World Organisation for Animal Health (OIE). Since the appearance of IHNV in the Columbia River Basin in 1981, government hatcheries destroyed well over 70 million fish and eggs due to IHN. A conservative estimate of these lost revenues at that time was well over $350 million [41]. More currently, the rainbow trout farming industry in Hagerman Valley is worth approximately $70–90 million US dollars annually, with production rates of 18–23 million kg of fish per year. IHN epizootics in this industry have resulted in 1–50 % direct loss of total annual fish production with an additional marketing loss of 1–4 % due to spinal deformities occurring in surviving fish [43]. Losses of juvenile sockeye salmon in Alaska during 1978 and 1992 resulted in conservative estimated losses in adult revenues of $4.7 and $8.6 million, respectively [44]. A more recent outbreak of IHN in 2001–2003, affecting Atlantic salmon (*Salmo salar*) farms in British Columbia, caused a loss of 12 million fish from mortality or culling, with a net worth of USD$40 million in inventory representing $200 million in lost sales [45].

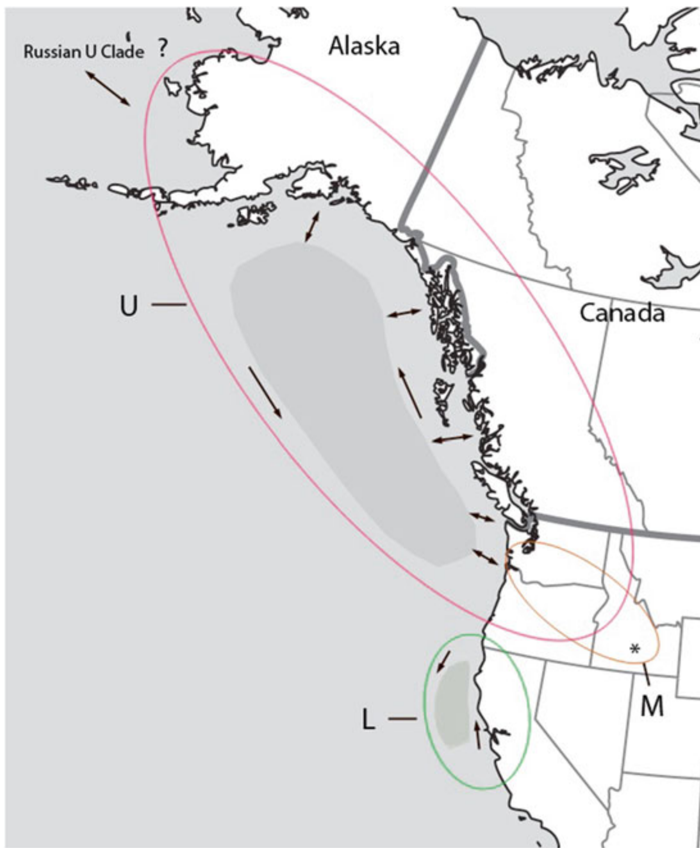

Fig. 11.2 The geographical ranges of the U, L, and M genogroups of infectious hematopoietic necrosis virus in the Pacific Northwest of North America. The *star* indicates the rainbow trout farming area in Hagerman Valley, Idaho. The *shaded areas* and *arrows* within two of the genogroup ranges indicate general migrational patterns of anadromous Pacific salmon during ocean maturation (modified from Kurath) [22]. The identical U clade strains existing in sockeye salmon on the Russian Kamchatka Peninsula (indicated by "Russian U Clade ?" in the figure) may extend the contiguous range of the U genogroup [27]

2.1.4 Natural History, Transmission, and Host Range

The principal fish hosts for IHNV are members of the Salmonidae. The three major genotypes of IHNV within the endemic range of Pacific Northwest and Idaho have a strong predilection for naturally infecting the host that each genotype has evolved with over time: U clade—primarily sockeye salmon with some chinook salmon and steelhead trout; M clade—non-anadromous rainbow trout; L clade—Chinook salmon and some steelhead trout (see Table 11.1). However, these viruses can infect non-preferred fish hosts (when provided the opportunity) under selective pressures

Table 11.1 Host range of IHNV and potential invertebrate vectors

Fish	Species	Fish	Species
Susceptible to disease			
Sockeye salmon	*Oncorhynchus nerka*	Chinook salmon	*O. tshawytscha*
Kokanee salmon	*O. nerka*	Chum salmon	*O. keta*
Cherry salmon	*O. masou masou*	Biwa salmon	*O. masou rhodurus*
Amago salmon	*O. masou macrostomus*	Yamame trout	*O. masou*
Rainbow trout	*O. mykiss*	Steelhead trout	*O. mykiss*
Cutthroat trout	*O. clarki*	Atlantic salmon	*Salmo salar*
Brook trout	*Salvelinus fontinalis*	Brown trout	*Salmo trutta*
Japanese char	*S. leucomaenis*	Sea bream	*Sparus aurata*
Turbot	*Scophthalmus maximus*	Burbot[a]	*Lota lota*
Pike (fry 1–2 mo)[a]	*Esox lucius*		
Limited susceptibility to disease			
Lake trout	*Salvelinus namaycush*		
Refractory to experimental infection[b]			
Arctic char	*Salvelinus alpinus*	Arctic grayling	*Thymallus arcticus*
Pink salmon[b]	*O. gorbuscha*	Coho salmon[b]	*O. kisutch*
White sturgeon[b]	*Acipenser transmontanus*	Sea bass[b]	*Morone labrax*
Northern squawfish	*Ptychocheilus oregonensis*	Pacific herring[b]	*Clupea pallasii*
Largescale sucker	*Catostomus columbianus*		
Whitefish[b]	*Prosopium williamsoni*		
Lamprey	*Entosphenus tridentatus*		
Other transient marine hosts[a]			
Tubesnout	*Aulorhynchus flavidus*	Shiner perch	*Cymatogaster aggregate*
Transient invertebrate mechanical vectors			
Salmon leech	*Piscicola salmositica*	Mayfly nymph	*Callibaetis sp.*
Parasitic copepods	*Salmincola* spp.,		
	Lepeophtheirus salmonis		

[a]Experimental studies have been conducted with burbot by Polinski et al. [295], with pike by Dorson et al. [296], and other transient marine hosts by Kent et al. [297]
[b]May carry the virus transiently

present during intensive fish culture, sometimes producing severe mortality [46]. However, a genogroup-specific virulence pattern has been demonstrated in the U and M clades in sockeye salmon and non-anadromous rainbow trout, expressed as reduced lethality in non-preferred hosts. The higher lethality in the natural fish host may involve early replication of the virus to threshold levels beyond the control of the host's innate immune system [47]. Fish of certain species are completely refractory to IHNV [48], whereas others, such as larval Pacific herring (*Clupea pallasi*), are able to become transiently infected without observable signs of disease [49]. However, results of host susceptibility studies performed prior to the discovery of IHNV genotypes in 2003 may be varied due to differences in genogroup-specific virulence. For example, Arctic char (*Salvelinus alpinus*) exposure to U clade viruses

did not result in virus infection [48], whereas another study exposing the same host to a rainbow trout IHNV from Idaho (most surely an M clade virus) resulted in lethality of up to 41 % with isolation of virus from survivors [50].

Several researchers have pursued the hypothesis that invertebrate biological reservoirs of IHNV could be important as intermittent amplifying hosts. However, invertebrate amplifying vectors that are essential to the natural history of IHNV have not been found. Instead, a few invertebrates have been identified as potential mechanical vectors capable of harboring the virus for brief periods of time. These vectors obtain the virus when exposed to water contaminated by nearby IHNV-infected fish, as occurs with mayflies (*Callibaetis* spp.) [51]. Alternatively, leeches (*Piscicola salmositica*), and ectoparasitic copepods (*Salmincola* sp.) receive virus directly with blood meals from IHNV-infected fish. IHNV is able to persist without replication in leeches for 16 days [52]. The salmon louse (*Lepeophtheirus salmonis*) is able to harbor IHNV for 12 h and successfully transmits IHN to naïve Atlantic salmon after uptake of the virus from water-borne exposure or after parasitizing Atlantic salmon infected with IHNV [53]. A list of fish hosts and possible invertebrate vectors of IHNV is provided in Table 11.1.

In salmonids, signs of IHN are typically seen during the juvenile fry, fingerling, and smolt stages or when the host becomes sexually mature and ready to spawn. In between these life stages the virus generally cannot be detected by conventional cell culture methods. In juvenile fish, infection is usually followed by disease, resulting in moderate to nearly complete mortality.

The virus spreads horizontally from fish to fish through direct contact, and from virus shedding into freshwater and less often in seawater, the primary route of transmission in juvenile and adult fish [54, 55]. The route of host entry during horizontal transmission of the virus is through skin, gills, gastrointestinal tract, and fin bases [41, 56]. Titers of virus as high as 1,000 plaque-forming units (pfu) per ml of water were detected when infected fish were held at high densities [57]. In experimental infections, virus shedding from exposed juvenile sockeye salmon preceded death at 7 days post exposure, whereas death occurs at 13 days. Viral shedding peaked at 14 days post challenge, reaching 4.87×10^3 pfu [58]. Conversely, some asymptomatic juvenile sockeye salmon had low virus titers [59, 60], thus supporting the possibility of a carrier state or subclinical persistence of IHNV. In some instances of wild sockeye smolt infections, disease may have been initiated in subclinical carrier fish by the physiological stress from smolt transformation, passing through weirs to enumerate outmigration, and infection by other pathogens [60, 61].

IHNV replicates to high titers in spawning fish and is shed externally with urine, feces, mucus, and sexual secretions or products, infecting many other adult fish in a hatchery raceway or on the spawning grounds. Virus concentrations of up to 1,600 pfu/ml water were measured in a river side channel used to hold maturing adult fish for spawning [55]. Outbreaks of IHN have been reported in juvenile wild and feral salmonids [41], in which horizontal transmission of the virus has been the major route of infection, either from contaminated spawning substrate or from surface egg-associated virus. In most cases, infected adult sockeye salmon usually die from spawning senescence before IHNV can overwhelm the host. This observation

is typical for clinically normal adult fish having virus titers greater than 10^7 pfu/ml of ovarian fluids, which appear normal when sampled during spawning at the hatchery or on natural spawning grounds (TR Meyers, unpublished data).

IHNV may also adsorb to organic materials from decaying fish and sediments, remaining viable for weeks to months [55, 62, 63]. A reduction of virus titer of 10^3 pfu/ml of ambient seawater or freshwater may take 14–25 days, respectively [64]. Transmission of the virus can also occur mechanically when virus particles on the surface of an egg infect the hatching embryo, unless particles are destroyed by application of a disinfectant.

True (biological) vertical transmission within the egg is supported by studies in fish hatcheries [44]. In vertical transmission, the virus supposedly enters embryonic tissues from the perivitelline space by pinocytosis through the yolk, or by intra-ovum deposition during egg formation. The probability of vertical transmission is enhanced in large scale hatchery programs where 20–30 million eggs are spawned from broodstock that are test-positive for IHNV [65, 66]. Factors that affect the carrier rate in juvenile fish include the prevalence of virus in the broodstock and the proportion of infected female parent fish having high viral titers. A 27-year study of Alaskan sockeye salmon hatcheries showed that the occurrence and degree of IHN outbreaks in juvenile fish closely followed the percentages of virus prevalence and virus titers in broodstocks [44]. This study also found that the overall IHNV prevalence in the ovarian fluids of spawning female sockeyes averaged 40.4 % with an annual high of 56 % to a low of 8.5 %. Higher virus titers ($\geq 10^4$ pfu/ml of ovarian fluid), considered a threshold for increasing the risk of vertical transmission, occurred overall in 42.6 % of female infected fish ranging from an annual high of 65.7 % to a low of 10 %. Compelling anecdotal descriptions of vertical transmission are found in several case reports [41, 62, 66]. IHNV also adsorbs to sperm, giving rise to another potential route for vertical transmission (during fertilization) whereby the virus could enter the egg through the micropyle while attached to a sperm cell [67]. Despite numerous experimental studies, vertical transmission has not been demonstrated reliably in the laboratory, most likely because of the randomness of the event and low probability of its occurrence (e.g., within a few eggs and from a small number of female test fish).

The concept of carrier state is another controversy regarding the natural history of IHNV and its perpetuation within a population of host fish. A popular notion is that a water reservoir exists that would account for the survival of the virus during the eclipse of IHN after the juvenile stage and before its reappearance again in the adult spawning fish. A true marine or freshwater fish, or nonfish reservoir (e.g., an invertebrate), that can maintain the virus has never been conclusively demonstrated, other than the known, primary fish hosts listed in Table 11.1. Alternatively, a considerable body of evidence supports the hypothesis that fish surviving epizootics, and progeny receiving virus vertically transmitted from overtly virus-positive parents, harbor virus that is undetectable by cell culture. This subclinical carrier state may be due to colder water temperatures, less stress on the host, exposure to a low infectious dose, and increasing age. Specific and nonspecific immune responses then become more effective in controlling virus infection.

Support for a carrier state is both factual [41] and circumstantial information [66]. The most compelling evidence includes detection of IHNV in tissues of fish surviving experimental IHNV infections, including both the detection of viral proteins [68, 69] and detection of viral RNA by PCR [70]. Additionally, truncated defective interfering particles of IHNV from kidneys and livers of surviving fish were detected by immunogold transmission electron microscopy [69]. These particles were biologically active when explants of the tissues from survivors were reexposed to IHNV. Liver explant tissues from re-exposed fish produced more defective particles than explant tissues from naïve fish that produced standard virus particles, thus significantly decreasing virus titers in re-exposed fish by comparison [41, 71]. More recently, investigators have demonstrated the presence of IHNV in the brains of juvenile sockeye salmon that survived a laboratory exposure, and in asymptomatic juvenile fish from the marine environment. A comparison of the transcriptional responses of carrier fish and control asymptomatic fish revealed different gene expression profiles [72]. Collectively, these results support the hypothesis that some individual fish within a population can be lifelong carriers of IHNV. This persistence would explain the epizootics of unknown origin that affect juvenile fish, as well as the presence of the virus in adults that were previously test-negative. However, the mechanism of defective particle reactivation has yet to be identified.

2.2 Pathogenesis and Clinical Features

2.2.1 Pathogenesis and Immunology

IHN disease is an acute, systemic infection of mostly juvenile fish, primarily targeting the hematopoietic tissues and endothelium of the blood capillaries of the kidney and spleen. However, the liver, pancreas, gut, and other major tissues and organs also are affected. Histologically, the virus causes extensive necrosis of affected tissues, beginning with the interstitial cells of the kidney and progressing to full involvement of all cellular elements with macrophage infiltration (Fig. 11.3). Extensive necrosis is also found in the spleen, pancreas, liver (with ceroid deposition), and gastrointestinal tract, where mucosal sloughing gives rise to externally visible fecal casts. One pathognomonic feature of the disease not observed in other fish viral infections is degeneration and necrosis of the granular cells in the lamina propria, stratum compactum, and stratum granulosum of the alimentary tract [11, 73]. Another clinical feature of presumptive diagnostic value is the appearance of necrotic cells and debris, known as necrobiotic bodies, observed within peripheral blood smears or impression smears of kidney, along with numerous blast cells and poikilocytosis [11]. Other clinical and biochemical changes in the serum and blood include longer clotting times, low hematocrit, and a normocytic aplastic anemia accompanied by decreases in bicarbonate, bilirubin, calcium, chloride, osmolality, and phosphorous [11, 41]. The disease results in systemic organ dysfunction, including renal dysregulation with osmotic imbalance that results in high losses of susceptible fish in the hatchery, as well as in juvenile feral and wild adult fish. Upon virus entry into

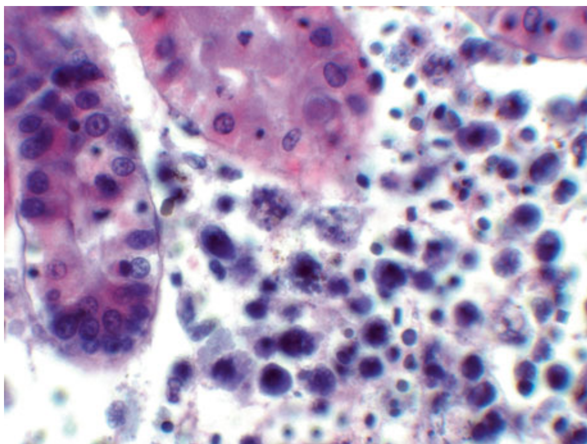

Fig. 11.3 Histopathology of sockeye salmon smolt infected with infectious hematopoietic necrosis virus. *Left figure*: kidney tissue with intact renal tubules and rounded interstitial hematopoietic cells showing nuclear pyknosis (*arrows*) and karyorrhexis (*arrowhead*). *Right figure*: liver tissue with focal necrosis (*circle*) and rounded hepatocytes showing nuclear pyknosis (*arrow*) and karyorrhexis (*arrowhead*); Hematoxylin and eosin (400×)

the body, leukocytes (small lymphocytes, monocytes, polymorphonuclear cells) may serve as target cells in the early phase of infection [75].

Some surviving fish are lifelong carriers of the virus, whereas others may clear the virus through innate and adaptive host responses (interferon and T-cells) and induction of adaptive humoral immunity. Development of humoral immunity usually takes several weeks after infection [76], although transcriptional activation in the immune cells may occur as early as 7 days post-exposure [77]. Based on virus detection by cell culture, clearance of virus from fish surviving an epizootic may take up to 46 days after water-borne exposure [71]. Rainbow trout surviving experimental and natural IHNV-caused epizootics produce high levels of complement-dependent neutralizing antibodies 6 weeks after infection. These antibodies protect against similar strains of IHNV, either by reinfection or after passive transfer to another host [41]. Virus-neutralizing activity has also been observed in the cutaneous and lower intestinal tract mucus from virus-exposed fish and normal, unexposed fish. This antiviral activity is not associated with humoral antibodies but rather with a nonspecific innate host resistance that acts as one of the first lines of defense to infection [78]. Antibody production against IHNV in adult fish after IHNV exposure is variable, with some fish producing increased antibody titers, whereas others are unresponsive, including fish that tested positive for virus [41]. Despite this variability, the overall picture created by the observations to date indicates that adult fish are immunocompetent against IHNV, and that maternal antibodies are transferred to progeny fry. Immunization of adult rainbow trout with a fragment of IHNV glycoprotein produced increased survival in their 7-day-old-fry after exposure to IHNV that persisted for at least 25 days [79].

Fig. 11.4 Acute infectious hematopoietic necrosis mortality of sockeye salmon fry in a hatchery raceway

2.2.2 Incubation Period, Clinical Signs, and Gross Lesions

The incubation period after viral exposure varies for fish of different species and is also influenced by the age of the fish, water temperature, viral dose, and exposure route (immersion or injection). Younger fish are more susceptible (Fig. 11.4). The optimum water temperature for IHNV replication is 10–12 °C. Fry losses generally increase with viral doses from 10^2 to 10^5 pfu/ml of water [41]. Generally, immersion exposure of susceptible fry to a lower viral dose at an optimum water temperature results in clinical signs and death within 3–10 days, [11] with peak mortality occurring in 8–14 days. However, IHN epizootics among incubating alevins in Alaskan sockeye salmon hatcheries occur at water temperature(s) as low as 1–2 °C, but time to peak mortality may be longer than normal [80].

The disease often causes premature emergence of alevins and fry from incubators containing substrate and is accompanied by clinical signs of cephalic swelling (Fig. 11.5), lethargy, and listless drifting in the water column. When stimulated, these fish may exhibit erratic corkscrew swimming behavior. Affected salmon smolts are often darkly pigmented and may have some external hemorrhaging that may include fins, vent, ventral abdomen, gills, and eyes (Fig. 11.6). Gills are pale from the anemia caused by hemorrhaging and hyperplastic due to early viral replication in gill epithelium. Exophthalmia is common, with occasional abdominal swelling due to ascites. Internal signs include petechial hemorrhages in the mesentery, adipose tissue, and swim bladder. Fingerlings and pre-smolts may present with similar but lesser external signs, including trailing fecal casts and yellow watery

Fig. 11.5 Cephalic swelling in sockeye salmon fry infected with infectious hematopoietic necrosis virus. *Top fish* with relatively normal appearing head. Varying degrees of cranial swelling are shown in the fry below

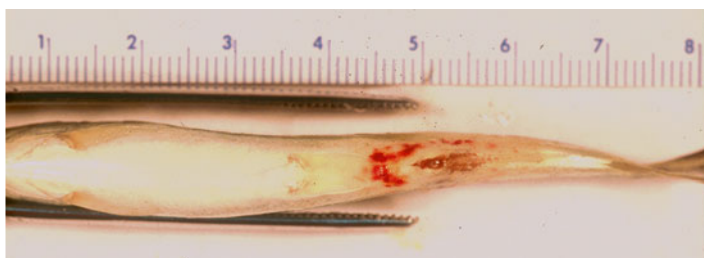

Fig. 11.6 Ventral petechial hemorrhages around the vent of a sockeye salmon smolt infected with infectious hematopoietic necrosis virus

fluid in the intestine. One clinical sign reportedly unique to chinook salmon fry is a subdermal hemorrhagic area immediately behind the head [73, 81]. A small (1–4 %) percentage of fish surviving IHNV epizootics develop scoliosis, which has been a marketing problem in the USA Idaho trout industry [43].

2.2.3 Morbidity and/or Case-Fatality Rates and Prognostic Factors

Morbidity and mortality rates are dependent on fish age and size, host species, infecting viral genotype, water temperature, viral dose, and exposure route. Under optimum host, viral, and environmental conditions, losses in juvenile fish may approach 100 %. Losses are reduced, and clinical disease is more chronic (i.e., less fulminant) in older fish and at higher water temperatures equal to or above 15 °C [41].

An illustrative example of a natural outbreak of IHN in pen-farmed Atlantic salmon is described by Saksida [82] during which 12 million fish were destroyed. The virus source was likely migrating wild sockeye salmon passing near the farm sites with horizontal transmission among the farm sites, either by seawater or farming practices. The overall fish case-fatality rate averaged 58 %. The highest mortality occurred in smaller smolt populations and decreasing with increases in both fish size and age. The outbreak of clinical disease within the farms occurred over a period of 20–22 weeks, with peak mortality occurring 6–8 weeks after onset of IHN in the index case.

The prognosis for infected fish is poor, and there is no available therapeutic intervention that is effective against IHN. Infected fish generally die, but may recover and clear the virus, or become lifelong carriers. In many hatchery operations, infected fish are destroyed to contain virus transmission regardless of mortality rate.

2.3 Diagnosis and Control

2.3.1 Diagnosis and Epidemiology

The current gold standard for diagnosis of IHN is isolation of the virus from infected fish by use of cell culture, followed by genetic identification with molecular methods such as RT-PCR or quantitative RT-PCR, with possible sequencing for genotype identification [42]. Virus isolation is accomplished by inoculating supernatants of homogenized and centrifuged kidney/spleen pools from infected juvenile fish or centrifuged ovarian fluids from spawning adult female fish into cultures of susceptible cell lines. Such lines include the fathead minnow (FHM), epithelioma papulosum cyprini (EPC) epithelial cells or Chinook salmon embryonic cells (CHSE-214). Detection of virus occurs by observation of cytopathic effect (CPE) in the inoculated cell monolayer, followed by harvest of the supernatant containing progeny virions for nucleic acid-based tests. Several antibody-based tests are available that have also been used for virus identification, either directly from infected tissues or from infected cell cultures, including virus neutralization, immunofluorescence, immunohistochemistry, ELISA, staphylococcal co-agglutination, immunoblot, and western blot [41]. Detection of virus becomes problematic in subclinically infected carrier fish, therefore cell culture and PCR or other molecular methods used in parallel may or may not be useful for surveillance.

The occurrence of IHNV within fish populations is unpredictable, but several circumstances and variables are important to better understand the epizootiology of the virus. Not surprisingly, if the virus is present in the untreated water supplies of fish culture operations, susceptible host populations will become infected with IHNV in a random and unpredictable fashion.

In fish hatcheries with secure water supplies, future broodstock populations may become infected as progeny through vertical transmission despite surface egg disinfection and water hardening in iodophor. When IHNV is present in an unknown number of asymptomatic carrier juvenile fish, environmental variables such as

water temperature, interrupted or low water flows, low dissolved oxygen, high fish densities, gas supersaturation, and handling are stressors that can precipitate IHN outbreaks [66]. Complacency by hatchery staff in following proper disinfection procedures (i.e., failed biosecurity) is also directly correlated with IHN incidence and severity [44].

Among sexually mature adult salmonids, the prevalence of IHNV is most studied in adult sockeye salmon [41]. A retrospective study of IHNV in Alaskan sockeye salmon from 1978 to 2000 indicated that all anadromous sockeye stocks tested positive for IHNV using cell culture at varying levels, with no predictable cycle from year to year [44, 65]. The annual virus prevalence in ovarian fluids of spawning fish and titers in such fluids paralleled each other closely during most years of the study. A subsample of post-spawned fish, when compared to spawning fish during the same time period, had significantly higher virus prevalence and titers, indicating continued virus replication after spawning. An earlier study in Alaska found that milt from male fish had significantly lower prevalences of virus than female ovarian fluids, but viral titers of infected males were comparable to females [65]. The different levels of virus present in sexual secretions from males and females may be related to hormonal differences during spawning or lack of detection in males due to viral sequestration by adsorption to sperm. Additionally, physical factors of the spawning habitat may increase or decrease horizontal transmission of IHNV, leading to variable prevalence and titers. A factor that leads to a low prevalence of IHNV is high gradient flow in short spawning tributaries that may be tidally influenced, causing more flushing of adult carcasses, organics and/or virus from the system and less organic substrate overall to adsorb virions. In addition, virus dilution occurs in larger lakes where sockeye migrate to mature, spawn, and sometimes just to die and decompose. Factors that lead to a high prevalence of IHNV include low water flows and higher water temperatures, both of which increase stress, fish densities, and (initial) virus concentrations in the spawning grounds.

Genetic timing of maturation and spawning could also influence fish densities and other environmental variables. Such timing could be compressed into a short period (results in more fish) or prolonged (results in less fish), or occur earlier in the summer (produces warmer temperatures, lower flows) or later in the fall (produces cooler temperatures, higher flows). Although minor increases of overall virus prevalence and titers in Alaska sockeye salmon populations have occurred over the last 20 years, these changes have not been attributed to any selective effect from hatchery practices. Both wild and hatchery virus isolates have been genetically stable during the time of these studies [83, 84].

2.3.2 Vaccination, Control, and Eradication

The development of a vaccine for IHN has been a high research priority for the last 32 years. Despite investigations of live attenuated vaccines (LAV), whole inactivated vaccines, purified subunits (proteins or glycoproteins) of the virus, purified proteins from cloned genes, and DNA vaccines, at this writing no commercial,

licensed vaccine is on the market in the USA. Some of these formulations have been experimentally successful in the laboratory and in field trials, but failed in large scale production testing. The DNA vaccines are the most promising and are highly effective in stimulating early neutralizing antibodies that protect vaccinated rainbow trout from disease (100 % survival at 3 months post vaccination). This immunity declines, but 47–69 % of fish survive virus challenges after 6–25 months post vaccination [85]. Injection is an impractical delivery method for fish, and this route is an impediment for use of these vaccines in juvenile stages of fish. Another significant regulatory obstacle involves public perception of nucleic acid vaccines. Perception varies among countries regarding whether a DNA-vaccinated animal becomes a genetically modified organism or not. A complete review of the vaccines used in aquaculture is provided by Lorenzen and LaPatra [86]. Autogenous killed and DNA vaccines are licensed by Canada for use in the Atlantic salmon pen farming industry on the west coast of North America, where injection of larger fish is practical [42]. Although the bacterin and autogenous vaccines available during the 2001–2003 IHN outbreak in British Columbia, were not effective in protecting vaccinated farm populations [82], the currently approved DNA vaccine is considered effective by the Canadian government and commercial industry [87].

Effective control of IHNV and the disease can only be achieved through avoidance of the virus, since there is no therapeutic treatment once fish are exposed. Simple biosecurity measures are exemplified by the Alaska Sockeye Salmon Culture Policy (SSCP) that was initiated in 1981 to control losses from IHNV in sockeye salmon hatcheries operated by the Alaska Department of Fish and Game [44, 88]. The cornerstones of this policy include a virus-free water supply (either fishless or depurated), rigorous surface disinfection of spawned broodstock, and single family egg disinfection/iodophor water hardening to eliminate surface-associated virus and reduce vertical transmission. To contain inevitable disease outbreaks due to vertical virus transmission, eggs and fry are compartmentalized, and infected fish are destroyed regardless of the severity of mortality or clinical signs followed by rigorous disinfection to prevent infection of the remaining hatchery production and release of virus into the environment [44, 88]. These procedures do not eliminate the virus from the hatchery but provide control to reduce losses of fish during production. This strategy has allowed the US state of Alaska to successfully “farm around” IHNV, and to conduct the largest sockeye salmon production program worldwide over the last 32 years.

Other control procedures used in Canadian pen farming of Atlantic salmon to reduce the possibility of IHN outbreaks (or control those in progress) include maintaining fish in single-year (age) class and single-species sites with fallowing before restocking with new inventory. Restricting movement or judicious disinfection of boats, utensils, and external clothing of personnel moving between farms are other common-sense precautions [82].

There has been some success regarding development of salmonid hybrids that are resistant to IHNV. However, most hybrids would be unlikely candidates for commercial culture. These hybrids have more value in studying mechanisms of resistance to IHN [41].

Eradication of IHNV may be possible if stringent biosecurity measures are followed. This has been demonstrated in Denmark with the related rhabdovirus viral hemorrhagic septicemia virus (VHSV) [74]. One major difference between the two viruses' natural histories is that IHNV can be vertically transmitted in the egg. Therefore, fish stocks must originate from virus-free sources, and a virus-free water supply must be available. The importance of this virus-free concept was demonstrated by an exchange of sockeye salmon from the Fraser River in Canada as a single shipment of eggs to New Zealand in 1901. Although IHNV had been indigenous in the Fraser sockeye, subsequent use of the progeny from those eggs resulted in a virus-free stock of fish at the Glenariffe Hatchery and for the non-anadromous kokanee salmon established in the Waitaki River system of New Zealand. IHNV continues to be exotic to New Zealand and apparently was not introduced with the small number of eggs imported, leading to the conclusion that they were likely free of the virus [89].

In cases of IHNV introduction outside its geographic range in the USA, the virus apparently never became established. Eradication has never been reported in other countries into which IHNV was introduced and became enzootic in wild fish population.

2.3.3 Public Health/One Health Crossover

IHNV is a poikilothermic virus. The virus poses no human or animal health threat from contact with or from consuming potentially infected fish.

3 Infectious Salmon Anemia Virus

3.1 Etiologic Agent and Natural History

3.1.1 Definition

Infectious salmon anemia (ISA) is a significant, generalized viral disease of farmed Atlantic salmon and was first reported in Norway in 1984 [90]. Outbreaks of ISA have had severe effects on the farmed salmon industry.

3.1.2 Etiology and Evolution

ISA is caused by infectious salmon anemia virus (ISAV), the only known member of the genus *Isavirus* in the family *Orthomyxoviridae*. The two glycoproteins embedded in the ISAV particle envelope, the hemagglutinin esterase (HE) glycoprotein and the fusion (F) glycoprotein, are important for virus uptake and cell tropism [91]. The receptors of ISAV are 4-*O*-acetylated sialic acids [92], expressed on endothelial cells and red blood cells in the host [93].

The segmented ISAV genome is highly conserved. The two gene segments with the highest variability are those coding for HE and F. Phylogenetic analyses of ISAV isolates have revealed two major clades, one European clade and one North American clade [94]. In addition, ISAV has been characterized and typed based on amino acid patterns of a highly polymorphic (HPR) region, consisting of 11–35 amino acid residues in HE [91]. The HPR variants may be explained as differential deletions [95] of a putative full length ancestral sequence (HPR0), first identified in a wild salmon in Scotland [96]. Whereas all ISAV isolates from ISA disease outbreaks have deletions in the HPR region, the HPR0 subtype has not been associated with clinical or pathological signs of ISA [91, 97]. Furthermore, the HPR0 type is frequently detected in gills during RT-PCR surveys of farmed Atlantic salmon [97, 98]. Thus, it seems that the highly pathogenic variant causing the severe anemia is less common than the gill-associated HPR0. The transformation of HPR0 subtype into virulent HPR-deleted types remains to be fully elucidated, including the risk associated with this transition.

3.1.3 Geographic Distribution and Economic Effects

Spontaneous outbreaks of ISA have only been found in farmed Atlantic salmon, and the majority of cases have occurred during the seawater stage of the salmon lifecycle. However, virus replication without clinical disease has been demonstrated experimentally in a range of fish of other species, including brown trout (*Salmo trutta*), rainbow trout, Arctic char, chum salmon (*Oncorhynchus keta*), Coho salmon (*O. kisutch*), herring, and Atlantic cod (*Gadus morhua*) [91]. ISAV has also been detected in healthy wild Atlantic salmon and sea or brown trout [99, 100]. As subclinical infections are difficult to detect in the marine environment, these wild fish or fish of other species may act as carriers or reservoirs of the virus.

Epizootics of ISA have had significant impact on the economy of the Atlantic salmon aquaculture industry, and have led to implementation of large scale biosecurity measures. Outbreaks have been reported in most Atlantic salmon farming areas including the east coast of Canada and the USA, Scotland, Norway, the Faroe Islands, and in Chile [91]. In Chile and the Faroe Islands, the disease caused major economic setbacks, leaving the entire industry with an uncertain future, similar to Norway in and after 1989 [91]. ISA is now listed as a reportable disease by the World Organisation for Animal Health (OIE).

3.1.4 Natural History, Transmission, and Host Range

ISA epidemics often start as small, local outbreaks separated in time and space, suggesting horizontal transfer [98, 101]. Biosecurity measures targeting horizontal transfer through management practices are successful in curbing epidemics. However, the mechanism by which the infection is first introduced into the farmed salmon population is unknown. Unknown reservoirs, maintenance vectors, and/or vertical transmission of asymptomatic/subclinical infections in Atlantic salmon are

possibilities. Alternatively, episodic, in situ emergence of virulent ISAV (through deletions in the HPR region) from the enzootic, low virulence HPR0 (full length HPR) is considered likely.

3.2 Pathogenesis and Clinical Features

3.2.1 Pathogenesis and Immunology

ISA is a systemic disease affecting the blood and the circulatory system. Examination of blood from moribund fish during field outbreaks revealed leukopenia, in particular lymphocytopenia and thrombocytopenia, and increased red blood cell fragility with an increased number of degenerate, “smudge” erythrocytes [102]. Hepatocellular necrosis and/or renal tubular necrosis with hemorrhage in the surrounding tissue are often observed histologically.

Using immunohistochemistry, we have recently documented that the major target cells for the virus are endothelial cells lining blood vessels of all organs, including sinusoids, endocardium, and scavenger endothelial cells in the hematopoietic compartment of the kidney [93]. In addition, we also documented that this cell tropism could largely be explained by the cellular distribution of the 4-*O*-acetylated sialic acid receptors [93]. The necrotic parenchymal cells in the liver and kidney tubules do not express the sialic acid receptor and appear to be uninfected in otherwise heavily infected fish. The specific mechanistic link from the initial endothelial infection to the severe anemia and necrosis remains to be resolved; however, necrosis is likely due to ischemia as a result of the progressing anemia.

ISAV isolates from both field outbreaks and experimental trials vary in virulence, as observed by differences in disease development, fatality and clinical signs [103–105]. However, specific virulence factors or markers other than the HPR deletion pattern have not yet been identified.

Both innate and adaptive cellular and humoral immune responses against ISAV have been experimentally demonstrated in Atlantic salmon [105–109]. Partial protection against experimental ISAV infection was demonstrated in fish injected with sera from fish that had previously survived an ISAV infection (i.e., passive immunization), suggesting the significance of humoral factors for immunity [106]. Experiments with DNA vaccines expressing the HE-protein have also demonstrated the importance of this antigen for a protective immune response [110, 111].

3.2.2 Incubation Period, Clinical Signs, and Gross Lesions

The incubation period in natural outbreaks has been estimated to be as low as a few weeks to several months [112, 113]. A peculiarity of ISA is that clinical disease often spreads slowly from net-pen to net-pen within a farm, possibly reflecting the extended time from infection to development of severe anemia and clinical disease. ISA can be experimentally reproduced in Atlantic salmon by intraperitoneal injection,

cohabitation with intraperitoneally infected fish, or by immersion challenge [114]. The incubation period for experimental infection is usually 10–20 days [91].

Diseased fish appear physically normal, but swim sluggishly at the water surface or hang listlessly at the net-pen wall. The progressive anemia results in "watery blood" with a hematocrit below 10 %. Prominent disease findings reflect the anemia and circulatory disturbances. Externally, these findings may include pale gills, localized hemorrhage of eyes and skin, exophthalmia, and scale edema (Fig. 11.7).

Necropsy reveals ascites, swollen spleen, edema, and petechial bleeding on serosa as constant findings. More variable, but very obvious when present, are severe hemorrhagic lesions in liver, kidney, or gut. The liver may turn almost black and show zonal hemorrhagic necrosis histopathologically (Fig. 11.8). The fish kidney contains hematopoietic tissue which is primarily affected by the hemorrhages, but kidney tubules can become necrotic in severe cases. The kidney hemorrhages are most easily

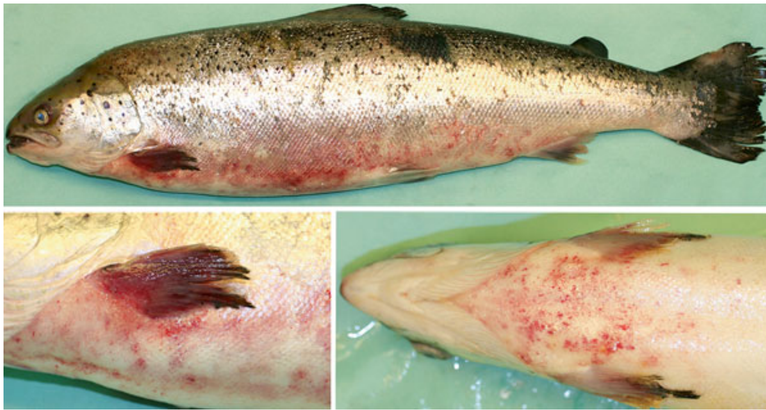

Fig. 11.7 Hemorrhages in skin and exophthalmia (*lower right figure*) in moribund Atlantic salmon from a confirmed outbreak of infectious salmon anemia in Norway

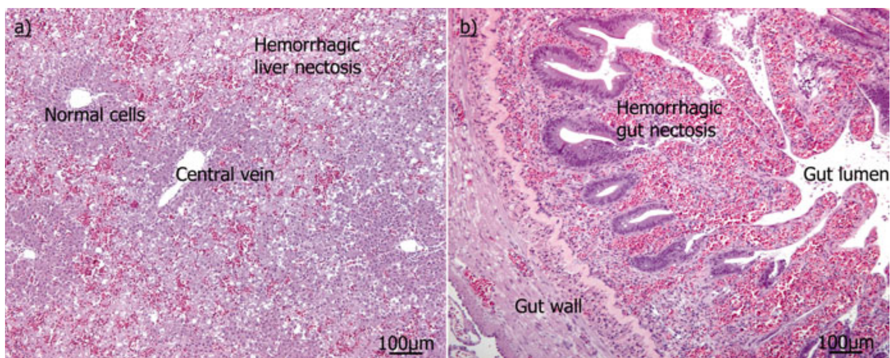

Fig. 11.8 Hemorrhagic necrosis: Hematoxylin-eosin stained tissues from Atlantic salmon naturally infected with infectious salmon anemia virus. Liver (**a**) and gut (**b**)

discovered by histology as the kidney is normally diffusely dark in color. Intestinal hemorrhages may resemble hemorrhagic enteritis. However, on fresh specimens no blood is present in the lumen, but histological sections are characterized by extensive bleeding within the lamina propria in the absence of inflammation, Fig. 11.8. These findings elegantly demonstrate that ISA is a disease of the blood and circulatory system with different organ manifestations. For unknown reasons, some ISA outbreaks can be dominated by just one or a subset of specific organ manifestations, but in other outbreaks a mixture of all manifestations can be found [91].

3.2.3 Morbidity and/or Case-Fatality Rates and Prognostic Factors

At the farm level, ISA is a slowly developing disease. Initially, diseased fish are only found in a few net-pens on a farm, and daily fatality is typically 0.05–0.1 %. However, if nothing is done to limit disease development, spread to other cages will occur, and accumulated fatality in a farm may reach more than 80 % during a period of several months. Episodes of high, sudden fatality occur, but are rare. Factors contributing to sudden fatalities are not known, and these infections could be due to a more malignant infection, or (for example) stress-induced mortality in a population of already anemic fish. Transportation of highly infected fish to slaughter has led to high fatality rates.

Implementation of general biosecurity measures aimed at lowering infection pressure and interrupting spread of infection have proven to be efficient in controlling the disease. These measures include early detection, isolation and slaughter of diseased populations, general restrictions on transport, disinfection of offal and waste from slaughterhouses, year-class separation at farming sites, and improved health control and certification [115].

3.3 Diagnosis and Control

3.3.1 Diagnosis and Epidemiology

Diagnosis is based on clinical signs, gross lesions, and histopathological findings supplemented with immunohistochemical examinations for endothelial infection. Positive immunohistochemical findings are confirmed by qPCR testing and virus isolation. The ISAV HE gene (i.e., HPR type) is sequenced for use in epidemiological evaluations.

3.3.2 Vaccination, Control, and Eradication

An experimental trial using an adjuvanted, inactivated ISAV cell culture preparation prior to challenge with ISAV via cohabitation with infected fish [116] resulted in relative percent survival (RPS) of 84–95. Lauscher et al. [109] also tested a virus cell culture preparation in experimental infections, and found an RPS of 86.

Vaccination against ISAV was first attempted on the East Coast of Canada in 1999. Vaccination has also been implemented in the Faroe Islands (since 2005) and in Chile. However, the efficacy of the vaccine was questioned when ISA outbreaks were recorded among vaccinated populations,

Large scale sanitation was seemingly successful in Scotland and the Faroe Islands, as ISA outbreaks are no longer found in these countries, and the salmon industry has recovered. In Norway, the number of outbreaks fell sharply following introduction of biosecurity measures. A few outbreaks still occur yearly, and affected fish are usually diagnosed early and contained effectively.

3.3.3 Public Health/One Health Crossover

The virus poses no human or animal health threat from contact with or from consuming potentially infected fish.

4 Nairobi Sheep Disease Virus

4.1 Etiologic Agent and Natural History

4.1.1 Definition

Nairobi sheep disease (NSD) is a tick-borne viral disease of sheep and goats caused by Nairobi sheep disease virus (NSDV). NSD presents as an acute hemorrhagic gastroenteritis associated with high case fatality rates [117–120]. NSDV is capable of causing rare, mild disease in humans and is classified as a Risk Group 3 agent [121].

4.1.2 Etiology and Evolution

NSDV is the prototype of the NSDV serogroup in the genus *Nairovirus*, family *Bunyaviridae* [117]. The *Nairovirus* genus is comprised of 46 mostly tick-borne viruses classified into 12 presumed serogroups [122]. The most important of these serogroups are the Crimean–Congo hemorrhagic fever virus (CCHFV) group and the NSDV group, which includes NSDV, Dugbe, and Kupe viruses [117, 118, 122, 123]. Nairovirions are small, spherical or pleomorphic, enveloped particles containing single-stranded negative-sense RNA arranged in three segments known as S (small), M (medium), and L (large) [117–119, 124]. The S segment encodes the viral nucleocapsid protein, the M segment encodes the viral glycoproteins, and the L segment encodes the viral RNA-dependent RNA polymerase [119, 122, 125]. NSDV is sensitive to lipid solvents and detergents, and it is rapidly inactivated at high and low pH [120]. The half-life of NSDV at optimal pH (7.4–8) is 6.8 days at 0 °C and 1.5 h at 37 °C [120].

4.1.3 Geographic Distribution and Economic Effects

NSD can cause large economic losses during outbreaks due to its high pathogenicity in naïve sheep and goats [126]. NSDV was first isolated from sheep in 1910 in Nairobi, Kenya [117, 119, 122, 127] by inoculating sheep with the blood of sheep suffering from acute gastroenteritis [119]. The virus was later found to be prevalent in Uganda and the Congo basin [122] and is now considered endemic in Eastern and Central Africa [119, 122]. In addition, serologic surveys suggest that NSD may also occur in Botswana, Ethiopia, Mozambique, and Somalia [117].

Serologic and genetic analyses have recently confirmed the existence of an Asian variant of NSDV, known as Ganjam virus (GANV). Serologic evidence indicates that GANV is present in India and Sri Lanka, but outbreaks of GANV infection on the same scale as NSD outbreaks in Africa have not been reported [117, 118, 122, 128]. GANV was first isolated in 1954 from ticks collected from goats suffering from lumbar paralysis in Orissa, India [117, 125]. The virus is antigenically and genetically closely related to NSDV; the two viruses differ by only 10 % at the nucleotide level and 3 % at the amino acid level [125].

4.1.4 Natural History, Transmission, and Host Range

Sheep and goats are the only known vertebrate reservoirs and amplifying hosts of NSDV [117, 129], although a few human cases of mild field and laboratory-related NSD have been reported [119, 130]. Other domestic animals, including cattle, horses, donkeys, pigs, poultry, and dogs, are not susceptible to NSDV infection [129]. Goats are generally regarded as less susceptible than sheep, but lethality may still be high [120].

In Africa, NSDV is transmitted primarily by the hard tick *Rhipicephalus appendiculatus* [118, 119], although it has also been isolated from *Rhipicephalus pulchellus*, *Rhipicephalus simus*, and *Amblyomma variegatum* [118]. A large number of ixodid ticks are possibly involved in the maintenance of the virus in nature [125]. NSDV is transmitted transstadially and transovarially by *Rhipicephalus appendiculatus* and can survive for at least 871 days in adult ticks [131]. In India, GANV has been most commonly isolated from ticks of the *Haemaphysalis* genus, primarily *Haemaphysalis intermedia* [117, 125, 128]. GANV was isolated from a *Rhipicephalus haemaphysaloides* tick and from a pool of 100 mosquitoes of the *Culex vishnui* complex [122, 125]. NSDV and GANV are shed in the urine and feces of sheep and goats, but are not spread by contact, aerosolization, or fomites [118].

4.2 Pathogenesis and Clinical Features

4.2.1 Pathogenesis and Immunology

NSD is the most pathogenic viral disease of sheep in Eastern Africa [131]. NSDV targets the reticuloendothelial system, multiplying in the lymphoid tissue, liver, lungs, spleen, and vascular endothelium [120]. In endemic areas, young animals

are protected by maternal antibodies until natural immunity develops after early (subclinical) infection [118, 132]. NSDV antagonizes the innate immune system by inhibiting interferon induction and action [119] and induces a Th_1 pro-inflammatory response [133].

4.2.2 Incubation Period, Clinical Signs, and Gross Lesions

NSD is characterized by fever that develops after an incubation period of 2–6 days [118]. NSD may persist for 2–8 days, and leukopenia may develop [120, 131]. A severe gastroenteritis develops 1–4 days after the onset of fever [118, 131] and is characterized by diarrhea. Diarrhea is initially watery but becomes mucoid and bloody [118, 120]. Other clinical signs include depression, dyspnea, collapse, injected conjunctiva, serosanguinous nasal discharge, enlarged and palpable lymph nodes, and inappetence that progresses to anorexia [118, 120, 131]. Pregnant animals may abort during or after the febrile phase [131].

Death may occur at any time after the onset of fever, although most deaths occur in the first few days [131]. Animals dying in the early stages of disease may have unremarkable gross lesions [118]. When present, gross lesions may include congestion of most organs, including petechial and ecchymotic hemorrhages on the serosal surfaces of organs and lymph nodes. Enlarged and edematous lymph nodes and splenomegaly may also occur [120]. Animals that die later in the course of disease may also exhibit inflammation of the gastrointestinal tract, including mucoid or hemorrhagic ulcerative enteritis of the abomasum, duodenum, cecum, or colon [118, 120].

4.2.3 Morbidity and/or Case-Fatality Rates and Prognostic Factors

NSD lethality in sheep may range from 75 to 90 % during outbreaks in naïve populations [122, 124]. Disease in goats is usually less severe, although 90 % lethality has been reported [120]. In Eastern Africa, indigenous breeds of sheep, such as hair sheep and Persian fat-tail sheep, are most susceptible to the disease. Imported wool sheep, such as the Romney and Corriedale, appear less susceptible to disease (lethality 30–40 %) [118, 120]. In contrast, native breeds of sheep and goats in India are less susceptible to GANV, whereas cross-bred and exotic breeds are more susceptible and die more often [120].

4.3 Diagnosis and Control

4.3.1 Diagnosis and Epidemiology

Infestation with the primary tick vectors, notably *Rhipicephalus appendiculatus*, is an important finding in support of a diagnosis [120]. In Eastern Africa, animals born in areas where the primary vector is present appear to be immune to NSD [119], as the majority of sheep and goats in these areas have serological evidence of infection

without disease [118]. Epizootics occur when naive animals from nonendemic areas move into endemic areas [118, 119], often following natural trade movements from drier surrounding pastures to population centers with high densities of infected *Rhipicephalus appendiculatus* [127]. Outbreaks may also occur when increased rains allow for expansion of the vector tick into areas with previously naïve animals [118, 129]. NSD should be considered whenever clinical signs consistent with NSD and lethality are seen along with tick infestation in a population of sheep or goats, especially those recently transported to an endemic area.

Virus isolation and serology are the primary tools available for laboratory diagnosis. Appropriate samples for submission include blood, mesenteric lymph nodes, lung, and spleen [118, 120]. Virus isolation may be performed on serum, plasma, or a 10 % homogenate of mesenteric lymph node, liver, or spleen collected post mortem [118]. NSDV may only be isolated from the blood during acute stages of illness [118]. Laboratory sheep, newborn mice, or cell culture (baby hamster kidney [BHK], lamb testis and kidney, or Vero cells) can be used for primary viral isolation [118, 134]. CPE can often be seen on the first passage of NSDV in BHK cells at 3–6 days post inoculation [118, 134]. Other cell lines may require multiple subinoculations before CPE develops [134]. Immunofluorescent staining can detect the virus as soon as 1–3 days postinoculation [118]. Serological tests include indirect immunofluorescence, complement fixation, ELISA, immunodiffusion, or hemagglutination inhibition tests [118, 134]. One disadvantage of serology is that cross-reactions can occur with other nairoviruses, including Dugbe and Crimean–Congo hemorrhagic fever viruses [117, 118]. Recently, a NSDV/GANV-specific real-time PCR has been developed for use in research [133]. Real-time PCR is reportedly more sensitive than virus isolation and may have diagnostic applications in the future [133].

4.3.2 Vaccination, Control, and Eradication

There is currently no treatment or vaccine available for NSD. Routine treatment with costly acaricides is not recommended due to the negative environmental impact, as well as the development of resistance among tick populations [120, 129]. Experimental vaccines have been developed, but none have been tested in field trials [127]. In endemic areas, control is best achieved by establishing high levels of herd immunity through continuous, low-level exposure to the tick and virus [118, 127]. In non-endemic areas where climatic factors may encourage the spread of nearby infected tick populations, aggressive tick control can be a useful short-term strategy to protect naïve populations [118, 127]. NSD is an OIE notifiable disease and is considered a foreign animal disease by many countries [118, 127].

4.3.3 Public Health/One Health Crossover

NSDV and GANV can cause infections in humans in laboratory settings, whereas naturally occurring disease is rare [118, 125]. Human infections are characterized by a self-limiting, mild febrile disease [117], but may include other symptoms such

as fever, shivering, abdominal pain, back pain, and headache [129]. Neutralizing antibodies to GANV have been found in serological surveys of humans in southern and northern Indian states [122] and in workers on a goat farm in Sri Lanka [118]. Neutralizing antibodies to NSDV have been found in laboratory workers and the general population in Uganda [118]. Whereas laboratory infections with NSDV in Africa are apparently rare despite reports of accidental needle sticks [127], a number of laboratory-acquired infections with GANV have been recorded in India [129]. Some infections were attributable to needle sticks [119] whereas others were associated with handling contaminated glassware [118]. There is one report of a naturally acquired clinical case in a 16-year old Ugandan boy [130]. Overall, the cause of differences in laboratory-acquired infections rates between India and Africa are unclear but may be due to varying levels of surveillance, variations in virulence between NSD and GANV, or other host factors.

In India, GANV is likely to be circulating more widely than has been recorded. The virus can be isolated from a variety of tick vectors found across the country, and there is wide serological evidence of infection in both humans and animals [125]. In Africa, the greatest challenge to restricting geographic spread of NSDV is preventing movement of infected vectors, either on imported animals or through expansion of range from climatic change, such as increases in temperature and/or rainfall. Finally, NSD and Ganjam disease might be considered or may become emerging diseases in sub-Saharan Africa and the Indian subcontinent, respectively, due to the complex interactions between the hosts and the environment. The ability of NSDV to replicate in multiple arthropod vectors, an anticipated expansion of tick habitats due to climatic change, and an increase in small ruminant populations to meet growing demand for animal protein could lead to a favorable environment for expansion of NSDV into areas previously considered free from the disease.

5 Reston Virus

5.1 Etiologic Agent and Natural History

5.1.1 Definition

Reston virus (RESTV) infection is a viral disease characterized by acute onset of generalized illness, with or without hemorrhage, in captive macaques and domestic pigs. RESTV was first discovered in captive crab-eating macaques (*Macaca fascicularis*) imported from the Philippines during a VHF epizootic in the USA in 1989. Outbreaks involving this disease are rare, but recent recognition of domestic swine as possible hosts raises concerns about introduction of RESTV into the human food chain.

5.1.2 Etiology and Evolution

RESTV is a member of the *Ebolavirus* genus, family *Filoviridae*, order *Mononegavirales* [135]. Its virions are enveloped and have a distinct filamentous shape characteristic of all filoviruses.

5.1.3 Geographic Distribution and Economic Effects

RESTV is one of only two members of the family *Filoviridae* that has not been associated with clinical disease in humans (the other being Lloviu virus). Until its re-emergence in 2008 in swine in the Philippines, RESTV was only known to infect captive crab-eating macaques. All three outbreaks, recorded in the USA and in Italy, were associated with crab-eating macaques imported from the Philippines islands, making this filovirus the only known ebolavirus that routinely circulates outside of Africa.

5.1.4 Natural History, Transmission, and Host Range

The first documented outbreak occurred in 1989 and was associated with a primate research facility in Reston, Virginia, USA. Outbreaks occurred simultaneously in Alice, Texas and Philadelphia, Pennsylvania. The source of the virus was traced back to a primate breeding and export facility in the Philippines. Coinfection of nonhuman primates with the arterivirus simian hemorrhagic fever virus (SHFV) was also noted during this outbreak.

In 1992, a similar incident occurred in Siena, Italy. This outbreak also involved crab-eating macaques purchased from the same vendor in the Philippines involved in the 1989 epizootics. Two individuals that worked on the farm during this time were found to have immunoglobulin G antibodies to RESTV, but they did not develop clinical disease [136, 137].

RESTV was once again imported into the USA from the same vendor in the Philippines in 1996. This outbreak involved a private quarantine facility in Alice, Texas. SHFV was once again isolated from a proportion of the RESTV-positive animals. This outbreak was discovered through laboratory testing requirements instituted after the 1989 outbreak and was contained by strict quarantine measures. No illness or seroconversion occurred in humans at the facility [136].

In 2008, RESTV re-emerged in domestic swine (*Sus scrofa domesticus*) in the Philippines. The animals involved in this outbreak were concurrently infected with a highly pathogenic strain of porcine reproductive and respiratory syndrome virus (PRRSV), an arterivirus related to SHFV. Coding-complete RESTV genome sequences were obtained for several isolates during this outbreak, but no discernible evolutionary relationship to the RESTV isolates from macaques was evident [138, 139].

As both SHFV [140] and PRRSV [138] have been isolated from animals infected with RESTV, researchers speculate that arteriviruses may act as facilitators of RESTV infection or (severe) disease.

5.2 Pathogenesis and Clinical Features

5.2.1 Pathogenesis and Immunology

Because of the nature and hazard of animal experiments involving live filovirus work, the pathogenesis and immunological response to RESTV infection has not been fully elucidated [136, 141].

Generally, RESTV pathogenesis is related to an impairment of the immune response along with vascular dysfunction, the degree of which is correlated with the severity of the clinical signs. Compared to experimental infection with other ebolaviruses, RESTV lesions appear less severe in nonhuman primates. Viral replication occurs in macrophages, interstitial fibroblasts, intestinal epithelial cells, hepatocytes, salivary gland epithelium, transitional epithelium of the urinary bladder, and renal tubular epithelial cells. Viral inclusions have been infrequently found in eosinophils, adrenal cortical cells, and villous or crypt epithelial cells of the gut. Antigen has been detected in splenic mononuclear cells. Experimentally challenged animals have exhibited hepatocytic inclusion bodies and extensive fibrin deposition in the splenic red pulp. Systemic coagulopathy was demonstrated by the observation of fibrin thrombi in the hepatic and splenic sinuses and the capillaries of the renal medullae. Vascular dysfunction and loss of endothelial barrier function contributes to the outcome of RESTV infections. Ischemic necrosis is generally present upon gross examination of the carcass and is related to the extent of viral replication in the tissues [136, 141, 142].

5.2.2 Incubation Period, Clinical Signs, and Gross Lesions

During the Reston outbreak in 1989, clinical manifestations of disease in nonhuman primates included an abrupt onset of depression, lethargy, anorexia, diarrhea, swollen eyelids, enlarged spleens and kidneys, and generalized respiratory signs, including cough and nasal exudates. Hemorrhage was only present in 1 % of the animals infected. Death typically occurred within 2–7 days after the onset of clinical disease. Leukocytosis with accompanying lymphopenia was also a prominent finding [137, 143]. Experimental challenge studies in nonhuman primates have documented dramatic increases in blood urea nitrogen values when the animals became moribund, suggesting that renal failure is a terminal event in RESTV disease. Few of the experimentally infected animals survived long enough to develop a neutralizing antibody response [136].

Viral challenge studies in pigs have demonstrated replication and shedding of RESTV from the nasopharynx in the absence of clinical disease, confirming the

possibility of asymptomatic infection. Nasopharyngeal involvement in shedding of the virus suggests an aerosol route of transmission from pig to pig. In six of eight infected animals, the highest levels of virus replication were observed in the lung and lymphoid tissues, with infectious virus also being isolated from muscle tissue samples. Linking the outbreak in pigs to the challenge study, findings suggest that preexisting respiratory disease might facilitate an increase in RESTV viral replication in pulmonary tissue [139]. Fecal shedding of virus was also observed.

5.2.3 Morbidity and/or Case-Fatality Rates and Prognostic Factors

A wide range of clinical outcomes has been demonstrated through both natural and experimental infections with a number of filoviruses, including RESTV. Relatively minor genetic differences between variants of the ebolavirus may result in significantly different outcomes of infection. Rapid changes associated with the virulence of a number of variants have been documented following relatively few passages in either cell culture or animal models [144]. During the Reston outbreak in 1989, nonhuman primate lethality was greater than 80 %.

5.3 Diagnosis and Control

5.3.1 Diagnosis and Epidemiology

Bats are suspected as potential reservoirs for RESTV. The host range of RESTV has not been fully elucidated. Based on available data, nonhuman primates, pigs, bats, and humans should all be considered as potential hosts. In the laboratory setting, an extensive range of cell lines originating from a wide variety of vertebrates can be infected by the virus [144, 145].

RESTV infections can be diagnosed by detecting antigens with immunostaining or with an antigen-capture ELISA. Viral RNA can be detected by RT-PCR, and numerous cell lines are useful for viral isolation. Electron microscopy can be used for virus particle identification in tissues. Serologic tests include IFA, ELISA, and immunoblotting techniques. The current recommendation is to work with potentially infectious RESTV under Biosafety level-4 (BSL-4) precautions [146].

5.3.2 Vaccination, Control, and Eradication

Clinically approved and reliable RESTV pre- and/or post-exposure prophylaxis is not currently available. Guidance for infection control measures for VHFs, including ebolavirus infections, are available [144].

Quarantine of nonhuman primates and strict biosecurity measures during importation protect healthy nonhuman primates and humans from potential exposure. During epizootics, suspect animals should be isolated and then euthanized once infection has been confirmed.

Effective measures to prevent infection of domestic swine have not yet been established. Normal biosecurity measures, with the addition of protection from bats and nonhuman primates, are recommended.

5.3.3 Public Health/One Health Crossover

The emergence of RESTV in the human food chain, through the involvement of domestic pigs, has prompted public health concerns. Serologic studies indicate the virus can be transmitted to humans, mainly those occupationally exposed to infected pigs. To this date, no clinical disease in humans has been described; exposure has only been documented through serological testing. Differences in the swine isolates from the 2008 outbreak from previous nonhuman primate isolates suggest either a distinct spillover event from an unidentified host, or that pigs could possibly act as asymptomatic reservoir hosts. The studies performed by Marsh et al. demonstrate the need to further understand RESTV infection in pigs to better assess the potential risks posed to humans [139, 147].

The World Health Organization's expert consultation group concluded that RESTV should be considered potentially pathogenic for humans [144].

6 Rift Valley Fever Virus

6.1 Etiologic Agent and Natural History

6.1.1 Definition

Rift Valley fever (RVF) is an arthropod-borne viral disease caused by Rift Valley fever virus (RVFV) that may be an acute, severe disease in animals and humans characterized by high rates of abortion, fever, and weakness. The disease results in significant economic losses due to "abortion storms" in RVFV-infected livestock (Fig. 11.9).

6.1.2 Etiology and Evolution

RVFV is an ambisense, single-stranded RNA virus of the family *Bunyaviridae*, genus *Phlebovirus*. All isolates to date are serologically similar, but strains exhibit variable virulence. RVF was first described in sheep in the African Rift Valley around 1900, but the virus was not isolated until 1930 [148–151].

RVFV appears to evolve significantly during interepidemic periods, as well as during outbreaks [152, 153]. However, strains of RVFV are surprisingly closely related given the evolutionary potential of the virus [150, 151]. This relatively low sequence variation in RVFV strains could be due to the constraints of replication in

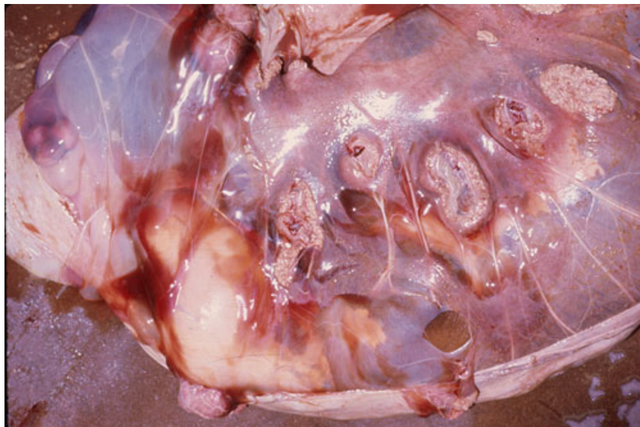

Fig. 11.9 Aborted bovine fetus and fetal membranes, edema and hemorrhage (hydramnion) from cattle infected with RVF. *Source*: Plum Island Animal Disease Center

phylogenetically diverse species to complete the alternating host (chordates and arthropods) cycle [150, 154], a long-term maintenance strategy of RVFV under dry conditions (i.e., extended survival of transovarially infected eggs from *Aedes* mosquitos), recent dispersal [150, 151], or other as yet unrecognized factors.

6.1.3 Geographic Distribution and Economic Effects

RVFV can be found throughout Africa, including the island of Madagascar [149, 151, 155]. Endemic areas are mostly in Southern and Eastern Africa [156]. Endemic boundaries are defined by the location of infected mosquito eggs. Outbreaks occur in endemic areas following conditions of heavy rain and sustained flooding that favor hatching and growth of transovarially infected mosquito eggs (see below). In the past, outbreaks of RVF occurred in Africa at 5–15 year intervals. In 1950–1951, a major epizootic occurred in South Africa that resulted in 500,000 sheep abortions and 100,000 sheep deaths [267]. The first RVF cases affecting large numbers of livestock and humans identified outside of Africa occurred in Saudi Arabia and Yemen in 2000. Serologic evidence in domestic and wild ruminants suggests that unrecognized outbreaks or low-level transmission may occur between mosquitos and wild ruminants in interepidemic periods [157–159].

The economic effects of RVF are due to abortion and direct losses of adult animals (mostly sheep and goats). Additionally, the trade restrictions and ancillary effects on agriculturally related industries (e.g., transport, slaughter, feed) are significant, as well as the costs of rebuilding devastated herds [160, 161]. The 2007 outbreak in Kenya was estimated to have cost the economy KSH 2.1 billion (US$ 32 million) in total [161], and these losses prompted fears of proportional losses in the USA [162].

6.1.4 Natural History, Transmission, and Host Range

RVFV can infect many animals of many species, including sheep, cattle, goats, camels, primates, wild ruminants (e.g., African buffalo, wildebeest, antelopes), and some rodents (e.g., gray squirrels). The primary amplifying hosts are wild and domestic ruminants. Viremia without severe disease has been described in adult cats, dogs, horses, and some nonhuman primates, but severe disease can occur in newborn puppies and kittens. Rabbits, pigs, guinea pigs, chickens, and hedgehogs do not become viremic. RVFV has also been isolated from bats, but their role in the natural cycle remains unclear [149, 163].

RVFV is arthropod-borne and infects a wide range of vectors. However, differences in vector competence probably condition the geographical distribution of the disease. Host selection by epidemic vectors clearly conditions the exposure of humans or animals by arthropods, and will affect incidence rates in an outbreak [164–166].

RVFV is transmitted primarily by mosquitos and is amplified in ruminant hosts. Serological surveys indicate that RVFV regularly circulates silently in endemic areas between wild ruminants and susceptible mosquitos of various species [157, 159]. However, the principal mechanism of long-term maintenance of the virus in dry endemic regions is hypothesized to be through viral survival in transovarially infected mosquito eggs that require a period of dehydration before the eggs will hatch [151]. Barriers at any step can decrease or eliminate transovarial transmission, and physical or biological barriers probably define which mosquitos are responsible for long-term maintenance and/or epidemic transmission of RVFV [151]. Mosquitos of many different species (e.g., *Aedes*, *Anopheles*, *Culex*, *Eretmapodites*, *Mansonia*) can act as epidemic vectors of RVFV, with varying levels of competence. However, certain *Aedes* species most likely act as reservoirs for RVFV during interepidemic periods.

Long-term maintenance of RVFV in endemic locations begins when transovarially infected eggs are laid by infected mosquitos and survive extended dry periods (many years). Grassland depressions known as “dambos” are thought to play a role in this process, as they flood and retain water during heavy rains, and are ideal egg-laying locations for *Aedes* mosquitoes [151]. Other areas that may experience alternating flooding periods and dry periods such as rainwater drainage (i.e., “wadi”) agricultural systems or other fields play a role in outbreaks as well [160, 166, 167].

A “typical” outbreak scenario for RVFV begins with large numbers of infected mosquito eggs hatching after a period of unusually heavy rains and sustained flooding. Then, RVFV is amplified in wild or domestic ruminants, spreads to the larger constellation of secondary transmission (epidemic) vectors, and spreads through local animal and (possibly) human populations. The 2007 outbreak in Kenya linked to flooding in the affected area provides a good example for this scenario [165].

Transovarial transmission from mosquitos is most likely not sufficient to maintain the virus over time [168]. Infected mosquitos must hatch and infect ruminant amplifying hosts to ensure broad horizontal spread to populations of local arthropods, which will then lay infected eggs at a rate probably less than 10–15 % [169].

Culex and *Anopheles* mosquitoes likely serve as the main secondary biological vectors [165]. Ticks and biting flies such as ceratopogonids, phlebotomids, stomoxids, and simulids may serve as mechanical vectors of the virus. RVFV has also been found in raw milk from infected animals [170, 171].

6.2 Pathogenesis and Clinical Features

6.2.1 Pathogenesis and Immunology

Wild and domestic ruminants are exposed by the bite of an infected vector. After exposure, the virus travels to and infects many tissues, but the hemorrhagic effects are due to the infection of the liver [149]. In neonatal lambs, gross lesions include an enlarged, yellow to orange, friable liver with subcapsular hemorrhages, extending to a severe necrosis sometimes called "hepatic liquefaction" [172]. The livers of infected adult sheep and cattle are typically darker with scattered small pale foci of necrosis (Fig. 11.10). Multifocal hepatic necrosis spreads to include the entire liver, and hemorrhage occurs secondary to consumption of clotting factors in the absence of replacement by normal hepatic metabolism.

Widespread cutaneous hemorrhages may be present. Hemorrhagic enteritis, petechial to ecchymotic hemorrhages and edema, and/or hemorrhagic diathesis may be present on the visceral serosa (Fig. 11.11). Edema, hemorrhage, and necrosis may be present in the gallbladder and lymph nodes. In rare instances, latent neurotropism can lead to hydranencephaly [35, 36, 76]. High viral loads can be found in infected animals (allowing infection of naïve vectors), aborted fetuses, and fetal membranes.

A strong innate immune response is critical for control of the initial phase of virus replication [173–175]. An adaptive immune response develops rapidly following infection, and is necessary for prevention of neurological disease in mice injected subcutaneously with RVFV [176]. By approximately days 4–8 post inoculation, neutralizing antibodies are detectable, and they are critical to clearance of

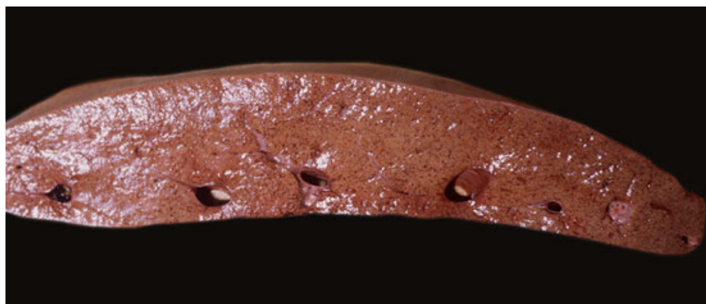

Fig. 11.10 Adult bovine liver, massive necrosis from cattle infected with Rift Valley fever virus. *Source*: Plum Island Animal Disease Center

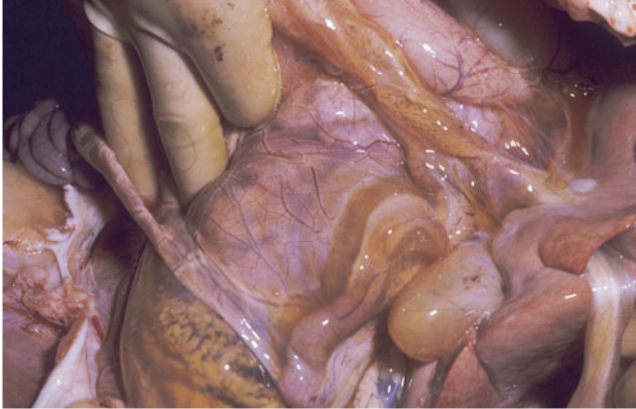

Fig. 11.11 Adult bovine intestine, marked mesenteric and serosal edema from cattle infected with Rift Valley fever virus. *Source*: Plum Island Animal Disease Center

virus from tissues. Neutralizing antibodies are also an easily measurable correlate of response to vaccines in sheep and mice [176–178], but they are not prognostic. Rather, it would appear that a well-regulated proinflammatory cytokine response is necessary to the overall resolution of RVFV infection [175, 176].

6.2.2 Incubation Period, Clinical Signs, and Gross Lesions

Several studies have detailed the clinical and pathological course of disease and symptoms in naturally infected humans and animals [179–181]. The incubation period of RVF can be as long as 3 days in sheep, cattle, goats, and dogs. In newborn lambs, the incubation period is approximately 12–48 h [296]. Experimental infections usually become evident after 12 h in newborn lambs, calves, kids, and puppies [149, 182–184].

Clinical signs of RVF vary with the age, species, and breed of the animal [149], and can range from a mild or asymptomatic infection to a severe and lethal acute infection. Long-term sequelae may occur in survivors with either type of initial infection [151]. In endemic regions, epidemics of RVF can be recognized by high lethality in newborn animals and abortions in adult sheep and cattle (i.e., “abortion storms”). In young lambs and calves, a biphasic fever, anorexia, lymphadenopathy, and hemorrhagic diarrhea may be followed by weakness and death within 36 h. Hemorrhagic diarrhea may be observed.

Abortion storms are the most characteristic sign in adult sheep and cattle (Fig. 11.9) [181]. Both adult sheep and cattle may have nasal discharge, excess salivation, loss of appetite, weakness, and/or diarrhea [17, 54, 224]. More severe symptoms that may occur in adult sheep include fever, weakness, a mucopurulent nasal discharge (sometimes bloodstained), melena, hemorrhagic or foul-smelling diarrhea, and vomiting. Compared to cows and goats, viremia in adult sheep was highest, and therefore sheep

pose the greatest risk for human or vector infection [172]. In adult cattle, fever, anorexia, weakness, excessive salivation, fetid diarrhea, and decreased milk production have been reported. Icterus may also be seen, particularly in cattle [149].

Similar but milder infections occur in adult goats [185]. Adult camels do not develop signs other than abortion, but young animals may have more severe disease. Viremia without severe disease may be seen in adult cats, dogs, horses and some monkeys, but severe disease can occur in newborn puppies and kittens [184, 186].

The livers of infected adult sheep and cattle are typically darker with scattered small pale foci of necrosis. There may be widespread cutaneous hemorrhages. Petechial to ecchymotic hemorrhages may be present on the visceral serosa. Hemorrhagic enteritis and hemorrhagic diathesis may also be present. Edema, hemorrhage, and necrosis may be present in the gallbladder and lymph nodes. In rare instances, latent neurotropism can cause hydranencephaly.

6.2.3 Morbidity and/or Case-Fatality Rates and Prognostic Factors

RVF is most consistently known for presentation with abortion storms and high lethality that can approach 100 % in neonatal lambs and can range from 10 to 70 % in calves [17, 224]. For animals over a week of age, lethality is typically reduced to 20–30 %. A similar lethality is seen in infected ewes, and the case-fatality rate is about 10 % in adult cattle [179, 180, 187].

6.3 Diagnosis and Control

6.3.1 Diagnosis and Epidemiology

In areas where the disease is known to occur, RVF may be suspected based on clinical signs, insect activity, concurrent disease in animals and humans, rapid spread of the disease, and the presence of environmental factors that favor hatching of infected mosquito eggs. Laboratory tests are required to confirm the diagnosis.

RVF can be diagnosed by isolation of the virus from the blood of febrile animals. RVFV can also be recovered from the tissues of dead animals and aborted fetuses; the liver, spleen, and/or brain are generally used. RVFV can be grown in numerous cell lines including BHK cells, grivet kidney (Vero) cells, chicken embryo reticulum, and primary cultures from cattle or sheep. Hamsters, adult or suckling mice, embryonated chicken eggs, or 2-day-old lambs can also be used for virus isolation [151, 188].

Viral titers in tissues are often high in infected animals, and a rapid diagnosis can sometimes be made with complement fixation, neutralization, or agar gel diffusion tests on tissue suspensions. Viral antigens can also be detected by immunofluorescent staining of impression smears from the liver, spleen, or brain. RT-PCR testing can detect viral RNA [149, 188, 189].

Commonly used serologic tests include virus neutralization, ELISA, and hemagglutination inhibition tests. Immunofluorescence, complement fixation, radioimmunoassay, and immunodiffusion are used less frequently. Cross-reactions with other phleboviruses can occur in serologic tests other than virus neutralization [149, 151, 190, 191].

The epidemiology of RVFV is defined by its natural history. The genome structure and promiscuous host range of RVFV would seemingly indicate a large, but as yet unrealized, evolutionary potential for RVFV. However, viral spread seems restricted, possibly by interepidemic maintenance in mosquitos of one or a few species, or host selection by insects during endemic and epidemic periods. Outbreaks "burn out" by killing or generating immunity in susceptible hosts (thereby removing amplification of the virus).

6.3.2 Vaccination, Control, and Eradication

Vaccines are generally used to protect domestic animals from RVF in endemic regions. LAV and inactivated RVF vaccines are both available. Administration of LAVs produce better immunity than inactivated RVF vaccines, but abortions and birth defects can occur in pregnant animals [192]. Development of a reverse genetics system for RVFV [193] led to the generation of novel LAV based on natural mutants (e.g., clone 13) that are safer than previous LAVs such as MP-12 [174, 194]. Recent tests of one "second generation" vaccine in pregnant sheep indicate both safety and efficacy [177]. Subunit, inactivated, and virus-like particle (VLP) vaccines are also in development [178, 195].

Surveillance to monitor RVF in animal populations and immediate notification upon detection are essential elements for the prevention and control of RVF [196]. However, human cases have been known to occur before the recognition of cases in animals [197]. Less commonly used preventative measures include vector control efforts (e.g., larvicides, dips, sprays), movement of stock to higher altitudes, and the confinement of stock in insect-proof stables. Spraying and management of mosquito breeding grounds have both been tried, but these measures require a long-term commitment to be effective. Vector control efforts are often impractical or are ineffective because the efforts are instituted after an outbreak has begun. However, systems used to monitor variations in climatic conditions can provide advance warning of conditions that favor mosquito hatching and signal the need to implement enhanced vector control measures in endemic areas [156].

The movement of animals from endemic areas to RVF-free regions can result in epidemics through spread of the virus to local vectors [198]. During epidemics, vaccination of susceptible animals can prevent amplification of the virus and protect people as well as animals [151].

6.3.3 Public Health/One Health Crossover

RVF is a zoonosis. Humans are susceptible to RVFV infection and may become infected through bites from infected mosquitos, through contact with the blood, other body fluids, or tissues of infected animals, as well as consumption of uncooked meat or raw milk from infected animals. Humans working in slaughter facilities in endemic areas, laboratories, or hospitals are therefore at high risk of acquiring infections [149, 151, 199, 200].

The use of the RVFV as a bioterrorism agent has been examined, and RVFV was found to have similar characteristics to other possible biothreats [201]. However, unintentional introduction of RVFV by an infected human or animal movement is a more likely scenario. Several researchers have examined this possibility, especially in the context of annual religious pilgrimages or festivals that bring travelers and animals from many different areas into a single, concentrated focus. Additionally, some of these activities involve the ritual slaughter of animals, increasing the risk of RVFV transmission to humans through tissue or blood exposure [159, 202, 203].

Although rare, the hemorrhagic fever form of RVF is very serious. Signs appear 2–4 days after the onset of illness, and are related to the severe liver dysfunction caused by massive hepatocellular lysis due to RVFV infection. Blood-tinged vomit and/or feces, ecchymoses on the skin, frank bleeding from the nose or gums, menorrhagia, and bleeding from venipuncture sites can all be seen, but none of these signs are characteristic of RVFV infection [196]. Case-fatality rates for humans in outbreaks can be as high as 50 %, with death occurring 3–6 days after the onset of severe symptoms. The overall case-fatality rate for RVFV in humans is generally recognized as less than 1 % in documented outbreaks, with fatalities occurring mostly in patients who develop the hemorrhagic form of the disease [196].

7 Viral Hemorrhagic Septicemia Virus

7.1 Etiologic Agent and Natural History

7.1.1 Definition

Viral hemorrhagic septicemia virus (VHSV) is a rhabdovirus infecting a wide range of fish of different species in both the marine and freshwater environments in Europe, Japan, and North America. The disease is listed as reportable to the World Organisation for Animal Health (OIE).

7.1.2 Etiology and Evolution

VHSV has a linear, single-stranded negative-sense RNA genome of 11,158 nucleotides with six genes in the order of 3′-N-P-M-G-Nv-L-5′ encoding nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), non-virion protein

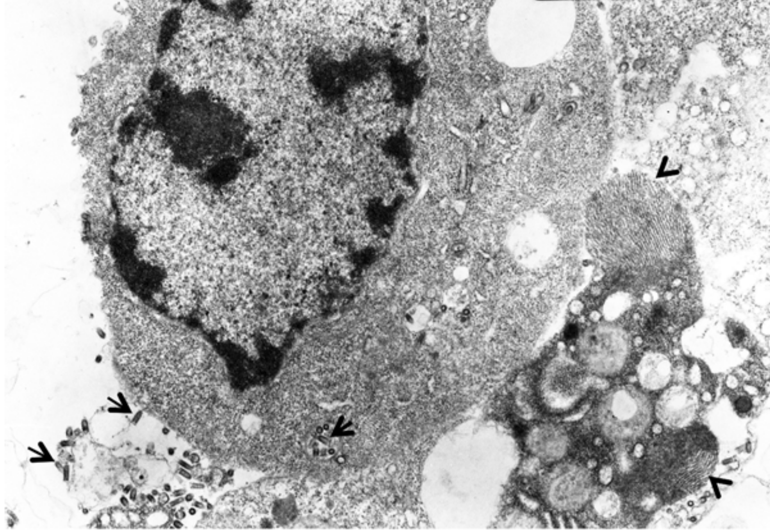

Fig. 11.12 Transmission electron micrograph of epithelioma papulosum cyprini (EPC) epithelial cells inoculated with viral hemorrhagic septicemia virus type IVa from a Pacific cod. Virions on the outside of an EPC cell and within cytoplasmic vacuoles (*arrows*). Unassembled nucleocapsid protein near the cell surface in the cytoplasm (*arrowheads*). Uranyl acetate and lead citrate (10,000×)

(Nv), and RNA-dependent RNA polymerase (L) [204]. Similar to other rhabdoviruses, G is the most effective antigen for producing neutralizing and protective antibodies [205–207]. The presence of the unique Nv gene places this virus into the *Novirhabdovirus* genus of the family *Rhabdoviridae* along with infectious hematopoietic necrosis virus (IHNV) [15]. The bullet-shaped virus particles that measure 70 nm in width by 180 nm in length are enveloped (Fig. 11.12), heat, acid and ether labile, and have a replicating temperature range of 4–20 °C with an optimum replicating temperature of 10–15 °C [74].

The disease caused by VHSV was first described in 1931 by Schaperclaus in rainbow trout (*Oncorhynchus mykiss*) known then as "infectious kidney swelling" [208]. Schaperclaus suspected the disease was caused by a virus based on his transmission studies using filtered homogenate from infected fish. The viral etiology was confirmed in 1962 by isolation of the virus in cell culture by Jensen [209, 210]. Jensen reproduced the disease in inoculated rainbow trout, fulfilling Koch's postulates, and the disease became known as Egtved Disease, named after the village in Denmark where the disease was first recognized. VHS is widely known as an infectious disease of rainbow trout in Europe, where the virus causes significant losses in commercial aquaculture [74, 211].

Before the late 1980s, VHSV was considered to be a freshwater virus of rainbow trout restricted to continental Europe. In 1988, VHSV was detected in the ovarian fluids of hatchery-spawned Chinook and coho salmon in Washington State, USA. This isolate was not of European origin based on ribonuclease fingerprinting

and comparative differences in the NP gene [212]. DNA probes were constructed based on this information to examine the identity of other virus isolates [213]. Subsequent isolation of the same virus (subsequently designated VHSV type IVa) from Pacific cod (*Gadus morhua*) and from Pacific herring (*Clupea pallasii*) [214] in the early 1990s and later in other marine forage species established that the virus was enzootic in the Pacific Northwest. This discovery prompted European investigators to examine marine fish species in their home waters [212]. In early 2000, VHSV was isolated from dead marine and freshwater fish species in eastern Canada, forming a distinguishable subgroup [215] later designated as IVc [36, 216]. By 2005, VHSV was recognized as the cause of extensive epizootics in freshwater fish in four of the five Laurentian Great Lakes, including Lake St. Clair and five of the inland lakes outside the Great Lakes Basin in Wisconsin, Michigan, New York, and Ohio [216, 217]. Additionally, viral nucleic acid was detected by qRT-PCR in the 5th Great Lake— Lake Superior—in 2009 [218]. Archived samples indicated the virus had been present in the USA as early as 2003 [219]. Viral isolates from the 2005–2007 outbreaks show very low genome diversity [217] and were genetically distinct from all other VHSV. They were subsequently classified as a new sublineage known as VHSV IVb [219, 220].

Earlier phylogenetic analyses suggested that VHSV diverged into two primary clades about 500 years ago, forming the North American strain IV in the Northwest Atlantic and strains I–III in the Northeast Atlantic near Europe [221, 222]. Additional sequencing investigations using three genes that have high mutation rates (G, N, and Nv) supported the earlier phylogenetic analyses, but further elucidated the biogeographical evolution of VHSV that has produced the observed diversity of populations and/or sublineages [216]. However, more recent phylogenetic analysis [36] using the entire G gene sequences of the four known novirhabdoviruses suggests that VHSV and snakehead rhabdovirus (SHRV) likely share a common ancestor that is sister to that of infectious hematopoietic necrosis virus (IHNV) and Hirame rhabdovirus (HIRRV). Estimated bifurcation between VHSV and SHRV may have occurred about 1,000 years ago following a more ancient divergence of the genus. Because these two divergences involved relatives endemic to the Pacific (IHNV, SHRV, HIRRV), the bifurcations likely occurred in the Pacific Ocean rather than the North Atlantic as previously postulated. The primary clade divergence of the European VHSV lineages and that of the North American genotypes is now postulated to have occurred less than 300 years ago. The virus then disseminated from the Pacific westward to East Asia, eastward to the North Atlantic/Baltic Sea, the Great Lakes, Atlantic Canada watersheds, and the Atlantic coast. Although the North Atlantic is still considered to be a major viral reservoir for continental Europe, it is much less so for North America [36] as suggested by isolations of the European genotype III from the nearby offshore Flemish Cap in 1994 [221, 223]. Spread of the virus to North America has not been reported since all North American isolates have been genetically type IV [36]. Regardless of where VHSV originated, dissemination and divergence are likely associated with natural fish migrations, human fish culture activities and transport of invasive fish, such as the round goby (*Neogobius melanostomus*) as is implicated for transport of genotype IVb into the

Great Lakes [224]. Although contaminated ballast water is another possible source for VHSV, this route has not been implicated [225]. Collectively, this information provides credible evidence to support earlier hypotheses that the ancestral VHSV was likely of marine origin, and gave rise to the four genotypes [212].

Varying reports estimate the divergence of the European freshwater and marine strains to have occurred between 60 [226] and 150 years ago [36] from a basal member of marine strain II, again indicating a marine origin of the European clade. Strain II diverged in the Baltic Sea while strains I and III are united by a common ancestor from which strain III diverged in the North Atlantic and North Sea, while strain I evolved in both freshwater and marine waters, producing several sublineages. Strain I has been divided into five sublineages, 1a–e [227]. Sublineage 1a may be the most divergent, probably due to rapid evolution driven by selective pressures in aquacultured freshwater fish and might have originated in France [36]. This sublineage occurs in brown trout (*Salmo trutta*) and rainbow trout in freshwaters of several European countries and may represent three different subgroups. Sublineage 1c occurs in rainbow trout exclusively from Denmark [216] and may have been the second subtype to diversify before the first VHSV isolate DK-F1 was reported [36]. Further studies may place the Denmark sublineage in sublineage 1a but other studies using the full length G gene report that Danish isolates are distinct from other trout isolates originating in other European countries [228]. Sublineage 1b apparently originated from asymptomatic marine fish and includes the rhabdovirus isolate associated with cod ulcus syndrome, one of the first VHSV variants to be isolated from a marine fish that was originally thought to be a laboratory contaminant [229, 230]. Subtype 1b is likely a sister to 1a rather than a progenitor. Both possibly originated from a pathogenic freshwater rainbow trout strain that diverged around 1950 [36]. Sublineage 1d is a group of isolates largely from marine sources and likely evolved from the progenitor of the two sister taxa, 1a and 1b [36]. Sublineage 1e likely originated from marine fish in the Black Sea and is closely related to a rainbow trout isolate from Denmark [231. Further sequence analysis is warranted to verify this conclusion [216]. Apparently, sublineage 1e may have been the first subtype that had already diversified before VHS was reported in the literature in 1938 [36].

The divergence of the New World strain IV sublineages a, b, and c are less clear but IVb and IVc are possibly sisters diverging during the late 1980s from an ancestor that diverged from that of IVa 20 years earlier [36]. The occurrence of sublineages IVa (from the east) and Ib (from the west) in Japan and Korea likely occurred from two separate introductions in the mid-to-late-1990s [216, 232].

7.1.3 Geographic Distribution and Economic Effects

VHSV type I sublineage 1a occurs in fish of 13 species in Europe, mostly in freshwater brown and rainbow trout. Sublineage 1b occurs in fish of ten marine and estuarine species of the Baltic and North Seas. A single Japanese isolate from farmed Japanese flounder (*Paralichthys olivaceus*), identified as Type Ib, was accidentally introduced

into Japan [233]. Sublineage 1c occurs in rainbow trout in Denmark. Sublineage 1d includes isolates from rainbow trout at a marine site and from Baltic herring (*Clupea harengus*). Sublineage 1e occurs in marine and estuarine brown trout and turbot (*Scophthalmus maximus*) in the Black Sea [231]. VHSV type II infects fish of several marine species in Baltic Sea estuarine waters [231, 234]. VHSV Type III infects marine and estuarine fish of several species in the North Sea from the Flemish Cap [235] to the Norwegian coast [236], Skagerrak, and Kattegat, but has caused epizootics in marine-farmed rainbow trout in Norway [236].

VHSV type IVa infects Northeastern Pacific salmonids [237, 238] and several marine fishes [212, 239, 240]. The southernmost hosts of type IVa in the Pacific Northwest are likely to be Pacific sardine (*Sardinops sagax*) and Pacific mackerel (*Scomber japonicas*) from southern California, where the transition to higher seawater temperatures restricts viral replication further south [239]. In Asia, type IVa was first detected in the Japanese olive flounder [241, 242], and subsequently the virus has been detected in fish of several other species in Japanese and Korean waters [233, 243, 244]. Type IVb is enzootic in freshwater fish of at least 31 species in the Great Lakes [234], and a newly designated subtype—IVc [216]—occurs in marine and estuarine fishes in North Atlantic waters.

Fish of approximately 82 species are susceptible to VHSV and encompass the bony fish orders of: Salmoniformes (salmon, trout, whitefish, grayling); Esociformes (pike, muskellunge); Clupeiformes (herring, pilchard, sprat); Gadiformes (cod, hake, burbot, pollock); Pleuronectiformes (flounders, soles, plaice, dab, halibut, turbot); Osmeriformes (smelt); Perciformes (perch, drum, sand lance, sand eels, gobies, temperate basses and sunfish); Scorpaeniformes (rockfishes, sculpins); Anguilliformes (eels); Cypriniformes (minnows, carp); Cyprinodontiformes (mummichog); and Gasterosteiformes (sticklebacks, tubesnouts). Many of these fish develop only subclinical infections.

In addition to fish, VHSV IVb has been isolated from a leech (*Myzobdella lugubris*) [245] and from *Diporeia* amphipods [246]. Experimentally, freshwater turtles could be infected by feeding them infected bluegills (*Lepomis macrochirus*) [247]. However, the ubiquity of natural fish hosts infected by VHSV likely precludes non-fish vectors as important hosts in the natural history of VHSV, either for transmission or maintenance. A complete list of known and suspected VHSV hosts is provided by the OIE [234].

VHS has caused serious economic losses in the rainbow trout industry within continental Europe, resulting in estimated annual losses of £40 million in sterling [211]. In Denmark, VHS has been virtually eradicated [228] through the application of strict biosecurity measures, but these measures have also been costly, sometimes causing a 50 % decrease in rainbow trout production [248]. The cost of VHS outbreaks at two Danish Farms in 2000 has been estimated at €211,000 [249]. The Pacific herring roe fishery in Prince William Sound, Alaska traditionally supported five commercial fisheries with an annual ex-vessel value of USD $8.3 million. The fishery collapsed in 1993 due to several different factors [250], but some analyses have indicated that VHSV type IVa was a minor contributing factor [251]. Although in subsequent years VHSV has only been sporadically detected in Prince William

Sound herring, the fishery has never recovered from those initial losses. The 2005–2007 epizootic emergence of VHSV type IVb in the Laurentian Great Lakes region of the USA caused losses of tens of thousands of native fish, and caused the US and Canadian governments to place stringent restrictions on the movement and sale of live food fish, baitfish, and sport/game fish that might disseminate the virus. This caused a significant economic hardship industry-wide in the Great Lakes states and Canadian provinces, which likely cost hundreds of thousands of US dollars in lost sales. This is a conservative estimate based on the most recent year available (1998 agriculture census). Additionally, these data described the value of the industry in only four states bordering the lower Great Lakes (New York, Pennsylvania, Michigan, and Ohio) where VHSV was isolated. Total industry sales in these states, consisting of live food fish, baitfish and sport/game fish, were estimated to be worth $17.4 million in a 2006 analysis of the industry [252].

7.1.4 Natural History, Transmission, and Host Range

Transmission of VHSV is primarily horizontal via contaminated fomites, through direct contact from fish to fish, or from virus released into ambient fresh or seawater from decomposing infected fish. The virus can be fairly stable for short periods of time outside the host, which facilitates environmental transmission. In a study investigating the transmission of VHSV subtype IVb via fomites, glass beads, two kinds of fishing line, plastic bottle pieces, and metal pieces from soft drink cans could sustain infectious virus (when kept wet) for up to 10 days [253]. Urine and female sexual secretions from clinically diseased or carrier fish may also transmit the virus. Juvenile fish are usually more susceptible than older fish.

The average time to inactivate 99 % of VHSV particles (strains I and IV) in raw freshwater was 13 days at 15 °C and 4 days in raw seawater. VHSV survival was greatly enhanced in filtered freshwater held at 4 °C, in which some of the strains remained infectious for 1 year [254]. In a similar study, infectivity of subtype IVa virus in seawater at 15 °C was reduced by 50 % after 10 h, but viable virus could still be recovered after 40 h [255]. Naturally infected herring can shed up to $10^{6.5}$ pfu/h while the minimum concentration required for horizontal transmission is much less at $10^{1.5}$–$10^{2.0}$ pfu/ml [255]. The titer of recoverable virus is also reduced by 90 % when VHSV-infected fish are subjected to commercial freezing and thawing [256]. Sexual secretions of Pacific herring are a potential source of horizontal transmission, where type IVa has been found at concentrations of 15 pfu/ml in seawater near spawning free-ranging herring [257]. Vertical transmission within fish eggs has not been demonstrated, but egg-associated virus on the surface does occur in trout. This surface-associated virus takes about 10 days to be eluted by flowing water during incubation, promoting water-transmission to other susceptible hosts [258]. The primary portal of entry of VHSV in trout is considered to be the gills.

VHSV has not been isolated from fecal material of either sex, nor from male seminal fluids in trout [74]. Experimental oral transmission in juvenile pike (*Esox lucius*) [74] and juvenile rainbow trout [259] is possible using infected fish or

infected feed homogenate. VHSV replication in the stomach was suspected to be the portal of entry for systemic infection and suggests the potential for infectious virus to be shed in the feces [259]. The presence of VHSV subtype IVa in the feces of juvenile Pacific herring is unknown, but the virus replicated in the submucosa of the intestinal tract [260]. Should the virus gain entry into the intestinal lumen of Pacific herring, the virus may be capable of surviving passage through the gut. VHSV-infected fish regurgitated by piscivorous birds are still infectious, but the virus does not survive passage through the avian gastrointestinal tract [261].

7.2 Pathogenesis and Clinical Features

7.2.1 Pathogenesis and Immunology

Some of the marine strains of VHSV are relatively apathogenic, whereas others are highly virulent for some of their hosts. The occurrence of VHS in general is dependent on the optimum host, life stage, and water temperature. Sublineages Ia and Ic affecting rainbow trout are highly virulent and represent most of the descriptions in the older literature. In trout, VHSV is endotheliotropic, causing a systemic infection and producing histopathological changes in affected organs. Pyknosis and karyorrhexis occur in tissues of the kidney, pancreas, and spleen. Focal necrosis of liver hepatocytes (coagulative) and pancreatic acinar cells (lesser degree than liver) is noted. Some accompanying hemorrhage in the dorsal skeletal musculature may be present, but necrosis of muscle bundles is not apparent [74].

The disease caused by sublineage IVa in the extremely susceptible Pacific herring exposed to water-borne virus is an acute systemic infection that transitions into a chronic neurologic phase. Immunohistochemical staining demonstrates the presence of the virus in affected tissues (Fig. 11.13) [260]. In the acute phase, epidermal thickening is followed by infection of fibroblasts within the fin bases and dermis, causing severe cell necrosis and some hemorrhaging (Fig. 11.14) [260]. At this stage, systemic infection causes cell necrosis of the kidney interstitial cells, diffuse splenic necrosis, and focal necrosis of liver hepatocytes (coagulative), exocrine acinar cells of the pancreas, and connective tissues cells of the submucosa. Minor cell degeneration is also present in the meninges of the brain. Immunohistochemical staining demonstrates viral persistence in the meninges and parenchyma of the brain (Fig. 11.13) and sometimes peripheral nerves with clearance of the virus from other tissues [260]. Cellular changes in the brain may consist of varying degrees of diffuse nuclear pyknosis and karyorrhexis to very small multifoci of necrosis in the granular layer of the optic tectum, most often near lobe attachment to the cerebellar valvula (TR Meyers, unpublished).

The systemic disease caused by subtype IVb results in histopathological changes that include general vasculature congestion of the fins, gills (with necrosis in some species), spleen, and ovary. Skeletal muscle and subcutaneous hemorrhages are noted (e.g., swimbladder). Other manifestations include multifocal vacuolation and

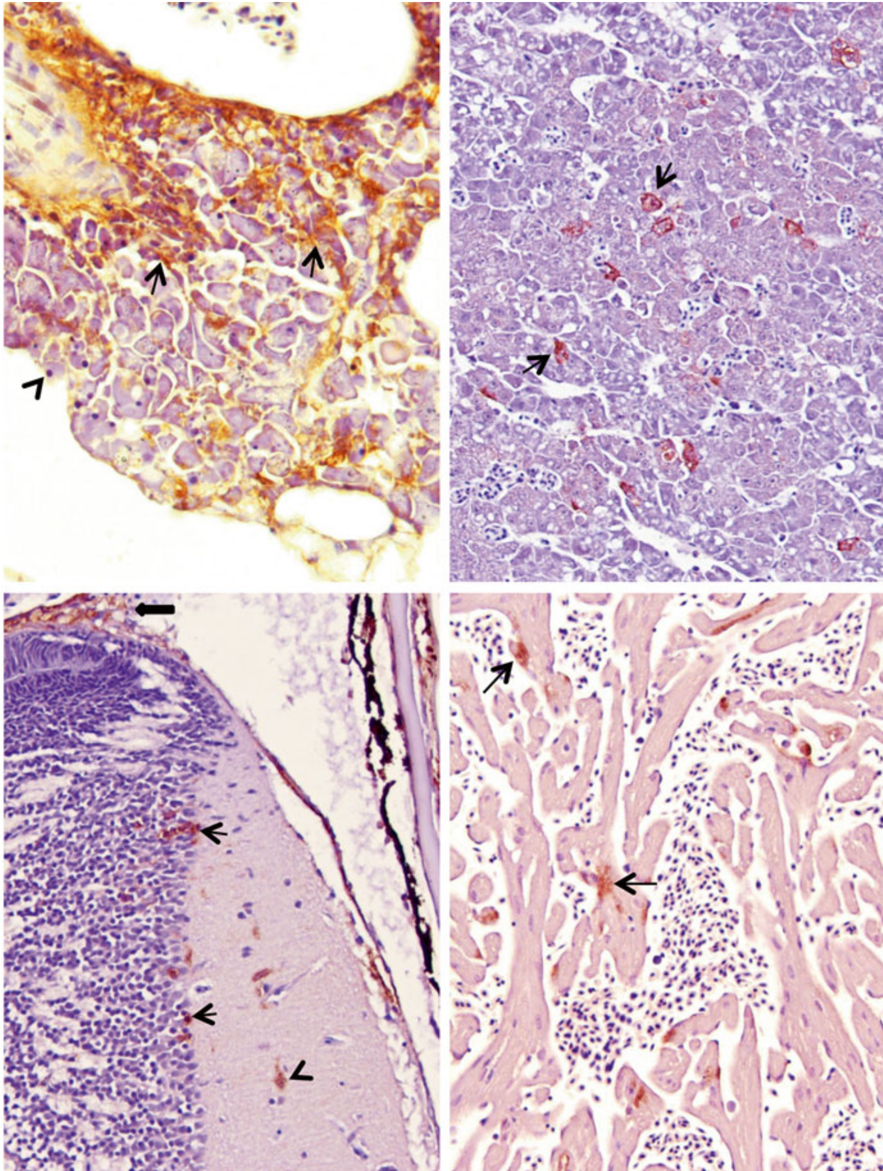

Fig. 11.13 Immunohistochemical staining (brown foci) demonstrating viral hemorrhagic septicemia virus (VHSV) type IVa antigen in tissues of experimentally infected juvenile Pacific herring at 7 and 8 days post-exposure. *Top left*: exocrine pancreas with abundant viral antigen (*arrows*) and pyknotic cells (*arrowhead*) (400×); *Top right*: several hepatocytes in liver containing viral antigen (*arrows*) (200×); *Bottom left*: optic lobe of the brain with viral antigen in cells of the outer granular layer (*arrows*), in neurons of the myelinated parenchyma (*arrowhead*), and meninges (*filled arrow*) (200×). *Bottom right*: trabecular muscle fibers of the heart ventricle with viral antigen (*arrows*) (200×). Procedure: Primary anti-VHSV monoclonal antibody, biotin conjugated secondary anti-mouse IgG, streptavidin horseradish peroxidase and color development with 3,3′-diaminobenzidine [260]

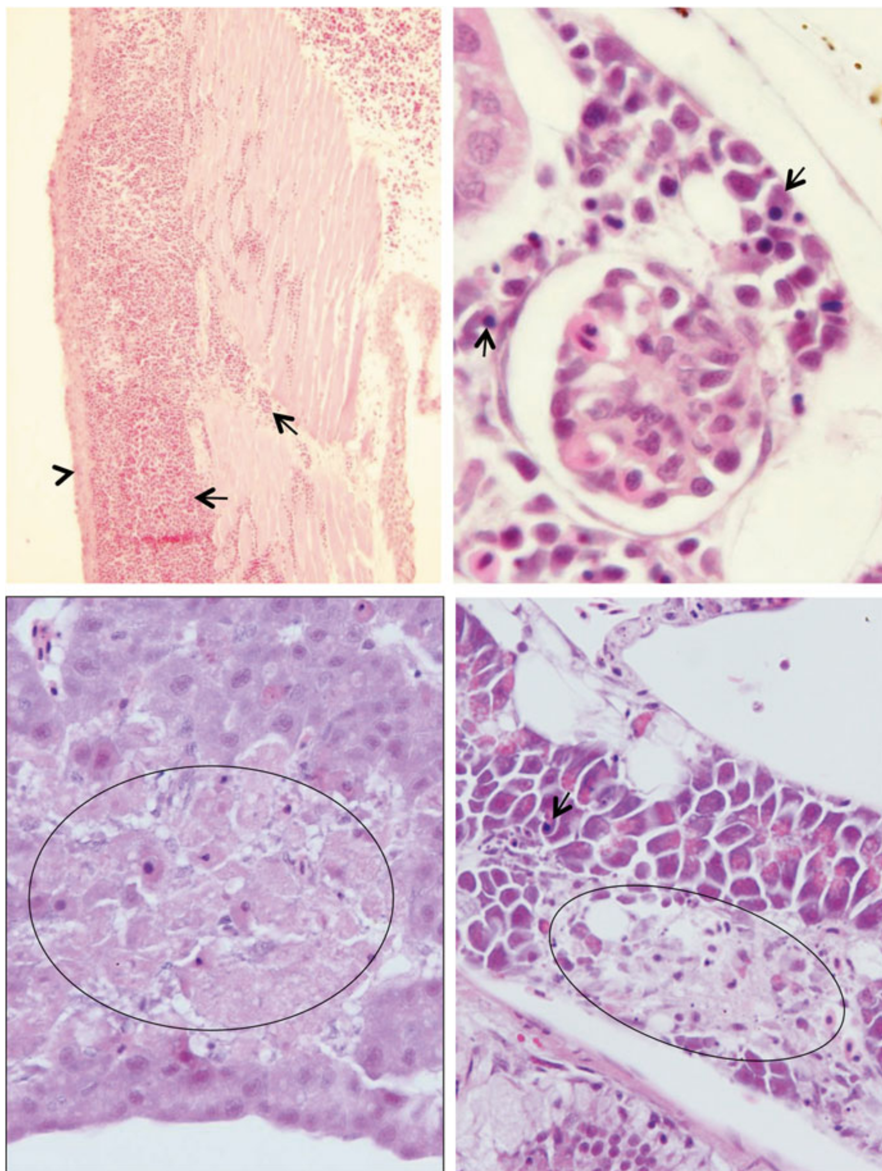

Fig. 11.14 Histopathology of juvenile Pacific herring experimentally infected with VHSV type IVa sampled at various days post-exposure (PE). *Top left*: intact epidermis (*arrowhead*) with hemorrhage (*arrows*) in the dermis, subcutis, and underlying skeletal musculature at 8 days PE (200×). *Top right*: nuclear pyknosis and necrosis of renal interstitial hematopoietic cells (*arrows*) at 7 days PE, normal glomerulus in *center*, and tubule *top left corner* (1,000×). *Bottom left*: focal coagulative necrosis (*oval*) in liver at 21 days PE (400×). *Bottom right*: focal necrosis of exocrine pancreas (*oval*) and single cell nuclear pyknosis (*arrow*) at 7 days PE (40×). Hematoxylin and eosin

necrosis of liver hepatocytes, and interstitial edema and congestion with tubular necrosis in the kidney [262, 263]. The muskellunge (*Esox masquinongy*) was found to be the most susceptible host for this subtype [263].

Type III VHSV in 1- and 3-year-old tank-reared turbot causes hepatocellular necrosis and hemorrhage, vacuolation of kidney tubules, and focal hemorrhaging behind the retina and within skeletal musculature. A feature not reported in other VHSV infections is marked necrosis of the ventricular muscle fibers of the heart [264].

Early on, trout growers in Europe learned that fish surviving VHS were immune to reinfection. Experimental work in France found that trout produced nonspecific interferon early in the infection (peak at 3 days), followed later by specific acquired immunity with significant titers of both non-neutralizing and neutralizing antibodies [74, 76]. Standardization of infection procedures reproduced the disease with predictable results and facilitated immunological investigations [265]. Early attempts at developing vaccines by immersion with inactivated or attenuated virus were successful in producing protection against challenge with virulent virus. However, not all of the virus strains generated protective immunity by these methods. In later studies, three serological subgroups of VHSV (I, II, III) emerged based on a panel of four monoclonal antibodies and one polyclonal neutralizing antibody, indicating that VHSV subgroups share several antigenic epitopes [266]. This serogrouping does not correspond to the genotypes determined by nucleic acid sequencing. More recently, specific monoclonal antibodies have also been developed for genogroup isolates IVa and I-1b [233].

Naïve juvenile Pacific herring are highly susceptible to VHSV sublineage IVa by immersion exposure [267], but fish that survive infection are refractory to the disease [257, 268]. Unlike rainbow trout, DNA vaccination of herring against VHSV infection has not produced early nonspecific protection due to interferon, nor has it resulted in significant measurable neutralizing antibody [269]. However, injection of plasma of herring that survived experimental epizootics of VHSV confers passive immunity to the disease to naïve herring, thus providing strong evidence for the presence of an adaptive humoral immune response [270].

Neutralizing antibodies against type IVb have been detected in experimentally infected muskellunge that produced peak titers at 11–17 weeks post-exposure [271]. Additional field studies testing the sera from 13 fish species collected from Lake St. Clair, Michigan, found neutralizing antibodies against sublineage IVb in fish of four of the species. These results demonstrated that detection of neutralizing antibodies may prove useful as a nonlethal approach for assessing the prevalence of prior IVb exposure in wild fish of several species in the Great Lakes [271].

7.2.2 Incubation Period, Clinical Signs, and Gross Lesions

Clinical signs of VHSV infection (for sublineages Ia and Ic) by water exposure in rainbow trout occur within 4 days of infection, with shedding of virus in urine by 3 days [211]. The disease in rainbow trout can occur in three forms: acute infection

with high mortality, chronic infection, and infection affecting the nervous system. External signs for acute infection include anorexia and lethargy to hyperactive swimming, darkened body, pale gills due to anemia, exophthalmia in one or both eyes, ascites, and petechial hemorrhages in the eye orbits, fin bases, and the skeletal musculature. Internal signs vary with the severity of the disease, from mild petechial hemorrhages in the internal organs, body wall, and cavity to profuse bleeding. The kidney may be swollen, pale, and grossly necrotic, the spleen may be red and enlarged, and the liver may appear mottled with hyperemic areas or petechial hemorrhages. The gut is usually empty and contains a yellow mucoid cast. No external signs are present in rainbow trout with chronic infection, but some visceral pathology is present. Infection affecting the nervous system is indicated by aberrational swimming behavior and viral tropism for the brain, where the virus may be detected in very high titers. Survivors of any form become carrier fish from which virus can be isolated from tissues, such as the kidney and brain [211].

The incubation period for water exposure to a high dose ($10^{5.5–6.5}$ pfu/ml) of VHSV sublineage IVa in Pacific herring is 4–6 days. At the peak of infection at day 7, external signs include 1–2 mm hemorrhages on the lower jaw, isthmus (throat), and around the eye. This dose is associated with very high mortality. Occasionally, fish exhibit extensive subcutaneous hemorrhaging. Fish exposed to a low dose of virus ($10^{1.5–2.5}$ pfu/ml) die later, at 6–12 days post-exposure with peak mortality at 10–11 days. Those fish surviving to 21 days post-exposure have low or undetectable levels of virus [267]. Neurologic signs of erratic swimming behavior and darkened dorsal skin develop in the chronic phase in surviving infected fish [260]. Fish surviving the disease either clear the virus or become asymptomatic carriers [257, 268].

The progression of disease caused by sublineage IVb varies in susceptible fish hosts, but generally occurs within 4–7 days of water exposure to high virus concentrations (10^5 pfu/ml). Lower exposure concentrations (10^3 pfu/ml) resulted in clinical disease in 8–26 days. In peracute mortal disease, clinical signs may be absent. In acute and chronic mortal infections, external signs may include lethargy, petechial hemorrhages on most fins, diffuse dermal erythema on the ventral abdomen, perianal swelling with partial anal prolapse, focal hemorrhages of the gill arches, and pale gills. Internal signs may include intramuscular hemorrhages, petechial hemorrhages of the swimbladder, serosanguinous ascites, swollen testes and ovaries, and severe hepatomegaly [262, 263].

VHSV in trout causes a hypochromic normocytic anemia with low erythrocyte counts, low hematocrit, and low hemoglobin values when compared to normal fish [272].

7.2.3 Morbidity and/or Case-Fatality Rates and Prognostic Factors

Disease and mortality caused by VHSV has occurred mostly in farmed European trout, turbot, and Japanese flatfish, in wild marine fish in the Northeast Pacific, and wild freshwater fish in the Great Lakes region of North America. Morbidity and mortality rates are dependent on host age, size, and species, infecting viral

genotype, water temperature, viral dose, exposure route, and stress level from environmental conditions. Such conditions include as capture and handling, high population densities, spawning, nutritional deprivation, harassment by predators, and infection by other pathogens [214]. Generally, VHS causes greater losses in smaller, younger fish, the exception being reported in rainbow trout, turbot, and some Great Lakes fish in which VHS outbreaks caused significant mortality in larger fish that were suspected to be naïve hosts. With the presence of a susceptible host for a given viral genotype and under optimum viral and environmental conditions, mortality in juvenile rainbow trout, Pacific herring and various Great Lakes fish may approach 90–100 %. Case-fatality rate in turbot may range from 0 to 68 % when exposed to freshwater and marine VHSV isolates [249]. In disease outbreaks in Japanese flounder, mortality can reach 50–70 % [242]. In general, salmonids have very low or no mortality when exposed to marine and North American VHSV isolates, whereas injection may cause high mortality [249]. Experimental immersion exposure of juvenile Atlantic cod (*Gadus morhua*) and Atlantic halibut (*Hippoglossus hippoglossus*) to various types of VHSV (Ia, Ib, II, III, IVa) demonstrated that these fish can become infected at a very low incidence with almost no VHSV-related fatality (0.12–0.08 %) [273], whereas injection of type III into cod produced mortality over 80 % [274]. Observed mortality has been somewhat higher in one outbreak of VHSV in captured juvenile Pacific cod (*Gadus macrocephalus*) naturally infected with type IVa. Affected fish developed external hemorrhages (Fig. 11.15) and sustained a cumulative mortality of 9 % from a population of 450

Fig. 11.15 Captive Pacific cod that died from natural infection from viral hemorrhagic septicemia virus type IVa infection. External hemorrhage (*arrows*) of the anal fin and caudal peduncle (*left figure*) and ventral opercular area (*right figure*)

over a 12 day period (TR Meyers, unpublished case report). Additional exposure and lethality/mortality studies for VHSV are further reviewed in Skall et al. [249].

The prognosis for infected fish is poor as no therapeutic option is available for treatment of disease caused by VHSV. Infected fish generally die, may recover and clear the virus, or become lifelong carriers. In many farmed operations, infected fish must be destroyed regardless of mortality rate to contain the spread of virus.

7.3 Diagnosis and Control

7.3.1 Diagnosis and Epidemiology

Standard diagnosis of VHS is by isolation of the virus based on viral CPE in susceptible fish cell lines, and identification of the virus by serological or nucleic acid detection methods. Cell lines used include the bluegill fry (BF-2) and EPC [249] that are inoculated with homogenized kidney/spleen pools, heart, or brain encephalon from larger affected fish, or homogenized whole fry less than 4 cm in length, or centrifuged ovarian fluids from spawning fish. BF-2 and EPC cells are typically more sensitive for the European genotypes or North American genotypes, respectively. Cell cultures are maintained in a slightly alkaline environment, with pH minimums of 7.6 for European strains and 7.0 for North American strains. Common serologic methods to identify VHSV include virus neutralization, immunofluorescence, and ELISA using antisera that have been validated for sensitivity and specificity. For instance, the monoclonal antibody IP5B11 [275] recognizes all VHSVs that have been isolated to date [276]. Current molecular methods used consist of standard RT-PCR and quantitative RT-PCR.

Detection of virus becomes problematic in subclinically infected carrier fish for which cell culture and PCR or other molecular methods used in parallel may or may not be useful for surveillance. Quantitative RT-PCR with confirmatory cell culture has been validated for surveillance of type IVb in the Great Lakes [277].

Natural outbreaks of VHS have been reported in farmed rainbow trout [74], farmed turbot [264], in both farmed and wild Japanese flounder [241, 242], in wild Pacific herring, pollock (*Theragra chalcograma*), hake (*Merluccius productus*), black cod (*Anoplopoma fimbria*), pilchards in the Pacific Northwest [214, 240, 278], and in wild fish of various species in the Great Lakes region of the USA. Mass mortalities in wild marine fish from which VHSV has been isolated have not been reported from Europe [249].

VHS is a cool water disease most frequently occurring in the spring during fluctuating water temperatures. Temperatures above 15 °C are largely nonpermissive for viral replication [74]. The requirement for cool temperatures shortens the time course of the disease and decreases the cumulative lethality of outbreaks in warmer regions or times of year.

Rates of endemic, subclinical virus in wild marine fish of several species have varied from zero to upwards of 69 % (found in Atlantic herring) based on qRT-PCR

testing of samples, some of which were confirmed by isolation of virus in cell culture [249]. These reservoirs represent point sources for infection and occasional disease for farmed trout and other susceptible cultured fish in a marine net-pen environment. The population effect of VHSV infection in wild marine fish is also relatively unknown.

The epizootiology of VHS in wild marine fish populations is probably best studied in Pacific herring in Prince William Sound, Alaska. VHSV is most prevalent in large schools of young-of-the-year herring during springtime transitional seawater temperatures, sometimes causing disease and mortality. At the same time, sexually mature fish that are spawning on kelp may also be positive for the virus with no overt disease [279]. By the fall, the virus cannot be detected in either juvenile or adult fish [251]. Several factors may help to perpetuate the virus in the population: cohabitation of juvenile infected herring in nursery and spawning areas; shedding of virus from small numbers of adult infected herring and other reservoir species; and increased viral shedding during the spring spawning or other times of stress. Conversely, other factors influence the early stages of VHSV infection and determine whether or not epizootics in juvenile fish will occur at all, such as exposure to low virus concentrations in water, effect of ambient water temperature on virus amplification, rates of fish-to-fish transmission, and herd immunity [280, 281].

VHSV subtype IVb emerged in the Great Lakes in 2005, causing a large, multispecies epizootic in wild fish through 2007. In 2008 and through 2010, endemic areas had overall lower rates of overt disease (detected by cell culture and qRT-PCR) than that occurring during the epizootic. This disease pattern suggests an initial outbreak in naïve populations of fish, which then developed some degree of acquired immunity. This hypothesis is supported by a medium-to-high prevalence of neutralizing antibodies in fish of some susceptible species [271]. Currently, VHSV subtype IVb has not been detected in any hatchery or cultured stocks of fish.

7.3.2 Vaccination, Control, and Eradication

Despite investigations of killed vaccines, LAV, a recombinant vaccine in both prokaryotic and eukaryotic expression systems, and DNA-based vaccines, no commercial vaccine is approved for use. Among the vaccines tested, the G-gene DNA plasmid constructs have been the most promising. Administration of 1 μg of vaccine to rainbow trout fingerlings (3–4 g) significantly protected against disease as early as 8 days post vaccination (pv) to 168 days when the experiment was terminated. Early protection is attributed to activation of nonspecific defense mechanisms (such as interferon) associated with elevated Mx gene transcription. Later protection was assumed to be due to stimulation of the adaptive immune system and production of neutralizing antibodies [282]. This rapid activation of nonspecific innate immunity of short duration was further illustrated by another vaccination study in which fingerlings vaccinated with either VHSV or IHNV vaccines were protected from VHS at 4 days pv. This cross-protection was either lost or present at intermediate levels in IHNV-vaccinated fish at 60 days pv, depending on fish size [283, 284]. Later investigations established that VHSV DNA vaccination in 100 g rainbow trout

resulted in protection up to 9 months after an injection dose of 0.5 μg [285]. Similar DNA vaccination studies conducted with Pacific herring did not result in early protection, and much less robust overall protection at 6 and 15 weeks pv. The observed responses were assumed to be due to some form of adaptive immunity, although neutralizing antibodies have yet to be confirmed in Pacific herring surviving infection by VHSV [269].

The need for a mass delivery system (rather than impractical manual injection) has been an impediment to the use of vaccines in juvenile fish, in which the disease is most acute and economically debilitating. The success of intramuscular injection of nanogram doses of vaccine in conferring very high protection in fry as small as 0.5 g could spur further development of an alternative delivery system [286]. Another significant regulatory obstacle involves the varied public perception as to whether a DNA-vaccinated animal becomes a genetically modified organism (GMO). A complete review of vaccines used in aquaculture is provided by Lorenzen and LaPatra [86].

As with all infectious diseases, avoidance of VHSV provides the most reliable control strategy. Denmark has been exemplary regarding successful eradication in its rainbow trout industry, where the first outbreaks of VHS were observed in the 1950s. An eradication program started in 1965 required biosecurity measures including virus-free water supplies, removal of infected fish stocks, fallowing, and disinfection of facilities and eggs, repopulating with fish stocks from registered VHSV-free producers, and annual surveillance inspections for the virus [74]. There has not been a case of VHSV detected in Norway since 2009 [228] despite to active surveillance systems. Denmark's eradication of the virus has been an example which reemphasizes that VHSV is mostly disseminated by transport of infected farmed fish and that the current trade regulations and biosecurity programs are working successfully to maintain approved VHSV-free zones [249]. Since the disease is not transmitted vertically, egg disinfection with iodophor compounds (50 mg/l) provides reasonable assurance of inactivation of any surface virus [287]. However, care must be exercised when relying on strict biosecurity to control a disease. In the Great Lakes region of the USA, investigators have found that the tannic acid used as an anticlumping agent for eggs destroys the virucidal properties of iodophor against VHSV sublineage IVb unless it is thoroughly rinsed from the eggs [287].

Eradication of VHSV has been achieved in various rainbow trout farming locations in Europe through very stringent biosecurity measures. In other areas without such biosecurity, VHSV has become established in farmed fish stocks and in associated watersheds, where the virus and disease becomes enzootic in both cultured and wild carrier fish.

Selective breeding of rainbow trout is a promising approach for developing stocks resistant to VHS, but no resistant stocks are available commercially [234]. Various genetic crosses of rainbow trout with other char, trout, and salmons have generally resulted in varying degrees of resistance to VHSV [288, 289]. Most hybrids developed to date would be unlikely candidates for commercial culture and have more value in studying resistance mechanisms.

Control of the risk of reintroduction of VHSV from sea water net-pen culture of fish to freshwater aquaculture requires measures designed to minimize this risk.

Circumstantial evidence indicates that avirulent strains of VHSV could mutate into virulent forms by acquiring only a few amino acid substitutions [249]. Proposed control measures include the prohibition of raw fish used as feed despite the absence of an alternative artificial diet for certain fish species (trout are tolerant to artificial diets). Additionally, prohibitions on co-cultivation of rainbow trout with VHSV-susceptible marine fish and the introduction of farmed fish from seawater to freshwater are important elements of a control program, even though broodstock can be spawned in seawater and the eggs disinfected before transfer to freshwater areas. Finally, approved virus-free status of coastal zones should remain unaltered to prevent any free trade between marine and freshwater approved or unapproved sites. If the virus-free status is removed, the resultant trade could easily introduce virus into areas that are free of virus. However, a "virus-free coastal zone" should be further defined to both protect the industry, as well as facilitate the detection of and response to legitimate outbreaks of disease.

7.3.3 Public Health/One Health Crossover

VHSV is a poikilothermic virus. Contact with or consumption of infected fish poses no human or animal health concern.

8 Other Negative-Stranded RNA Viruses Causing Viral Hemorrhagic Fever-Like Diseases

8.1 Avian Influenza Virus (AIV)

Avian influenza (AI) ranges from an asymptomatic or mild infection up to a fatal disease of domestic and wild fowl (e.g., chickens, turkeys, migratory waterfowl), Generally, AI is considered a gastrointestinal disease in fowl [290]. However, with a highly pathogenic subtype of the virus, severe or fatal disease can occur. In chickens, vascular disturbances can occur in many organ systems, and edema of the face and neck and associated structures (e.g., swollen combs, wattles, periorbital tissues) is a common clinical finding in affected flocks. However, petechiae, ecchymoses, cyanosis, and/or necrotic foci of those same structures can occur. Areas of diffuse hemorrhage on the legs and a hemorrhagic tracheitis similar to that seen with infectious laryngotracheitis may be observed. Similarly, petechiae may be observed on the internal surfaces of many structures in or associated with the gut, and kidneys can be congested, indicating a fluid disturbance in the tissue itself with a secondary effect on the body (i.e., dehydration). Ovaries may be hemorrhagic and/or necrotic. Lesions in turkeys and domestic ducks are similar, but may be less severe than that observed in chickens [291].

8.2 Bovine Ephemeral Fever Virus (BEFV)

Bovine ephemeral fever (BEF) is a severe disease that paradoxically usually resolves with a rapid recovery in the majority of cases. Part of a group of antigenically related rhabdoviruses, BEF has only been observed to cause disease in cattle and water buffalo. The disease is less severe in water buffalo (*Bubalus bubalis*), whereas many other wild ruminants have neutralizing antibodies and therefore appear to be protected. Bovine ephemeral fever virus is endemic in tropical, subtropical, and temperate countries in Africa, Asia, Australia, and Japan, and follows the range of its arthropod vectors from the genera *Culex* or *Anopheles* (mosquitos) or *Culicoides* (gnats) [292].

Clinical signs can be quite severe, and generally follow the signs seen with any severe inflammatory disease. Vascular disturbances may be commonly seen as submandibular edema or foci of edema anywhere on the head or neck. In a small number of cases, greater hemostatic disturbances are evidenced by fibrin-rich fluid in body cavities, including the pericardium and joint capsules. Petechial hemorrhages in lymph nodes are rare, as is necrosis of muscle. These findings are likely secondary to the rise in serum fibrinogen concentrations seen in clinical disease, but these concentrations return to near normal within 2 weeks of recovery. Inappropriate clotting could lead to exhaustion of the hemostatic system and to overt hemorrhage similar to that seen in other VHF-causing pathogens that attack the liver. Given the inflammatory source of the signs, cattle usually respond to intensive treatment with anti-inflammatory drugs and/or calcium to counteract the paralysis that develops secondary to calcium imbalances [291].

8.3 Peste Des Petits Ruminants Virus (PPRV)

PPRV, a morbillivirus, causes a subacute/acute disease of goats and sheep that rarely exhibits hemorrhagic enteritis, consistent with its relationship to another morbillivirus, rinderpest virus. PRR is generally considered a disease of the upper respiratory and gastrointestinal tract [293].

In the acute form, the incubation period is 4–5 days, leading to a high fever for 5–8 days before occasionally returning to normal. Coincidentally, a serous nasal discharge evolves, which may progress to a mucopurulent discharge. This discharge can be severe enough to physically impair breathing. Small areas of necrosis may be seen on the nasal mucous membranes or the stoma, which may spread but rarely involve the basal layer of epithelium. In the upper respiratory tract, small erosions and petechiae can be seen on several structures, probably due to attack of lymphatic structures. The conjunctivae are usually congested, and severe diarrhea is often present.

Of the four chambers of the stomach, the abomasum will commonly have regularly outlined erosions that will ooze frank blood. Deficits in the small intestine are generally limited to small streaks of hemorrhages, probably sec-

ondary to the (sometimes severe) necrosis and/or ulceration seen in Peyer's patches. In the posterior colon and rectum, "zebra stripe" lesions may form, similar to those seen in rinderpest. In fatal cases, death may occur 5–12 days after onset of disease.

PPRV transmission requires close contact. Upper GI or ocular secretions, and feces, are the main sources of transmissible particles. There is no known carrier state; infected animals only transmit the disease during clinical episodes [291].

8.4 Rinderpest Virus (RPV)

RPV is a highly contagious virus of domestic and wild cloven-footed animals (ruminants and pigs) causing disease with a high case-fatality rate. Field strains of RPV vary widely in virulence, sometimes leading to self-limited disease outbreaks caused by virulent strains, but endemic maintenance of mild strains. Depending on multiple virus and host factors (e.g., virus strain, innate or acquired resistance of the animal, concurrent infections), RPV infection is associated with peracute, acute, or mild clinical signs. Hemorrhagic manifestations may be only present in the acute form as hemorrhagic diarrhea or gastroenteritis with intestinal mucosal hemorrhages. However, similar to PPR, the walls of the cecum and colon become edematous, with blood possible in the lumen, and adherent blood clots on the mucosa. These signs are usually more severe in the upper colon and/or concentrated at the cecocolic junction. "Tiger striping" occurs at the colonic ridges due to congestion. In contrast to PPR, the omasum is the part of the stomach that will rarely show erosions and hemorrhage [291].

RPV has only one serotype, and immunity is lifelong, which allowed an eradication effort that was declared successful in 2011 [5]. No natural reservoir, vertical transmission, arthropod vector, or carrier state is recognized for RPV.

8.5 Spring Viremia of Carp Virus (SVCV)

Also called infectious dropsy of carp or infectious ascites, SVC is a severe viral disease of some coolwater and warmwater fish which may have serious economic consequences. Morbidity can be as high as 100 %, and lethality of 70 % has been observed. Clinical signs include inflammation, congestion, and hemorrhage of the swim bladder and some other internal organs, ascites, and petechial hemorrhages of gills and skin. Vasculitis and necrosis may be seen microscopically. However, these signs are not specific to SVCV (see IHNV, ISAV, and VHSV sections), complicating field diagnosis [294].

SVCV spreads by horizontal transmission through diseased or dead fish, and some fish may become carriers with variable virus shedding and/or recrudescence (usually related to stress). Additionally, vector transmission through fomites, birds, or arthropods may be important. Finally, water temperature affects transmission, probably through its effect on the host immune system. Prevention of SVC (in aquaculture facilities) occurs through stringent biosecurity and complete depopulation of affected populations as there are no effective vaccines or treatments for SVC [291].

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Chapter 12
Viral Hemorrhagic Fevers of Animals Caused by DNA Viruses

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Core Message Here we outline serious diseases of food and fiber animals that cause damaging economic effects on producers all over the world. The only vector-borne DNA virus is included here (i.e., African swine fever virus), and the herpesviruses discussed have a complex epidemiology characterized by outbreaks that are linked to differing susceptibility of related animals to infection and/or disease. Much work remains to be done to fully explain the genetic and ecological determinants of disease for these complex viruses.

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1 African Swine Fever Virus

1.1 Etiologic Agent and Natural History

1.1.1 Definition

African swine fever (ASF, also East African swine fever) is a contagious hemorrhagic viral disease of swine.

1.1.2 Etiology and Evolution

ASF is caused by African swine fever virus (ASFV), a large and complex double-stranded (ds) DNA virus that produces enveloped virions. ASFV is classified as the sole member of the species *African swine fever virus*, genus *Asfivirus* (sigil *asfi*: *A*frican *s*wine *f*ever v*i*rus), family *Asfarviridae* (sigil *asfar*: *A*frican *s*wine *f*ever *a*nd *r*elated [viruses]) [1].

The ASF virion, approximately 200 nm in diameter, is complex with an icosahedral shape, and is composed of several structural layers containing more than 50 polypeptides [2–5]. This structure contains a core ("nucleoid") that is approximately 80 nm in diameter [6] and that is surrounded by two lipid bilayers [6, 7]. The icosahedral capsid is mainly composed of the structural protein p72 [6, 8, 9]. An external membrane that originates from the plasma membrane coats the capsid during the budding process [2, 3, 10, 11].

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1.1.3 Geographic Distribution and Economic Effects

ASF is classified as a reportable disease by the OIE (World Organisation for Animal Health). The disease is of a worldwide concern due to its detrimental effect on swine breeding in enzootic areas or on international pork trading with countries free of the disease.

ASF was first described in Kenya during the 1920s and was then recognized in other sub-Saharan countries [12], where the virus is endemic. During the 1950s, the virus spread from Africa to the Iberian Peninsula, where it remained endemic until its eradication in the mid-1990s [13]. Since then the European Union (EU) has been considered free of the disease, with the exception of the island of Sardinia. Outbreaks of ASF were reported in countries in the Western hemisphere including Haiti and the Dominican Republic (1978), Cuba (1971), and Brazil (1978). These outbreaks were successfully controlled and the disease was eradicated each time there was an outbreak in the West. Recent ASF outbreaks (2007) in domestic and wild pigs that originated in the Caucasus region, including Georgia, Azerbaijan, and Armenia, and southern and western Russia, Ukraine, Belarus, and Iran have brought concerns about the disease re-emerging outside the African continent.

The spread of ASF outside of Africa has resulted in important economic losses to swine industries wherever it occurred, mainly due to bans on international trading of pigs and pork products from affected countries, together with the implementation of costly control measures necessary to eradicate the disease [13]. The cost of control and eradication of ASF in Cuba during 1980s is estimated to have totaled $9.4 million USD [13, 14], whereas the last 5 years of the eradication program in Spain led to expenditures of $92 million USD [13, 15]. Preventing the introduction of ASF into the USA is estimated to lead to a net benefit of $450 million per potential outbreak [13, 16].

1.1.4 Natural History, Transmission, and Host Range

Two types of transmission cycles are recognized in ASF epidemiology: "sylvatic" and "domestic" cycles [13]. In the sylvatic cycle, ASFV is maintained in the environment by actively circulating between desert warthogs (*Phacochoerus africanus*), bushpigs (*Potamochoerus larvatus*), and soft ticks of the genus *Ornithodoros*. The presence or absence of competent ticks of various species in a particular geographical area conditions the distribution and maintenance of ASFV in that environment [17]. Warthogs and red bushpigs only develop inapparent, subclinical infections with intermittent viremia [18–20]. The sylvatic cycle may occasionally be spread into domestic swine herds by infected ticks or direct contact with infected wild suids [21, 22]. In the domestic cycle, ASFV is maintained in swine herds mainly due to efficient direct transmission of the virus among animals (for review, see [23]).

Persistent infections with ASFV are observed in warthogs and bushpigs, and in domestic pigs surviving acute viral infections [24, 25]. Similar long-term persistent infections in domestic pigs can also be generated by experimental inoculation [26].

In these animals, genomic DNA (but not infectious virus) can be detected in peripheral blood monocytes by polymerase chain reaction (PCR) for up to 500 days post-inoculation.

1.2 Pathogenesis and Clinical Features

1.2.1 Pathogenesis and Immunology

The severity of the clinical presentation of ASF in domestic pigs depends on the virulence of the circulating virus. Presentations of ASF in infected herds range from acute and highly lethal to subclinical infections. The clinical appearance of ASF in swine herds may resemble that of other pig diseases such as classical swine fever, thus hampering diagnostic efforts that depend solely on clinical signs [12, 23, 27].

In domestic pigs, ASFV infection mainly occurs via the oronasal route, with primary replication of the virus in tonsils followed by a period of viremia and further replication of the virus in organs of the hemolymphatic system. The primary cell types targeted by ASFV belong to the mononuclear-phagocytic system, including tissue macrophages and specific reticular cells [28–31].

The role of various genes in the virulence of ASFV is not well understood. For instance, specific genes within the multigene families (MGFs) act as virulence determinants. A large deletion, in the left variable region of the genome, encompassing several genes within MGF360 and MGF530, significantly reduces viral replication in macrophages and virulence in pigs [32]. Similarly, deletion of the thymidine kinase (TK) genes from ASFV reduced viral replication in macrophages and led to virus attenuation in pigs, suggesting an association between the ability to replicate in macrophages in vitro and virulence [33]. Besides some of the MGFs and TK, other ASFV genes have been linked to virus virulence, but do not affect virus replication in macrophages in vitro. Deletion of UK (DP96R), a gene expressed early in replication, from virulent ASFV isolates results in marked attenuation of the virus in pigs [34]. Similarly, deletion of the 23-NL gene from ASFV isolate E70 significantly reduces virulence of ASF in swine. 23-NL, which encodes the NL protein, is similar to the human herpesvirus 1 (herpes simplex virus 1) neurovirulence factor ICP34.5, but does not affect in vitro ASFV replication in macrophages [35, 36].

1.2.2 Incubation Period, Clinical Signs, and Gross Lesions

In the acute form of ASF, the incubation period ranges from 5 to 15 days. Affected animals exhibit fever and anorexia followed by congestion, ecchymoses, hemorrhage, and cyanosis of the skin, increased respiratory and heart rates, nasal discharge, incoordination, bloody diarrhea, vomiting, and finally coma, followed by death (Fig. 12.1). The duration of survival after the development of clinical signs for animals infected with virulent ASFV isolates ranges from 2 to 9 days [37–41]. Typical hematological and clinical chemistry findings in acute ASF include leukopenia

Fig. 12.1 Swine: Multifocal cutaneous ecchymosis and hemorrhages associated with thrombocytopenia, with moderate to severe congestion, and cyanosis of the ears. *Source*: Plum Island Animal Disease Center

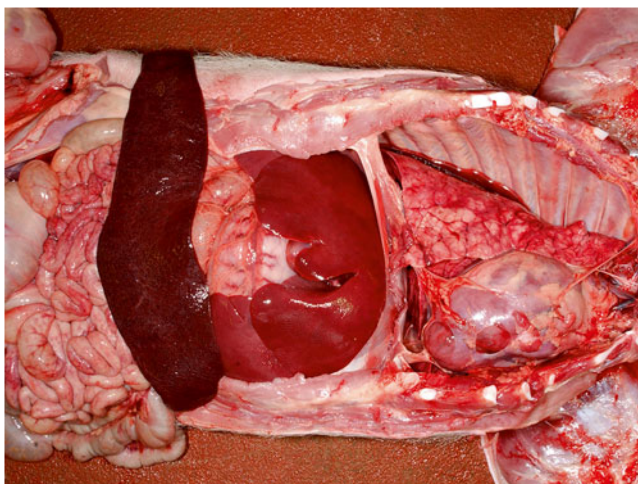

Fig. 12.2 Spleen: Marked splenomegaly with severe splenic congestion. *Source*: Plum Island Animal Disease Center

[25, 42, 43], B and T cell lymphopenia [44, 45], thrombocytopenia [42, 46, 47], and increases in concentration of acute-phase proteins [48, 49]. Pathological findings include lymphocyte and mononuclear cell apoptosis [8, 50–53]; hemorrhage in lymph nodes, spleen, kidneys, heart, and respiratory and gastrointestinal tracts; congested serosae; and severe interlobular lung edema (Figs. 12.2, 12.3, and 12.4) [12, 24, 54–60]. The extensive necrosis observed in affected tissues and the severe

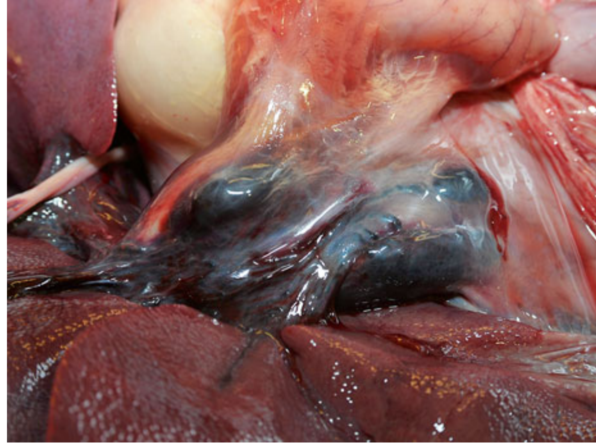

Fig. 12.3 Lymph node, gastrohepatic: Marked enlargement with diffuse hemorrhage. *Source*: Plum Island Animal Disease Center

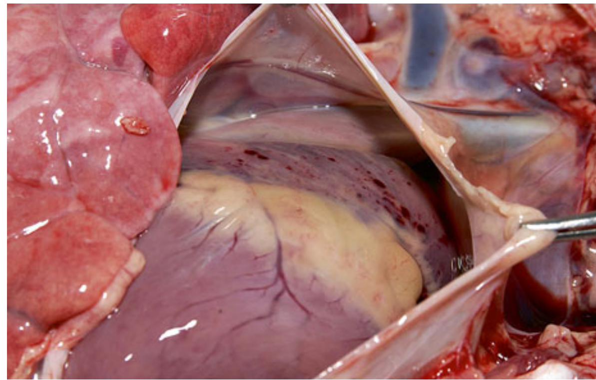

Fig. 12.4 Heart: Moderate, multifocal, ecchymotic epicardial hemorrhages. *Source*: Plum Island Animal Disease Center

hemostatic and hemodynamic changes caused by the infection are likely the leading causes of death.

The duration of subacute ASF usually is 3–4 weeks. The most prominent clinical signs include remittent fever, loss of body condition, pneumonia, dyspnea, cardiac insufficiency, and swelling of the joints. Hemorrhages in lymph nodes and other tissues may be observed at necropsy but are not as prominent as those present in acute ASF [31].

1.2.3 Morbidity and/or Case-Fatality Rates and Prognostic Factors

Acute, subacute, and persistent forms of ASF can occur. Morbidity rates in outbreaks can approach 100 % in previously unexposed herds, and case fatality rates range from 0 to 100 % depending on the virus isolate, immune status of the herd, and other uncharacterized factors.

1.3 Diagnosis and Control

1.3.1 Diagnosis and Epidemiology

Phylogenetic typing of ASFV strains based on the *(p72)* (*B646L*), *(p54)* (*E183L*), and *B602L* genes segregates temporally and geographically distinct ASFV isolates into 22 genotypes [61, 62]. Additionally, genomic heterogeneity has been reported among African isolates of ASFV associated with disease outbreaks in swine relative to isolates from ticks [63, 64]. Phylogenetic studies revealed relative strain homogeneity among isolates from all sources in Western Africa, Europe, and America, but relative strain heterogeneity among isolates from Southern and Eastern Africa [61, 65, 66]. A correlation between ASFV virulence, induction of heterotypic protection from infection with different ASFV strains, and ASFV genotype(s) has not been established. Outside the African continent, only isolates belonging to the West African p72 genotype I were usually detected until June 2007 when an ASF outbreak was reported in the Republic of Georgia, in the Caucasus region, presumably originating from feeding pigs with ASFV-contaminated pork [67]. The Georgia 2007 isolate is related to p72 genotype II that is usually circulating in southeastern Africa. This outbreak confirmed that the threat of ASF spreading to countries outside the African continent is high, and such a spread could be potentially devastating for the global pig industry.

A variety of techniques are available for detecting ASFV virions, nucleic acid, or antibodies to ASFV. ASFV can be readily detected in samples from several tissues, including lymph nodes, kidney, spleen, lung, blood, or serum by means of virus isolation, hemadsorption, viral antigen detection (direct immunofluorescence, DIF), or viral DNA detection (PCR). Serum is the preferred sample for antibody detection. Tissue exudates can also be used for viral detection by PCR and for antibody detection.

The persistence of anti-ASFV IgG antibodies for long periods of time in infected pigs provides the primary strategy for detection of subacute and/or chronic forms of the disease, which is essential for ASF eradication programs. Several techniques have been adapted to ASF antibody detection, but those most commonly used are enzyme-linked immunosorbent assay (ELISA), followed by confirmatory tests such as immunoblotting, indirect immunofluorescence, and the immunoperoxidase test. The most commonly used techniques for virus detection and identification are the hemadsorption test, direct immunofluorescence test, and molecular detection by PCR [68, 69].

1.3.2 Vaccination, Control, and Eradication

Currently, no vaccine is available against ASF. Therefore, detection with elimination of infected pigs and preemptive slaughter of animals in contact with infected pigs are the only methods available to control/eradicate ASF [13, 15].

To date, experimental inactivated and/or subunit vaccines based on crude or semi-purified extracts have been consistently unsuccessful in protecting pigs against ASF [30, 70–72]. However, pigs administered live-attenuated vaccines (LAV) such as low-virulence ASFV isolates, or tissue culture-adapted or genetically modified viruses can develop protective immunity to challenge with homotypic viruses [13, 33, 34, 36, 73]. Protective immunity induced through survival of disease or by vaccination rarely induces long-term resistance to heterotypic virus infection [74, 75]. Both antibodies and cellular immunity are thought to be important in the development of a protective immune response against ASFV. Passive protection can be achieved by transferring ASFV-specific antibodies into animals [74–76]. While the overall importance of ASFV-neutralizing antibodies in protection is controversial [77, 78], there is evidence that $CD8^{+}$ lymphocytes are important in the protective response to ASFV infection [79].

The ASFV genome encodes for proteins that both affect the degree of cytopathology and modulate the host immune response. Changes in the secretion of chemokines play a role in pathogenesis by inducing altered states of coagulability and changes in vascular permeability [80–82]. ASFV infection of pig macrophages interferes with the innate immune response by affecting expression of interleukin-8 (IL-8), interferon-α, and tumor necrosis factor-α [83]. These effects have been confirmed in studies using ASFV deletion mutants. Deletions in the MGF 360 or MGF 530 genes render viruses attenuated through stimulation rather than downregulation of interferon-α expression [84, 85]. The ASFV hemagglutinin (8DR), an analog of cellular surface T-lymphocyte antigen CD2, inhibits peripheral blood mononuclear cell response to mitogens [86, 87]. pA238L, a cellular Iκ-B analog [83, 88], affects NF-κB function leading to changes in the expression of several host antiviral mechanisms [89].

ASF control programs are based on early detection of infected animals and depopulation of susceptible animals in the affected area. Depopulation is the primary countermeasure aimed at limiting viral "shed and spread" from infected animals. The speed with which infected herds and contacts are slaughtered, including proper disposal of carcasses and disinfection of premises, may have an effect on disease spread, duration of the outbreak, and overall effectiveness of the control measures [90]. At a minimum, control measures should include elimination of infected herds, active surveillance of herds within an established control zone, and restriction of animal and human movement within those zones. Eventually, depopulation of contact herds and/or neighboring herds might be necessary. Depopulation as a control measure will be effective in countries or geographic areas where pigs are housed in well-defined premises or pig farms. In areas where domestic pigs are kept in free-range systems or engage in scavenging, control through depopulation will be more difficult to achieve.

1.3.3 Public Health/One Health Crossover

ASFV is not a zoonotic threat.

2 Malignant Catarrhal Fever-Causing Viruses

2.1 Etiologic Agents and Natural History

2.1.1 Definition

Malignant catarrhal fever (MCF) is an acute systemic viral disease caused by members of the so-called MCF virus (MCFV) group [91]. MCF is primarily a disease of ungulates of the order Artiodactyla, primarily those of the families *Bovidae*, *Cervidae*, and *Giraffidae* [91, 92]. MCF occurs worldwide wherever reservoirs are sympatric with clinically susceptible animals.

2.1.2 Etiology and Evolution

At least 10 members of the MCFV group have been identified, all of which are closely related gammaherpesviruses assigned to the genus *Macavirus* [91, 93]. The viruses coevolved with and are well adapted to their respective natural (reservoir) hosts. Adaptation of the viruses to clinically susceptible hosts varies significantly, likely due to the duration of this coevolution. The adaptive process in non-reservoir hosts is generally expected to decrease the virulence of the disease, similar to the adaptation to coexistence of the virus with its reservoir host. Of the 10 MCFV members, six are pathogenic in susceptible, non-reservoir hosts under natural conditions (Table 12.1) [94]. Additional members are likely to be recognized in coming years as ungulates are examined for MCFV-type viruses. The range of known susceptible animals is likely to expand given the increasing availability of specific laboratory tests to identify MCFVs. Membership in the MCFV group is defined by the presence of the 15A antigenic epitope and appropriate similarity in conserved regions of the DNA-dependent DNA polymerase gene [91]. The recent rapid growth of membership in the MCFV group notwithstanding, most natural outbreaks of MCF are still due to the two viruses originally incriminated in early outbreaks: alcelaphine herpesvirus 1 (AlHV-1) and ovine herpesvirus 2 (OvHV-2) [92, 95]. AlHV-1 is responsible for wildebeest (*Connochaetes*)-associated ("African") MCF [93, 96] and OvHV-2 causes sheep (*Ovis aries*)-associated MCF [91, 92].

2.1.3 Geographic Distribution and Economic Effects

Wildebeest-associated MCF distribution corresponds to the distribution of free-ranging black (*C. gnou*) and blue (*C. taurinus*) wildebeest in Eastern and Southern Africa (see Fig. 12.2 of [93]). Sheep-associated MCF occurs worldwide wherever sheep are raised alongside clinically susceptible end-stage hosts. Relative economic effects of the disease are listed in Table 12.1. Morbidity as high as 50 % in herds of American bison and cervids can be economically catastrophic to producers.

Table 12.1 Members of malignant catarrhal fever virus group

Virus (abbreviation)	Reservoir host	Clinically susceptible hosts[a]	Virus isolated	Economic importance
Alcelaphinae/Hippotraginae subgroup				
Alcelaphine herpesvirus 1 (AlHV-1)	Black and blue wildebeest (*Connochaetes taurinus* and *C. gnou*, respectively)	Domestic cattle (*Bos taurus*)	Yes	Moderate
Alcelaphine herpesvirus 2 (AlHV-2[b])	Hartebeest (*Alcelaphus buselaphus*)	Barbary red deer (*Cervus elaphus barbarus*)[c]	Yes	Minimal
	Common tsessebe (*Damaliscus lunatus*)	American bison (*Bison bison*)	NA	NA
	NA	Domestic cattle (*Bos taurus*)	NA	NA
Hippotragine herpesvirus 2 (HipHV-1)	Roan antelope (*Hippotragus equinus*)	NA	Yes	Minimal
	Scimitar-horned oryx (*Oryx dammah*)	NA	NA	NA
Gemsbok-MCFV	Gemsbok (*Oryx gazella*)	NA	No	None
Caprinae subgroup				
Ovine herpesvirus 2 (OvHV-2)	Sheep (*Ovis aries*)	Domestic cattle (*Bos taurus*)	No	Moderate
	Wild sheep including mouflon (*Ovis aries* orientalis group)	Most cervids	No	NA
	Bighorn sheep (*Ovis canadensis*)	Eurasian elk (*Alces alces*)	No	NA
	NA	American bison (*Bison bison*)	No	Potentially severe
	NA	European bison (*Bison bonasus*)	No	NA
		Red deer (*Cervus elaphus*)	No	Important
	NA	Domestic pigs (*Sus scrofa domesticus*)	No	NA
	NA	Water buffalo (*Bubalus bubalis*)	No	Possible
	NA	Banteng (*Bos javanicus*)	No	Important
Caprine herpesvirus 2 (CpHV-2)	Domestic goats (*Capra hircus*)	Domestic pig (*Sus scrofa domesticus*)	No	Minimal
	NA	Sika (*Cervus nippon*)	NA	NA
	NA	European roe (*Capreolus capreolus*)	NA	NA

(continued)

Table 12.1 (continued)

Virus (abbreviation)	Reservoir host	Clinically susceptible hosts[a]	Virus isolated	Economic importance
	NA	Eurasian elk (*Alces alces*)	NA	NA
	NA	White-tailed deer (*Odocoileus virginianus*)	NA	NA
	NA	Water buffalo (*Bubalus bubalis*)	NA	NA
	NA	Pronghorn (*Antilocapra americana*)	NA	NA
MCFV-WTD[d]	Domestic goats (*Capra hircus*)	White-tailed deer (*Odocoileus virginianus*)	No	Minimal
		South American red brocket (*Mazama americana*)	NA	NA
Ibex-MCFV	Nubian Ibex (*Capra nubiana*)	Bongo (*Tragelaphus eurycerus*)	No	Minimal
	NA	Mountain anoa and anoa (*Bubalus quarlesi* and *Bubalus depressicornis*, respectively)	NA	NA
Muskox-MCFV	Muskox (*Ovibos moschatus*)[e]	NA	No	None
Aoudad-MCFV	Barbary sheep (*Ammotragus lervia*)	NA	No	None

Abbreviations: *NA* not available
[a]Additional information about clinically susceptible animals in [128]
[b]AlHV-2 from hartebeest and tsessebe may be different viruses [93]
[c]It is unclear whether the AlHV-2-like agent causing MCF in Barbary red deer and American bison is identical to original isolates of AlHV-2 from hartebeest and topi [130]; the agent causing MCF in bison was a topi isolate
[d]Proposed name is caprine herpesvirus 3 (CpHV-3) [91]
[e]There is circumstantial evidence that muskox-MCFV may be pathogenic in some muskox

2.1.4 Natural History, Transmission, and Host Range

The range of MCF-susceptible animals is remarkably broad. Each member of the MCFV group asymptomatically infects a particular reservoir host and (for those which are known to be pathogenic) one or more animals that develop clinical disease (Table 12.1). This division is not absolute. Lesions and/or disease can be induced in some reservoirs when the challenge dose is sufficiently high [97]. Nevertheless, as general rule, reservoir hosts are well adapted to their specific

MCFV, have only subclinical infections, and efficiently shed cell-free virus, while MCF-susceptible animals are poorly adapted and shed little or (more commonly) no cell-free virus. This absence of viral shedding is the reason that the latter are considered end-stage hosts [93, 96].

Although the classical forms of MCF due to AlHV-1 and OvHV-2 are essentially identical in clinical and pathological presentation, the natural epidemiology of the two agents is significantly different. AlHV-1 is transmitted to cattle by newborn wildebeest calves during calving season [93, 96]. Most newborn wildebeest calves are infected and continuously shed virus via oculonasal secretions until 3–4 months of age. Those calves are the primary source of AlHV-1 for transmission. Essentially all age classes of wildebeest are infected, but adult wildebeest usually do not shed virus [96]. Sheep-associated MCF can occur any time in clinically susceptible animals are exposed, particularly when sheep are present in large numbers. Unlike wildebeest, and contrary to long-standing assumptions, the majority of lambs are not infected with OvHV-2 under natural flock conditions until after 2 months of age. Lambs aged 6–9 months are the most important source of virus in outbreaks [98, 99]. Adult sheep can shed virus at any time in unpredictable patterns. Nasal shedding is the predominant mode for transmission; each shedding episode from nasal secretions is short lived, typically less than 24 h [91, 99].

2.2 Pathogenesis and Clinical Features

2.2.1 Pathogenesis and Immunology

Researchers generally agree that MCF is a disease of dysregulated cell-mediated immunity [95, 100]. Beyond this point there is little agreement about the precise mechanisms resulting in clinical illness and lesions. A widely accepted hypothesis is that infection results in T-lymphoblast hyperplasia with unregulated cytotoxic activity by natural killer cells [95]. The failure of cyclosporine A to modulate the outcome of infection suggests that the pathogenesis is more complex than simple, unregulated lymphoid hyperplasia [93]. An interesting observation is that lesions of MCF mimic those seen in IL-2-knockout mice that develop inflammatory autoimmune disease, indicating a possible role for IL-2 (or other cytokine modulators) in the pathogenesis for MCFV [101, 102]. Experimental studies using different viruses and hosts have generated conflicting results with regard to viral gene expression. Gene expression is either minimal with a latent profile (e.g., AlHV-1 in cattle or laboratory rabbits) [95, 100, 102] or progresses to the point of lytic viral gene expression (OvHV-2 in rabbits, domestic pigs, bison, or laboratory rabbits) [91, 103].

2.2.2 Incubation Period, Clinical Signs, and Gross Lesions

In general, MCF is characterized by high fever, depression, ulceration of mucosal surfaces, and a progressive clinical course generally culminating in death or requirement for euthanasia [96]. The incubation period following natural exposure

or experimental intranasal challenge in most clinically susceptible hosts is 1–2 months [104]. The incubation period of the disease during outbreaks varies. Two factors affecting the length of the incubation period are host genotype and challenge dose [105, 106]. Intravenous challenge results in a shorter incubation period (2–3 weeks) [93, 96, 104, 107]. Initial signs in symptomatic animals are depression and fever (40–42 °C) followed by mucoid-to-mucopurulent nasal discharge, dyspnea with open-mouthed breathing, pharyngitis, mucosal lesions of the palate and tongue, and progressive bilateral keratitis (Figs. 12.5 and 12.6). Severe progressive bilateral keratitis starting at the limbus is considered a classical feature of the disease [108, 109]. Ulcerative colitis, typhlocolitis, and diarrhea may occur with passage of blood-tinged feces; these signs are particularly common in bison and most cervids (Fig. 12.7). Some animals display neurological signs [108]. Lymphadenopathy is prominent in domestic cattle [93, 96], but not other hosts (most cervids, American bison) [110]. The development of ulcers on the muzzle and in the oral cavity is a helpful clinical feature.

Terminally ill animals experience a fall in body temperature. Gross lesions involve multiple organs and reflect three basic changes: generalized arteritis-phlebitis of medium caliber vessels, lymphoid proliferation with generation of atypical lymphoblastoid cells, and mucosal ulceration in the digestive, urinary, and respiratory tracts [93, 96]. The floridity of these features tends to be species-specific, but the histological presence of this trifecta, regardless of severity, should prompt the diagnostic consideration of MCF. Among other changes, hemorrhagic cystitis is

Fig. 12.5 Bison with acute MCF (experimental disease, as are Figs. 12.6, 12.7, and 12.8): Ocular lesions are characteristic and bilateral. In this animal there is a zone of limbal edema (blue-discolored area) at corneoscleral junction due to keratoconjunctivitis

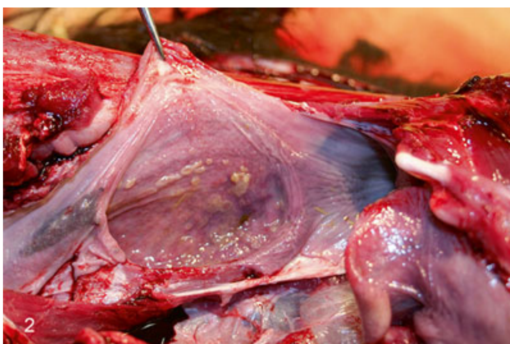

Fig. 12.6 Bison with acute MCF: Acute multifocal pharyngitis. Oral lesions are generally present, particularly in mucosa of pharynx, palate, and tongue

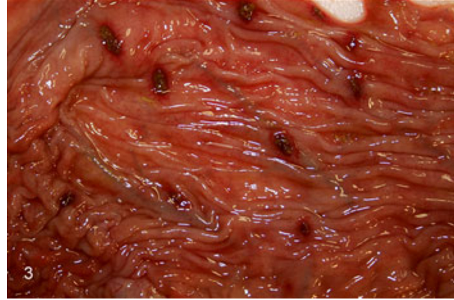

Fig. 12.7 Bison with acute MCF: Acute multifocal ulcerative colitis. Typhlocolitis is characteristic of MCF in some species, including bison and most cervids

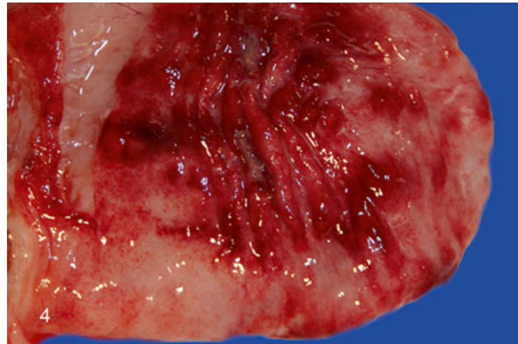

Fig. 12.8 Bison with acute MCF: Acute multifocal hemorrhagic ulcerative cystitis. Few other viral diseases cause this lesion. Its presence should be considered of presumptive evidence that death was due to MCF, with appropriate samples taken to confirm the diagnosis using molecular probes (PCR)

a useful diagnostic feature since it is uncommon to occur in other febrile diseases of ungulates presenting with MCF-like disease (Figs. 12.8) [92]. Most cattle die or are euthanized after a clinical course of 2–7 days [104].

A proportion of acutely affected cattle survive to develop chronic disease or to make an apparent recovery [108, 111]. Characteristic lesions of acute infection persist in such animals, particularly arteritis in medium-caliber arteries. Resolution with scar formation in inflamed vessels leads to a disseminated obliterative arteriopathy. “Recovered” cattle remain latently infected. A poorly understood aspect of MCF is the occurrence of asymptomatic, serologically positive animals with low concentrations of circulating MCFV DNA in blood and no antecedent history of clinical MCF [112]. Clinical signs in ungulates such as bison and deer are often behaviorally masked as an anti-predatory survival instinct (i.e., attempting to appear healthy, so one is not targeted by predators). In such cases, a common history upon presentation is a seemingly rapid course of disease that was actually a masked, normal course of disease. Such animals may one day go off food, stand separate from herdmates, and display dysuria and/or hematuria. The next day, the animal dies [98, 112]. On the other hand, truly atypical forms of MCF can occur in some animals when infected with viral agents other than OvHV-2 and AlHV-1 [94, 113, 114] (Fig. 12.9).

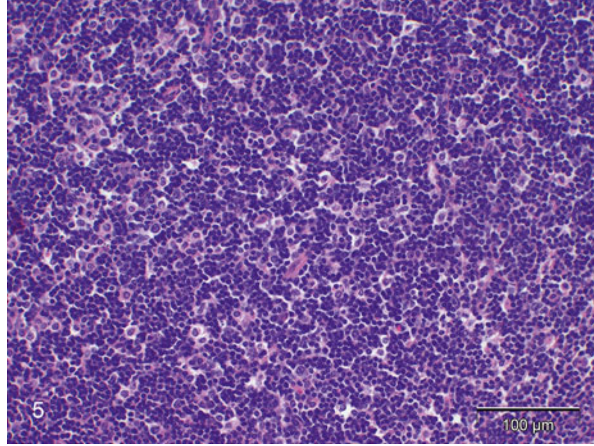

Fig. 12.9 Ox with acute MCF (natural disease, as are Figs. 12.10 and 12.11): Lymph node. Moderate diffuse lymphoid hyperplasia in paracortical zone. Lymphoid hyperplasia is prominent in affected cattle. Hematoxylin and eosin (HE)

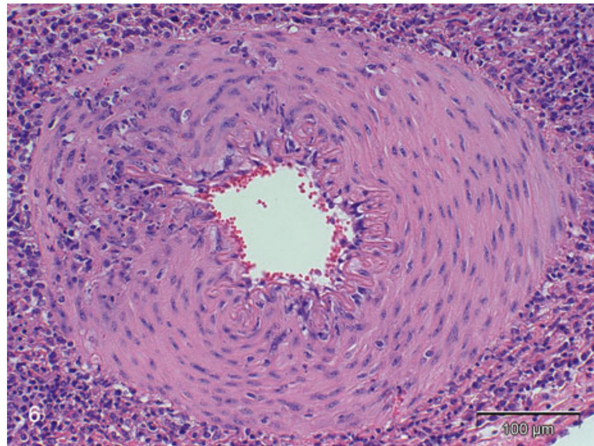

Fig. 12.10 Ox with acute MCF: Kidney. Moderate necrotizing arteritis with periarteritis. Arteritis is one hallmark of MCF, affecting multiple organs (HE)

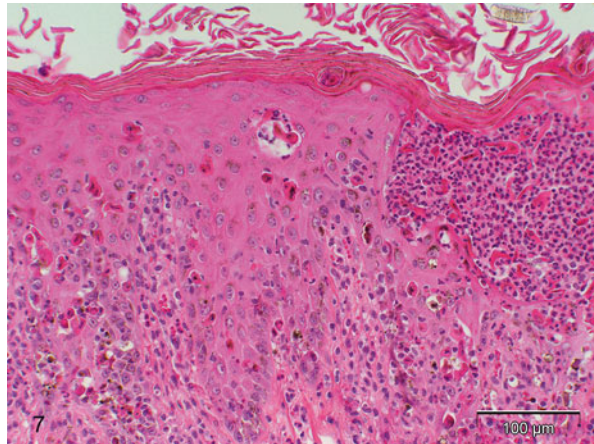

Fig. 12.11 Ox with acute MCF: Skin. Severe acute dermatitis with scattered apoptotic keratinocytes throughout epidermis. Similar epithelial lesions in digestive, respiratory, and urogenital tracts result in widespread erosions and ulcers (HE)

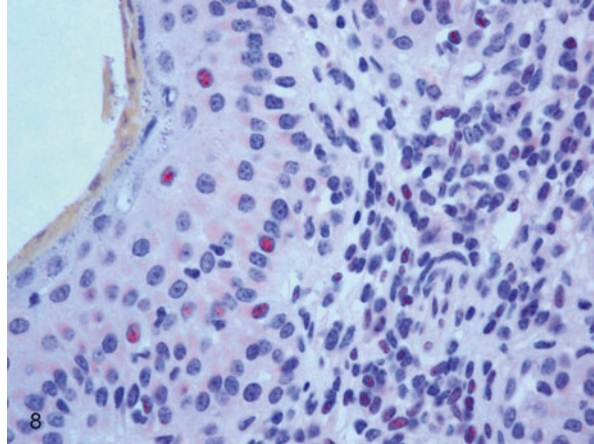

Fig. 12.12 Bison with acute MCF (experimental disease): Rumen. Expression of caspase 3 in epithelium and in scattered lymphoid cells in lamina propria. Apoptosis is one basis for ulcerative lesions in epithelium in affected animals. Red stain = immunohistochemical demonstration of caspase 3

2.2.3 Morbidity and/or Case-Fatality Rates and Prognostic Factors

Morbidity in cattle herds is typically low (<5 %) [93, 96]. Higher morbidity occasionally occurs in cattle herds, but is atypical and for that reason tends to be overrepresented in the veterinary literature. By contrast, morbidity in herds of American bison and most cervids is generally around 50 % or more [98, 110, 112, 115–117]. Case-fatality rates are high for all affected animals. Those exhibiting clinical signs are likely to die or require euthanasia. Researchers used to assume that death was invariant in domestic cattle showing clinical signs. Recent investigations of natural outbreaks show that a proportion of affected cattle can recover (some completely) and remain persistently infected. Recovery is rare in bison and cervids, and an attempt to experimentally define a low challenge dose that establishes infection in the absence of disease was unsuccessful [105]. Major histocompatibility complex (MHC) class IIa polymorphisms appear to determine susceptibility to fatal clinical disease in American bison [106]. Extending this observation to domestic cattle would be interesting, as they are relatively resistant to infection with OvHV-2 [118] (Fig. 12.12).

2.3 Diagnosis and Control

2.3.1 Diagnosis and Epidemiology

The most common clinical form of MCF in domestic cattle is "head and eye," a clinically distinctive presentation. Once owners or veterinarians have seen a confirmed case they are unlikely to confuse this form of MCF with other diseases presenting with high fever, depression, and oculonasal signs. Differential diagnoses for

cattle in temperate climates are bovine viral diarrhea-mucosal disease, infectious bovine rhinotracheitis ("red nose"), epizootic hemorrhagic disease, bluetongue, and foot-and-mouth disease. By contrast, in bison and most cervids, the clinical onset is acute, signs are masked, and animals are typically found dead or dying. Differential diagnostic considerations are similar to those in cattle and include any acutely fatal disease, including anthrax.

The development of molecular diagnostic assays has improved the detection and differentiation of MCF-causing viruses and increased the accuracy of laboratory assays to confirm MCFV infection and/or disease in animals of various species [119–122]. Since a significant percentage of clinically susceptible animals of different species can be subclinically infected with MCFV, the presence of circulating antibody supports a diagnosis of disease only when antibodies are associated with clinical and/or histopathological evidence suggestive of MCF. As few other viral diseases include hemorrhagic cystitis, detection of this lesion should be considered presumptive evidence that death was due to MCF. Appropriate samples should be taken to confirm the diagnosis using molecular probes (PCR). When using PCR to confirm a clinical diagnosis of MCF, especially in mixed species operations such as zoological collections or game farms, it is helpful to use multiplex PCR or several PCRs specific for multiple MCF-causing viruses. The development of improved assays has been a major factor in increasing our understanding of the biology of MCFV infection, and in recognizing additional susceptible hosts.

Serological assays are valuable for epidemiological studies, especially of asymptomatic animals in the field; a positive result is indicative of infection. Large outbreaks of MCF often have a long, tapering epidemiological tail after point-source exposure [98, 123] due to the variation in incubation period.

2.3.2 Vaccination, Control, and Eradication

No commercially available vaccines exist for wildebeest-associated or sheep-associated MCF [93, 96], and earlier attempts (1960–1970s) to develop vaccines were unsuccessful. Recently, interest in vaccine development for both diseases has been renewed due to their potential value to bison producers in North America and cattle farmers in Africa [124–126]. Control efforts (at present) are based on spatial separation of clinically susceptible animals from reservoirs. Appropriate safe distance(s) varies (vary) depending on many factors influencing transmission, such as number of reservoir animals, age, and climate [98, 115, 123]. Given the high proportion of infected domestic sheep and wildebeest, eradication is not practical at this time. However, OvHV-2-free flocks of sheep have been generated [127, 128].

2.3.3 Public Health/Animal Health Crossover

No members of the MCFV group are known to be zoonotic.

3 Other DNA Viruses Causing Viral Hemorrhagic Fever-Like Diseases

3.1 Lumpy Skin Disease Virus

Lumpy skin disease is a skin disease of cattle that exhibits vascular disturbance as focal necrosis of the typical "inverted conical" lesion. The disease is caused by a capripoxvirus, lumpy skin disease virus (LSDV), and is characterized by high morbidity but a low case-fatality rate. The virus can persist in necrotic skin for approximately 1 month, and is viable in hides for half that time at room temperature. Arthropods can transmit the virus mechanically, and are considered the main route of transmission.

LSDV can cause nodules anywhere on the body that involve all the layers of the skin, the subcutis, and occasionally underlying muscle and/or draining lymph nodes. In severe disease, the presentation of nodules is a continuum of disease from minor lesions to congested, hemorrhagic, edematous nodules that may exhibit vasculitis that leads to necrosis. Nodules are called "sitfast" after the scab forms. If the disease becomes generalized, widespread edema can occur. In mucous membranes in the oronasal area, lesions can coalesce, leading to hemorrhage. While severe LSD cases could lead to overt blood loss, these large lesions are more likely to "weep" as no overt hemostatic deficiency is present [129].

3.2 Sheeppox and Goatpox Viruses

Sheeppox and goatpox are acute-to-chronic, subclinical-to-clinical diseases of domestic and wild sheep and goats. Sheeppox and goatpox are caused by two distinct capripoxviruses closely related to LSDV, sheeppox virus and goatpox virus, and the two viruses cannot be distinguished serologically. Strains of the two viruses vary widely in that some cause disease in both sheep and goats, and some only cause disease in sheep but not in goats or vice versa. The viruses replicate in cattle but do not cause disease. Both viruses can be transmitted by many routes, including mechanical transmission by arthropod vectors, but direct contact is the main means of transmission.

While the incubation period of sheeppox/goatpox ranges between 4 and 8 days, recovery may take up to 90 days for full resolution of lesions. Within a few days of the prodrome, sheeppox and goatpox lesions will develop. As a general rule, more severe skin lesions are correlated with increasingly severe disease. Adult sheep and goats with underlying deficiencies or lambs and kids under 1 month of age may suffer from a very severe generalized form of disease. Vasculitis with congestion, hemorrhage, edema, and necrosis will develop in all layers of the skin and in severe cases the muscle layer. Lymph nodes will show similar gross pathological findings, including hemorrhage [129].

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Chapter 13
Viral Hemorrhagic Fevers of Animals Caused by Double-Stranded RNA Viruses

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Core Message Here we outline serious diseases of both wildlife and food and fiber animals that cause damaging economic effects on producers all over the world. All of these diseases are vector-borne (i.e., transmitted by arthropods). Their epidemiology is complex in that outbreaks are often linked to importation of animals and to particular geographic strains of animals (e.g., "North American" sheep vs. "European" sheep) that differ in their susceptibility to infection and/or disease. The genetic and ecological determinants of the described diseases remain to be fully explained.

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1 African Horse Sickness Virus

1.1 Etiologic Agent and Natural History

1.1.1 Definition

African horse sickness (AHS) is a noncontagious arthropod-borne viral disease of equids, European horses, mules and donkeys (in order of decreasing severity of disease), which can have a case-fatality rate as high as 95 %. The principal vector for the virus causing AHS, African horse sickness virus (AHSV), are biting midges in the genus *Culicoides* that can be found between western sub-Saharan and Eastern Africa.

1.1.2 Etiology and Evolution

AHSV is a member of the genus *Orbivirus*, family *Reoviridae*. The virus has a double-stranded RNA genome consisting of ten segments surrounded by a double capsid composed of four viral proteins [1], and produces virions that lack envelopes. There are nine serotypes of AHSV.

1.1.3 Geographic Distribution and Economic Effects

AHS was first recognized in what is now South Africa in 1791 where it caused high fatality in imported European equids, including horses, mules, and donkeys. Today, AHSV is endemic in the central and southern tropical regions of Africa, with AHS occurrence and prevalence influenced by climate and conditions affecting populations of the biological vectors *Culicoides imicola* and *Culicoides bolitinos* [2]. Incursions have occurred into Northern Africa and countries of the Iberian Peninsula, where the disease was imported through an infected zebra [3, 4]. Culicoid vectors that are competent to harbor and transmit AHSV have been found throughout other parts of the world, including the USA and Canada, in the absence of recognized AHSV transmission. Other insects, such as mosquitoes, have been found to be infected with AHSV but are not known to be important in the natural virus transmission cycle [5]. Economic impacts of AHS result from direct loss from death, debilitating disease of animals, and movement restrictions on live animals and animal products. AHS is reportable to the World Organisation for Animal Health (OIE), and can result in serious economic consequences inside established containment zones, which may extend across international boundaries. In endemic countries, the added expense of vaccination programs to control disease further impacts producers and equine hobbyists [6, 7].

1.1.4 Natural History, Transmission, and Host Range

AHSV infects equids, causing disease of increasing severity from zebra's to donkeys to horses [8, 9]. Zebras are thought to be a reservoir host and are important in the epidemiology of the disease in Africa. Antibodies to AHSV have been found in a variety of non-equid Africa-specific mammals, including elephants [10], but these animals do not play a significant role in the epidemiology of AHS. Mortality in dogs has been associated with ingestion of AHSV-infected meat. AHSV antibodies in wild carnivores are thought to be induced through exposure to meat of infected prey [11].

1.2 Pathogenesis and Clinical Features

1.2.1 Pathogenesis and Immunology

AHSV infects endothelial cells of the microvasculature, resulting in loss of cellular junctional integrity and formation of microthrombi, factors thought to be the source of severe pulmonary edema, pleural and pericardial effusions, and/or edema of subcutaneous tissues [12]. Like bluetongue virus, pro-inflammatory and vasoactive mediators may also play a role [22]. Animals surviving infection form neutralizing antibodies that protect against reinfection with the same serotype, and limited cross-protection against different serotypes exists in vaccinated horses [13].

1.2.2 Incubation Period, Clinical Signs, and Gross Lesions

Four clinical forms of AHS occur: pulmonary, cardiac, mixed (cardiac and pulmonary manifestations), and febrile. The pulmonary form has an incubation period of 3–5 days and is characterized by rapid onset fever, severe respiratory distress, and death in nearly 100 % of cases [14]. Upon death, copious frothy fluid exudes from the nose. Gross lesions include dramatic pulmonary edema and occasionally pleural effusion. The subacute, cardiac form is characterized by fever and edema, and results in death in 50–70 % of the cases 4–8 days following onset of disease. Edema may be found in the conjunctivae, supraorbital fossa, and subcutaneous tissues of the intermandibular space and anterio-dorsal regions. Gross lesions include hydropericardium, petechial and ecchymotic hemorrhages of the epicardium and endocardium, and pronounced edema in subcutaneous, facial, and intermuscular regions in the head and neck [15]. Myocardial necrosis develops occasionally [16]. The mixed form of disease has characteristics of both the cardiac and pulmonary disease. A mild or subclinical form of disease can also occur with a variable incubation period from 4 to 14 days, and a low grade fever that may be accompanied by congestion of the mucous membranes, anorexia, and depression. No gross or microscopic lesions are usually found with this form of AHS.

1.2.3 Morbidity and/or Case-Fatality Rates and Prognostic Factors

Case-fatality rates of 0–90 % have been observed in horses. This broad spectrum is primarily due to variations in viral virulence determinants, but host susceptibility factors also contribute [14, 17]. Zebras and African Wild Donkeys may be infected but are refractory to disease.

1.3 Diagnosis and Control

1.3.1 Diagnosis and Epidemiology

Diagnosis of AHS is based on clinical signs and laboratory testing. Tests available for virus detection include sensitive and specific RT-PCR, ELISA, and virus isolation. AHSV serotypes can be determined using serum neutralization assays and type-specific RT-PCR. Serologic testing for antibodies includes ELISA, serum neutralization, and hemagglutination inhibition tests.

1.3.2 Vaccination, Control, and Eradication

One of the best methods to control AHS is prevention of vector contact with susceptible animals, including housing equids in insect-proof barns. Vaccines are commercially available and widely used in high-risk areas. Modified live virus vaccines and killed virus vaccines are in use, and subunit vaccines are currently in development [18, 19].

1.3.3 Public Health/One Health Crossover

Under natural conditions, AHSV has not been found to cause disease in humans.

2 Bluetongue Virus

2.1 Etiologic Agent and Natural History

2.1.1 Definition

Bluetongue virus (BTV) causes hemorrhagic disease in domestic and wild ruminants, and is transmitted by biting midges of the genus *Culicoides*. Infections are associated with high case-fatality rates in infected European breeds of sheep and wild cervids, especially white-tailed deer. Recently emerged BTV strains have dramatically increased the global distribution of infections and display an altered host range and virulence.

2.1.2 Etiology and Evolution

BTV is a member of the genus *Orbivirus*, family *Reoviridae*, and therefore has the same physical characteristics as AHSV. The wide genetic and phenotypic diversity of BTV is exemplified by 24 established serotypes. Novel viral strains emerge through genetic variation that arises from a combination of genetic drift and genetic shift caused by genome segment reassortment between members of a serogroup [20–24]. These processes generate subpopulations that can be selected for optimal fitness. However, the need for the virus to remain fit in both the insect and animal host restricts the mutation rate. Random fixation of specific genetic variants through genetic bottlenecking occurs as the culicoid vectors ingest small numbers of virus particles in a blood meal [22].

2.1.3 Geographic Distribution and Economic Effects

The distribution of BTV is limited to climates suitable for its culicoid vectors. Historically, BTV has a worldwide distribution between latitudes 35°S and 40–50°N. However, in the past decade, virus distribution has extended into Northern Europe [1]. The extension is due to northern expansion of competent vectors into areas with warmer climate and to transmission of novel strains by new vectors resident in colder regions. Bluetongue can be severe in European breeds of sheep and livestock, resulting in a high case-fatality rate and significant economic losses to the producers. As bluetongue is a "List A" disease according to the World Organisation for Animal Health (OIE), the trade barriers imposed on the export of live animals and semen have severe economic impact [25–27].

2.1.4 Natural History, Transmission, and Host Range

Bluetongue was first recognized in Africa after importation of European breeds of sheep [28]. Severity of disease varies with the susceptibility of the host and strain of the virus [69, 21], and large outbreaks may occur in naïve populations [25]. The virus is transmitted by *Culicoides* of diverse species [29, 30]. Domestic and wild ruminants are susceptible to infection [31]. Dogs and wild canids may be infected with BTV through eating meat of infected animals, but these animals are not thought to be part of the natural transmission cycle.

2.2 Pathogenesis and Clinical Features

2.2.1 Pathogenesis and Immunology

BTV preferentially infects microvascular endothelial cells, but can also infect mononuclear phagocytes and dendritic cells [32, 33]. Direct vascular damage, as well as cytokine response(s), result in increased vascular permeability, thrombosis,

tissue infarction, necrosis, and hemorrhage [34]. Surviving animals develop neutralizing antibodies [35] and lifelong immunity against reinfection with viruses of the same serotype but have little or no cross-protection against other serotypes. As BTV closely associates with the surface of red blood cells during late infection, long-term viremia can occur even in the presence of neutralizing antibodies [36].

2.2.2 Incubation Period, Clinical Signs, and Gross Lesions

The incubation period ranges from 5 to 10 days. Clinical disease is typically most severe in naïve sheep of European breeds and mild or asymptomatic in cattle and goats [31]. Severe disease and high lethality is also possible in some wild ruminants, including North American cervids and pronghorns [37, 38]. Clinical signs can vary from mild to severe, and include fever, bloody oral/nasal discharge, pulmonary and facial edema, oral erosions and ulcers, hyperemia of the coronary band, and lameness. Lesions include ulcerations; hemorrhage; and necrosis of the mucosal lining of the upper gastrointestinal tract, and edema and hemorrhage of subcutaneous tissues, lymph nodes, and fascial planes of muscles; pleural and pericardial effusions; and muscle necrosis [31]. Cattle may develop oral erosions and ulcers, conjunctivitis, and subcutaneous edema. New BTV strains with increased virulence in cattle have recently emerged, such as BTV-8 in Europe [39]. This new virus and some attenuated vaccine strains can also cross the placenta causing abortions, still births, or malformed offspring with severe central nervous system deformities [40, 41].

2.2.3 Morbidity and/or Case-Fatality Rates and Prognostic Factors

In animals developing clinical disease, case fatality rate may range from 2 to 30 % and may be as high as 70 % in some outbreaks [31, 38].

2.3 Diagnosis and Control

2.3.1 Diagnosis and Epidemiology

Clinical signs caused by BTV can mimic other diseases, so laboratory confirmation is required for diagnosis. Assays for viral detection include virus isolation in cell culture or in embryonated chicken eggs, RT-PCR, and antigen detection with ELISA. Serologic tests, such as agar gel immunodiffusion (AGID), may cross-react with related orbiviruses, such as epizootic hemorrhagic disease virus. ELISA has improved sensitivity and specificity for antibody detection.

In tropical regions, BTV circulates between vectors and animal hosts through all or most of the year. In temperate climates, infections are distinctly seasonal with prolonged periods without any observed transmission. Adult vectors maintain

bluetongue infection for life, but this population does not survive through most winters. Researchers are not clear how the virus "overwinters" (i.e., is maintained in these regions). A small adult population can possibly survive in protected hibernacula, perhaps even inside of buildings [42]. Transovarial transmission in culicoids was suggested after detection of viral RNA in pools of larvae from field collection sites. However, BTV was not isolated from these samples, and the epidemiological relevance of this finding remains to be determined [43]. The possibility of a reservoir animal host has been investigated; however, infection in ruminants (particularly cattle) has been found to be prolonged but not persistent [44].

2.3.2 Vaccination, Control and Eradication

Modified live and killed virus vaccines are used widely in areas with high risk for infection or regions with recent BTV incursions [45]. Of concern is the potential for attenuated live virus vaccine strains to cause abortions and fetal defects [46] and to reassort with endemic viruses resulting in new strains with altered biological properties [47]. New recombinant subunit vaccines are in development [48].

2.3.3 Public Health/One Health Crossover

Bluetongue is not a zoonotic disease.

3 Epizootic Hemorrhagic Disease Virus

3.1 Etiologic Agent and Natural History

3.1.1 Definition

Epizootic hemorrhagic disease virus (EHDV) is an arthropod-transmitted virus that can infect many wild and domestic ruminants. In wild ungulates, infection can result in severe disease with high lethality, particularly white-tailed deer (*Odocoileus virginianus*) in Northern America. In domestic ruminants, most infected cattle may have mild or unrecognized disease, but cattle-virulent strains can cause severe disease [49, 50].

3.1.2 Etiology and Evolution

EHDV is a member of the genus *Orbivirus*, family *Reoviridae*, and therefore has the same physical characteristics as AHSV and BTV. EHDV is less studied than BTV and AHSV. Genotyping of EHDV indicates heterogeneity in field samples [51].

Genetic diversity arises through a combination of genetic drift and genetic shift through reassortment of gene segments between related viruses. An example of virus reassortment is a new virus that was recognized in the USA in 2006 with gene segments from an endemic EHDV-2 and an exotic EHDV-6 virus [52]. New strains produced by point mutations or genetic reassortment have the potential for altered virulence, host and vector preference, and environmental distribution.

3.1.3 Geographic Distribution and Economic Effects

EHDV can be found throughout much of the world, including the Americas, Africa, Australia, and Asia between the latitudes 35°S and 49°N. In the USA, EHD was first described in 1955 in white-tailed deer in Virginia [53], but the disease was likely recognized by hunters as early as the 1800s in the southeastern states as "black-tongue." Distribution and seasonality of outbreaks is determined by conditions that support the breeding success of the vector populations. Cattle-virulent strains include an EHDV-2 serotype (Ibaraki virus) found in Japan and Asia [50, 54], EHDV-7 strains that emerged in 2006 in Israel [55], and EHDV-6 (strain 318) in Mediterranean countries (2004 and 2006) [56]. Occasional outbreaks of cattle-virulent disease have been reported in the USA. Disease in cattle can mimic foreign animal vesicular diseases, such and foot-and-mouth disease and vesicular stomatitis. Recent outbreaks of EHD in cattle had major economic impact on the livestock industry in multiple countries bordering the Mediterranean Basin, including Morocco, Turkey, and Israel [25, 56]. These strains and emerging new strains can cause economic losses to beef cattle and dairy industries [49, 50, 57, 58].

3.1.4 Natural History, Transmission, and Host Range

EHDV infects wild and domestic ruminants and wild cervids. Domestic livestock found to be susceptible to infection include cattle and sheep. Like the orbiviruses BTV and AHSV, EHDV is transmitted by arthropod vectors, the biting midges of the genus *Culicoides*. Thus, distribution of the disease is limited to regions with competent vectors. Disease outbreaks in North America occur in the late summer or autumn, coinciding with the highest vector populations, and tend to continue until the first frost reduces or eliminates insect transmission [27].

3.2 Pathogenesis and Clinical Features

3.2.1 Pathogenesis and Immunology

Following infection by the bite of the biological vector, EHDV replicates in endothelial cells of the lymphatic system and local lymph nodes [59] and then disseminates throughout the body by hematogenous spread. Secondary sites of infection include the

spleen and lymph nodes. As EHDV closely associates with the surface of red blood cells during late infection, long-term viremia occurs even in the presence of neutralizing antibodies [60]. In most infected deer, the virus could be detected in the blood for less than 3 weeks, but in a few animals it could be found for up to 56 days postexposure [61, 62]. In cattle, viremia is typically detectable for less than 2 weeks, but in a minority of infected animals viremia is present for up to 50 days postexposure [63, 64]. After infection, serotype-specific antibodies are produced that protect against future infections and may be detected over the entire lifespan of the animal. The number of EHDV serotypes is not clearly defined, but seven to eight serotypes are generally accepted. There is limited cross protection between US serotypes 1 and 2 [65].

3.2.2 Incubation Period, Clinical Signs, and Gross Lesions

The incubation period ranges from 5 to 10 days postexposure. Clinical signs vary depending on the host. Disease is especially severe in wild cervids, with highest lethality in Northern American white-tailed deer. In deer, three disease forms have been described; peracute, acute, and chronic. These forms cannot be clinically distinguished from disease caused by bluetongue virus. Deer suffering from the peracute form die rapidly, typically within 6–36 h of onset of disease. Signs can include high fever, anorexia, respiratory distress, and rapid onset of swelling due to edema of the head and neck. Hemorrhagic diathesis with bloody diarrhea, hematuria, and dehydration are found post mortem. Animals are often found dead with no apparent sign of illness in or near bodies of water such as creeks or ponds (Fig. 13.1).

Fig. 13.1 A white-tailed deer found dead in a creek with no outward signs of disease. This is a typical presentation of peracute death caused by epizootic hemorrhagic disease virus or bluetongue virus

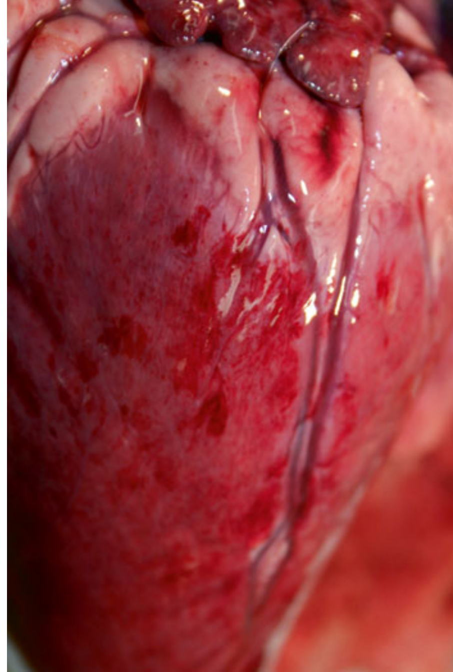

Fig. 13.2 Discrete hemorrhage on the heart of a pronghorn. Widespread hemorrhages are typical of hemorrhagic disease in cervids caused by epizootic hemorrhagic disease virus or bluetongue virus

This phenomenon is thought to be due to animals seeking relief from a high fever in the cool water. The acute form of EHD has the same signs and lesions seen among peracute cases, but the deer (and other close relatives such as pronghorn) typically live longer and develop more extensive hemorrhages in many tissues, including the skin, heart, and the gastrointestinal tract (Fig. 13.2). There may be swelling, hyperemia, and/or ulceration in the oral cavity; excessive salivation; and nasal discharge. Chronic disease is seen in some acutely ill deer that recover. Clinical signs include growth interruption in the hoof wall, lameness, and potential sloughing of the hoof. Gross lesions may include scarring from healed ulcers in the mouth, rumen, and other portions of the gastrointestinal tract. Histologic lesions include widespread vasculitis and thrombosis, endothelial swelling, hemorrhages, degenerative changes, and/or necrosis in many organs.

In endemic areas, clinical disease may be infrequent due to a combination of herd immunity and differences in host genetic susceptibility [66]. Most cattle infections are mild, but disease caused by cattle-virulent strains can cause abortions; signs of fever; swollen conjunctiva and tongue; redness, ulceration, or erosions of the nose and lips; dyspnea; difficulty swallowing; lameness; and decreased milk production [50, 67]. Lesions may include oral ulceration, degeneration of muscles of the esophagus or throat, or edema and hemorrhages in the mouth, lips, and digestive tract [25, 58]. These lesions and signs mimic those caused by foot-and-mouth disease and vesicular stomatitis (Fig. 13.3), possibly triggering a foreign animal disease investigation and quarantine of affected farms.

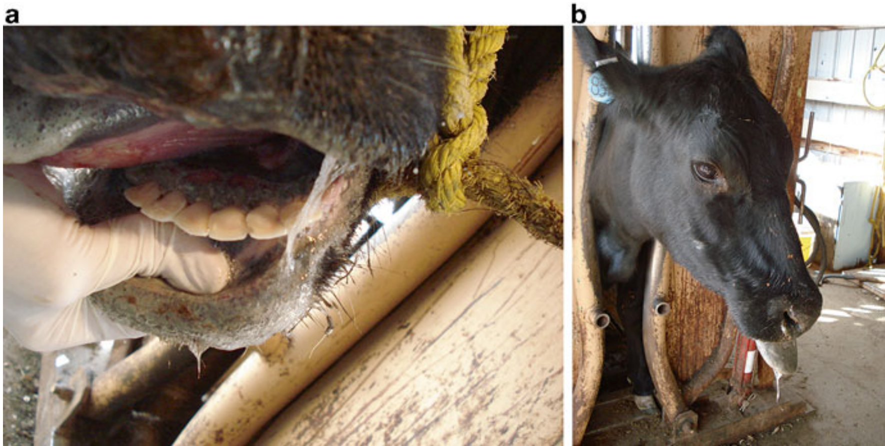

Fig. 13.3 Cow infected with epizootic hemorrhagic disease virus. Hypersalivation, oral ulcers, and swollen tongue mimic foreign animal diseases, such as foot-and-mouth disease and vesicular stomatitis

3.2.3 Morbidity and/or Case-Fatality Rates and Prognostic Factors

Since EHD occurs primarily in wild ruminant populations and since outbreaks occur in epizootic cycles, good estimates of morbidity and lethality are not available. A population survey of one outbreak allowed an estimation of certain epidemiological parameters. A total population infection rate was estimated at 29 % with a mortality of 20 %, leading to a case fatality rate of 67 % [68]. However, anecdotal reports describe die-offs of over 90 % of a local population, such as in Montana in 2012 and 2013 (USA Today News posted 1/8/2012).

3.3 Diagnosis and Control

3.3.1 Diagnosis and Epidemiology

EHD lesions are indistinguishable from BTV infection in wild ruminants, making laboratory testing necessary to establish the diagnosis. Virus isolation from blood cells or tissues [69] or RT-PCR can be used to detect the virus [70–72]. The serotype of virus can be determined using serum neutralization assays or RT-PCR targeting serotype-specific genes [73]. For EHDV antibody testing, AGID assays are available, but they lack sensitivity and may cross-react with BTV antibodies [74]. ELISAs are more sensitive and specific for detection of EHDV than AGID, and do not cross-react with BTV [75, 76].

3.3.2 Vaccination, Control, and Eradication

Modified live virus and killed virus vaccines have been used in Israel and Mediterranean countries. In the USA, cervid grower organizations use killed virus autogenous vaccines. Vector control methods may be attempted in the case of outbreaks involving cattle, but are not practical for wildlife.

3.3.3 Public Health/One Health Crossover

EHD is not a zoonotic disease.

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Chapter 14
Viral Hemorrhagic Fevers of Animals Caused by Positive-Stranded RNA Viruses

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Core Message Here we outline serious diseases of wildlife, food and fiber animals, and non-human primates that cause damaging economic effects on producers all over the world. While some zoonotic viruses that occasionally cause serious disease and death in humans are mentioned, the positive sense RNA viruses generally cause economic damage that can have serious societal implications for humans. Finally, honorable mention is given to yellow fever virus, a success of vaccine development efforts. This virus once caused similar serious effects (in humans) during the construction of the Panama Canal, but has been relegated to a footnote in textbooks because of a cheap and effective vaccine.

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1 Bovine Viral Diarrhea Virus

1.1 Etiologic Agent and Natural History

1.1.1 Definition

Bovine viral diarrhea (BVD) was first described as a distinct acute febrile disease of cattle in 1946 [1]. The presence of blood in diarrheic feces and nasal exudate was noted in these early reports along with a fever (40–42 °C) and leukopenia, followed by abortions and the birth of congenitally deformed and weak, nonviable calves. The disease was associated with erosive, ulcerative lesions of the gastrointestinal, oral, and pharyngeal mucosae, and hemorrhages in lymph nodes, gastrointestinal mucosa, subcutaneous tissues, pericardium, and vaginal mucosa. This early association of BVD with bleeding manifestations was overshadowed by the discovery of fetal infections, and the role of immune tolerance in the generation of persistently infected (PI) animals. Cases of hemorrhagic syndrome (HS) associated with BVD likely occurred in the interim, but were not reported again until 1989 and in 1993–1995 [2, 3]. As a result of these outbreaks of severe hemorrhagic disease with significant lethality, interest in BVD HS was reinvigorated [3, 4]. Sequencing of the viruses isolated from HS cases revealed a distinct genotype of BVD viruses, BVDV-2, associated with this syndrome. HS cases are typically caused by BVDV-2 [5].

1.1.2 Etiology and Evolution

BVDV-1 and BVDV-2 are positive-sense, single-stranded RNA viruses with genomes approximately 12.3 kb in length contained within enveloped icosahedral capsids. BVD viruses are members of the genus *Pestivirus* within the family

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Flaviviridae, but are classified in two distinct species. BVDV-1 and BVDV-2 are further classified into several subtypes and multiple strains thereof [6]. Each pestivirus appears to have evolved with its primary ruminant host, but is capable of infecting additional ungulate species. Both viruses occur as two distinct biotypes, noncytopathic (ncp) and cytopathic (cp). Most isolates are ncp viruses (i.e., they do not cause morphologic changes or cell death in infected cell cultures). In animals that are persistently infected with ncp viruses, various spontaneous mutations render the ncp viruses cytopathic in cell culture. Isolation of pairs of cp and ncp viruses is associated with the development of mucosal disease (MD) in PI animals [6]. The BVDV-2 isolates from HS cases are ncp.

1.1.3 Geographic Distribution and Economic Effects

BVD viruses are distributed in cattle worldwide with the exception of countries from which the viruses have been eradicated [7, 8]. On an individual herd basis, monetary losses range widely according to the specific circumstances. The financial losses due to BVD in an individual dairy herd were calculated to be £2,295 and £4,115 in 1982 [9]. Economic losses specifically due to BVDV HS cases have not been calculated. Estimates for the economic impact of BVD at the national level vary from $20 million per million calvings in Danish cattle to a conservative estimate of greater than $400 million for US cattle based on approximately 34 million calves born in 2012 and 2013 [10, 11].

1.1.4 Natural History, Transmission, and Host Range

The persistence of BVDVs in bovine populations is due to the ncp replication and immunosuppressive qualities of the viruses and their ability to be transmitted from susceptible female hosts to developing fetuses with great efficiency. The consequences of fetal BVDV infection include infertility, abortion, stillbirth, weak calves, congenital defects, and growth retardation in calves [12]. Most importantly, infection of the fetus during the first 125 days of gestation results in the generation of PI calves. The survival of PI animals into the breeding season is the key to the maintenance of virus in cattle populations. PI animals shed large quantities of BVDVs in secretions throughout life, and are the source of the infection for susceptible cattle. In nature, infection of cattle occurs via the inhalation of BVDVs in aerosolized respiratory secretions, or ingestion of urine, feces, or saliva from PI-animals, or from contact with aborted infected fetuses and fetal fluids. Inadvertent infection can occur through the administration of live vaccines, contaminated biological products, or through contaminated insemination equipment or semen. BVDVs infect cattle, other domestic and wild ruminants, camelids, pigs, and wallabies [13]. Rabbits can be infected experimentally, but do not develop disease.

1.2 Pathogenesis and Clinical Features

1.2.1 Pathogenesis and Immunology

In acute BVDV infections, virus is inhaled or ingested and replicates in the lymphoid tissues of the nasopharynx. Virus-infected lymphocytes and macrophages spread the virus to other lymphoid tissues via the blood, including the bone marrow. Specifically regarding the mechanisms of the hemostatic disorders involved in BVD HS, evidence exists for: (1) direct infection and damage to blood vessels by the virus, and (2) virally induced thrombocytopenia with alteration of platelet function. Evidence for the first mechanism is the localization of BVDV-2 antigen in endothelial cells and smooth muscle cells in blood vessels that may be accompanied by necrotizing vasculitis [14, 15]. Infection and damage to the endothelium triggers thrombus formation and consumption of platelets and other clotting factors. Indirectly, cytokines, such as interleukin-1 and tumor necrosis factor α, produced by monocytes during infection [16] alter the expression of endothelial cell surface proteins, favoring development of disseminated intravascular coagulation (DIC) and vascular leakage.

In naturally occurring BVD HS, the number of platelets is severely diminished (2,000–33,000/μl) with little effect on clotting times (PT, aPTT) and fibrinogen plasma concentrations [4]. In experimental studies, platelets decreased 4–8 days after intravenous inoculation, reaching a nadir at 14–16 days post-inoculation. Hemorrhage was noted when platelet numbers were <5,000/μl [17]. Potential mechanisms for BVDV-2-induced thrombocytopenia include: (1) viral infection of megakaryocytes resulting in decreased production and function of platelets, (2) DIC with consumption of platelets and clotting factors, and (3) immune-mediated destruction or sequestration of platelets. The first mechanism is supported by the identification of viral antigen on platelets by immunofluorescent antibody (IFA) and on megakaryocytes by immunohistochemical (IHC) staining [17–20], and by the observation of decreased numbers of megakaryocytes with degenerative, necrotic changes in the bone marrow [4, 20, 21]. Infection and necrosis of megakaryocytes, however, have not been observed in all studies [18]. Megakaryocyte hyperplasia, possibly compensatory due to platelet consumption and without evidence of virus infection, has been reported by other investigators. These contrasting results may reflect various factors, such as a difference between virus strains in their tropism for megakaryocytes [20, 22, 23], the time post-inoculation at which bone marrow was examined, and/or the age and breed of calves examined [22, 24]. In addition to direct effects of BVDVs on megakaryocytes, platelets from infected calves have altered morphology [20] and a decrease in the aggregation response compared to controls [25]. Recreating BVD HS experimentally is difficult, evidenced by the fact that the hemorrhagic lesions are inconsistently produced even with viruses isolated from HS cases [23]. Clearly, additional factors such as nutritional management and environmental conditions may influence the development of hemorrhagic lesions and the outcome of infection in nature.

The impact of BVDVs on the bovine immune system was initially observed by noting the decrease in leukocyte counts and depletion of lymphoid tissues in the first

reports of the disease [26]. Later, the systemic nature of infections and the viral tropism for T- and B-lymphocytes and macrophages was determined [27, 28]. Acutely infected cattle respond with specific T helper cell, CTLs, and B cell responses. However, the detection of BVDV-specific antibodies in serum may be delayed for 24–49 days in acute infections with HS strains [18]. Once the virus is cleared, surviving cattle maintain strain-specific immunity for years.

In general, viruses that infect these key cells of the immune system significantly reduce the host's defense against a variety of infectious organisms. Consequently, multiple concurrent bacterial, parasitic, and/or viral infections occur in BVDV-infected animals that are not responsive to treatment. The isolation and culture of mixed populations of microbes from cases of pneumonia and enteritis in cattle should trigger suspicion of BVDV infection [29].

The consequences of fetal infection depend on the specific pathogenicity to the fetus of the virus strain, and on the gestational and developmental age of the fetus [30]. Pathogenicity in the fetus is not necessarily similar to infection with the same BVDV strain in adult cattle, and can be unrelated to the biotype of the viral strain as well. Early in gestation, the fetus responds to infection with elements of both the innate and adaptive immune responses; however, these immune functions are not sufficient to clear the BVDV infection [31]. Importantly, the lack of viral clearance results in a PI fetus. When the infection occurs after approximately 150 days of gestation, the fetus is able to develop virus-specific antibodies, mount a virus-specific response, and clear the infection. These are present at birth virus-specific antibodies.

1.2.2 Incubation Period, Clinical Signs, and Gross Lesions

The majority of BVD HS cases occur as a result of acute, postnatal infections following an incubation period of 2–12 days, and should be considered when cattle present with a high fever (40–42 °C), depression, and death within 24–48 h after the onset of signs [15, 18, 23]. Affected animals may also exhibit dyspnea and diarrhea with or without blood in the feces. A complete blood count (CBC) may reveal lymphopenia within 3–18 days [18] and thrombocytopenia within 14 days post exposure [17].

At necropsy, petechial and ecchymotic hemorrhages may be noted on oral or vaginal mucosae, in subcutaneous tissues, and on serosal surfaces of multiple organs [4, 32, 33]. The grossly visible hemorrhages of HS may be accompanied by thymic atrophy in young animals; diffusely reddened, fluid filled lungs reflective of an interstitial pneumonia; and diffusely red segments of the small intestines. The wall of the intestine is thickened by edema and the intestinal contents are scant, blood-tinged, and mucoid in consistency. Peyer's patches are translucent reflecting depletion of lymphoid populations accompanied by small hemorrhages. Mesenteric lymph nodes may be enlarged and edematous. None of these lesions are pathognomonic for BVDV infections and may be mistaken for a number of diseases caused by bacterial and toxic agents. The primary alternative (differential) diagnosis of extensive hemorrhagic lesions in aborted fetuses and neonatal calves is dicoumarol toxicosis

following ingestion of moldy sweet clover hay by the dams [34]. In adults and older calves, clostridial diseases, salmonellosis, leptospirosis, anthrax, and other bacterial septicemias should be considered.

In general, the clinical signs of acute BVD are dependent on the strain of virus and the epizootiological status of the herd. Distinguishing between acute, chronic, and fetal infections, based on clinical signs and gross or histopathologic lesions, is often difficult [17, 32]. For each clinical outcome (e.g., abortion), the list of differential diagnoses is lengthy, and lesions (if present) are not pathognomonic. BVD should be suspected when multiple diseases occur within a herd, such as infertility, abortion, or weak calves with diarrhea and pneumonia. Clinical disease that is refractory to treatment should raise suspicion of underlying immunosuppression and, therefore, BVDV infection.

1.2.3 Morbidity and/or Case-Fatality Rates and Prognostic Factors

As BVD is not a reportable disease in the USA, information on prognosis, morbidity, and lethality of BVD cases with HS is scant. A 1977–1987 retrospective case review of 146 cattle with acute BVDV infection at New York State College of Veterinary Medicine revealed that 15 (10 %) had BVD associated with thrombocytopenia and bleeding and that 9 of the 15 died or were euthanized [4]. Another study demonstrated that an ncp-BVDV could induce thrombocytopenia that was severe enough to observe a morbidity rate of 50 % and case-fatality rate of 20 % [18].

1.3 Diagnosis and Control

1.3.1 Diagnosis and Epidemiology

An array of laboratory diagnostic tests for detecting BVDV proteins (IFA, IHC, antigen-capture enzyme-linked immunosorbent assay [ELISA]), viral RNA (reverse transcriptase-polymerase chain reaction [RT-PCR], real-time RT-PCR) or infectious virus (virus isolation) from individual animal serum, whole blood, ear notch samples, and/or pooled milk is available, as well as serologic tests (serum neutralization, ELISA) [35]. The appropriate choice of samples and tests and the selection of animals to be tested are dependent on the peculiarities of each case. Individual case factors that affect the choice of tests and testing strategies include the management system (dairy, feedlot, beef cow-calf) of the herd, the manifestation of disease (enteritis, bovine respiratory disease, abortions), and whether one is attempting to detect BVDVs in PI or acutely infected animals. In HS cases resulting from acute infection with BVDV-2, whole blood samples from multiple live animals during the febrile phase of disease, or lung and lymphoid tissues obtained from necropsy, should be tested for the presence of infectious virus, viral proteins or viral RNA. BVDV may be detected in the buffy coat of whole blood or tissue samples by virus isolation,

RT-PCR, or real-time RT-PCR in as few as one of ten acutely infected cattle in a HS outbreak, whereas antigen may not be detected by ELISA, IFA or IHC staining in this same scenario. Further evidence of infection may be demonstrated by a fourfold or greater increase in BVDV-specific antibody titers (by serum neutralization or ELISA) in surviving animals. However, interpretation of serologic data is frequently complicated by the prior or concurrent use of BVDV-containing vaccines in cattle.

The epidemiology of BVDV centers on the PI animal, which sheds BVDV in oral and respiratory secretions, feces, and placental fluids and milk, all in high concentrations. PI animals are generated through the infection of susceptible pregnant cows during in the first 125 days of gestation. For this to occur, PI cattle must survive into the breeding season of the herd. PI animals may be inadvertently purchased or be introduced into a herd through the purchase of previously infected female cattle that later give birth to PI calves. Cows may also become infected while pregnant when they encounter PI animals from other herds in shared pasture.

In herds in which breeding is synchronous (as in the US beef industry), one PI animal can infect a large number of cows. The results of this first event can be catastrophic, with losses due to abortions, stillbirths, and weak, non-viable calves. The high proportion of BVDV-associated disease is characteristic of the epidemic phase of herd infection. Any PI calves that are born in this event cause additional morbidity and mortality due to acute BVDV-induced diarrhea and pneumonia within their cohort. Infection with BVDV renders the cow herd largely immune to the specific strain of virus. Births of female offspring by immune dams in subsequent years provide a new generation of cows susceptible to infection. This susceptible cohort serves as the continual source of PI calves in the endemic phase of the herd infection as these heifers are bred and become infected with BVDV.

In herds with year round breeding (e.g., dairy herds), cows are in different stages of gestation. The introduction of PI animals into the herd will result in fewer losses due to abortion, but with the occurrence of characteristic and more easily recognized congenital defects. The reproductive losses may be preceded by a spike in cases of pneumonia and diarrhea in adults and young stock 4–7 months before the birth of defective and PI calves. The epidemiology of BVDV is reviewed by Van Campen in [36].

1.3.2 Vaccination, Control, and Eradication

Ultimately, BVD control is achieved by the identification and removal of PI animals, thereby preventing further infection of pregnant cattle and the perpetuation of PIs. Where instituted, BVD control programs have successfully eradicated BVDVs from domestic cattle [7, 8]. In North America, BVDV-containing vaccines are commonly used to prevent BVD [37–39], but control programs based on PI identification and removal are voluntary. However, under field conditions, BVDV vaccines do not provide complete fetal protection in all vaccinated cows; therefore, PI cattle continue to be generated and perpetuate the infection. Despite a large array of BVDV-containing inactivated or modified live vaccines, the viruses continue to infect cattle and cause disease [36].

1.3.3 Public Health/One Health Crossover

Pestiviruses are uniquely adapted to ruminants of various species, and to pigs. Although humans have been exposed to pestiviruses via viral vaccines contaminated with BVDV through fetal bovine serum, there is no evidence that humans can become infected by them [40].

2 Classical Swine Fever Virus

2.1 Etiologic Agent and Natural History

2.1.1 Definition

Classical swine fever, also known as hog cholera, is caused by classical swine fever virus (CSFV), a virus of great economic and health importance to the global swine industry. CSFV, discovered in 1903, is enzootic in much of the world, persists in wild swine populations, but has been eradicated from the USA and several other countries.

2.1.2 Etiology and Evolution

CSFV is a member of the genus *Pestivirus*, family *Flaviviridae* [41] along with BVDV-1, BVDV-2, and border disease virus (BDV) [42].

As is the case for all pestiviruses, the spherical CSFV particle (40–60 nm diameter) is comprised of an icosahedral nucleocapsid enclosed by a lipid membrane that contains three structural glycoproteins [43, 44]. The approximately 12.5 kb CSFV genome is a single-stranded RNA of positive polarity. The genome consists of a single large open reading frame that encodes four structural and eight nonstructural proteins [45]. Structural proteins, encoded in the 5′ third of the genome, include capsid protein C and envelope (E) glycoproteins E^{rns}, E1, E2. Nonstructural (NS) proteins include N^{pro}, p7, NS2, NS3, NS4A-B, NS5A-B [45]. E2 and E^{rns} play important roles in the attachment of the virion to the cell surface [46, 47], and each protein interacts with a different cell receptor involved in virion attachment and cell-to-cell spread [46].

2.1.3 Geographic Distribution and Economic Effects

CSF was first reported in 1833 from Ohio in the USA [48]. By 1889, 36 US states had reported a disease in pigs with clinical presentation similar to CSF. In the same time frame, outbreaks of a disease resembling CSF were affecting pig herds in Great Britain and across continental Europe.

CSF is classified as a reportable disease to the OIE (World Organisation for Animal Health). CSF is a global concern due to the effect of the disease on pig breeding activities in enzootic areas and as a threat to the pork industry and international pork trading in countries free of the disease.

CSF has been eradicated from Australia, Canada, the USA, and almost all member states of the European Union (EU). Routine vaccination of pig herds has been banned in these countries. However, outbreaks of CSF still occur intermittently in European domestic pigs, leading to significant economic losses. In the EU, from 1992 to 2008, close to 20 million pigs were euthanized due to control measures imposed to combat CSF epizootics, causing total costs of about 5 billion euros. In many countries, CSF is still a major problem [49, 50], and vaccination of pig herds is applied to control spread. Currently, CSFV is considered enzootic in domestic herds in Eastern European countries and Russia, Asia (predominantly in Southeast Asia), in some countries of South and Central America, southern Mexico, and in the Caribbean.

2.1.4 Natural History, Transmission, and Host Range

The only known natural hosts for CSFV are all members of the Suidae [51]. While transmission in domestic pigs is relatively well studied, the epidemiological role of European wild boar is the best understood among wild suids. Wild boars are a source of direct or indirect infection for domestic pigs [52–54]. CSFV infections of wild boar populations can be self-limiting or persistent, with virus circulating within infected populations for years [55]. In wild boars, CSF has a similar clinical presentation as in domestic pigs, including transplacental transmission of CSFV followed by the birth of persistently infected piglets [56, 57]. The most frequent cause of spread of CSFV into a population is probably due to swill feeding [58].

Direct transmission (either horizontal or vertical) is the primary mode of CSFV spread. Horizontal transmission occurs via pig-to-pig by direct contact. In both acute and chronic forms of the disease, virus is constantly shed from infected animals via secretions and excretions even before the onset of clinical signs. Additionally, in adult boars, infections with CSFV lead to excretion of the virus in semen [59]. Subsequently CSFV can be transmitted via artificial insemination. Inseminated sows seroconvert, and virus can be detected in both sows and fetuses [59]. Vertical transmission of CSFV is also common and may occur at any time during gestation when pregnant sows become infected. During an outbreak of CSF in the Netherlands (1997–1998), it was estimated that 17 % of CSFV spread between an infected herd and neighboring herds during the high-risk period (i.e., before the detection of the first infected herd) was due to direct contact. After the high risk period, direct contact transmission decreased to about 1 %, mainly due to the implementation of control measures [60]. Regardless of the source of the virus, it has been observed that during a natural CSF outbreak, the probability of secondary outbreaks of the disease decreases with increased distance from an infected herd [61–63].

Transmission rates for CSFV can be quantified by estimating the reproductive ratio (R_0), described as the average number of secondary cases caused by one infected animal in a fully susceptible population. Experimentally, R_0 values ranging from 81.3 to 100 were estimated for weaned pigs [64, 65]; 13.7 to 15.5 for slaughter pigs [64, 65]; and 13.0 for gilts [66]. From interpen transmission rates of CSFV in weaned and slaughter pigs, R_0 values were estimated to range from 3.39–7.77 [64].

Indirect transmission of CSFV from wild boars to domestic pig herds is a common event [67, 68]. Common sources of infection for domestic pigs are: feed containing meat from wild pigs, silage originating from areas frequented by wild pigs, contact with hunters, and contaminated vehicles. In addition, other indirect modes of CSFV transmission include artificial insemination, swill feed, livestock trucks, personnel, and pig slurry from infected farms. Indirect transmission mediated by animals other than suids, such as ruminants, rodents, birds, or insects has not been documented.

Experimentally, airborne transmission of CSFV over short distances is possible [69–71]. The precise distance that the virus can spread via air is still unknown.

2.2 Pathogenesis and Clinical Features

2.2.1 Pathogenesis and Immunology

Historically, CSF has been characterized by sudden death of pigs or by CSF's most common appearance of red to purple discoloration (hemorrhage) of the skin covering the nose, abdomen, inside of limbs, ears, and pubic regions (Fig. 14.1).

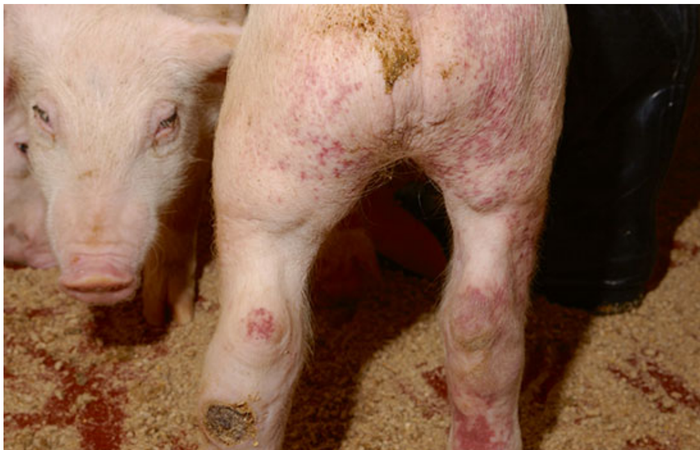

Fig. 14.1 Skin: numerous petechial and ecchymotic hemorrhages along the caudal aspect of the hind limbs in a pig with classical swine fever. Conjunctivitis is present in the second pig pictured (*left pig*). *Source*: Plum Island Animal Disease Center

Postmortem findings are characterized by hemorrhage and congestion in the spleen (Fig. 14.2), lymph nodes (Fig. 14.3), cecum, colon (Fig. 14.4), and lungs.

CSFV primarily causes a hemorrhagic fever characterized by vascular lesions, including splenic infarcts (Fig. 14.2), hemorrhages in lymph nodes (Fig. 14.3) and the urinary system, and disseminated microthrombosis with necrosis of lymphocytes, particularly in the B-cell areas of the lymphoid organs.

The clinical presentation of CSF varies depending mainly on the virulence of the infecting virus. After infection via the oronasal route, CSFV actively replicates in tonsils [72]. Within 2–6 days post exposure, CSFV antigen can be detected in tonsilar crypt epithelium, lymphoid follicles, and para-follicular regions. The virus spreads through the lymphatic vessels into regional lymph nodes, and from there into the bloodstream. Viremia is readily detected between 4 and 6 days post exposure. A second wave of virus replication takes place in several organs, particularly

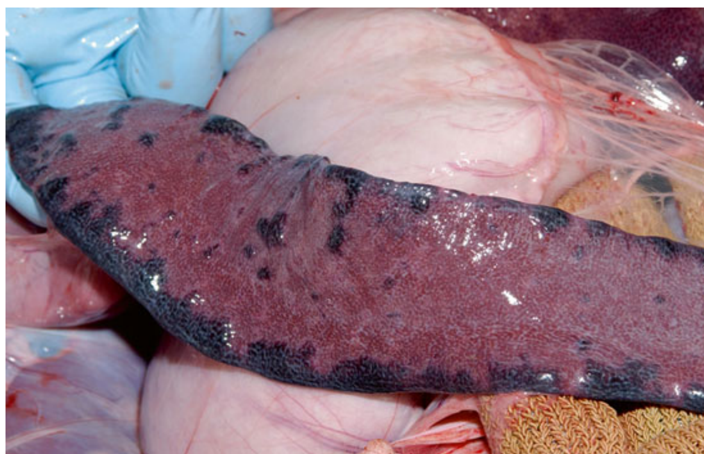

Fig. 14.2 Spleen: severe multifocal splenic infarcts in a pig with classical swine fever. *Source*: Plum Island Animal Disease Center

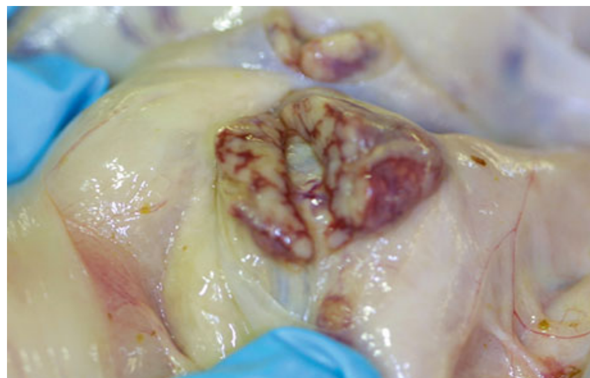

Fig. 14.3 Lymph node, mesenteric: swollen edematous cut surface with typical cortical congestion in a pig with classical swine fever. *Source*: Plum Island Animal Disease Center

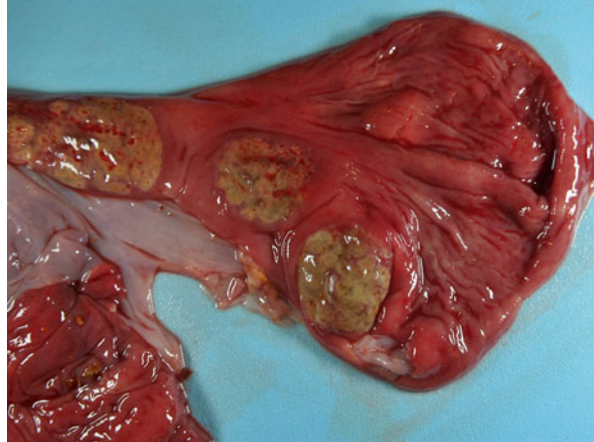

Fig. 14.4 Colon: severe multifocal ulcerative colitis ("button ulcers") in a pig with classical swine fever. *Source*: Plum Island Animal Disease Center

in spleen, bone marrow, and visceral lymph nodes. Lymphoid depletion in the spleen is generally observed with poorly defined peri-arteriolar lymphoid sheaths, and abundant viral antigen accumulation in peri-arteriolar regions and follicles. At this stage of the infection, CSFV is excreted into the environment via nasal, conjunctival, and oral (saliva) routes, as well as via urine and feces.

A hallmark of the infection is the progressive lymphopenia and thrombocytopenia observed during the course of the disease [73]. During the infection, there are profound changes in the bone marrow that may account for decreased white blood cell (WBC) counts [74–78]. The decrease in WBCs also may be mediated by active replication of CSFV in mononuclear cells [79–81], as virus replication leads to changes in monocyte/macrophage gene expression that results in the release of cytokines, some of which can be immunomodulatory or immunosuppressive. At later stages of the disease, infected lymphocytes (as well as granulocytes) are observed. Infected monocytes and macrophages release factors that coincide with the observed onset of fever and coagulation disorders in infected pigs [74]. Observed hemorrhages are the result of vascular changes that affect arterioles, postvenules, and capillaries [82], including a direct effect of CSFV on endothelial cells [83], on platelets leading to thrombocytopenia [73], or by inducing DIC [83, 84] and microthrombosis leading to endothelial damage [84]. Although the mechanisms of CSFV pathogenesis are not well understood, the development of infectious full-length copies of CSFV genomes have led to a better understanding of the role of viral proteins in mechanisms of attenuation and virulence [85–90], and will provide insights into mechanisms of disease progression.

General immunosuppression occurs early after infection with CSFV as indicated by dramatic decrease of peripheral B- and T-cells. Knowledge about the innate immune response triggered by CSFV infection is limited. As with other viral infections, pigs infected with virulent CSFV isolates react with a significant increase in serum concentrations of interferon-α (IFN-α) (500–4,500 U/ml) that is detectable

days 2–5 post-exposure [91, 92]. A viral protein (N^{pro}) has a well described role in inhibiting the type I IFN response [93–95]. Transcriptional analyses of tonsil, retropharyngeal lymph node, and spleen tissues obtained from pigs infected with CSFV strains of different virulence revealed differential expression of 44 host genes by day 3 post exposure [96]. Gene expression changes included those involved in mechanisms of innate and adaptive immune response (including specific antiviral genes), regulation of IFN, apoptosis, ubiquitin-mediated proteolysis, oxidative phosphorylation, and cytoskeleton formation.

Three CSFV proteins are the main targets of antibody response elicited after exposure: envelope glycoproteins, E^{rns} and E2, and a nonstructural protein, NS3. The envelope E2 glycoprotein is the most immunogenic viral protein and induces a strong viral neutralizing antibody response. The neutralizing antibodies induced by envelope glycoprotein E^{rns} are more limited in their avidity than E2 glycoprotein, whereas NS3 protein induces non-neutralizing antibodies. The antibody response is usually detected 2 to 3 weeks post exposure/vaccination, increasing until 4–12 weeks post exposure [97–99]. The critical role of glycoproteins E2 and E^{rns} in the induction of a protective immune response has been elucidated using recombinant vaccinia viruses expressing envelope protein E2 and/or E^{rns} [100]. Based on these findings, subunit vaccines have been developed by expressing the E2 envelope protein in baculovirus/insect cells systems [51].

In contrast to observations of antibody-based protective immunity, pigs vaccinated with live attenuated vaccines (LAV), such CSFV C-strains, mount an early protective cellular immunity (3 days post vaccination) in the absence of circulating neutralizing antibodies. Inoculation of pigs with CSFV C-strain induces virus-specific T-cell responses [101, 102] targeting the E2 and NS3 proteins [103–107]. Furthermore, a close temporal correlation between T-cell responses and the rapid protection induced by a CSFV C-strain inoculation has been observed as early as 3 days post vaccination [108, 109].

2.2.2 Incubation Period, Clinical Signs, and Gross Lesions

The incubation period in individual animals is usually 3–10 days [72]. The course of a CSFV infection varies depending on host characteristics and the virulence of the infecting virus [72]. Acute, chronic, or prenatal forms of the disease are recognized in pigs. At early stages of the acute infection animals show nonspecific signs of disease, such as anorexia, lethargy, increased body temperature, huddling, conjunctivitis, respiratory distress, vomiting and diarrhea. After few days, reddened and purple skin discoloration (hemorrhage) manifest (Fig. 14.1). Some animals may develop signs of neurological disorders evidenced by staggering gait, weakness of hind legs, incoordination, and convulsions.

Chronic forms of CSF are often fatal and usually develop in a low proportion of infected animals [72]. Animals harbor the infection for up to 2–4 months before death. Initially, infected animals develop signs similar to those observed in acute forms of the disease. Skin hemorrhage(s) are manifested as purple discoloration on the ears, tail,

abdomen, and the lower parts of the limbs, usually during the second or third week post exposure. Later, generalized signs of disease may be seen sporadically, but chronic diarrhea and progressive wasting of the animals is consistently observed through to the terminal stage. The severe leukopenia and immunosuppression caused by CSFV infection often leads to secondary enteric or respiratory infections.

In acute cases of CSF, hemorrhage is the predominant manifestation of vascular compromise. Pathological changes can be readily visible in tonsils, lymph nodes, spleen, and kidneys (Figs. 14.2, 14.3, 14.5, and 14.6). Petechial hemorrhages, and even necrotic foci, may be detected on the palatine tonsils (Fig. 14.6). Lymph nodes

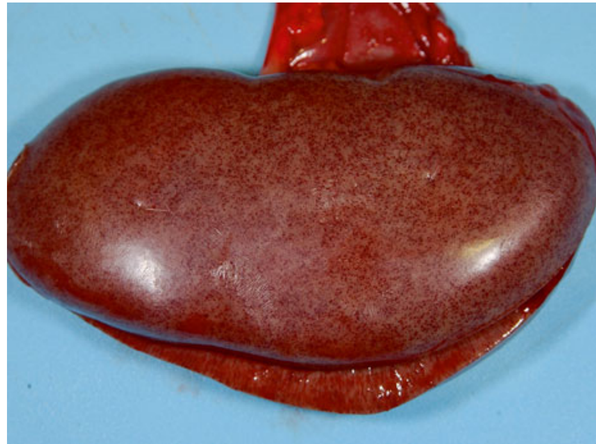

Fig. 14.5 Kidney: multifocal cortical petechial hemorrhages referred to as "turkey egg kidney" in a pig infected with classical swine fever. *Source*: Plum Island Animal Disease Center

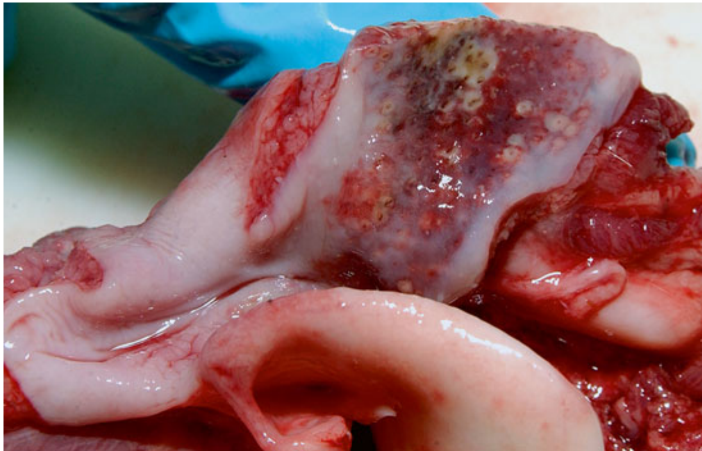

Fig. 14.6 Tonsil: multifocal crypt necrosis and diffuse congestion in a pig with classical swine fever. *Source*: Plum Island Animal Disease Center

are usually enlarged, edematous, and hemorrhagic; the mandibular, retropharyngeal, gastro-hepatic, and mesenteric lymph nodes (Fig. 14.3) are usually the most affected. Enlargement of the spleen and the presence of infarcts in the periphery of the organ are frequently observed (Fig. 14.2). The parenchyma of kidneys may display a yellowish-brown discoloration and petechial hemorrhages (Fig. 14.5) might be observed. Petechial hemorrhages or ecchymoses are also often present in the urinary bladder, heart, and serosae of the abdominal and thoracic cavities. Gross lesions associated with encephalitis include hyperemic and congested blood vessels of the brain. Non-suppurative encephalitis might be observed microscopically in brains of animals showing signs of neurological disorders.

In chronic cases of CSF, gross pathological changes tend to be less accentuated than in acute cases of the disease. Purple skin discoloration might be observed due to hemorrhages in subcutaneous tissues. At this stage, inflammation in the respiratory, gastrointestinal, and urinary tract are often seen as the consequences of secondary infections. Animals usually display chronic diarrhea as a consequence of necrotic and ulcerative lesions on the ileum, the ileocecal valve, and the rectum. "Button" ulcers in the large intestine and colon are considered typical (Fig. 14.4) [72].

In cases of congenital infection, a proportion of piglets may show incomplete development of the cerebellum or other developmental abnormalities, such as atrophy of the thymus [72]. Fetal mummifications, malformations, and stillbirth are a consequence of transplacental infections [110].

2.2.3 Morbidity and/or Case-Fatality Rates and Prognostic Factors

The severity of pathological lesions caused by CSFV depends on time of infection, age of the animal, and virulence of the infecting virus [111]. Case-fatality rates in acute cases of CSF tend to be high particularly among young animals. Acute forms of CSF are associated with virulent isolates of the virus; however, the chronic form of CSF is associated with virus isolates of moderate-to-low virulence. Congenital forms of CSF caused by transplacental infections may occur at any stage of the pregnancy. The course of CSFV infections in sows is mainly subclinical, with animals having transient anorexia and reproductive failures. Infections during the first trimester of gestation often lead to abortions, and repeat breeding could lead to subsequent abortions. Persistently viremic piglets are born to sows that became exposed to CSFV, usually during the second trimester of gestation. These piglets are clinically normal although they may show poor growth, wasting, and tremors. These animals survive for a long period of time (30 days or more) while constantly shedding virus [112]. The outcome of this type of infection, known as "late onset of CSF," is always fatal. Pregnant sows or "carrier sows" usually do not manifest the disease, although they shed virus, particularly at farrowing. Infections that occur in the third trimester of gestation result in abortion, fetal malformation, or birth of weak or dead litters.

2.3 Diagnosis and Control

2.3.1 Diagnosis and Epidemiology

CSF is diagnosed tentatively on clinical grounds. However, this approach is rather limited since CSF clinically resembles other diseases of pigs such as African swine fever, erysipela, porcine reproductive and respiratory syndrome, coumarin poisoning, purpura hemorrhagica, postweaning multisystemic wasting syndrome, porcine dermatitis and nephropathy syndrome, *Salmonella* or *Pasteurella* infections, or any enteric or respiratory syndrome with fever not responding to antibiotic treatment.

Techniques for the detection of CSFV and virus-specific antibodies are established and described in the OIE's Manual of Diagnostic Tests and Vaccine for Terrestrial Animals [113]. Traditionally, virus isolation and detection of viral antigens in frozen tissue sections using antibodies have been used to diagnose CSF. Currently, antibody-detection ELISA, targeted to anti-E2 antibodies, is commonly used for CSF surveillance and for diagnostic purposes. ELISAs based on the detection of antibodies against E^{rns} have also been developed, mainly for distinguishing vaccinated animals from naturally infected animals when subunit vaccines are used to immunize pigs. Antigen detection ELISAs are also available for detecting CSFV antigens in tissue samples. The advent of nucleic acid-based tests, such as RT-PCR and qRT-PCR, has significantly improved the simplicity and sensitivity of tests for detecting CSFV. These techniques are rapid and highly sensitive, capable of detecting low amounts of virus even before the onset of clinical signs.

Genetic typing of CSFV [49, 114, 115] targeting the 5′ untranslated genomic region [49] or E2 and NS5B genes [116, 117] has been widely used for identifying and comparing isolates. Phylogenetic analysis grouped CSFV isolates of different chronological and geographical origins into three distinctive groups, with three or four subgroupings: groups 1.1, 1.2, and 1.3; groups 2.1, 2.2, and 2.3; and groups 3.1, 3.2, 3.3, and 3.4 [72]. Group 1 includes historic CSFV isolates and vaccine strains. Most of these viruses were isolated from around the world in the period dating from 1920s to 1990s, although CSF outbreaks caused by these isolates have been reported in Cuba and Colombia in the early 2000s. Group 2 includes all CSFV isolates that have been circulating in the EU in the last 30 years. The earliest appearance of subgroup 2.3 viruses was reported in West Germany in 1982 [49]. Subsequently, isolates with the characteristics of subgroups 2.1 and 2.2 have been detected in domestic and wild pig populations in different EU countries. The 2.1 viruses have been only sporadically reported in Europe. These isolates were first reported in West Germany in 1989, and thereafter in the Netherlands (1992) and Switzerland (1993). During the major CSFV outbreak in the EU in 1997 and 1998, the virus is believed to have been introduced from Germany into the Netherlands, with subsequent spread to Italy, Belgium, and Spain [118, 119]. Isolates from this group have been detected in Africa and Asia. Group 3 isolates are the most diverse CSFV isolates, and seem to be confined to Asia, particularly Southeastern Asia, where all groups and subgroups have been detected at one point in time.

2.3.2 Vaccination, Control, and Eradication

The first attempts to develop a vaccine against CSF in the USA were made in 1907 by the US Department of Agriculture Bureau of Animal Industry. Vaccination consisted of the inoculation of pigs with anti-CSF serum, followed by inoculation with virulent virus. In 1935, Dorset et al. developed a vaccine killed with crystal violet. In 1965, Baker developed a live attenuated vaccine (LAV) that conferred effective protection against the disease [120]. In 1961, the US Congress authorized a CSF eradication program, but no diagnostic test for CSF was readily available. In 1963, Mengeling et al. developed and described a fluorescence-based rapid diagnostic test for CSF that took less than a day to perform.

LAVs were derived from wild-type CSFVs that were attenuated through repeated passages in either cell culture or experimental animals [98, 121, 122]. CSFV subunit marker vaccines have been developed using recombinant E2 envelope protein [86, 123, 124]. A key feature is that these subunit vaccines induce an antibody response that differentiates *i*nfected from *v*accinated *a*nimals (*DIVA* principle). However, these subunit vaccines are not as efficacious as traditional LAVs, particularly when animals are exposed shortly after vaccination [86, 123, 124]. Solid protection is usually observed after 14 days post vaccination, evidenced by the appearance of circulating neutralizing antibodies.

Other experimental vaccines against CSFV have been designed including peptides, DNA vaccines, vectored vaccines, and trans-complemented, deleted CSFV genomes (replicons; for review see ref. [125]. Vaccines based on immunogenic peptides (mostly derived from the E2 glycoprotein) have been used experimentally [126–129]. DNA vaccines based on the E2 protein have been formulated in combination with cytokine genes to enhance their immunogenicity [130–134]. Vaccinia virus vector-based vaccines have been constructed expressing E2 and/or E^{rns} glycoproteins [100]. E2-bearing viral vectors, including pseudorabies virus [135–138], porcine adenovirus [139–142] swinepox virus [143], and parapoxviruses [144], have been used in pigs. In general, viral vector-based vaccines have DIVA capabilities and induce protection against clinical disease. Alternatives to the use of LAVs (e.g., *trans*-complemented viruses) have been constructed due to the hypothetical potential of any live virus to revert back to its virulent parental strain [145–148]. These viruses lack the E^{rns} or E2 proteins and are complemented in *trans* after their RNA is transfected into helper cell lines expressing the lacking viral gene [146]. When inoculated in pigs, these viruses can enter cells, but they are unable to produce viable viral progeny.

CSF control programs in enzootic areas are based on vaccination of domestic pigs. The most currently used vaccines are LAVs. Vaccination with LAV strains C, GPE−, Thiverval, or PAV-250 induces protective immunity in pigs within a few days after vaccination. In general, these LAV vaccines provide lifelong immunity against disease [98]; however, they do not allow serological differentiation of vaccinated from infected animals. This makes eradication efforts and demonstration of freedom of disease difficult, both of which are prerequisites for removing barriers to animal trade.

With the advent of reverse genetics, experimentally improved LAVs with DIVA capabilities have been developed. Chimeric pestivirus vaccines (CP7_E2alf) and genetically modified FlagT4v CSFV vaccines were constructed [145, 149–153] and included some combination of positive antigenic markers and/or negative markers suitable for differentiation from natural infection. These vaccines confer protection against CSF as with conventional LAV while allowing distinction between vaccinated and infected animals.

In countries free of CSFV, control policies involve depopulation (slaughter) of exposed animals [51]. In the EU, outbreaks of CSF lead to depopulation of infected farms and the destruction of cadavers. During outbreaks, protection zones (3 km radius) and surveillance zones (10 km radius) are established in affected areas, restricting pig movements. An epidemiological investigation is established with the purpose of tracing a CSFV isolate to the source of infection. If considered appropriate, emergency vaccination could be an option for controlling an outbreak of the disease in countries previously free of CSFV.

April 1973 was the first month over a period of 100 years without a CSF outbreak in the USA. In January of 1978, the US was declared free of CSF [79]. Similarly, after implementation of strict control measures, several countries, including Australia, Canada, New Zealand, and member states of the EU, were able to eradicate the disease.

2.3.3 Public Health/One Health Crossover

CSFV is not a risk to humans, other than those public-health effects induced by the economic hardship associated with an outbreak response.

3 Rabbit Hemorrhagic Disease Virus and European Brown Hare Syndrome Virus

3.1 Etiologic Agent and Natural History

3.1.1 Definition

Rabbit hemorrhagic disease (RHD) is caused by the calicivirus rabbit hemorrhagic disease virus (RHDV). RHD was first described in 1984 as a disease of European rabbits (*Oryctolagus cuniculus*) imported from Germany into the People's Republic of China [154], but whether these rabbits were carrying the virus or exposed to a local virus is unclear. The case-fatality rate in these rabbits was over 90 %.

European brown hare syndrome (EBHS) is also caused by a calicivirus, European brown hare syndrome virus (EBHSV), and was first reported in Sweden in 1980 [155]. EBHSV infects European brown hares (*Lepus europeus*) and mountain hares (*Lepus timidus*), causing an acute hepatitis with lower case-fatality rate compared to that of RHD in rabbits.

3.1.2 Etiology and Evolution

Both RHDV and EBHSV are single-stranded, positive-sense RNA viruses in the genus *Lagovirus* within the family *Caliciviridae* [156]. The genomes of both viruses are approximately 7.5 kb long and code for nine viral proteins [157–160].

The hepatotropic, lethal RHDV has likely evolved from closely related but non-pathogenic lagoviruses, which are widely distributed in wild and domestic rabbits [161–164]. These viruses replicate in the gut causing little or no pathology [162, 165, 166]. The mutation or mutations that have altered the cellular tropism of the virus and allowed the very rapid replication in the liver have not been defined. Additionally, the timeframe of this evolutionary step is unclear; researchers still debate whether the switch to pathogenic forms happened once or several times during the evolutionary history of the RHDV [167–169]. Recently, moderately pathogenic lagovirus strains have also been described [170–172].

The origins of EBHSV are unclear. However, viral antigen was detected in tissues of healthy European brown hares in Argentina. The absence of notable hare lethality [173] suggests that non-pathogenic lagoviruses may also exist in hares.

3.1.3 Geographic Distribution and Economic Effects

RHDV was first described in farmed European rabbits in the People's Republic of China in 1984. Over the next few years, RHD occurred in South Korea, Europe, the Americas, Northern and Western Africa, Western Asia, Cuba, and Réunion, and was probably spread by trade of rabbits and rabbit products [154]. Coincident with the emergence of RHD in rabbits, EBHS was reported in Northern Europe in 1980 [155].

RHD causes substantial commercial losses in rabbitries due to the direct costs of deaths in outbreaks. Millions of rabbits have died, and when combined with the associated costs of control and eradication, RHDV is a significant economic burden to producers [154, 174, 175]. RHDV has been established in the wild European rabbit populations of Europe where it has been responsible for an ecologically significant decline of free-living wild rabbit populations [154, 176]. This decline has impacted higher order predators, such as the Spanish imperial eagle (*Aquila adalberti*) and Iberian lynx (*Lynx pardinus*), recreational hunting, and maintenance of traditional landscapes through rabbit grazing [177]. In contrast, RHDV was released in Australia and subsequently New Zealand as a biological control for wild European rabbits, which are major introduced vertebrate pests causing serious agricultural and ecological damage [178, 179]. In these countries, RHDV has had a significant positive economic and ecological benefit [180]. In addition, RHDV appears to have maintained high virulence after its introduction, unlike myxoma virus which was deliberately spread in Australia and Europe in the 1950s [181]. However, emerging genetic resistance to RHDV in Australian rabbits has been reported [182, 183].

3.1.4 Natural History, Transmission, and Host Range

The only known host of RHDV is the European rabbit. Other animals of more than 20 species, including many animals native to Australia, were experimentally infected as a prerequisite for the release of RHDV as a biocontrol agent for rabbits, but no productive infection was observed [178]. Lagomorphs other than European rabbits, including American leporids such as the volcano rabbit (*Romerolagus diazi*), black-tailed jackrabbit (*Lepus californicus*), and eastern cottontail (*Sylvilagus floridanus*), also were not susceptible [184]. This observation suggests that RHDV is unlikely to become endemic in the Americas outside of commercial rabbitries or possibly free-living feral European rabbit populations.

EBHSV has been reported to be less species-specific. While the main host of the virus is the European hare, EBHSV can also infect mountain hares where the host ranges of the two leporids overlap [185], as well as eastern cottontails [178]. Recently, a new lagovirus genetically distinct from RHDV and EBHSV has been described in Europe [171, 172] that causes lethal infections in both European rabbits and Cape hares (*Lepus capensis*) [186].

RHDV is infectious orally, nasally, and conjunctively, as well as by injection. Virus is present in most discharges from infected rabbits, including feces and urine, and in the carcasses of infected dead animals. International spread occurs by direct contact between infected and susceptible rabbits, contaminated rabbit products or other fomites, or by virus present in the environment. Mechanical transmission by flies, especially Australian bush flies (*Musca vetustissima*) and blowflies (*Calliphora* spp.), is of particular importance for distance transmission in the field in Australia and also other countries [154, 187, 188]. Virus can adhere to the legs and mouthparts of flies, and also pass through the gut unchanged [187]. Under laboratory conditions, RHDV could also be transmitted by mosquitoes or fleas that fed on infected rabbits [189].

RHDV is highly stable in the environment. Virus in rabbit carcasses can remain infectious for more than 3 months, particularly inside rabbit warrens [190]. As rabbit carcasses with extremely high virus loads effectively transmit RHDV, selective pressure is probably low for attenuation of RHDV in the field. This hypothesis could explain why field isolates continue to be highly virulent. The frequent reemergence of RHD in rabbit populations, together with detection of viral RNA in recovered rabbits, has led to suggestion of persistent infection [191, 192]. However, although viral RNA can be detected in rabbit tissues months after recovery from disease [193], viral antigen or virus transmission could not be demonstrated. Virus could also not be reactivated by immunostimulation or immunosuppression [194]. Results from recent epidemiological studies suggest that viruses initiating natural outbreaks vary genetically between years in the same location, indicating that the virus does not persist on-site between outbreaks [195].

3.2 Pathogenesis and Clinical Features

3.2.1 Pathogenesis and Immunology

The major target organ of RHDV is the liver, but hemorrhage due to DIC and loss of clotting factors is an inconsistent feature of clinical disease seen in the field. RHDV binds in a strain-specific manner to oligosaccharide histo-blood group antigen (HBGA) receptors expressed by epithelial cells of the gut and upper respiratory tract [183, 196, 197]. Researchers have suggested that these receptors provide an initial site of attachment for RHDV. However, the role of these potential receptors has not been clearly defined. These receptors on epithelial cells are not expressed at high levels in young rabbits [196] even though these animals are readily infected [189, 198, 199], and these antigens are also not expressed in the liver.

The sites of initial replication following oral/nasal inoculation have not been identified. RHDV can be detected in hepatocytes as early as 8 h after oral/nasal inoculation [200]. Rapid virus replication to extremely high titers causes massive hepatic necrosis and fulminant liver failure within 36–96 h after infection. Hepatic encephalopathy may occur as a result of the liver failure. Histopathologically, coagulative necrosis of hepatocytes is observed, starting at the periphery of the lobule and moving inward as the disease progresses. Over 60 % of hepatocytes may be positive for viral antigen [201], and viral RNA can be detected by PCR in the liver [194].

DIC has been observed with thrombus formation in the blood vessels of the liver, kidneys, and lungs, as well as widespread ecchymotic and petechial hemorrhages. Hemorrhage and edema of the alveoli may also be present in the lung [202, 203]. Virus antigen can be detected in alveolar macrophages by in situ hybridization, and viral RNA can be detected by PCR in the lungs [194, 204].

Nephrosis with impaired renal function (elevated BUN and serum creatinine concentrations) occurs late in infection, characterized by congestion of glomerular tufts and the renal medulla, hemorrhages within the renal corpuscles and the interstitium of the cortex, hyaline thrombi within small blood vessels, and hydropic degeneration of the tubules [202, 205]. Depletion of lymphocytes from the white pulp of the spleen is characteristic of RHD. Virus antigen also can be detected in macrophages and lymphocytes in the spleen, blood monocytes, and glomerular mesangial cells by immunohistochemistry or in situ hybridization [201, 204] and in the feces and bile by hemagglutination [194]. Viral RNA can be detected by PCR in bile, spleen, lymphoid tissues, kidneys, WBCs, urine, and feces [194].

3.2.2 Incubation Period, Clinical Signs, and Gross Lesions

The incubation period can be as short as 12–24 h following injection of RHDV. Clinical presentation can be peracute, expressed as sudden death with no premonitory signs: rabbits may be observed grazing then squeal, convulse, and die. In the acute

form of the disease, the infected rabbit may appear depressed and reluctant to move around. These animals will show elevated temperature (up to 42 °C, 107.6 °F), and heart and respiratory rate may be increased. Ataxia, lateral recumbency, convulsions, and coma may occur prior to death and 2–5 days after exposure. Blood-stained discharge from the nose and bloody diarrhea or hematuria may be seen.

Infected rabbits that survive longer than 4–5 days are considered to express a subacute form of the disease during which obvious icterus may be present. Depending on the degree of hepatic damage in these rabbits, death may occur days to weeks later; hepatic cirrhosis has been described in subacutely affected rabbits [202, 206]. Subclinical infection may occur in a small proportion of infected adult rabbits and in young rabbits under 10 weeks of age. These rabbits are infected and seroconvert, but clear the virus with few or no clinical signs of disease. An age-related resistance to disease occurs; infected kits less than 4–5 weeks old rarely show clinical disease although they shed virus [189, 198, 199]. Mild hepatic pathology is present with elevated liver enzyme serum concentrations, and occasional deaths do occur [199]. This resistance is gradually lost, and by 10 weeks of age, rabbits are fully susceptible [207].

The most consistent finding at autopsy is a pale swollen liver, usually with a strong lobular pattern. The spleen is enlarged and black, and the kidneys may be dark. Lungs may be congested and hemorrhagic, with fluid or froth in the trachea and bronchi, and the walls of the trachea are commonly hyperemic due to dilation of the blood vessels. Ecchymotic and petechial hemorrhages may be scattered over all the internal organs, mucosal surfaces, subcutaneous tissues, and muscle, but are not a consistent finding [189]. In subacutely infected rabbits that have survived for some days, the subcutaneous tissue may be yellowish.

Clinical hematology and hemostasis findings include lymphopenia, neutropenia, decreased thrombocytes, increased prothrombin time, and decreased factor V and Factor VII. Clinical chemistry findings include extremely elevated liver transaminase concentrations in serum (aspartate amino transferase >100 times normal; alanine amino transferase >10 times normal), marked elevation of total bilirubin concentrations, elevated BUN and serum creatinine concentrations, elevated serum Na^+ and K^+ concentrations, hypoglycemia, and hyperlipidemia [199, 203, 205, 208].

3.2.3 Morbidity and/or Case-Fatality Rates and Prognostic Factors

Historically, case-fatality rates in RHD-infected adult rabbits were generally 90–95 %. Recently, a complex interaction between clinical outcome, virus genotype, and HBGA receptor type has been demonstrated. Rabbits of particular HBGA types are more likely to survive infection at low doses of particular RHDV strains, although this resistance can be overcome at higher virus doses [183]. In addition, evidence from France and Australia indicates that this natural resistance may be leading to selection in wild populations of rabbits that are negative for the HBGA receptor type that facilitates infection of the predominant virus strain in a specific

geographic region [182, 183]. Emerging genetic resistance to RHDV has been described in Australian wild rabbits, which manifested as resistance to infection rather than resistance to disease [182].

3.3 Diagnosis and Control

3.3.1 Diagnosis and Epidemiology

In commercial rabbitries, RHDV exposure typically leads to a history of rapid onset of high fatality in unvaccinated adult rabbits and survival of younger animals. Gross autopsy findings are largely confirmatory. RHDV does not replicate in cell cultures tested to date [113], so laboratory diagnosis is based on detection of virus, virus antigen, or viral nucleic acid. Virus can be detected in virtually all tissues and secretions using RT-PCR. Liver is the most useful tissue for diagnosis because of the extremely high titers of RHDV in this tissue. Virus can also be demonstrated in liver extracts using antigen-capture ELISA, western blot or by hemagglutination using human erythrocytes. However, some RHDV strains have been reported not to hemagglutinate possibly due to variations in HGBA binding [113, 183]. RHDV RNA can be detected by RT-PCR for prolonged periods in recovered animals, and cross-reaction can also occur with related rabbit lagoviruses. Indirect or competition ELISA can demonstrate antibody to RHDV. Isotype ELISAs are used for detection of IgG, IgM and IgA but are less specific and can cross-react with other rabbit lagoviruses. IgM can be detected as early as 3–4 days after infection. Hemagglutination inhibition can also be used for antibody detection [113].

In Europe, Australia, and New Zealand, RHD is enzootic in wild rabbit populations [178]. The timing of outbreaks is closely linked to the immune status of a population, juvenile resistance, and rabbit breeding patterns, which in turn strongly depend on climatic factors [154]. Young rabbits are resistant to lethal RHDV infection but can become infected and acquire lifelong immunity. Therefore, natural outbreaks of RHD are usually observed towards the end of the breeding season, when a sufficient density of seronegative rabbits of a susceptible age is available [154]. Maternal antibodies can prevent infection [207]. Such antibodies also help to delay exposure of rabbits until they have reached a susceptible age, thereby delaying, but not preventing, outbreaks. Acquired immunity to RHDV is life-long, but boosts in antibody titers have been observed, indicating that rabbits probably get reinfected and mount an anamnestic immune response [209].

Avirulent rabbit lagoviruses related to RHDV occur in both wild and domestic rabbit populations in Australia and Europe. Some avirulent strains may provide a degree of cross-protection against RHD, thus further modifying the epidemiology of the disease. This protection is seen in areas where these avirulent relatives of RHDV are present, which in Australia are predominantly the cooler temperate climatic zones [161–164]. Serological studies are often confounded by the presence of these nonpathogenic lagoviruses, such as rabbit calicivirus (RCV)-A1 in Australia [164].

These nonpathogenic lagoviruses cross react in serological assays for RHDV [209, 210], so specific serological assays have been developed for studying the epidemiology of RHDV in areas where these viruses circulate [211, 212].

3.3.2 Vaccination, Control, and Eradication

In countries where European rabbits are uncommon in the wild, such as the USA, eradication following entry of RHDV has been successful, although further incursions have occurred [174]. In Europe, where wild European rabbits provide a reservoir of infection, eradication is not feasible. Control in commercial rabbitries relies largely on quarantine and vaccination. For meat breeding, control of infection may mean vaccinating the breeders and relying on maternal antibody to protect the meat rabbits prior to slaughter. Inactivated, adjuvanted whole virus vaccines produced from infected rabbit livers are commercially available and provide good protection following a single injection at 8–12 weeks of age, followed by an annual booster. It is not clear if maternal antibody can interfere with vaccination, so delaying vaccination until 12 weeks of age or boosting vaccination at 10–12 weeks may be sensible depending on the situation. A combination of a live myxoma virus vaccine with inactivated RHDV is available commercially (Dercunimix, Merial), and a recombinant attenuated live myxoma virus expressing the RHDV capsid protein was released in 2013 (Nobivac Myxo-RHD, MSD Animal Health). Vaccination in the face of an outbreak is considered effective [113] but should be combined with isolation/culling of affected rabbits, strenuous hygiene, and other biosecurity measures. As noted above, kits may not show clinical signs but will shed virus.

An antigenic variant of RHDV termed RHDVa appears to be replacing RHDV throughout most of its range [167, 213]. Conventional RHDV vaccines are still protective against this variant, although it can overcome suboptimal vaccine doses [214]. However, vaccines appear largely ineffective against the recently described variant strain RHDV2 [172], and immune responses to natural infection with this strain are only partially protective against challenge with classical RHDV [215]. Antigenic variants that overcome vaccination against RHDVa are present in China [216].

Vaccination to protect wild rabbit populations is logistically and economically infeasible although recombinant myxoma viruses expressing RHDV capsid protein have been tested in the field [217]. Nonpathogenic rabbit caliciviruses that provide some cross-protection against RHDV and spread naturally could provide a possible means of immunization of wild rabbits [218, 219].

3.3.3 Public Health/One Health Crossover

The deliberate release of RHDV in Australia as a biological control agent generated controversy because of the broad host range of some caliciviruses and the perception (at the time) that the virus had possibly jumped species into rabbits [174, 220, 221].

However, specific testing and nearly 30 years of experience have demonstrated that RHDV is highly specific for European rabbits, and that it is closely related to (and probably evolved from) a nonpathogenic rabbit calicivirus. The only exception is a recent report describing RHDV2 infection of European rabbits and Cape hares, but not other hares [186]. Despite this, the emergence of a highly virulent virus from an apparently avirulent progenitor provides a warning of how easily new viruses may emerge.

4 Simian Hemorrhagic Fever Virus

4.1 Etiologic Agent and Natural History

4.1.1 Definition

Simian hemorrhagic fever virus (SHFV) is classified as an arterivirus in the family *Arteriviridae*, order *Nidovirales*. Like other arteriviruses, SHFV has a positive-sense RNA genome approximately 15 kb in length, and differs from other arteriviruses by having three additional open reading frames [222].

4.1.2 Etiology and Evolution

SHFV is a relatively uncharacterized virus that causes simian hemorrhagic fever (SHF), a severe disease of Asian macaques (*Macaca* spp.) characterized by fever, facial edema, anorexia, adipsia, petechiae, diarrhea, hemorrhages, and up to 100 % lethality. All outbreaks of SHF have occurred at primate research facilities, but the means of introduction into primate colonies remains undefined. Dependent on the strain, the virus causes subclinical, persistent infection in Kibale red colobus (*Piliocolobus rufomitratus*), red-tailed monkeys (*Cercopithecus ascanius*), and possibly patas monkeys (*Erythrocebus patas*), grivets (*Chlorocebus aethiops*), and Guinea baboons (*Papio papio* spp.), which serve as natural hosts in Africa [223–227].

4.1.3 Geographic Distribution and Economic Effects

Asian macaques, which probably are not exposed in nature to SHFV, are highly susceptible to SHFV infection and disease, whereas African monkeys may carry the virus persistently without clinical signs. The overall distribution of SHFV in wild primates remains unclear. SHFV was a major concern for primate research centers in the past, but physical separation of Asian from African monkeys and improved screening methods have been effective control measures. The last outbreak of SHF was recorded in 1996.

4.1.4 Natural History, Transmission, and Host Range

Much of what is known about SHFV is derived from experiments with the prototype variants LVR 42-0/M6941 and Sukhumi-64. These prototypes were first identified in 1964 during almost simultaneous outbreaks of febrile hemorrhagic disease in Asian macaques that occurred at the Sukhumi Institute of Experimental Pathology and Therapy in the Georgian Soviet Socialist Republic and at the National Institutes of Health (NIH) in Bethesda, MD, USA [224, 228, 229]. Macaques in both institutes were housed in close proximity to African primates, including patas monkeys, baboons, and grivets [226, 229, 230]. During the Sukhumi outbreak, animals presented clinically with a hemorrhagic diathesis and encephalomyelitis. Lethality reached 100 % over 2 months [228, 229]. Conversely, the macaques housed at NIH also presented with hemorrhagic diathesis, but had high fevers in the absence of encephalomyelitis. Transmission was thought to have occurred as a result of reusing needles while tattooing and/or tuberculosis testing the African-origin primates and macaques [224–226]. The lethality during the NIH outbreak did not reach quite the extent seen in the USSR, as there were a few animals that survived infection. Blood and tissue samples from a such a survivor successfully induced hemorrhagic fever in macaques not associated with the initial outbreak, satisfying Koch's postulates [226].

Sporadic SHFV outbreaks of iatrogenic origin have occurred semi-regularly since 1964 [226, 230–232]. During SHFV outbreaks in 1972 and 1989, the virus was thought to be spread by both direct and indirect contact between macaques [232, 233]. Curiously, there seems to be a connection between SHFV and ebolaviruses, as SHFV was found together with Reston virus (RESTV) in macaques with viral hemorrhagic fever in four out of five RESTV emergences [234].

4.2 Pathogenesis and Clinical Features

4.2.1 Pathogenesis and Immunology

Initial exposure of a nonhuman primate colony occurs through infected blood or tissue from carrier animals. The virus is transmitted through direct contact and fomites. Specific subsets of macrophages are the principal target cell for viral infection.

Very little is published characterizing the pathogenesis or immune response to SHFV in English [222, 235, 236]. Similar to other arteriviruses, SHFV principally replicates in macrophages, although there is considerable variation in the cellular tropism, virulence, and immunogenicity of individual strains of SHFV in African monkeys.

Infected monkeys produce complement-fixing and neutralizing antibodies. The humoral immune response of persistently infected patas monkeys varies with the infecting strain of SHFV. Patas monkeys infected with low virulence variants (P-248 and P-741) had minimal or no antibody response and persistently low viremia, whereas a more virulent variant (LVR 42-0/M6941) induced antibodies within

7 days after infection [237, 238]. Additionally, the appearance of these antibodies was associated with clearance of the virus from blood circulation 21 days post inoculation. Neutralizing antibodies against one variant of SHFV do not completely neutralize other variants, suggesting that variation in the neutralization determinants of individual variants of SHFV exists. However, these determinants have yet to be characterized.

4.2.2 Incubation Period, Clinical Signs, and Gross Lesions

The incubation period of SHFV is approximately 3 days, and most animals succumb to the infection within 10–15 days post exposure. Clinical signs begin with depression, ataxia, anorexia, dehydration, edema, cyanosis, and petechial rash. Hemorrhage in the form of epistaxis, hematemesis, ecchymosis, retrobulbar hemorrhage, and melena is common. Hematologic signs of coagulopathy including abnormal coagulation factors with fibrin degradation products are typical. Concentrations of liver enzymes, including LDH, GGT, AST, may be elevated. Elevations of BUN and creatinine concentrations may indicate kidney involvement, but the elevations are variable. Typical gross lesions include random hemorrhage and congestion throughout the gastrointestinal tract, liver, renal capsule, retrobulbar tissue, subcutis, and lung. The proximal duodenum may contain focally extensive congestion, hemorrhage, and necrosis with sharp demarcation at the pylorus. Splenic infarction with significant absence of white pulp may be present. Microscopically, extensive lymphoid necrosis in the spleen is observed, with perifollicular hemorrhage and fibrinous exudate. A unique feature of SHFV is cortical thymic necrosis of the spleen, with sparing of the medulla. Systemic lesions consistent with DIC, including necrosis, fibrin thrombi in glomeruli, hepatic sinusoids, and lung are also present in severe cases.

4.2.3 Morbidity and/or Case-Fatality Rates and Prognostic Factors

Asian macaques are very susceptible to even small doses of SHFV, and usually uniformly develop disease with lethality usually reaching close to 100 %. Other nonhuman primates (NHPs) either do not get infected at all or develop subclinical infections.

4.3 Diagnosis and Control

4.3.1 Diagnosis and Epidemiology

Serologic tests cannot distinguish carriers from previously infected animals, and virus isolation is generally unreliable for diagnosis. Molecular detection by RT-PCR has become the method of choice for detection of SHFV.

4.3.2 Public Health/One Health Crossover

SHFV has not been linked to human disease. SHFV and RESTV were isolated concurrently and repeatedly in crab-eating macaques (*Macaca fascicularis*) maintained in Philippine and US quarantine facilities. SHFV has caused sporadic outbreaks of hemorrhagic fevers in macaques at primate research facilities [234, 239]. Any severe disease of closely related animals such as NHPs must be viewed with caution, as the potential for adaptation of the virus to humans should always be a concern given the common phenotypic and genotypic features of both animals.

5 Other Positive-Stranded RNA Viruses Causing Viral Hemorrhagic Fever-Like Diseases

5.1 Feline Calicivirus

A strain of feline calicivirus (FCV-Ari, named for the second observed case) was observed by Pederson et al., to cause disease in domestic cats with hemorrhagic features and this disease could be induced in experimental infections [240]. While vasculitis was an inconsistent finding, severe edema and local necrosis of skin and subcutaneous tissues were present. “Loss of vascular integrity” was considered to be the best explanation for the observed gross and biochemical findings in both the naturally and experimentally infected animals. This loss was supported by finding viral antigen in endothelial cells [241] and identifying a cellular junctional adhesion molecule as a putative cellular receptor [242]. However, it was unclear whether the vascular disruption was due to direct cytopathology by live virions, disruption of tight junctions, or simply antigen uptake and cellular damage in the context of significant epithelial damage. Persistence of viremia appeared to be a prognostic factor. The genetic sequence of FCV-Ari was shown to be within the predicted range of variability when compared to either vaccine or field strains of FCV, but commercial vaccines against FCV were shown to be only partially protective. In an outbreak of FCV-Ari, the virus was suspected to have been transmitted from a shelter cat to the cats of employees and a client of a veterinary practice.

5.2 Venezuelan Equine Encephalitis Virus

Venezuelan equine encephalitis virus (VEEV) is an arthropod-borne zoonotic virus affecting both horses and humans [243]. Vectors are typically mosquitoes of the genus *Culex*, but during epidemics members of the genera *Aedes*, *Anopheles*, *Deinocerites*, *Mansonia*, and *Psorophora* may also transmit the virus. The disease in equids can be severe, resulting in a fulminant fatal disease before encephalitic signs have time to develop. In less severe cases, encephalitis with neurological signs

develops during disease progression. In horses that express neurological signs, CNS lesions can be extensive, with necrosis and hemorrhage. Hemorrhage is likely secondary to endothelial destruction and not vascular leakage, as necrotic lesions have been seen to involve the walls of small-to-medium vessels. In fulminant and less severe cases, the viremias in horses are very high, and these horses act as critical amplifying hosts in epizootics. In humans, influenza-like signs and symptoms with a fever and severe headache are seen in the proportion of cases that show any clinical signs (attack rates can be as high as 0.02 %). The virus is normally maintained in a sylvatic (enzootic) cycle, with few differences seen between enzootic viruses and the epidemic (epizootic) viruses recognized in horses or humans.

5.3 Yellow Fever Virus

The disease caused by yellow fever virus (YFV) in nonhuman primates (NHPs) is essentially indistinguishable from the disease in humans, including the hemorrhagic manifestations observed [244]. The main difference is that the disease in NHPs appears to have a more rapid course, with death occurring at 7 days post exposure, instead of the typically longer recurrent fever course seen in fatal human cases. Due to both hepatic and immunologic consequences of infection, yellow fever appears to resemble an inappropriate and overwhelming immune response, similar to that seen in severe sepsis. Conversely, experimental YFV infection of hamsters more closely resembles the hepatic impairment of yellow fever in humans, during which the degree of liver insult is prognostic for lethal disease.

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Chapter 15
Flaviviruses: Introduction to Dengue Viruses

Margot Carocci, Jens H. Kuhn, and Priscilla L. Yang

Core Message Flaviviruses are a major concern of the World Health Organization, and dengue virus is now the most widespread arthropod-borne viral disease infecting humans. Currently no vaccine or specific antiviral drugs are available to combat dengue fever. Thus, investigations and research to better understand this virus and its relation with its host are of great importance.

1 Flaviviruses

1.1 Etiologic Agent and Natural History

1.1.1 Definition

Flaviviruses *sensu lato* are all the members of the viral family *Flaviviridae*, which includes the four genera *Flavivirus*, *Hepacivirus*, *Pegivirus*, and *Pestivirus*. Members of these different genera are distantly related but their genomes share a similar gene order and conserved nonstructural protein motifs [1]. Important and well-known members of the family are bovine viral diarrhea virus, classical swine fever virus, dengue viruses, hepatitis C virus, and yellow fever virus.

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1.1.2 Etiology and Evolution

This chapter addresses the members of the genus *Flavivirus* (flaviviruses *sensu stricto*). The first bona fide flavivirus characterized was yellow fever virus (YFV), the causative agent of yellow fever (named after the prominent icterus of infected patients). Together with YFV, more than 70 viruses are currently classified in the genus, which also is the taxonomic home of other important human and animal pathogens such as dengue viruses (DENV1-4), Japanese encephalitis virus, Murray Valley encephalitis, St. Louis encephalitis virus, tick-borne encephalitis virus (TBEV), and West Nile virus (WNV) [1]. Flaviviruses can be categorized into three groups based on phylogenetic analysis of genomic sequences, and these groups correlate largely with their respective insect vector: the mosquito-borne viruses, the tick-borne viruses, and viruses for which no vector has been identified and that may spread without a vector. Table 15.1 summarizes the major characteristics of some well-known flaviviruses. Dengue fever, transmitted by mosquitoes, is currently the most widespread arthropod-borne viral disease of humans. Due to this prevalence, we here use DENV as an example to discuss, introduce, and describe flaviviruses.

1.1.3 Geographic Distribution and Economic Effects of Dengue Virus Infection

In the past 50 years, dengue viruses have become a major global human health threat. The World Health Organization estimates that about 100 million human infections occur annually, whereas a recent study estimated this number to actually be closer to 300 million [2]. Dengue fever is predominantly found in tropical and subtropical regions. In 2013, more than half of the world's human population lived in areas at risk of infection [3, 4]. Currently, there is no specific treatment for or vaccine against these viruses. Control of the primary DENV vector, the *Aedes aegypti* mosquito, is currently the principal measure available to prevent and control DENV transmission.

1.2 Pathogenesis and Clinical Features

1.2.1 Pathogenesis and Immunology

Four antigenically and phylogenetically distinct dengue viruses are known to be established in humans although sylvatic viruses that lie outside of these four serotypes can also infect humans. Immunoglobulin M (IgM) can be detected at the end of the febrile phase of dengue fever, followed by a moderate IgG response that is thought to confer lifelong protection against that DENV serotype. During subsequent infection with a heterologous serotype of dengue virus, IgG against both serotypes arise rapidly, but antibody titers are higher against the first virus serotype than against the second virus serotype [5].

Table 15.1 Characteristics of clinically important flaviviruses

Viruses	Vertebrate host	Clinical signs	Prevalence/epidemiology	Ref.
Dengue virus 1-4 (DENV1-4)	Humans	See text	See text	See text
West Nile virus (WNV)	Birds; humans and horses are accidental hosts	• Approximately 20 % develop influenza-like signs for 3–6 days • Less than 1 % develop severe (meningitis, encephalitis, myelitis) or lethal disease, especially in elderly and immunodeficient patients	• US 2012 outbreak: total of 5,387 cases (243 deaths) in humans • Enzootic and endemic in Africa and Asia. More sporadic in America and Europe	[98–100]
Japanese encephalitis virus (JEV)	Birds, pigs, and humans	• Incubation period: 5–10 days. • Most cases are asymptomatic or mild • About 1/300 infections induce disease ranging from febrile illness to severe meningoencephalitis (lethal in 25 %) and long-term neurological defects	• 30,000–50,000 total cases/year • 10,000–15,000 deaths/year in Asia and Australasia	[9, 101–103]
Yellow fever virus (YFV)	Humans, nonhuman primates	• Fever, chills, malaise, headache, back pain, nausea, dizziness, jaundice and hemorrhage • ≈15 % of cases develop severe disease with hemorrhage • ≈20–50 % of patients develop fatal liver and kidney failure	• ≈200,000 worldwide cases/year, causing 30,000 deaths • Increase observed over the last 10 years • Endemic in tropical areas of Africa and South America	[9, 104]
Tick-borne encephalitis virus (TBEV)	Humans, small mammals, birds, and livestock	• Influenza-like illness with severe headache, nausea, vomiting, and severe back pain; often focal epilepsy, flaccid paralysis (e.g., shoulder girdle) • ≈30 % of cases develop a second phase with high fever, CNS signs, (meningoencephalitis, paralysis), with possible sequelae or death • The case-fatality rate: ≈20 % with Asian strains, 1–5 % European strains	• 10,000–12,000 cases/year • Endemic in forested areas of Europe, northern China, Mongolia, and Russia	[9, 105]

Clinical signs of dengue fever appear 3–14 days after a bite by a DENV-infected female mosquito. These signs vary from unapparent, mild febrile illness to severe dengue infection that can result in complications and death. About 1–70 % of the infections lead to influenza-like disease characterized by high fever, rash, joint and muscle pain, and mild hemorrhagic manifestations, such as petechiae, purpura, ecchymoses, and nose bleeds [3, 6–8]. Dengue fever can progress to a painful and debilitating disease commonly referred to as "break bone fever." Severe dengue, formerly referred to as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), is characterized by a significant increase in vascular permeability, plasma leakage, microvascular bleeding, and reduced functioning of the coagulation cascade. Hemorrhages occur in multiple organs, frequently including the gastrointestinal tract, and fluids may pool within body cavities. Severe dengue is characterized by a sudden drop in blood pressure due to vascular leakage that leads to hypovolemia and collapse of the vascular system. Since there is currently no specific antiviral treatment for DENV infection, supportive care that maintains the patient's body fluid volume is critical [9]. Under the supervision of experienced medical personnel, supportive care can reduce fatality rates for severe dengue from more than 20 % to less than 1 %.

Despite much study, the determinants of disease severity in dengue infection are complex, and the absence of a fully immunocompetent animal model that faithfully reproduces all aspects of human dengue virus infection has delayed scientific progress in this area. Considerable evidence exists that disease is correlated with greater viral burden [7], although some patients experiencing secondary infection with a heterologous serotype of DENV exhibit high viremia in the absence of severe disease [3]. Prior infection with a heterologous serotype of DENV has been identified as a major risk factor for severe dengue fever. Enhancement of secondary dengue fever is thought to be due to non-neutralizing antibodies that promote uptake and productive entry of virions (reviewed in ref. 10). The antibody-dependent enhancement (ADE) of viral infection is a phenomenon that has been described for several flaviviruses [11–13]. In particular, antibodies that recognize the DENV structural precursor membrane protein (prM) are thought to promote ADE by increasing uptake of immature virions. Since the presence of infection-enhancing antibodies is not always correlated with disease severity [14], other variables including viral [13, 14] and host genetics [11, 12] and kinetics (i.e., time between infections) are also likely to be important.

1.3 Flavivirus Characteristics

1.3.1 Flavivirions

Flavivirions are spherical particles of approximately 50 nm in diameter. Each virion contains a single copy of the positive-sense RNA genome surrounded by multiple copies of the viral core protein to form the viral nucleocapsid. This nucleocapsid is

protected by a lipid bilayer bearing 180 copies of the viral envelope protein (E) arranged as a well-ordered lattice of 90 homodimers organized in a herringbone pattern on the virion surface [15]. A glycoprotein, E, functions in viral entry by mediating the virion's interactions with entry factors and receptors and undergoing conformational changes. These conformational changes catalyze fusion of viral and cellular membranes following exposure to acidic pH in the endosome (see below). On immature virions, the E glycoprotein forms trimers in which each E monomer is protected by a copy of the chaperone premembrane protein, prM [16]; each trimer of E-prM heterodimers forms a "spike" on the virion surface [17] (schematic representation in Fig. 15.1). This association of E with prM prevents premature fusion of immature virions with cellular membranes in the acidic environment of the Golgi [18, 19]. The prM protein is processed to pr and M by furin, and pr is released by the virion once in the neutral pH of the extracellular space. This release produces the smooth-surfaced, mature virion observed in cryo-electron microscopy reconstructions [20, 21]. Studies using neutralizing antibodies indicate that E protein on the virion surface undergoes dynamic structural changes at physiological temperatures, and these changes have a significant effect on the accessibility of epitopes and neutralization of virus [22].

1.3.2 Flavivirus Genomes

The DENV genome is a single-stranded linear RNA of positive polarity ((+)ssRNA)) of approximately 11 kb (Fig. 15.2). Like that of other flaviviruses, the genome encodes a single open reading frame (ORF) flanked by highly structured 5′ and 3′ untranslated regions (UTRs) of about 100 and 400 nucleotides, respectively. The 5′ end bears a type I cap structure (m^7GpppAmG) for cap-dependent translation of a single polyprotein. The 3′ UTR terminus lacks a polyadenylated tail but ends with a highly conserved stem loop structure (3′SL). A high number of *cis*-acting RNA elements, located in the coding and noncoding regions of the genome, act as promoters, enhancers, and circularization signals that are required for efficient RNA replication or translation (for reviews see refs. [23–25]). A RNA hairpin structure in the capsid-coding region (cHP) directs start codon selection and is also required for viral replication [26]. The highly conserved 3′SL is required for viral RNA replication [27–29] but also promotes translation by facilitating binding of the RNA to polysomes [30].

1.3.3 Flavivirus Proteins

The single polyprotein encoded by flavivirus genomes is cleaved into ten individual proteins by a combination of viral and cellular proteases and peptidases. For DENV, these cleavages are catalyzed by the viral NS2B-NS3 protease, host signal peptidase (a protease of the *trans*-Golgi network), and by furin [31, 32]. Collectively, these cleavage events lead to the production of ten mature viral proteins: three structural

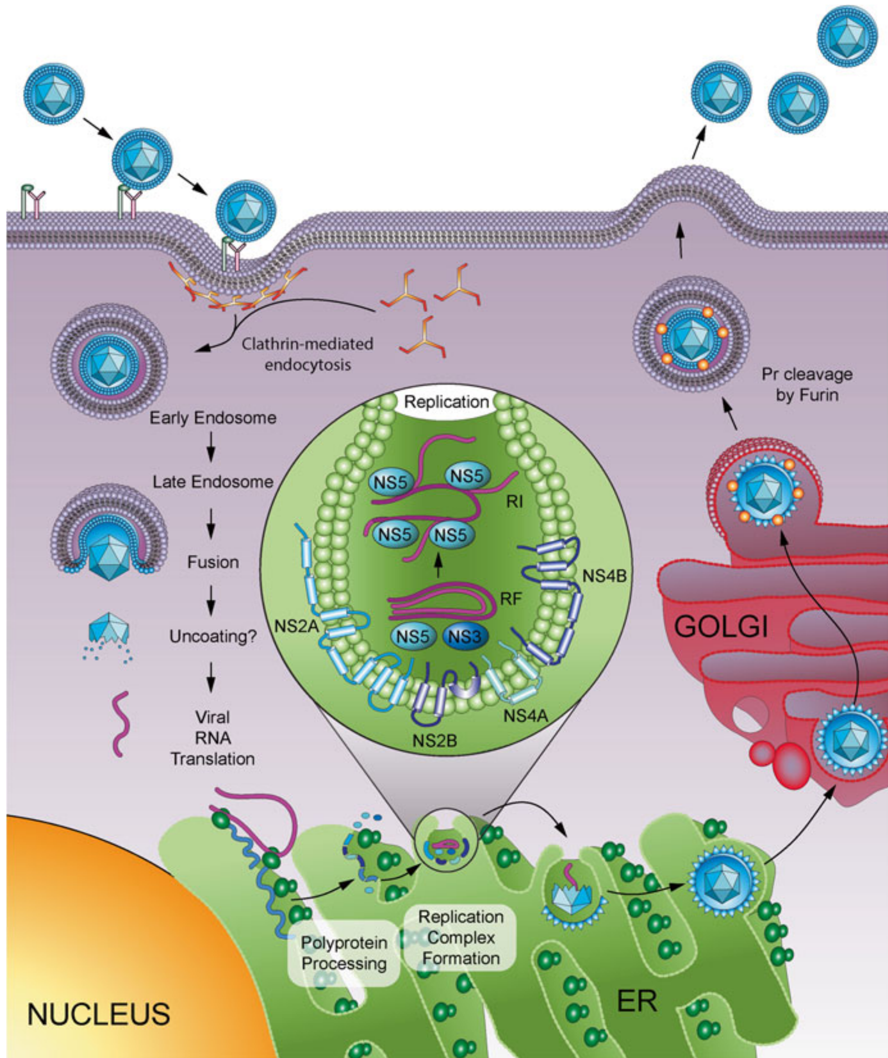

Fig. 15.1 Dengue cycle. Mature virus particles bind to host cell receptors and coreceptors and are internalized by clathrin-mediated endocytosis. In the late endosome, acidic pH triggers structural rearrangements of E that catalyze fusion of the viral and endosomal membranes. This fusion process is also affected by the presence of negatively charged lipids in the target endosomal membrane. The decapsidation process is not well known, but the released viral genomic RNA (*light pink*) is directly translated by the cellular ribosomes (*green*) to produce viral polyproteins (*blue*). The polyprotein is cotranslationally and posttranslationally processed by viral and host proteases. The nonstructural (NS) proteins produced from the polyprotein induce membrane rearrangement (invagination in the endoplasmic reticulum) to form the specialized compartment in which the replication complex is assembled and genome replication occurs. The viral genomic (+)ssRNA (light pink) is a template for the synthesis of a negative-sense RNA by NS5 and leads to the formation of double-stranded RNA (dsRNA) intermediates called the replication form (RF). The newly synthesized (−)ssRNA (dark pink) is used in turn as a template for the synthesis of multiple (+)ssRNA via a replication intermediate (RI). Newly synthesized (+)ssRNA can either serve as a template for translation or replication or undergo encapsidation. During encapsidation, RNA interacts with the capsid protein and buds into the lumen of the endoplasmic reticulum, thereby acquiring lipid bilayer and the precursor membrane (prM) and envelope (E) viral proteins.

Fig. 15.2 Flavivirus genomic organization. The genome of Dengue viruses is a positive, 5′-capped, single-stranded RNA. The genomic RNA consists of a single ORF that encodes a polyprotein (*dark grey*) flanked by 5′ and 3′ untranslated regions (UTRs). The polyprotein is cleaved during and after translation by the viral NS2B-NS3 proteases (*open triangles*) or host proteases, such as furin (*arrow*), signal peptidase (*closed triangles*), or unknown factors. Abbreviations: *AUG* translation initiation codon, *C* capsid, *cHP* capsid hairpin, *E* envelope, *NS* nonstructural, *PK* pseudoknot, *prM* precursor membrane, *SL* stem loop, *UAR* upstream of the AUG region

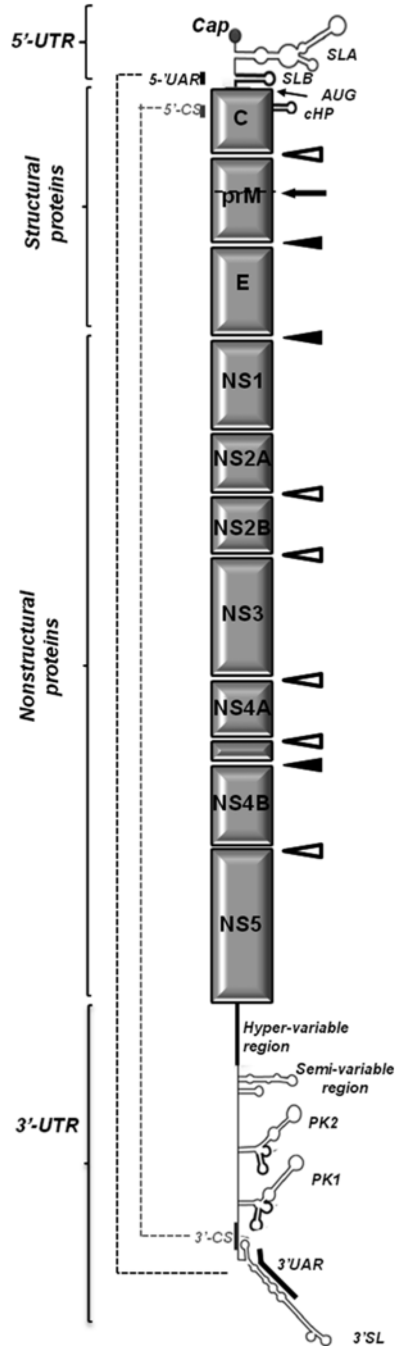

Fig. 15.1 These immature viral particles, recognized by the spikes of prM-E heterodimers on the particle surface, are transported along the secretory pathway to the Golgi. Proteolytic processing of prM by the host furin protease (*orange*) produces a pr peptide that remains associated with the viral particle until it reaches the neutral pH of the extracellular environment, where it is released from mature viral particles (smooth surface with E homodimers)

proteins (core/capsid (C), pre-membrane (prM), and envelope (E)) and seven nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). An overview of the characteristics of these structural and nonstructural proteins is provided in Tables 15.2 and 15.3, respectively.

1.4 Flavivirus Replication Cycle

1.4.1 Flavivirus Cell Entry

Flavivirion entry is complex, and the exact mechanisms involved are still not fully elucidated. The general events leading to productive flavivirus entry include attachment of the virion to the cell surface, particle endocytosis, fusion with the endosomal membrane, movement of the nucleocapsid into the cytoplasm, and delivery of the viral genome to the site of translation (Fig. 15.1).

The E glycoprotein mediates DENV entry and functions at several steps during this process, beginning with attachment of the viral particle to the plasma membrane of the target cell. This attachment to the cell surface is mediated by the putative receptor binding domain located in domain III of the E glycoprotein [33]. Domain II, which contains the hydrophobic fusion loop, is protected between domains I and III in mature virions. Exposure to acidic pH leads to significant structural changes including insertion of the fusion peptide into the target endosomal membrane and refolding of E as a post-fusion trimer. While conserved histidine residues in the E protein of TBEV function as a "switch" triggering membrane fusion upon protonation [34], this protonation is not required for WNV entry [35]. Likewise, fusion catalyzed by the DENV E protein appears to be regulated by networks of residues located (1) proximal to the fusion loop, (2) in the "latch" between E and M, and (3) in the hinge regions between domains I–II and domains I–III. Collectively, these residues stabilize the pre-fusion dimer with the fusion peptide protected at neutral pH and promote refolding of E into its post-fusion trimeric conformation upon exposure to acidic pH [36]. In vitro, an acidic pH is sufficient to trigger fusion and nucleocapsid release of DENV and other flaviviruses [37, 38]. In vivo, DENV fusion is thought to occur within a small endosomal vesicle in the late endosome via a process that requires the presence of negatively charged lipids [39]. Anionic lipids act downstream of the low-pH-dependent step and promote the steps of fusion from the earliest hemi-fusion intermediates to opening of the fusion pore [39].

Although DENV has broad cell tropisms, the target cells in humans are primarily dendritic cells (DC), monocytes, macrophages, and hepatocytes. Many different cellular proteins facilitate entry of DENVs including, heparan sulfate expressed at the surface of most cell types [40, 41], dendritic-cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) expressed by immature DCs [42], heat-shock proteins 70 and 90 [43], glucose-regulated protein 78 [44], laminin receptor [45], mannose receptor [46], and the T-cell immunoglobulin and mucin domain (TIM) and tyrosine 3-, AXL-, and MER-tyrosine kinase (TAM) family of phosphatidylserine receptors [47, 48]. The prevailing model today suggests a

Table 15.2 Overview of dengue virus structural proteins

Protein	Genomic sequence, protein properties and domains	Function	Ref.
Capsid (C)	• nt 97–396/438 • Immature C: 114 aa • Mature C: – 100 aa/11 kDa – Central hydrophobic region • 3 NLS • N- and C-terminal charged residues	• Forms nucleocapsid with viral genomic RNA • Immature C: anchored to ER membrane by 14 aa in C-terminal, cleaved by NS2B-3 to form mature C • Mature C: associates with ER membrane through internal hydrophobic region • 18 basic N-terminal aa required for viral encapsidation and possibly interaction with RNA • Binding to lipid droplets required for viral particle formation • Interacts with nucleolin during virion morphogenesis. Inhibition of nucleolin decreases viral titer but not RNA synthesis • Interacts with hSec3 to delay DENV translation and replication possibly through sequestration of eEF1α • 3 NLS interact with DAXX and induces apoptosis	[80, 81, 106–111]
Precursor membrane (prM)	• nt 439–936 • 166 aa/34 kDa • pr: 91 aa/26 kDa • M: 75 aa/8 kDa	• Interacts with claudin-1 for efficient virus entry • Forms heterodimer with E protein to prevent premature fusion of immature virion with host membrane • His residue at M39 in M protein influences virus assembly • Host furin in post-Golgi vesicles cleaves the prM into “pr” and “M” for virus maturation • prM and vacuolar-ATPase interaction influences efficient virion egress	[16, 112–114]
Envelope (E)	• nt 937–2421 • 495 aa/50 kDa • 3 domains (I–III)	• Class II fusion protein • Mediates attachment to cell surface entry factors • Undergoes structural changes at low pH that are coupled to fusion of viral and endosomal membranes during viral entry • Interacts with ER resident chaperones for folding and virus assembly • Among flaviviruses, Asn 67 (in DENV) associated with hemorrhagic fever • Major immunogenic protein; interacts with NKp44, a NK surface receptor, and activate NK cells	[16, 115–118]

Abbreviations: *Aa* amino acids, kDa kiloDalton, *DAXX* death domain-associated protein, *EF1α* elongation factor 1 alpha, *ER* endoplasmic reticulum, *His* histidine, *NLS* nuclear localization signal, *NS* nonstructural, *NK* natural killer, *nt* nucleotide

Table 15.3 Overview of dengue virus nonstructural proteins

Protein	Genomic sequence, protein properties and domains	Function	Ref.
NS1	• nt 2422–3477 • 352 aa/46–55 kDa • Glycosylated	• Cell-membrane-bound form: intracellular vesicular compartments, cell surface • Soluble form: secreted as hexameric lipoparticle • Implicated in disease pathogenesis and protection • Circulates in sera of DENV-infected patients • Important biomarker for early diagnosis • Interacts with NS4A, co-localizes with dsRNA • Involved in RNA replication but exact role undetermined	[71, 72, 122, 123]
NS2A	• nt 3478–4131 • 218 aa/22 kDa	• Transmembrane protein, associates with the ER membrane • Component of the viral replication complex • Functions in virion assembly • Along with NS4A and NS4B, inhibits type 1 IFN signaling upstream of STAT1 activation	[119, 120]
NS2B	• nt 4132–4521 • 130 aa/14 kDa	• Transmembrane protein, associates with the ER membrane • Component of the viral replication complex • Hydrophobic loop of NS2B necessary for NS2B-NS3 serine protease activity • NS2B-NS3 cleaves human adaptor molecule STING and inhibits type I IFN production • NS2B-NS3 interacts with IKKε to block IFN induction pathway	[121–123]
NS3	• nt 4522–6375 • 618 aa/70 kDa • C terminus domain: – NTPase/RNA helicase • N-terminal domain: – Serine protease – RNA 5’ triphosphatase	• RNA helicase and NTPase important for replication of the viral RNA genome • Activates fatty acid synthase and induces fatty acid production at the RC • NS2B-NS3 impairs type 1 IFN production • Interacts with La protein	[69, 124, 125]
NS4A	• nt 6376–6756 • 127 aa/16 kDa • C-terminal: 2 K fragment	• Associates with ER membrane • Component of the viral RC; may serve as a scaffold for formation of RC • Up-regulates autophagy in epithelial cells • Induces intracellular membrane rearrangements • Along with NS2A and NS4B, inhibits type 1 IFN signaling upstream of STAT1 activation	[67, 119, 126]
2 K	• nt 6757–6825 • 23 aa	• Regulates induction of intracellular membrane rearrangements by NS4A	[67]

(continued)

Table 15.3 (continued)

Protein	Genomic sequence, protein properties and domains	Function	Ref.
NS4B	• nt 6826–7569 • 248 aa/27 kDa	• Transmembrane protein • Component of the viral RC • Colocalizes with NS3, dissociates NS3 from ssRNA, and enhances the helicase activity of NS3 in vitro • Along with NS2A and NS4A, inhibits type 1 IFN signaling upstream of activation of STAT1	[119, 127]
NS5	• nt 7570–10269 • 900 aa/103 kDa • 2 NLS • 1 NES • RNA polymerase • MTase	• Mostly in nucleus (possesses 2 NLS), only hypophosphorylated NS5 in cytoplasm • RNA-dependent RNA polymerase, synthesizes positive and negative sense viral RNA • Caps 5′ end of viral genomic RNA through 2′O methyltransferase (MTase) activity • Interacts with and activates NS3 • Inhibits IFNα signaling via binding and inhibition of phosphorylation STAT2 • Interacts with La protein • CRM1-mediated nuclear export of NS5 modulates IL-8 induction and virus production	[73–76, 125, 128]

Abbreviations: *Aa* amino acids, *ds* double stranded, kDa kiloDalton *ER* endoplasmic reticulum, *IL* interleukin, *IFN* interferon, *NES* nuclear export signal, *NLS* nuclear localization signal, *NS* nonstructural, *nt* nucleotide, *RC* replication complex, *ss* single stranded, *STAT* signal transducer and activator of transcription, *STING* stimulator of interferon genes

multistep process in which E protein interacts sequentially with at least two cellular entry factors. Which host factor serves as the bona fide receptor for cell entry remains unclear. The initial interaction on the plasma membrane concentrates virions on the cell surface and thereby facilitates interaction of the virion with a high affinity, second receptor that mediates virion internalization through receptor-mediated endocytosis [49, 50]. The phenomenon of ADE, in which non-neutralizing antibodies increase infectivity by concentrating virus on the plasma membrane, is consistent with this model.

Following receptor binding, uptake of virions is a clathrin-dependent process [51, 52], and trafficking from early to late endosomes requires actin and microtubules [53–56]. Receptor binding of DENV on endothelial cells activates the RHO-family GTPases Rac1 and cell division control protein 42 (CDC42), which induce actin reorganization and formation of filopodia required for efficient virus entry [57, 58]. Following membrane fusion and creation of the fusion pore, the viral nucleocapsid traffics to the cytosol and is disassembled, and the RNA genome is delivered to the site on the endoplasmic reticulum (ER) membrane for translation. Although these events almost certainly are regulated by host factors, the specific molecular mechanisms remain poorly understood.

1.4.2 Flavivirus Translation

Flaviviruses, like most viruses, utilize the host cell machinery for translation of protein-coding ORFs. Flaviviruses have a capped, (+)ssRNA genome that is directly translated as a single polyprotein. DENV translation occurs mostly via cap-dependent initiation [59] although noncanonical initiation of translation has been described under conditions that inhibit cap-dependent translation in an internal ribosomal entry site (IRES)-independent manner [60]. During cap-dependent translation, initiation occurs when the eukaryotic initiation factor 4E (eIF4E) recognizes and binds the DENV genomic 5′ cap. Recruitment of the 43S preinitiation complex (composed of the 40S ribosomal subunit, eIF1A, eIF3, Met-tRNA-GTP) by eIF4F (composed of eIF4E, eIF4G, eIF4B, and the helicase eIF4A) leads to formation of the 48S complex. The 48S complex scans the viral 5′UTR, unwinding secondary structures until it reaches a start codon. Interestingly, the poly(A)-binding protein (PABP) can bind to the DENV genome 3′UTR (especially 3′SL) despite the absence of a poly(A) tail [61]. This finding suggests that circularization of the genome by the interaction of PABP with eIF4G is important for efficient translation. DENV and other mosquito-borne flaviviruses initiate translation of the C protein from a start AUG codon in a suboptimal context, and multiple additional in-frame AUGs are downstream from the start codon [26]. An RNA hairpin structure in the capsid coding region (cHP) (Fig. 15.2) directs translation start site selection [26]. The optimal distance from the start codon to the cHP is about 15 nucleotides [25], which corresponds to the footprint of a ribosome paused over a start codon [62]. Thus, the scanning initiation machinery is thought to pause at the structural cHP to unwind the cHP and, in this poor initiation context, the scanning initiation complex stalls momentarily over the first AUG [25, 26]. Association of the 60S subunit with eIF3 at the initiation codon forms the 80S ribosomal complex that is needed for translation elongation to proceed. The 3′SL present at the very end of the 3′UTR (Fig. 15.2) facilitates mRNA binding to polysomes and promotes efficient DENV mRNA translation, notably during the first round of translation in the absence of synthesized viral proteins [30].

Efficient translation of incoming genomes early in infection is an essential step in the flavivirus virus replication cycle. Synthesis of nonstructural proteins is a requirement for flavivirus replication since the cells do not possess an RNA-dependent-RNA polymerase capable of replicating flaviviral RNA. Deficiencies in translation can therefore significantly reduce viral replication and production of infectious particles [63].

1.4.3 Flavivirus Replication

The viral (+)ssRNA genome (light pink in Fig. 15.1) initially serves as the template for translation of the viral polyprotein, but eventually serves as a template for RNA replication. The transition between these two processes is not fully understood. The incoming (+)ssRNA is a template for the synthesis of a negative-stranded RNA that leads to the formation of double-stranded RNA (dsRNA) intermediates called

the replicative form (RF) [25]. The newly synthesized (−)ssRNA (dark pink in Fig. 15.1) is used in turn as a template for the synthesis of multiple (+)ssRNA via a replication intermediate (RI). Approximately five nascent (+)ssRNAs are present on an RI and 12–15 min are necessary to synthesize each strand [64]. This process leads to the production of ≈10–100-fold more (+)ssRNA than (−)ssRNA [29]. Thereafter, the (+)ssRNAs serve as substrates for a new round of translation, templates for production of (−)ssRNA, or are encapsidated for assembly into new virions. Replication of the flaviviral genome does not occur freely in the cytoplasm, probably to limit recognition of the dsRNA intermediates by intracellular cytosolic innate immune sensors such as retinoic acid-inducible gene 1 (RIG-1) and melanoma differentiation-associated protein 5 (MDA5). Concentration of the necessary substrates and catalysts within these specialized membrane-bound compartments also serves to increase the efficiency of RNA replication.

Extensive intracellular membrane rearrangement takes place in flavivirus-infected cells [65], and electron tomography, immuno-electron microscopy, and transmission electron microscopy have been used to characterize the architecture of DENV-induced membrane alterations [66]. DENV replication occurs inside replication complexes (RC) formed by membranous invaginated vesicles (≈90 nm in diameter) that are derived from the ER and are associated with most of the DENV NS proteins (schematic representation of the RC in Fig. 15.1). NS4A, along with additional viral and host factors, induces the membrane curvatures and rearrangements for invaginated vesicle formation [67, 68].

During DENV infection, fatty acid synthase re-localizes to the RC and is activated by interaction with NS3. The newly produced lipids are incorporated into RC, as these lipids co-purify with viral RNA in biochemical fractionation experiments. [69]. This incorporation may facilitate the extension of membranes and the formation of the RC. Virus-induced autophagy also leads to liberation of fatty acids from lipid droplets, and these fatty acids undergo beta-oxidation, producing ATP that fuel the energy demands of replication [70]. The hydrophobic NS2A, NS4A, and NS4B proteins are thought to anchor the RC to the ER membrane [67, 71, 72] although the molecular details of these interactions are still subject to investigation. Likewise, although the NS1 protein is known to interact with NS4B and to be essential for viral RNA replication, its function in this process remains obscure [71, 72].

NS5, a bifunctional protein with N-terminal methyl transferase and C-terminal RdRp-dependent RNA polymerase (RdRp), possesses two nuclear localization signals (NLS) [73] and a nuclear export signal (NES) [74]. NS5 is mostly located in the nucleus as a phospho-protein and only the hypophosphorylated form is located in the cytoplasm. Within the cytoplasm, NS5 replicates viral RNA and also interacts and modulates the enzymatic activity of NS3 [74–76]. NS3 is a multifunctional protein required for polyprotein processing and RNA replication. Besides its N-terminal protease activity, NS3 has RNA-stimulated nucleoside triphosphatase (NTPase), RNA helicase, and RNA triphosphatase (RTPase) activities that are absolutely required for viral RNA replication. RTPase activity is believed to be responsible for the dephosphorylation of the 5′ end of the genomic RNA before cap addition by NS5 [77]. NS3 binds to the 3′SL and NS5 enhances NS3's NTPase activity [78].

A number of *cis*-acting RNA elements, located in the coding and noncoding regions of flavivirus genomes, act as promoters, enhancers, and circularization signals necessary for efficient RNA replication [23–25]. The two inverted pairs of complementary sequences (5′-3′ CS and 5′-3′ upstream of AUG region [UAR], Fig. 15.2) at both extremities of the genome are required for genome circularization. The replication process begins with 5′-3′ UAR and 5′-3′ CS hybridization, which triggers circularization of the genome. NS5 binds specifically to the 5′ stem-loop A (SLA), and through this long range RNA-RNA interaction the 5′ promoter and the 3′ end of the genome are brought together. This enables transfer of NS5 to the site of initiation at the 3' end of the genome and the initiation of RNA synthesis. Circularization of the viral genome may also play an important role during ORF translation. Although both circular and linear forms of DENV RNA are necessary for virion production, viral RNA replication is highly vulnerable to changes that alter the balance between circular and linear forms of the RNA [79].

1.4.4 Flavivirion Egress

The last steps of the flavivirus replication cycle are the encapsidation of genomic progeny RNA, envelopment of the viral nucleocapsid, maturation of the virion surface proteins, and egress of infectious particles. Encapsidation of flavivirus genomes is thought to be directly linked to genome replication, as only nascent (+)ssRNAs from the RC are encapsidated [71]. Indeed, the presence of budding vesicles that may correspond to the formation of viral particles at the ER membrane is directly apposed to the RC [66]. Two clusters of basic amino acids that confer a high density of positive charges at the N-terminus of the C protein are essential for genome encapsidation in human cells [80]. Since encapsidation in mosquito cells still occurs when these residues are deleted, albeit in a less efficient fashion, interaction of the N-terminus of the C protein with a host factor likely differs between humans and mosquitoes.

The DENV C protein accumulates on the surface of lipid droplets (LD), and loss of LD targeting results in abrogation of particle formation [81]. This observation indicates a role of LD in virion assembly or release. In contrast to hepatitis C virus, no evidence of recruitment of lipid droplets to the RC or DENV RNA to the lipid droplets has been reported [66, 82]. An alternate explanation for the sequestration of C protein by LD is that this sequestration prevents the binding of C to newly synthesized viral RNA and thus averts premature encapsidation of the viral genome and the inhibitory effect this would have on replication of the viral genome [82]. Interestingly, DENV induces autophagy and degradation of the LD in autophagosomes to liberate fatty acid and produce energy [70, 82]. Although the kinetics and the sequence of these events have not yet been described in detail, coupling of virus-induced autophagy to the release of C protein is hypothesized to provide a mechanism for delaying viral assembly until sufficient genome replication has occurred.

Virus assembly occurs at the surface of the ER. The DENV C protein associates with newly synthesized RNA genomes liberated from the RC through a pore-like structure [66]. Newly formed immature virions contain a genomic RNA within an icosahedral capsid. Budding of this capsid into the lumen of the ER in close proximity

to the RC results in its envelopment within a lipid bilayer, yielding immature virions in which trimers of E and prM heterodimers appear as spikes on the particle surface. Individual virions travel toward distal sites of the ER lumen and are thought to be collected in dilated ER cisternae [66]. Maturation of these immature particles occurs as they traffic through the host secretory system. Processing of the N-linked glycan on the E protein by host enzymes in the ER and Golgi is required for efficient secretion of infectious virions in mammalian but not insect cells [83]. The prM portion of the trimer with E is cleaved by furin. The pr protein, corresponding to the N-terminal 91 residues of prM, dissociates from the virion upon exposure to neutral pH in the extracellular space while the M protein remains in the virion [16]. Processing of the dengue virion is inefficient, as a large proportion of secreted virions have unprocessed prM [84, 85].

1.5 Vaccines and Antiviral Agents

Vector control has been the most widely used strategy to prevent dengue virus infection. No FDA-approved vaccine is currently available to prevent dengue fever. The risk that an ineffective vaccine might exacerbate infection through ADE has provided an additional challenge to efforts to develop a vaccine providing pan-serotype protection. Several candidate antivirals show promise in vitro and in animal models of DENV infection (reviewed in ref. 86, 87). These include agents that act against viral targets, such as ST-148, which targets the C protein, and NITD-008, a nucleoside that inhibits the NS5 RNA-dependent RNA polymerase. NITD-451 and other benzomorphans are specific inhibitors of DENV translation. Some antisense morpholino-oligomers that directly or indirectly affect translation of DENV [88, 89] and other flaviviruses [90–92] are potent inhibitors of these viruses in cell culture.

Compounds that inhibit DENV translation and replication are currently under investigation as potential antivirals [93, 94]. Although some of these agents (e.g., NITD-008, NITD-451) have demonstrated antiviral activity in a mouse model of DENV infection, their therapeutic window is limited [87, 94]. In addition, fenretinide, a synthetic retinoid, has been shown to inhibit the replication of DENV and other flaviviruses in cell culture and in mouse models through effects on genome replication [95, 95b]. Celgosivir, which targets the host alpha-glucosidase, is thought to inhibit DENV pathogenesis and replication by causing misfolding of E, prM, and NS1 proteins [96, 97]. Now being tested in a phase Ib trial for treatment of dengue fever, celgosivir is currently the most advanced clinical candidate.

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Chapter 16
Flavivirus Encephalitis: Immunopathogenesis of Disease and Immunomodulation

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Core Message This chapter outlines innate and adaptive immune changes in the brain associated with flavivirus encephalitis. It reviews the data from various currently used models in vivo and in vitro and highlights some of the issues with interpretation of these. In the approaches to disease, it is argued that a carefully timed immunological intervention is possible by specifically reducing the massive influx of macrophages into the brain in encephalitis to ameliorate the often fatal immune damage done by these cells and enable normal immune components to clear the virus.

1 Background

The Flaviviridae are a family of single-stranded, positive-sense RNA viruses, representatives of which are found on all inhabited continents. Most have an enzootic cycle between an arthropod host, usually a mosquito, and an amplifying mammalian or bird reservoir, with humans inadvertently intersecting this. Although humans may themselves transmit these viruses to arthropod vectors, for some flaviviruses humans are incidental, or dead-end hosts, as they are thought in the main not to generate high enough viremias for transmission to arthropod vectors [1]. The abundance of mosquitoes in high-risk regions, the inability to restrict migration of

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reservoir hosts (mainly birds), the increasing extent of vector habitats due to expansion of human habitation and the effects of global warming, as well as the increased availability and ease of world travel, make it virtually impossible to prevent flavivirus spread [2]. Moreover, effective treatment and prevention options are lacking for many flavivirus infections, which include severe neurotropic or viscerotropic disease, depending on the particular virus and its target organ. Thus, flaviviruses pose both an emerging and ongoing global health threat. In this review we will focus on the neurotropic aspects of flavivirus infection involved in driving disease pathogenesis in encephalitis that offer possibilities for intervention.

The medically relevant neurotropic flaviviruses include West Nile virus (WNV), found in Africa, the Middle East, Europe, the Americas and Western Asia, Japanese encephalitis virus (JEV), restricted to Asia and the Subcontinent, Tick-borne encephalitis virus (TBEV), in Central Europe, Murray Valley encephalitis virus (MVEV), in Northern Australia and St Louis encephalitis virus (SLEV), in North America. Except for TBEV, these viruses are all members of the JE serogroup [1, 3]. Although non-pathogenic, the Kunjin strain of WNV (KUNV), also a member of the JE serogroup found in Northern Australia, is of interest, since it is genetically very similar to the Lineage I NY99 strain of WNV and induces an immune response in mice that cross-protects against this virulent strain [4].

WNV is probably the most widely spread flavivirus, geographically. However, while Lineage I WNV is now active in most regions, significant outbreaks of Lineage II WNV encephalitis in animals and man periodically occur in Africa and Europe and other lineages of WNV also cause neurotropic disease [5–8]. The incidence of Lineage I WNV infection has seen a substantial increase in the USA since a novel outbreak of encephalitis in New York in 1999, first attributed to SLEV [9, 10]. Within 10 years it had spread to all states of the USA and into Canada and Mexico. Recent estimates indicate that approximately three million people were infected with WNV between 1999 and 2010 in the USA, resulting in around 1,100 deaths [11, 12]. As most infections are asymptomatic and therefore not reported, these data are based on the number of WNV^{+} blood bank donations, with the irony that in the early phases of the outbreak in the USA, the unwitting transfusion of infected blood demonstrated unequivocal human-to-human transmission, despite viremias being too low to transmit to arthropod vectors [13, 14].

JEV is closely related to WNV, has a similar disease course and is endemic to more remote regions, making prevalence hard to determine, with estimates ranging between 20,000 and 68,000 cases a year, with up to 30 % fatality in encephalitis. Estimates suggest up to 50 % of survivors suffer from severe neurological sequelae and/or permanent disability [15, 16]. MVEV, also closely related to JEV, is endemic to Australia, generally confined to the northern part of the country, with occasional outbreaks related to periods of heavy rainfall [17]. Registered cases of KUNV infection are sparse and may be confused with MVEV infection, due to their serological cross-reactivity. However, MVEV causes more severe encephalitis and may be fatal, while none of the reported cases of KUNV infection have had a fatal outcome [18].

In contrast, the organotropic dengue virus (DENV), most prevalent in Asia, causes an estimated 50–100 million infections a year [12], with fatality rates for

patients with dengue hemorrhagic fever (DHF) ranging between 1 and 20 %, depending on the availability of medical care [19]. Furthermore, in recent years, infections with Yellow fever virus (YFV), the prototypic flavivirus endemic in Central Africa and Latin America, have risen dramatically, with about 200,000 reported cases a year, after relatively low occurrence in the past two decades [20].

1.1 Clinical Presentation

While most neurotropic flavivirus infections are asymptomatic, the clinical presentation of disease ranges from mild flu-like symptoms to severe encephalitic disease, which may be lethal, or result in life-long neurological sequelae. After a 2–14 day incubation period, approximately 20 % of WNV-infected individuals develop WNV fever, with symptoms such as headache, fatigue, fever and occasionally swollen lymph nodes. Ophthalmological symptoms may include blurred vision and vision loss, while chorioretinal streaks, chorioretinitis and occlusive retinal vasculitis may be seen on examination [21, 22]. These occur with greater frequency in patients with pre-existing ocular conditions, such as diabetic retinopathy [23]. The development of encephalitis, which occurs in about 1 % of infected individuals and has approximately 10 % mortality (~0.04 % of total infected), represents the most severe form of the disease. This is generally accompanied by high fever, disorientation, lack of coordination, convulsions and flaccid paralysis [24]. Similar to WNV, only about 1 % of JEV-infected individuals develop symptomatic encephalitic disease, but it usually presents more severely than WNV, with symptoms such as neck rigidity, convulsions and hemiparesis. About 1:500–1:1,000 MVEV-infected individuals develop encephalitis, but fatality rates of encephalitis are between 15 and 30 %, and only 40 % of patients recover fully, leaving many survivors with permanent neurological sequelae [18].

DENV infection can be asymptomatic, cause dengue fever or more severe DHF and dengue shock syndrome (DSS). With recent changes in the classification of DENV infection, disease is now ranked according to severity: dengue without signs, dengue with warning signs (elevated hematocrit, mucosal bleeding, persistent vomiting, liver enlargement) and severe dengue, (cases with extreme plasma leakage, bleeding or organ failure). Ocular complications have also been seen in DENV infection, with ocular pain being the most frequently reported symptom [25, 26], although macular haemorrhage and edema have also been observed [27]. However, cross-observational studies have not found any ocular manifestations in DENV cases, and it has been postulated that the development of DENV-mediated ocular conditions may be specific to certain dengue serotypes [28]. Although DENV is classically regarded as a hemorrhagic disease, infection can also have neurological complications, including encephalitis, neuro-ophthalmic disease and muscle dysfunction as well as immune-mediated syndromes. These have only recently begun to be recognised and studied in any detail.

1.2 Route(s) of Infection

Infection is initiated by the bite of an infected mosquito, which transfers virus to the dermis and the blood via its saliva. Mosquito saliva is argued to be immunosuppressive and may thus give the virus a replicative advantage before detection by the immune system [29], but this is still debated. Both Langerhans cells (LC) and dendritic cells (DC), derived from infiltrating Ly6C^{lo} monocytes in the skin, can be infected and may migrate to the draining lymph nodes (DLN) as the main carrier of viral antigen [30–32]. While WNV-productive replication in LC or DC has not been shown, DENV-infected DC support active replication of virus [33]. Although the exact mechanism of viral spread from the initial site of infection has not been defined, virus undoubtedly spreads via the bloodstream, resulting in systemic infection and febrile illness. There is much debate on the mechanism of subsequent viral entry into the brain. Three main possibilities exist: (1) immune- or virus-mediated breakdown of the blood–brain barrier (BBB), (2) “Trojan horse” transmission by infected monocytes and (3) centripetal nerve spread of virus from the periphery.

The BBB separates the CNS from the blood and is selectively permeable, preventing the entry of unwanted molecules and organisms. However, as flaviviruses evidently infect neurons in the brain, it is argued that the BBB may be breached directly or indirectly to achieve this. In JEV, direct endothelial infection has been shown in post-mortem brains [34] and this virus is transcytosed by CNS endothelium in suckling mice [35]. To our knowledge, WNV infection of endothelium in vivo has not been reported, despite its obvious susceptibility to infection in vitro [36] and the significant viremias that occur after intravenous (i.v.) or intraperitoneal (i.p.) inoculation in mouse models [37, 38] (King, Unpublished).

Indirectly, activation of toll-like receptor 3 (TLR3) during WNV infection has been implicated as a possible mediator of BBB disruption. Increased tumour necrosis factor (TNF) expression, induced by TLR3 activation, was implicated in BBB breakdown and TLR3 knockout (KO) mice displayed reduced BBB leakiness and lower viral titres in the brain than in wild type (WT) control mice [39]. Further findings in human monocyte-derived macrophages were taken to support this. Despite their much higher baseline levels of TLR expression, WNV inhibited in vitro macrophage expression of TLR3 in individuals in the third and fourth decade of life (20–36 years old), who are less prone to develop WNV encephalitis, while increasing TLR3 expression in cells from a more susceptible elderly (>55 years old) cohort [40]. The use of in vitro-differentiated adherent macrophages limits the conclusions of this study, however, since infected monocytes are more likely to participate in endothelial interactions in vivo. Moreover, replication of the murine work, using various routes of inoculation, as well as WNV grown in insect or mammalian cells, showed that TLR3 KO mice were more susceptible to infection, with increased viral loads in the brain, contradicting these findings [41]. A further problem with the involvement of TLR3 in BBB breakdown is that the correspondence of breakdown and CNS infection is poor in flavivirus models, indeed, it may not occur even after CNS infection [42–44], strongly suggesting that virus entry occurs via a different route than across the BBB and that breakdown of the BBB is not temporally

associated with the entry of virus, but rather the entry of inflammatory cells into the brain well after virus ingress.

Finally, astrocytes form a supportive component of the BBB, but have also been implicated as a possible mediator of BBB breakdown. WNV infection of astrocytes in vitro results in upregulation of matrix metalloproteinases (MMP), host proteases involved in the restructuring of tight junction proteins [45]. Therefore, it has been suggested that astrocyte infection results in MMP-mediated BBB breakdown. However, while astrocyte infection clearly occurs in vitro [46], in vivo infection has not been convincingly shown.

The second, so-called "Trojan Horse" scenario,[1] proposes that virus-infected monocytes cross the BBB, thus allowing the virus to enter the CNS undetected. This is thought to occur in HIV infection, where infection of the brain is argued to be due to the homeostatic replacement of HIV-infected perivascular macrophages from the bone marrow [47]. However, WNV infection is comparatively acute and although macrophages are susceptible to infection in vitro, these cells quickly control WNV, unlike HIV, making further spread unlikely [48]. Neutrophils are also susceptible to WNV infection and contain more WNV virions than infected macrophages and could be a more likely Trojan Horse for CNS entry by WNV [49]. However, neutrophils make up <3 % of the leukocyte infiltrate into the CNS, and while certainly present in the meningeal vasculature, are rare in the parenchyma [50]. We have never seen infection of T or B cells by WNV and to our knowledge, none has been reported, making lymphocytes dubious candidates for a Trojan Horse mechanism. While activated T cells are capable of circulating through the brain parenchyma [51], myeloid lineage cells are rare under normal homeostasis and are likely to be replenished in situ [42, 52, 53]. Furthermore, were leukocytes to transmit WNV into the brain by this route, it seems unlikely that only neurons would be infected, considering the susceptibility of virtually all cell types in the brain to infection in vitro. Finally, ignoring the likely altered function of an infected myeloid cell [54] and possible perturbation of its migratory capacity, in order for viral entry into the CNS to occur via a true Trojan Horse mechanism, chemokines for infected monocytes or neutrophils would have to be produced in the brain prior to viral entry into the CNS. To our knowledge, soluble factor profiles consistent with this scenario have not been reported.

The third hypothesis, centripetal nerve spread, suggests that virus spreads from the initial peripheral location of infection to the CNS via the nervous system. Evidence in support of this is seen in multiple models of infection. The footpad model of injection is used to mimic infection via the skin. Sensory fibres of the dorsal root ganglia of the peripheral nervous system innervate this part of the body. The capacity of WNV to infect both the dorsal root ganglia and peripheral nervous system neurons has been shown in vitro [55, 56] and use of microtubule inhibitor

[1] named after the giant wooden horse of Greek mythology, famously left outside the gates of Troy by the warring Greeks, ostensibly as an offering to the goddess, Athena, for their safe passage home after a 10 year siege of the city, in a ruse to make the Trojans think the Greeks had given up the war. Against the better judgment of some, the "Trojan Horse" was drawn into the city as a trophy, hiding in it a detachment of Greek soldiers who subverted the defences of Troy by night to let in the hidden Greek army and vanquish Troy.

nocodazole, which prevents axonal transport, was shown to significantly delay the distribution of WNV to the CNS, further supporting retrograde transport [57]. Intraperitoneal infection of mice showed detectable virus in the cervical cord 1–2 days before detection in the brain, thereafter spreading from caudal to rostral in the brain (King, unpublished). Detection of WNV in the brains of mice infected via the intranasal (i.n.) route, in which the virus is deposited onto the olfactory nerve, also indicates retrograde spread via known neural connections between different parts of the brain [42, 58].

Although evidence favours centripetal nerve spread as the most likely mechanism for viral infection of the CNS, several factors, such as viral strain, host age and especially immune status make more than one mechanism likely.

1.3 Experimental Models

The main in vivo model used to study flavivirus infection is the mouse. However, in addition to standard differences in experimental approaches by different investigators and the investigative modalities used, variations in mouse strain (e.g. BALB/c and C57BL/6 mice in TBEV [59] and WNV models [60]), mouse age (whether the BBB has fully formed, the maturity of the immune system), peripheral route of inoculation (subcutaneous [61], intravenous [37], intraperitoneal [62], intradermal [30, 31], intranasal [58], intravaginal [60] and oral [63] routes are all used for different reasons) and gene status (the use of transgenic mice or mice with inactivated, mostly immune response-related, genes), as well as virus strain, dose and provenance can make generalizable conclusions difficult. Peripheral inoculation at different sites with the same virus can produce markedly different results on rechallenge at a single site [60]. High and low dose inoculation produces immunologically different outcomes [37] and unlike infection by most other viruses, where mice survive according to a standard virus dose response curve, unless flaviviruses have direct access to the CNS they tend to produce ragged, unpredictable survival outcomes [64, 65]. Nevertheless, as models for WNV, TBEV and JEV disease, mice reliably develop active infection and CNS pathology similar to that observed in humans, with neuronal damage and mononuclear infiltration being the most prominent constituents of encephalitic disease [66]. In JEV peripheral inoculation models, the i.p. route may be more reliable if mice develop resistance to subcutaneous (s.c.) infection with age, although some results do not support this [67]. In DENV infection, on the other hand, one of the main obstructions to the study of neurological disease is the difficulty in establishing a suitable animal model. DENV is quickly controlled in immunocompetent mice and in order to study this virus, mouse-adapted virus strains, immunocompromised mice lacking Type-1 and -2 interferon (IFN) receptors, and humanised mice have been used to date, with consequent inconsistencies and difficulties in interpretation amongst published studies [68]. Thus, a suitable murine model for in vivo studies of neurologic DENV disease is still needed.

Other models for flaviviral disease include non-human primates, for example, i.n. inoculation of macaques with JEV resembles human encephalitic disease occurring

after natural infection, while other routes usually result in asymptomatic infection. Thus, these animals have been used for surrogate studies of viral persistence in the blood of asymptomatic human WNV infection [69]. Their closer genetic kinship to humans justifies the choice of macaques to test vaccines and other antiviral therapies [67] and much of the earlier work on YFV was done in macaques and other non-human primates, since they develop symptoms most closely resembling human disease. Stricter regulations and ethical concerns have driven the search for alternative models that have included the golden hamster as a model for YFV [70], as well as persistent WNV [71, 72]. Limitations associated with virus adaptation, however, have also resulted in the use of A129 mice, deficient in IFN-α/β receptor, in which viscerotropic disease is achieved without requiring YFV adaptation [73].

Although few animal models perfectly replicate human disease, much has been achieved using animal models, mainly mice, to study flaviviral disease. The use of genetically deficient mice has certainly contributed to the mechanistic understanding of disease process, but genetic absence of function throughout the disease course makes it difficult to understand what contribution(s), and in what disease time frame, are made by particular elements of the immune response in the normal course of virus control in the large majority of individuals infected by flaviviruses. Furthermore, the lack of reliable in vivo models for investigating some manifestations of human disease, for example flavivirus-induced retinal pathology, still largely restricts studies to in vitro approaches.

2 Role of Immunopathology vs. Virus-Induced Damage in Disease

Much discussion revolves around whether the main contributor to flavivirus-associated pathology in the CNS occurs via direct viral damage to neurons or by immune-mediated pathology. While it is accepted by definition that the immune system is central to tissue destruction in autoimmune disease, it has only relatively recently assumed its deserved importance in non-autoimmune diseases. This includes infectious diseases of all kinds, including viral infection, where the adaptive immune response is crucial to the eradication of the invading organism, and non-infectious inflammatory disease, typified by various models of tissue ischemia and/or reperfusion. The extent to which an over-enthusiastic immune response, particularly by innate immune cells, such as those of the myeloid lineage, can drive pathology, is only now becoming clear, but this understanding is providing exciting opportunities for the development of novel and potentially life-saving therapies.

2.1 Role of Virus

Although many cell types are permissive for flavivirus infection in vitro [31, 36, 46, 48, 64, 74–83] and occasional human post mortem studies have shown evidence of low level WNV infection of astrocytes, neurons remain the principle target for most

flavivirus infections in the CNS in mammals [9, 42, 58, 84–86]. Neurons have extremely limited regenerating capacity and exhibit the highest rates of apoptosis, compared to other CNS cell types when infected with flavivirus [84].

The capacity of flaviviruses to infect neurons and induce cell death in vitro suggests that encephalitis may be directly virus-induced [87, 88]. Apoptosis of human medulloblastoma cells directly correlates with JEV infection in a concentration- and time-dependent manner and administration of isolated viral protein NS2B-NS3 results in caspase-3 activation and mitochondrial-mediated apoptosis [89]. Similarly, JEV and WNV infection of neuronal cell lines induced a caspase cascade with an ensuing mitochondria-mediated apoptosis and the generation of reactive oxygen species (ROS) [90–92]. Death of DENV-infected neuroblastoma cells was linked to endoplasmic reticulum (ER) stress induced by the accumulation of viral proteins [86, 88], a result replicated in JEV infection of neurons [93]. Furthermore, WNV capsid protein induces ER stress to activate GADD153, a death-related ER stress gene, thereby inducing apoptosis [84]. On the other hand, Langat NS3 binding to caspase-8 in a neuronal cell line can also trigger apoptosis [94]. Thus, both virus and/or isolated viral components can act as a potent inducer of apoptosis in neural cells in the absence of additional immune stimulation and these mechanisms can be present even in a relatively non-pathogenic flavivirus, such as Langat.

While caution in interpretation of results in neuronal cell lines is important, since these cells are often immortalised and/or transformed and anatomically isolated, there is good evidence of virus-induced cell death in in vivo models. Injection of WNV capsid protein into muscle resulted in apoptosis as a consequence of the disruption of mitochondrial transmembrane potential and caspase-9 and -3 activation [95]. Neonatal mice infected with mouse brain-adapted DENV induced neuronal apoptosis concentrated in areas with high levels of viral replication [96] and demonstrable neuronal death also occurred in a hamster model of WNV infection [72]. In both cases there was little sign of CNS inflammation when virus was initially detected, emphasising the virus contribution to neuronal destruction. However, these few examples of in vivo flavivirus infection where pathological viral effects occur in the absence of, or before immune infiltration into the CNS contrasts with the overwhelming association of infection and leukocyte infiltration, making it difficult to determine if disease symptoms are primarily the result of virus-induced damage or mediated by inflammatory/immune responses to virus-induced neuronal stress and/or death.

2.2 Role of Immune Responses

The dual role of the immune system in combating infection and contributing to pathology was clearly inferred almost four decades ago [97]. Flavivirus encephalitis in mammals generally presents with neuronal infection and damage associated with a significant infiltration of virtually all leukocyte subsets, in particular, lymphocytes and macrophages, as well as the accompanying activation of CNS resident cells (microgliosis and astrogliosis) and a concomitant pro- and anti-inflammatory

mediator release, variously, from all cell types [9, 66, 84, 85, 98–100]. This cascade of events is triggered early by neuronal infection and is ultimately responsible for lethal encephalitis or survival and immunity [42, 50, 58, 101]. In humans, high levels of inflammatory mediators such as TNF, IL-8, IL-6, nitric oxide (NO) and CCL5 (RANTES) in the cerebrospinal fluid (CSF) and serum of JEV-infected patients are associated with a fatal outcome, whereas high IgM levels correlate with survival [102, 103].

2.2.1 Responses of Resident Cells of the CNS

Neurons

Although neuronal death is clearly a consequence of viral infection and/or ensuing bystander damage from activated inflammatory cells, pro-inflammatory mediators released by infected neurons also contribute directly, by potentially inducing apoptosis of neighbouring neurons or indirectly, by recruitment of pathological immune components. In vitro, neurons respond to WNV by producing pro-inflammatory cytokines such as CXCL10, TNF, IL-1β, -6 and -8, on infection. Neutralisation of TNF and IL-1β decreased apoptosis of WNV-infected neurons. These cytokines also induce activation of astrocytes, which are further implicated in disease pathogenesis [84, 104].

Infected neurons in vivo also produce significant amounts of the chemoattractant CCL2 (MCP-1), which recruits pathogenic Ly6C^{hi} inflammatory monocytes [42]. This may be a stereotypic response by infected cells, as large amounts of this chemokine are also secreted in vivo by infected fibroblasts outside the brain and recruitment of inflammatory monocytes to the skin and draining lymph node is similarly dependent on this chemokine [30]. Although neuronal contribution to pathogenesis may be limited, in an extended disease course, continuous recruitment of inflammatory cells mediating ongoing non-specific tissue damage in the brain may tip the balance from protective to pathogenic.

Astrocytes

Astrocytes are the main glial cell of the CNS, playing a supportive role in homeostatic conditions and producing myriad inflammatory mediators capable of regulating the surrounding CNS milieu in a neuro-stimulatory or -inhibitory manner, as reflected in distinct gene expression profiles [105, 106]. The intimate anatomical and functional relationship between astrocytes and neurons makes these cells obvious contributors to an immunopathogenic response during flavivirus infection.

Astrocytes are susceptible to flavivirus infection in vitro, producing Type-1 IFN and upregulating both Class I and II major histocompatibility complex antigens (MHC-I and -II) [46, 83, 107] and as mentioned above, occasional co-localization of WNV antigen with astrocytes has been observed in post-mortem human brain

from patients succumbing to WNV encephalitis [84]. However, astrogliosis is triggered principally by cytokines, such as IL-1β and TNF, released from infected neurons, rather than by infection with replicating virus [104]. Activation of astrocytes by JEV and WNV induces the secretion of several pro-inflammatory mediators, including CXCL10, CCL2, IL-1β and CCL5 [83, 84, 100, 107–110], although the extent to which this contributes to CNS protection or pathology is unclear. In vitro binding of CXCL10 to its cognate receptor, CXCR3, on foetal neurons results in elevated Ca^{2+} from the ER, the uptake of which by mitochondria leads to membrane permeabilisation, with cytochrome c release and its association with initiator caspase-9 that in turn activates effector caspase-3, ultimately inducing neuronal apoptosis [111]. Although IL-1β production by astrocytes has been shown to stimulate neurogenesis in homeostatic conditions [106], it may have the opposite effect during flavivirus infection. These cytokines activate glial cells and induce an array of other pro-inflammatory cytokines and chemokines whilst increasing their own production via an auto-feedback loop. Several studies have also implicated the IL-1 family of pro-inflammatory cytokines in the pathogenesis of acute and chronic neurodegenerative diseases such as Alzheimer's and stroke (Reviewed in refs. [112, 113]). The dysregulated production of these pro-inflammatory mediators induces apoptosis of neurons [107]. On the other hand, IL-1β-induced activation of astrocytes has also been shown to be protective against neuronal apoptosis [114], highlighting the intricate balance of neuroprotective versus pathogenic roles of various cytokines produced by astrocytes. IL-6 is also significantly upregulated in WNV encephalitis [58] and the production of IL-6 by astrocytes enhances the inflammatory immune response and subsequent pathology during experimental autoimmune encephalomyelitis (EAE), a murine model of multiple sclerosis [115].

In vitro, supernatant from activated astrocytes induces apoptosis in neurons, indicating that some of the products released by astrocytes are likely neurotoxic. However, activated microglia seem to be more toxic in this regard than activated astrocytes [100, 107], making it unlikely that astrocytes are the primary contributor to pathogenesis during flavivirus encephalitis. Nevertheless, their inflammatory mediators may amplify the pathogenic immune response, thus increasing the risk of neurodegeneration.

Microglia

Microglia are the resident innate immune cells of the CNS and populate the brain in early embryogenesis, replenishing themselves from this in situ population throughout life [116]. They function as local tissue macrophages and are activated to express cell surface markers and inflammatory mediators, similar to macrophages immigrating from the blood, in response to any processes causing inflammation, including necrosis, infection or autoimmunity. In the brain, microglia and immigrant inflammatory monocytes can be distinguished flow cytometrically by their differential CD45 and Ly6C expression. During infection, microglia upregulate CD45 expression from low to intermediate and become $CD45^{int}CD11b^{+}Ly6C^{int}$, whereas

macrophages are $CD45^{hi}CD11b^{+}Ly6C^{hi}$ [42, 117]. The presence of pathogen induces morphological changes in microglia associated with migration and secretion of various pro-inflammatory mediators. Although microglia play a significant role in neuroprotection and repair, there is a strong reported correlation between microglial inflammatory mediator expression and neurodegeneration during flavivirus infection.

Microglia in culture are permissive to flavivirus infection but do not support replication [83]. Infection activates microglia and induces several inflammatory mediators, including cytokines (TNF, IL-1β, IL-6, IFN-γ), chemokines (CCL5, CCL2), inducible nitric oxide synthase-2 (NOS2), cyclooxygenase-2 (Cox-2) and ROS [39, 50, 83, 100, 109, 118, 119]. Perhaps not surprisingly, supernatant from JEV-infected microglia induces more extensive neuronal death in vitro than that from infected astrocytes [100].

TNF and IL-18 released by lipopolysaccharide-activated microglia inhibited the neurogenesis of neural precursor cells (NPC) in vitro [120], as did the administration of TNF and IFN-γ, with IFN-γ increasing the rate of apoptotic cell death. Interestingly, TNF partially inhibited this apoptotic effect, indicating that at certain concentrations, TNF may have a neuroprotective role [121]. However, in a WNV model of infection, TNF and IL-6 directly mediated bystander damage to neurons; reduced TNF output in TLR3-KO mice coincided with reduced CNS inflammation and neurodegeneration [39], suggesting a role for TNF initiated by TLR3 in the immunopathogenic response to WNV infection. Raised TNF levels also were associated with mortality in TBEV infection [122]. Thus, while TNF is antiviral [123] high concentrations clearly facilitate disease pathogenesis in vivo.

Although direct viral activation of microglia is possible [100], it is more likely that initial virus-induced neuronal death and subsequent inflammatory mediator release is the main activator of glial cells. TNFR-1 associated death domain (TRADD) is a critical component of neuronal death in the CNS during JEV infection. Inhibiting TRADD synthesis resulted in decreased neuronal apoptosis and reduced activation of microglia and astrocytes. Attenuation of microgliosis resulted in reduced inflammatory mediator production and leukocyte infiltration [109].

Cox-2 and NOS2 both directly induce apoptosis in a JEV model of infection and minocycline-mediated inhibition of these products significantly reduces apoptotic neuronal cell death [119]. Significant NOS2 mRNA is also seen in the brain of WNV-infected mice with high levels of NO observed in both activated microglia and immigrating macrophages at the peak of infection. Inhibition of NO outputs using aminoguanidine extended survival in mice by several days, indicating a significant contribution of these soluble factors to immunopathogenesis [50].

Involvement of Cells in the Eye

In the eye, an extension of the brain, it is worth pointing out that the retinal pigment epithelium (RPE), which forms and maintains an outer blood-retinal barrier, separating the eye from the peripheral circulation, in particular, the fenestrated choroid plexus, is permissive for WNV infection. Microarray analysis of WNV-infected

RPE isolated from human cadaveric eyes showed upregulation of various inflammatory cytokines involved in innate and adaptive immunity. These include CCL5, CCL2, CXCL10, CXCL11, IL-6 and IL-8 and may play a role in oculopathies seen in WNV and DENV infection. Of particular interest is the significant upregulation of complement factor B (CFB) [79], which results in the deposition of complement factors C3a and C3 between the basal aspect of RPE and Bruch's membrane [124], creating a highly pro-inflammatory environment. The causative link between complement activation and age-related macular degeneration [125] implicates this as a likely contributor to ocular manifestations present in flavivirus infection. Moreover TNF, which positively regulates CFB [124], was also upregulated in infected RPE. Apart from exhibiting pro-angiogenic properties [126], TNF also increases MMP production [127] and apoptosis [128], which may be responsible for haemorrhage and edema in the vicinity of the compromised outer barrier, further encouraging damaging neovascular growth. Multiple MMP are also produced by WNV-infected human brain cortical astrocytes, and might play a role in compromising the BBB, thus contributing to immune infiltration and subsequent immunopathology [129].

2.2.2 Infiltrating Immune Cells

Leukocyte infiltration into the CNS is a hallmark of flavivirus encephalitis and is a result of the secretion of chemotactic inflammatory mediators by neurons as well as activated microglia and astrocytes, triggered by neuronal infection and viral induced death [87]. In humans, this infiltrate includes neutrophils and mononuclear cells like T-lymphocytes and macrophages in the most severely affected sites in the CNS and is often associated with a fatal outcome [98, 130]. This leukocyte infiltrate, with activation of glial cells, is thought to cause the clinical symptoms in flavivirus encephalitis. Mice developing meningoencephalitis during DENV infection show distinct behavioural changes occurring at the peak of inflammation in the CNS [131] and many studies show the close association of inflammation and development of clinical symptoms during flavivirus infection of mammals.

Myeloid Subsets

Monocytes and Macrophages

Flow cytometric identification of monocytes in humans and mice defines two principal $CD115^{+}$ $CD11b^{+}$ populations, based on further cell surface marker expression. Classical or "inflammatory" monocytes are defined as $CD14^{hi}CD16^{-}$ in humans and $Ly6C^{hi}$ ($CD43^{lo}CCR2^{hi}CXC3CR1^{-}$) in mice, whereas non-classical or "patrolling" monocytes are $CD14^{lo}CD16^{hi}$ in humans and $Ly6C^{lo}$ ($CD43^{hi}CCR2^{lo}CX3CR1^{hi}$) in mice [132, 133]. Circulatory monocytes can differentiate into either DC or macrophages in tissues, with this decision likely determined by signals encountered as

monocytes enter the target organ. In our laboratory we have used the i.n. model of WNV encephalitis to examine the anti-viral responses of the CNS, while minimising potentially confounding effects of the systemic immune response. In this model, nearly 50 % of leukocytes infiltrating the brain are monocyte-derived macrophages, almost 90 % of which are Ly6C^{hi}. There is minimal proliferation of resident microglia in vivo, but adoptive transfer of Ly6C^{hi} monocytes show a proportion of these cells differentiating into a phenotype indistinguishable from activated microglia, thus adding to the resident population [42].

The high concentration of CCL2 produced in the CNS during WNV infection is responsible for recruiting inflammatory monocytes, via CCR2, the cognate receptor expressed at increased levels on these cells. Neutralisation of CCL2 is associated with significant reduction in inflammatory monocyte immigration into the CNS, prolongation of survival by 2 days, with ameliorated clinical symptoms [42]. Immigration of Ly6C^{hi} monocytes into the CNS across the endothelium evidently occurs mainly via the integrin, VLA-4, on monocytes, since VLA-4 antibody blockade inhibits infiltration of these cells by approximately 60 %, resulting in the long term survival of 60 % of animals, with protective immunity in a group that would otherwise uniformly succumb. Interestingly, LFA-1 antibody blockade has no effect on survival notwithstanding some 30 % reduction in macrophage infiltration into the CNS. In these studies there was no reduction in viral titre, despite the reduction in infiltrating macrophages, thus relegating the notion that carriage of virus into the brain is mediated by these cells. Endothelial expression of VCAM-1, the ligand for VLA-4, and ICAM-1, the ligand for the integrin, LFA-1, increase substantially on the CNS during WNV infection in vivo in both i.n. and i.p. mouse models [50, 134]. Moreover, studies in vitro with WNV-infected human umbilical vein endothelial cells (HUVEC) show Th1 and inflammatory cytokines further increase the expression of VCAM-1, as well as ICAM-1, and E and P-selectin, crucial for rolling adhesion after leukocyte margination. Thus, a combination of neuronal infection, promoting specific recruitment via secreted chemokines, and cytokine-induced adhesion molecule upregulation on CNS endothelium, enabling diapedesis, evidently drives leukocyte infiltration into the CNS [36].

As mentioned above, many of these macrophages produce NO, which is in part responsible for pathology. NO is a highly reactive species, toxic for a wide variety of viruses, as well as bystander cells (reviewed in ref. [135]). In vitro, the potential role in bystander neuronal damage during JEV infection was studied in a population of thioglycollate-induced peritoneal exudate macrophages, in the supernatant of which, inflammatory mediators, IL-12, CCL2, TNF and IFN-γ were detected. As discussed, these factors can contribute to neuronal degradation, either by direct cytotoxicity, or indirectly by recruiting inflammatory cells. As with WNV [81], NO was also produced at high levels by JEV-infected macrophages, along with ROS and Cox-2. These soluble factors directly induced apoptosis of neuroblastoma and primary cortical neurons in culture [136].

In a DENV model of infection also, inflammatory macrophages produced significant levels of IFN-γ-dependent NOS2. High levels of NOS2 were associated with pathogenesis, as NOS2 KO mice exhibited improved survival, compared to

WT controls [137]. In contrast, IFN-γ-mediated NOS2 expression and NO production were crucial for survival in a mouse adapted DENV-3 infection [138]. NO production plays a crucial antiviral role against DENV in monocytes [139] and the antiviral function of NO protects against lethal JEV infection [140, 141] and WNV [64]. On the other hand, NO did not exhibit antiviral effects against TBEV in vitro and TBEV-infected mice treated with aminoguanidine showed no increase in survival rates but did have a longer mean survival time (MST), suggesting a contribution to immunopathology by NO [142]. Likewise, treatment of C57BL/6 WNV-infected mice with aminoguanidine at the peak of monocyte infiltration prolonged survival for approximately 3 days [50]. In contrast, in human DENV infection, low NO levels were associated with poor outcomes and development of the hemorrhagic form of disease, compared to patients with elevated NO [143]. In encephalitis, the concentration of NO in the CNS likely determines whether it exerts a protective or pathogenic role. This is related in turn to the number of IFN-γ-producing activated lymphocytes in the CNS and the number of inflammatory macrophages responding by producing NO. Discrepancies in animal models may be due to the use of different mouse strains, different flaviviruses or different strains of the same virus, different virus doses, as well as the temporal window of functional presence or absence of NOS2 during disease.

More recently, we have investigated another IFN-γ effector pathway, that of indoleamine 2,3 dioxygenase (IDO) induction in macrophages. Interestingly, although both IDO and NOS2 are strongly induced by IFN-γ, they appear to be tightly reciprocally regulated. IDO catalyses the destruction of L-tryptophan, a rate-limiting amino acid required for replication of lymphocytes and many viruses [144] Interestingly, work in vitro indicates that IDO is antiviral for WNV [48] and IDO upregulation has been found in the human CNS post mortem [84]. Of particular interest here is that in contrast to NOS2, IDO does not produce a highly, reactive, damaging chemical species and thus may exert its anti-viral and anti-proliferative effects with minimal bystander tissue damage and less inflammation as a result. This would be especially important in the CNS that is for the most part in G_0, since IDO would be expected to have little or no metabolically inhibitory effect on these cells. The temporal interaction of these two anti-viral effector pathways clearly could thus have important consequences for survival in encephalitis. However, how the decision is made at the cellular level as to which is differentially expressed and when, has yet to be elucidated.

Neutrophils

Neutrophils may contribute up to 3 % of the inflammatory infiltrate during lethal WNV encephalitis infection in mice [50] and have been implicated in pathology of flavivirus infection. High neutrophil numbers and IL-8 production are found in JEV-infected patients with severe forms of disease [98] and neutrophils recruited in an IL-1- and CXCL2-mediated manner are highly cytopathic in the ischemic brain [145].

Neutrophils, $CD8^+$ and $CD4^+$ T cells were present in significant numbers in the CNS of a lethal model of intracranial DENV infection, their presence was

accompanied by increases in several cytokines and chemokines, including IFN-γ, TNF, CCL2, CCL5, CXCL1, and CXCL2 [131]. Neutrophil infiltration and NOS2 expression were correlated with development of encephalitis in MVEV-infected young mice and neutrophil depletion resulted in significantly prolonged MST and decreased mortality, although the depleting antibody used also depletes monocytes. The inhibition of NOS2 with aminoguanidine also prolonged survival, but to a lesser extent than neutrophil depletion [62], emphasising the multifarious nature of disease pathogenesis and that where this population contributes significantly to disease, additional neutrophil-derived factors are likely to be involved [49].

Lymphoid Subsets

CD8⁺ T Cells

$CD8^+$ cytotoxic T cells (CTL) are the main specific anti-viral effector of the adaptive immune response in flavivirus infections. They recognise specific viral peptide antigens presented by MHC-I on infected host cells and release perforin and granzymes, which induce apoptosis of the infected or otherwise abnormal cells [46, 65, 146]. Effective lysis of infected host cells limits the spread of virus, but inevitably also affects the host and potentially leads to (irreversible) damage to tissue. This especially provides a challenge to fragile organs with slow or non-renewing cell populations, such as the CNS.

In TBEV, $CD8^+$ T cells producing granzyme B were found in close proximity to neurons expressing caspase-3 [147], suggesting that these cells may contribute to immune-mediated damage to neurons. A number of flaviviral models have shown $CD8^+$ T cells to have detrimental effects on disease outcome. In a s.c. model of TBEV infection, $CD8^{-/-}$ and SCID mice, which lack both B and T cells, survived 3–5 days longer than WT mice, despite having a higher viral titre in the brain [148]. MVEV-infected mice with gene deletions of perforin, granzyme and FasL, independently or combined, displayed better survival than WT mice for doses between 10^2 and 10^8 pfu of i.v. inoculated virus. Both NK cells and CTL produce these lytic factors and the authors do not distinguish between the two cell types; however, NK cell function is inhibited by MHC-I upregulation in flavivirus encephalitis, making $CD8^+$ T cells the likely mediators of pathology in this model [65].

In high dose i.v. infection with WNV Sarafend, mice that were antibody-depleted or genetically deficient in $CD8^+$ T cells showed increased survival and longer MST than WT mice. In low dose infections, however, WT mice had a survival advantage over mice deficient in CTL [37, 61]. Similar observations have been made in diseases without CNS involvement. In influenza virus infection, $CD8^+$ T cells, as in WNV, are detrimental in high dose infection, but beneficial to survival in low dose infection [149]. This suggests that the $CD8^+$ T cells recruited in low dose infection efficiently clear virus and have well-regulated cytokine production. In contrast, large amounts of virus attract enough $CD8^+$ T cells to result in overwhelming production of cytokines and non-specific cytotoxicity, which negates the benefit of

viral elimination. This is supported by research done in our laboratory, where uninfected mouse embryonic fibroblasts, on which MHC-I was upregulated by IFN-γ, were more efficiently lysed by anti-viral CD8+ T cells than untreated, uninfected cells [150]. In an in vivo scenario, this could translate to neighbouring cells upregulating recognition molecules for CD8+ T cells, as a result of the inflammatory milieu and proximity to infected cells, to such an extent that they become targets of low affinity CTL generated by the anti-viral immune response [151]. Transfer of virus-specific CTL accelerates viral clearance from the lungs of respiratory syncytial virus-infected mice but a direct positive correlation was found between the increasing amounts of transferred cells and respiratory distress in the mice [152]. Thus, the immune system has to manage a precarious balance between viral clearance and organ function and the cytopathic effect of CD8+ T cells. On the other hand, in the i.n. model of WNV encephalitis, the depletion of T cells does not result in extended survival, unlike the blockade of monocyte immigration. In this model, however, VLA-4 blockade also decreases T cell numbers by almost half, presumably diminishing any T cell-mediated immunopathology present, with the remaining T cells evidently able to clear the virus to produce permanent, protective immunity [50].

A comparison between the blood of children suffering from dengue fever and DHF showed that CD4+ T cells are activated in both forms of disease, though DHF patients show higher activation. However, CD8+ T cells were only found to be active in DHF and not in dengue fever patients, suggesting they may contribute to worsening disease [153]. In order to establish whether CD8+ T cells contribute to DENV pathology, SCID mice, which are normally resistant to DENV, were transplanted with DENV susceptible human HepG2 cells and i.p. infected with a DENV-2 strain isolated from a human patient. Simultaneously mice were injected with naïve thymocytes, DENV-specific CD8+ T cells or vehicle. Mice receiving naïve thymocytes showed the poorest survival with 100 % mortality within 14 days p.i. Transfer of DENV-specific CD8+ T cells resulted in 80 % mortality after 15 days, but the remaining 20 % survived long-term. Although there was a 100 % mortality rate in vehicle-treated experimental group, the disease course was extended significantly, compared to the other two groups. These results indicate that although virus-specific T cells are crucial for viral eradication, they may ultimately contribute to a more severe form of disease [154].

The role of CD8+ T cells is likely determined by a multitude of factors including severity of disease, phase of disease in which CD8+ T cells become activated and whether or not the recruitment of specific CD8+ T cells become dysregulated during the course of disease. Controlling the magnitude of the CTL response is key to managing the benefit of viral clearance against irreversible and potential fatal damage to the host. Of interest here is the upregulation of MHC-I by certain flaviviruses such as WNV [76, 77]; whilst increasing the likelihood of CD8+ T cell recognition and cytolysis, this also results in inhibition of NK cells. This highlights a controversial aspect of flavivirus infection, as the upregulation of MHC-I aids in immune evasion by inhibiting NK cell-mediated viral clearance, whilst also increasing CD8+ T cell immune-mediated pathology.

CD4$^+$ T Cells

CD4$^+$ helper T cells function mainly in enabling and enhancing the activation of CD8$^+$ T cells and class switching of B cells. Although there is evidence of direct CD4$^+$ T cell cytotoxicity [155], raising the possibility of helper T cell-induced immunopathological damage, either directly, or indirectly via stimulation of CD8$^+$ T cells, CD4$^+$ T cells appear to have a predominantly positive effect on survival and MST during flavivirus infection. For example, the transfer of CD4$^+$ T cells to TBEV-infected SCID mice increased both MST and survival rate [148]. Indeed, CD4$^+$ T cells are required for WNV clearance, as depleted or deficient mice show increased mortality and persistent infection [156]. Moreover, transfer of CD4$^+$ T cells alone was shown to be sufficient for viral clearance and survival of RAG-1$^{-/-}$ mice infected s.c. with WNV. CD4$^+$ T cells upregulated granzyme B expression and specifically lysed WNV-infected targets in a perforin- and FasL-dependant manner [155]. This suggests that CD4$^+$ T cells may have a much greater role in the immune response than just mediating CD8$^+$ activation: they may alone be sufficient to clear virus, while mitigating the potential immunopathological damage caused by CD8$^+$ T cells.

On the other hand, in DENV infection, CD4$^+$ T cells may contribute to immunopathology as well as viral clearance. Gagnon et al. isolated 6 CD4$^+$ CTL clones, with cytotoxic activity, from a donor infected with DENV-4, which had cross-reactivity with DENV-2 to analyse target and lysis mechanisms. They found that the clones lysed bystander cells as well as target cells. Specific inhibition of either FasL-mediated cytotoxicity or perforin release indicated that lysis of cells presenting antigen was more strongly affected by perforin blockade, whereas bystander cell lysis was reduced more by FasL blockade [157]. These results were obtained in vitro and with a limited number of CD4$^+$ CTL clones but suggest there may be an interesting difference between the targeting of infected and bystander cells via perforin-induced lysis and FasL-mediated lysis, respectively. The ability to differentiate beneficial and pathogenic actions of immune cells therapeutically in the pandemonium of responses during virus-mediated CNS inflammation could mean the difference between successful resolution of infection or a fatal outcome as a result of immunopathogenesis.

Regulatory T Cells

As regulatory T cells (Tregs) mainly suppress immune responses, they are not commonly implicated in immunopathology. However, evidence for lethal immunopathology driven by a dysregulated or amplified immune response during flavivirus encephalitis, stresses the crucial need for properly functioning Treg-mediated suppression. Defining the targets of inhibitory Tregs in patients effectively fighting off flavivirus infection may provide clues as to which subsets become dysregulated when Tregs fail to control the immune response.

The high proportion of asymptomatic WNV infections in humans prompted research trying to define what immune component differentiates between individuals with susceptibility to WNV (approximately 20 % of the population) and asymptomatic

infections. Comparison of the number of circulatory Tregs present in the blood of WNV$^+$ donors with asymptomatic and symptomatic infection showed that patients suffering from symptomatic infection had fewer Tregs up to 1 year post infection, compared to asymptomatic patients. This correlation was supported by a s.c. model of WNV infection, in which Treg-mediated suppression of CD8$^+$ T cell recruitment increased survival, while Treg depletion resulted in increased lethality [158].

The importance of Tregs in reducing immunopathology in other neurotropic virus infections, also, such as in ocular herpes simplex virus infection, which unlike WNV infection, causes recurrent infection due to latency in sensory ganglia [159] emphasise the importance of immune regulation by Tregs and may ultimately provide useful guidance for immune targeted treatment design.

NK Cells

NK cells can recognise and kill "abnormal" cells without being activated by antigen-presenting cells. They have both inhibitory and activating receptors, with their final status being determined by the balance of stimulating and supressing signals encountered. Viral infection of cells results in the expression of stress factors, which are ligands for NK cell activating receptors such as NKG2D. Binding of NK cells to the target cell via these receptors result in lysis of the infected cell. The function of NK cells in flavivirus infection is unclear, and a number of other viruses manage to avoid detection by this subset. Ligands of NKG2D were upregulated in the CNS of animals infected with a neurotropic strain of mouse hepatitis virus. Blocking of NKG2D in SCID mice (which although B and T cell-deficient, retain NK cell functionality) did not affect survival and the authors concluded that NK cells play neither a protective nor detrimental role in viral encephalitis [160]. In the case of flaviviruses, a likely explanation for this is the ability of flavivirus to upregulate MHC-I, which represents "self". Generally, virus-infected cells downregulate MHC-I expression, enabling NK cell recognition and activation. Flaviviruses, however, may have evolved to avoid NK cell detection by upregulating MHC-I expression on infected cells to inhibit NK cell activation by engaging inhibitory receptors. Indeed, experiments blocking the single inhibitory receptor present on NK92-44 cells resulted in NKp44-mediated activation of NK cells and subsequent lysis of WNV-infected Vero cells [161]. In vivo, NK cell depletion experiments have not shown any effect on survival in WNV infection, presumably because they are not essential in resolving infection [146]. However, NK cells make up a significant portion (up to 10 %) of the CNS infiltrating leukocyte population in WNV encephalitis and show a large influx into the CNS in an intracranial DENV infection model (King, unpublished results). Such a significant NK cell influx in the CNS seems too sizeable to be merely a side effect of the massive leukocyte recruitment and infiltration and suggests a possible cytokine helper function in flavivirus encephalitis. Indeed, these cells may contribute to the control of WNV replication in the liver in vivo [162]. However, in the current models it is possible that this function is concealed in the avalanche of bigger infiltrating cell populations that, once abrogated, have an obvious role in survival. Thus, a beneficial or immunopathological role for NK cells may remain to be discovered.

A genome wide association study attempted to identify genetic predisposing factors for DSS, the most severe form of DENV. Comparison of almost 2,000 children suffering from DSS and nearly 3,000 controls found a significant correlation between occurrence of DSS and presence of a single nucleotide polymorphism (SNP), MICB (major histocompatibility complex (MHC) class I polypeptide-related sequence B). The authors suggest that since MICB is a ligand for the activating NKG2D type II receptor, this mutation will result in strong activation of cytolytic NK cell responses, inducing severe immunopathology responsible for DSS [163]. A follow-up study by a different group included patients suffering from less severe dengue, as well as adults, whereas the original comparison only included children. They showed that the SNP in MICB also correlated to less severe dengue, albeit less strongly than DSS. Moreover, they suggest that the genotype may be more prone to DSS as a result of impaired, rather than enhanced, NK cell activity [164]. Thus, while some flaviviruses appear to avoid NK cell lysis, with the result that they appear to have little or no role in the pathogenesis of disease, clearly, further functional experimentation is needed to clarify the clinical significance of NK cells in flavivirus infection. While the possibility of clinically activating NK cells [165] at particular, e.g. early, time points in flavivirus disease may be attractive, as with monocytes and T cells, the benefit of such activation on the host in vivo should outweigh the potential detrimental effect.

3 Immunomodulatory Therapeutic Approaches

While it is clear that the ideal control of human infection by viruses that have multiple hosts and in which humans are often incidental to its life cycle, is an effective programme of immunisation, currently no approved vaccine for humans exists against WNV, and infections with other flaviviruses such as JEV and YFV, which have approved vaccines, still occur frequently. Clinically, the appearance of symptoms is often associated with significant immune system involvement, i.e. once there is already a well-established infection, shifting the focus of treatment from primary antiviral to immunomodulatory approaches.

Several studies have used minocycline, which not only exhibits antiviral properties but also significantly reduces inflammatory cytokine (IL-6, IL-12p70, IFN-γ), chemokine (CCL2), and ROS production by microglia and macrophages during JEV infection in vitro [82, 119], promotes neurogenesis [119], as well as effectively inhibiting WNV- and JEV-induced apoptosis of neurons [108, 166]. In vivo, promising results have been obtained with minocycline treatment of JEV-infected mice. Treatment prior to disease onset resulted in reduction of viral titre, neuronal death and subsequent microgliosis and pro-inflammatory mediator levels. Importantly, administration of minocycline after disease onset also promoted survival. Clearly, the ability to ameliorate established infection with developed clinical symptoms is relevant in a clinical setting. Recent studies with Simian immunodeficiency virus infection in non-human primates showed minocycline treatment resulting in less

severe encephalitis. The authors hypothesised that this was due to decreased CCL2 production and subsequent reduction of cytopathic monocyte recruitment [167]. As this tetracycline derivative has good penetration of BBB and is clinically approved it is a strong potential candidate for immunomodulatory therapy of flavivirus encephalitis.

Promising inhibitors of CCL2 such as Bindarit, have also been used to reduce the effects on monocyte infiltration in alphavirus diseases [168–170] and EAE [171] and several other inflammatory scenarios. This drug acts through modulating NF-κB [172], translocation of which is significantly increased by WNV [123, 173]. We have tested this agent in WNV encephalitis, but without observable ameliorative effect (King, Unpublished). This may not be surprising, as the direct inhibition of CCL2 by antibody neutralisation extended the life of mice with WNV for only 2 days [42] and likely emphasises the complexity of immunopathogenesis and perhaps the inflammatory intensity of flavivirus encephalitis.

Targeting soluble factors with inhibitors against ROS, Cox-2 and NOS2 has also been effective in reducing neuronal apoptosis during JEV infection [136]. Neutralising antibodies against TNF and IL-1β during WNV infection have a protective effect on neuroblastoma cells and reduce apoptosis of infected cells in vitro [104], but the importance of these innate cytokines in the initiation of the immune response may not make them ideal targets in vivo.

Inhibition of NO production with aminoguanidine proved to be protective in several models of flavivirus infection as determined by longer MST [50, 62] but did not improve final survival outcome. It is likely the sustained action not just of NO, but of a combination of inflammatory mediators produced by macrophages and possibly neutrophils ultimately skew the balance from a protective antiviral immune response to a pathogenic one. NO acts a neurotransmitter and high levels in the brain may in part be responsible for the generation of seizures that occur in mice with flavivirus encephalitis, leading to death, especially considering the high levels of IFN-γ and numbers of inflammatory macrophages in the brain at this time and the dependence of seizure generation on this cytokine [58]. In our model of WNV infection, Ly6C^{hi} inflammatory monocyte-derived macrophage infiltration into the CNS correlated with viral load, weight loss and increased severity of clinical symptoms. As mentioned above, blockade of this population by VLA-4 antibody at the onset of weight loss prevents these cells from getting across the BBB and results in 60 % long-term survival with protective immunity [50]. Importantly, however, VLA-4 blockade does not improve survival when administered prior to the development of clinical signs, emphasising the crucial nature of timing in immune responses to lethal infection. Furthermore, clodronate liposome-mediated monocyte/macrophage depletion throughout infection does not increase survival either [174], and despite widespread ablation of the myeloid lineage [42, 101], administration at particular time points does not improve survival as much as VLA-4 blockade (King, Unpublished). This indicates the importance of other, as yet undefined subpopulations in the control of infection and potentially immunopathology. In a recent breakthrough study from our laboratory, we have targeted Ly6C^{hi} inflammatory

monocytes prior to entering their target organs, i.e. prior to blockade at the endothelium, by using intravenously administered, negatively charged immune-modifying microparticles (IMP). These are phagocytosed by Ly6C^{hi} monocytes in the circulation of WNV-infected mice and quickly cleared from the blood. Experiments show that significant numbers of monocytes that phagocytose IMP subsequently express phosphatidylserine, a marker of incipient apoptosis and are as a result sequestered by the spleen as dying cells, and thus do not reach the focus of inflammation, resulting in significantly reduced inflammatory macrophage immigration into the brain. As in VLA-4 blockade, IMP are infused when animals have lost ~5 % weight. This approach results in up to 60 % survival of mice that would otherwise certainly succumb, with complete virus clearance within 3 days and protective immunity [101]. Interestingly, the reduction in inflammatory macrophages in the brain, like VLA-4 blockade, is also associated with a modest reduction in T cell numbers and this may also reduce the possible immunopathology caused by T cells [37]; irrespective, there are clearly sufficient T cells to clear the virus from the CNS. Notably, unlike VLA-4 blockade, IMP do not target T cells and their effect is dependent on the spleen. Thus, in WNV-infected splenectomised mice, monocytes are still recruited to the brain even when IMP are administered.

Interestingly, this approach rescues more animals inoculated with low dose virus, which exhibit slower kinetics of disease onset, than with high dose virus, which show accelerated disease onset. This emphasises the immunopathological nature of disease pathogenesis and the balance between the pathogenic macrophage population and the clearance of virus by T cells. Remarkably, this therapy has proven to be successful in other models of disease where there is significant contribution of inflammatory monocytes to pathology, such as EAE, myocardial infarction, ischemia-reperfusion injury and inflammatory bowel disease [101]. To our knowledge, this is the first time that temporally targeted interference with a cell subpopulation in a lethal virus infection has resulted in survival with protective immunity and this highlights the point that precise immunomodulatory therapies, an ideal just beginning to be realised in autoimmune therapy [175], may also be deployed in infections with a significant immunopathological component.

4 Conclusion

Despite massive urgent effort mobilised in the generation of safe vaccines for emerging flavivirus disease, progress has been slow. Notwithstanding the availability of vaccines for JEV, for example, many individuals remain unimmunised and are thus a target for infection in endemic areas. In many of these diseases, treatment is only supportive. Effective treatment design for flavivirus encephalitis requires a critical understanding of the cascade of events leading to and following virus-induced neuronal death. While viral infection of neurons may induce apoptosis, the inflammatory mediator release with subsequent activation of glial cells, as well as chemotactic recruitment of leukocytes ultimately concatenate to exert the greatest

pathological challenge to crucial organs like the brain. Bystander damage, induced directly by CNS resident cells can certainly be targeted by inhibitors that cross the BBB. However, the dramatic improvement in survival obtained by treatments diverting infiltrating Ly6C^{hi} inflammatory subsets in the peripheral circulation away from the CNS, places this subset in the spotlight for future treatment design. Moreover, the fine attenuation of peripheral immune subsets possible with this form of therapy will enable a rational, staged approach to a wide range of clinical disease potentially relegating the coarser subset deletion approaches used in non-infectious scenarios like autoimmunity or organ transplantation, that may leave patients immune deficient, with all its attendant infectious risks.

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Chapter 17
West Nile Virus

Sally F. Alrabaa, Charurut Somboonwit, and Paul Shapshak

Core Message West Nile virus is the most common cause of arboviral encephalitis in the USA at this time. The virus, originally discovered in 1937 in Uganda in Africa, west of the Nile, was of low virulence initially causing only minor illnesses in humans; however, since then a more virulent strain had emerged and spread to most continents, causing epidemics of severe central nervous disease. The evolution and spread of West Nile virus was studied extensively since its emergence in the western hemisphere in 1999 and this has provided an opportunity to better understand the factors that contribute to viral mutation and migration.

1 Introduction

It has been more than a decade since the first human case of West Nile virus (WNV) in the Western Hemisphere was documented. Before 1999, WNV was almost unknown to the public in North America. Today, it is widely distributed in the USA and has been detected in all continents except Antarctica, making WNV one of the

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most widely distributed arboviruses in the world [1]. Viruses constantly mutate, strains that are more virulent emerge, and West Nile Virus is no exception.

With the well-deserved attention toward other high virulence viruses such as influenza A and human immunodeficiency viruses (HIV), West Nile virus is beginning to slip from the attention of the public and health care providers. At a time of reemerging viral threats, this arthropod-borne illness remains important. This review summarizes what is known about WNV since it was first reported in New York more than a decade ago.

2 Virology

2.1 Classification and Structure

West Nile virus belongs to the family *Flaviviridae*, genus *Flavivirus*. It is a single stranded, positive sense, enveloped RNA virus with a genome that is approximately 11 kb [2]. Other members of the *Flaviviridae* family that are major human pathogens include Japanese encephalitis virus (JE), Saint Louis encephalitis virus (SLEV), Dengue viruses 1–4, and yellow fever virus. The WNV genome contains nine major proteins that are implicated in replication and pathogenesis [3]. Three proteins are structural including the capsid protein (C) that binds viral RNA, a premembrane protein (prM) that blocks premature viral fusion, and a protein (E) that mediates viral attachment, membrane fusion, and viral assembly [3]. Five other proteins are non-structural (NS1, NS2A, NS2B, NS3, NS4A, and NS5) and those regulate viral transcription, replication and attenuate the host antiviral responses [4–9].

Two major Lineages of WNV, 1 and 2, have been described. Lineage 1 covers a large geographic area including Africa, Middle East, Southern Europe, Australia, Asia, and the Americas [10]. Lineage 1 is more pathogenic with the potential to cause severe central nervous system infection and death. Lineage 2 is mostly confined to sub-Saharan Africa and its pathogenesis in humans is relatively low, mostly causing self-limited febrile illness.

2.2 Genotype Introduced to Western Hemisphere

Studies have shown that WNV Genotype NY99 was responsible for the 1999 New York City outbreak and that it was in Lineage 1 [11]. The subsequent spread of WNV across North America, however, was caused by a new genotype "North American dominant" or WN02 that emerged from the NY99 strain [12, 13]. WN02 genotype is characterized by 13 conserved nucleotides changes, 1 of which results in an amino acid substitution, Valine to aniline in position 159 (V159A), in the envelope (E) protein. This single amino acid change was shown to decrease the incubation period of the virus in mosquitoes and increase the virulence of the virus,

hence increasing transmission, infectivity rates, and the severity of the infection and can be viewed as a successful adaptation of the virus in its new environment [13].

More recent genomic sequences studies demonstrate further evolution of WNV, and potential emergence of a new genotype in the southwestern USA (SW/WN03 genotype); however, further experiments are needed to investigate potential phenotypic changes that occur in conjunction with the genotype changes and to determine if the SW/WN03 genotype will replace the current dominant NA/WN02 genotype [12].

3 Epidemiology

3.1 Virus Journey and Spread

WNV was first isolated in the West Nile region of Uganda in 1937 from a woman with fever [14]. Soon after that, mosquito transmission among vertebrate hosts was elucidated. Although at that time, the virus caused only self-limited febrile illness it was noted to be serologically related to the previously identified neurotropic viruses, Japanese Encephalitis and Saint Louis encephalitis viruses (JE and SLEV).

Between 1950 and 1990, periodic outbreaks with mild self-limited febrile illness and rare central nervous system involvement were documented [1]. In addition, sporadic cases and larger outbreaks were documented in rural areas in the Middle East, Israel, and southern France. Moreover, a WNV subtype (Kunjin virus) was isolated in Australia. After 1990, the epidemiology of WNV changed further with larger epidemics in Romania, Russia, Tunisia, and Israel [1, 10]. These strains now manifest with severe CNS involvement and high mortality. The first outbreak in the Western Hemisphere occurred in Northeastern USA. In New York City in 1999, 62 cases of encephalitis were reported including seven deaths [16]. The strain causing the epidemic, denoted as NY99, was in lineage 1 and similar to a strain circulating then in Israel (Isr98) [17]. The specific mode of introduction of WNV to the USA is still unknown, although infected birds are thought to be the most likely vehicle of transport. Possible theories of introduction include infected bird migration, illegal or legal importation of vertebrate hosts or vectors, intentional introduction as a bioterrorism attempt, or less likely by an infected human host [11, 17]. Soon thereafter the Virus rapidly spread across the continent, reaching the Pacific coast in less than 3 years (Fig. 17.1). By 2003, more than 2,000 cases of CNS disease and 200 deaths were reported in the USA. Although, since then, the incidence started to decline, mostly due to improved control measures, there are still more than 1,000 neurological cases per year. One interesting epidemiological phenomenon is that WNV appears to be displacing SLEV in its ecosystem in parts of western USA. WNV and SLE virus exploit the same avian host and vector species; however, the NS3 helicase mutation present in the WNV genotype confers elevated virulence for avian hosts and appears to have provided WNV a competitive advantage in this region [18, 19].

Fig. 17.1 Map showing origin and migration of West Nile Virus. Data to create Map obtained from [1, 10, 22]

Cases of WNV have also been reported in Canada. In addition, enzootic transmissions occurred in the Caribbean and Central America. Of note WNV activity has not been reported in tropical South America as yet. This may be due to cross-protection from other flaviviruses circulating in tropical regions, less competent arthropod and avian hosts than in temperate regions, and the greater diversity of host species in the tropics or reduced virulence of WNV in the tropics [1, 20]. Similarly, there have been no overt cases in the UK, although there is evidence of serological conversion in sentinel chickens. However, since the year 2000, WNV has been detected regularly in France, in the southern regions, with significant morbidity in horses. The lack of human cases in northern Europe, compared to southern Europe, may possibly be attributed to the feeding behavior of the predominant vector, *Culex pipiens* that exists as two strains in North Europe. One strain feeds primarily on humans and the other strain feeds on birds only, whereas hybrid mosquitos feed on both hosts in Southern Europe facilitating viral transmission [1, 21]. Additional factors, such as climate, likely play an important role in viral transmission; for example lower temperatures in northern Europe are not usually suitable for the development of large populations of competent mosquitoes capable of effective viral transmission to humans [22].

The dynamic relationship between vectors and hosts, including mosquito feeding and avian migratory patterns, has facilitated the distribution of WNV as one of the most widespread arboviruses in the world (Table 17.1). Currently WNV has been reported on all continents, with the exception of Antarctica. In North America alone, there are approximately 59 species of mosquitoes (predominantly the *Culex* species) and 284 species of birds that have been reported as having been infected

Table 17.1 Summary of epidemiology of WNV

Year	Geographical location	Viral strain	Clinical properties
1937	Uganda—region west of the Nile	WNV of lineage 2	WNF; low virulence virus.
1937–1949	Africa, Eurasia, Australia and the Middle East	WNV of lineage 2	Sporadic cases of disease and outbreaks of WNF mostly in rural population; low virulence virus.
1950–1990	Egypt and the upper Nile delta	WNV of lineage 2	Epidemics of WNF with rare CNS disease; low virulence virus.
1990–1998	Middle east, Romania, Southern Europe and Australia	Emergence of WNV of lineage 1, WNV subtype (Kunjin virus) was isolated in Australia	Frequent outbreaks associated severe disease including viral encephalitis, now with case-fatality rate of nearly 10 %; high virulence virus.
1999–2003	New York city, USA, Israel, Russia, and Tunisia	WNV of lineage 1, (NY99)	Outbreaks occurred now in major metropolitan cities with CNS disease and a case fatality rate of about 10 %; High virulence virus.
			Virus spread to Canada and parts of South America.
2003–2011	Virus spread across continental USA reaching endemicity level with periodic out breaks.	New genotype had arisen, denoted as North American or WN02 from, the 1999 introduced (NY99) genotype and had displaced the introduced virus.	Seasonal (Summer) outbreaks with CNS disease in about 1 % of infections and mortality of about 10 %. The WN02 dominance appears to be related to increased transmission efficiency in *Culex* spp. mosquitoes.

Summarized from [1, 10, 22]

with WNV. The virus is maintained in nature in an enzootic cycle between birds and mosquitoes. Some mammals (such as horses and squirrels) and reptiles (such as alligators) have also been shown to be viremic [1].

3.2 *Virus Transmission*

The majority of human infections are due to mosquito bites. Humans and many other vertebrates are accidental hosts with low-grade, transient viremia insufficient to infect mosquitoes and are considered “dead-end” hosts. The amplifying hosts are birds including crows (*particularly Corvus brachyrhynchos*), jays, blackbirds, finches, warblers, and sparrows, which generally remain asymptomatic. Crows and blue jays are particularly susceptible. Indeed, increased mortality in such birds can predict increased risk for human cases and can be a crude indicator of virus activity [23].

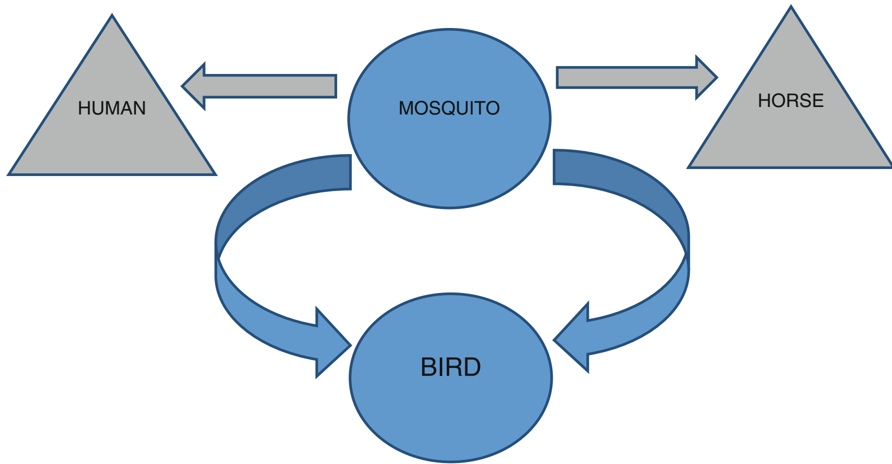

Fig. 17.2 West Nile Virus Transmission; the virus is maintained in nature in an enzootic cycle between birds and mosquitoes. Some mammals (such as humans and horses) are accidental "dead end" hosts

The *Culex* mosquito appears to be the most important mosquito species in the enzootic cycle, although the species varies by geographic location. Transovarial transmission of the virus in mosquitoes likely provides for viral over-wintering. After female mosquito takes a blood meal from an infected bird, the virus penetrates the gut, replicates, and travels to the mosquito's salivary glands. Then during subsequent feedings, mosquito injects virus-laden saliva into warm-blooded hosts and the cycle continues. Transmission season is more often from July to September. Multiple factors influence WNV transmission and infectivity, including advanced age, host immune and genetic susceptibility, and behavioral and environmental factors. Mortality among patients with meningitis and encephalitis is approximately 10 % and often in the elderly. The most significant outbreaks described in the 1990 serologic survey showed that severe complications are infrequent with only 1/150 infections resulting in WNV meningitis or WNV encephalitis. From data up to 2007, it has been estimated that from the 11,000 cases reported of invasive neurological disease in the USA that 1.6 million person were most likely infected. Serological surveys indicate that even in areas experiencing outbreaks, less than 10 % of the population is infected with WNV [24–29].

Rare but documented routes of viral transmission include transplantation of infected organs, the use of infected blood products, transplacentally, and possibly, through breast milk [30, 31]. At the peak of the 2002 epidemic in the USA, the risk for infection by transfusion was estimated to be as high as 21 per 10,000 donations [30]. Since then, blood-screening using real-time polymerase chain reaction (PCR) has been instituted and has significantly decreased the risk for contaminated blood.

Up to 2012 in the USA there were more than 37,000 reported (>350,000 estimated) human cases and over 1,500 (mostly in neuroinvasive cases) reported deaths since it was first detected in New York in 1999 [33] (Table 17.2).

Table 17.2 West Nile virus disease cases and deaths reported to CDC by year and clinical presentation, 1999–2012

Year	Neuroinvasive disease cases	Neuroinvasive disease deaths (%)	Non-neuroinvasive disease cases	Non-neuroinvasive deaths (%)	Total cases	Total deaths (%)
Total	16,196	1,443 (9)	20,892	106 (1)	37,088	1,549 (4)
2012	2,873	270 (9)	2,801	16 (1)	5,674	286 (5)
2011	486	42 (9)	226	1 (<1)	712	43 (6)
2010	629	54 (9)	392	3 (1)	1,021	57 (6)
2009	386	32 (8)	334	0 (0)	720	32 (4)
2008	689	41 (6)	667	3 (<1)	1,356	44 (3)
2007	1,227	117 (10)	2,403	7 (<1)	3,630	124 (3)
2006	1,495	162 (11)	2,774	15 (1)	4,269	177 (4)
2005	1,309	104 (8)	1,691	15 (1)	3,000	119 (4)
2004	1,148	94 (8)	1,391	6 (<1)	2,539	100 (4)
2003	2,866	232 (8)	6,996	32 (<1)	9,862	264 (3)
2002	2,946	276 (9)	1,210	8 (1)	4,156	284 (7)
2001	64	10 (16)	2	0 (0)	66	10 (15)
2000	19	2 (11)	2	0 (0)	21	2 (10)
1999	59	7 (12)	3	0 (0)	62	7 (11)

Source: CDC; ArboNET, Arboviral Diseases Branch, Centers for Disease Control and Prevention http://www.cdc.gov/westnile/resources/pdfs/cummulative/99_2012_CasesAndDeathsClinicalPresentationHumanCases.pdf

Although patients with WNV disease reported onset of illness throughout the year, more than 90 % of patients had onset of illness during July to September. The annual epidemic peak in the USA consistently occurs in August [33]. WNV has become endemic in the USA, with ongoing potential for seasonal epidemic transmission at the local, regional, or national level. Although annual WNV disease incidence varies substantially, the pattern of recurrence indicates that transmission is likely to continue [33].

4 Pathogenesis

WNV is maintained in nature in an enzootic cycle of mosquito-bird-mosquito. Mosquitoes serve as maintenance vectors in the cycle. The most common mosquito species responsible for transmitting WNV is the *Culex* species but this varies with geographic area. Of these, *Cx pipiens molestus*, *Cx univittatus* and *Cx tarsalis* have been implicated as important vectors of transmission in Africa, Middle East and the outbreaks in the USA [34]. Birds serve as amplifying hosts and usually remain asymptomatic, despite continuous viremia; however, occasionally high levels of viremia will lead to the bird's demise. Studies have shown a higher incidence of WNV infections in humans residing in high avian mortality areas, as avian mortality rates correlate with West Nile virus activity [23, 35]. The exact pathogenesis of

WNV in humans is not yet well understood but animal studies have shed some light into the interaction of the virus and the host immune system. Following *Culex* mosquito inoculation, the virus replicates in Langerhans *dendritic* cells and then disseminate to the regional lymph nodes where replication continues [36]. WNV then spreads to other peripheral organs including liver and spleen via the blood stream. As the virus is recognized by innate and adaptive immune responses, replication is limited in the periphery in most cases. Interferon-alpha and beta restrict viral translation and replication early in infection [37–41]. Both the humoral and cell-mediated immune systems are important in virus control [36].

Early CNS spread and high viremia are limited by B cells and primary IgM antibodies in conjunction with the complement systems. Specific activation of complement via innate recognition of proteins and secreted antibody interacts with a wide range of cell surface receptors on myeloid, lymphoid, and stromal cells [42–44]. Interferon-gamma-producing gamma-delta T cells also play a role in controlling viral replication, by stimulating the adaptive immune response. CD4 and CD8 T cells help viral clearance in the peripheral tissues [45–47]. If this peripheral control of the virus is inadequate, the virus travels to the CNS, via the blood or by retrograde axonal transport, where neuronal bodies are the primary target. In the CNS, INF alpha and beta with the aid of chemokines CXCL10 and CCL5 are responsible for controlling WNV infection and prolonging neuronal survival [37, 41, 48].

West Nile virus utilizes multiple mechanisms to survive, infect, and evade immune system recognition. Some of these mechanisms include the capacity to induce rapid cell death, diverse cellular tropism including the immune cells of the peripheral blood that may also serve in the early dissemination of the virus, masking of the virus RNA from the immune cells and the capacity to induce IFN resistance [49–51].

Human genetic factors are thought to influence the severity and outcome of the infection. For example in humans a 32-bp deletion in the coding region of the CC chemokine receptor 5 (*CCR5Δ32*) was reported to be associated with both increased susceptibility to WNV infection and death [52]. Certain single nucleotide polymorphisms (SNPs) of the genes that encode the antiviral enzyme 2′–5′ oligo-adenylate synthetase (OAS), *OAS1* and *OASL*, were found to be associated with WNV susceptibility or WNND [53]. Similarly, genetic SNPs in the interferon regulatory factors (*IRF3* and *MX1 genes*) alter the human interferon response to the virus pathway and were found to be associated with symptomatic WNV infection and disease progression [52].

5 Clinical Manifestations

WNV disease is a nationally notifiable disease with standardized case definitions. State and metropolitan heath departments report cases to CDC through ArboNET, an electronic passive surveillance system. The spectrum of WNV disease ranges from asymptomatic (~80 %) to neuroinvasive disease with morbidity and mortality of about 10 % of cases [10, 54]. When symptoms occur, they develop after an incubation period that typically lasts 2–6 days but may extend to 14 days, or even longer in immunosuppressed persons.

5.1 West Nile Fever

Roughly 20 % of those exposed to the virus develop West Nile Fever (WNF), symptoms of which include sudden onset of an acute, nonspecific, influenza-like illness lasting 3–6 days, with high fever, chills, malaise, headache, backache, arthralgia, myalgia, and retro-orbital pain, without overt neurologic signs [55]. In addition, generalized lymphadenopathy and a maculopapular or pale measles-like rash were reported. Incidence of rash is about 50–15 % of the cases, reported more frequently in children than adults and in WNF than with neuroinvasive disease which may point to a more robust host immune response to the virus. The rash usually presents approximately 5 days after the onset of symptoms, and lasts for about 1 week. Hepatomegaly, splenomegaly, myocarditis, pancreatitis, and hepatitis have also been described occasionally in severe WN virus infection [56].

5.2 West Nile Neuroinvasive Disease

Less than 1 % of those infected develop West Nile Neuroinvasive Disease (WNND), which may include encephalitis, meningitis, or meningo-encephalitis and flaccid paralysis (poliomyelitis-like syndrome) [10, 54]. WNND more frequently affects the elderly and immunocompromised population [57]. Patients typically have a febrile prodrome of 1–7 days, which may be biphasic, before developing neurological symptoms. Although in most cases, the prodrome is nonspecific, 15–20 % of patients have features suggestive of WN fever, including eye pain, and or a rash and about 5 % have lymphadenopathy [58, 59].

5.2.1 Meningitis and Encephalitis

Of those exhibiting WNND, roughly 40 % develop meningitis and 60 % encephalitis. Clinical features of meningitis include fever, nuchal rigidity, photophobia, headache, retro-orbital pain, and cerebrospinal fluid pleocytosis [55, 58, 59]. On the other hand, signs and symptoms such as altered mental status, focal weakness/numbness, seizures, or visual disturbances and diagnostic evidence of brain parenchymal involvement point towards the diagnosis of encephalitis [56].

5.2.2 Acute Flaccid Paralysis

Acute flaccid paralysis (poliomyelitis-like) caused by virus infection of the anterior horn of the spinal cord (myelitis) has been recognized and once paralysis is established, little long-term improvement has been described. Paralysis is frequently asymmetrical and may be associated with meningoencephalitis. Other neurological features include cranial neuropathies, optic neuritis, and ataxia. Stiffness, rigidity, spasms, bradykinesia, and tremors, associated with basal ganglia damage, have also recently been recognized in WNND [56–58] (Table 17.3).

Table 17.3 Clinical Manifestations of WNV infection

West Nile Fever:
Fever, chills, Flu-like illness; myalgia, arthralgia, retro orbital pain. Rash, lymphadenopathy, hepatomegaly, splenomegaly, myocarditis, pancreatitis, hepatitis.
West Nile Neuroinvasive disease (1 % of infections):
Flaccid (polio-like) paralysis
Meningitis syndrome
Encephalitis syndrome
Seizures
Extrapyramidal signs (tremors, ataxia, Parkinson's' like features)
Optic neuritis

Summarized from CDC; West Nile clinical evaluation
http://www.cdc.gov/westnile/healthCareProviders/healthCareProviders-ClinLabEval.html

6 Diagnosis

Diagnosis of West Nile virus infection (WNF and WNND) is based on clinical presentation, and confirmed with serologic or nucleic acid amplification testing [33, 60].

6.1 Clinical Criteria

Clinical Criteria for Diagnosis of Neuroinvasive disease requires the presence of fever and at least one of the following in the absence of a more likely clinical explanation: (1) acutely altered mental status (e.g., disorientation, obtundation, stupor, or coma), (2) other acute signs of central or peripheral neurologic dysfunction (e.g., paresis or paralysis, nerve palsies, sensory deficits, abnormal reflexes, generalized convulsions, or abnormal movements), or (3) pleocytosis (increased white blood cell concentration in cerebrospinal fluid) associated with illness clinically compatible with meningitis (e.g., headache or stiff neck) [33].

Non-neuroinvasive disease requires, at a minimum, the presence of fever, the absence of neuroinvasive disease, and the absence of a more likely clinical explanation for the illness. Involvement of non-neurologic organs (e.g., heart, pancreas, or liver) should be documented using standard clinical and laboratory criteria [33].

6.2 Laboratory Criteria

Laboratory Criteria for Diagnosis of neuroinvasive disease include at least one of the following: (1) Isolation of virus from or detection of specific viral antigen or genomic sequences in tissue, blood, cerebrospinal fluid, or other body fluid by PCR or (2) Detection of virus-specific immunoglobulin M (IgM) antibodies

demonstrated in cerebrospinal fluid by antibody-capture enzyme immunoassay (EIA); or (3) A fourfold or greater change in virus-specific serum antibody titer; or (4) Virus-specific IgM antibodies demonstrated in serum by antibody-capture EIA and confirmed by demonstration of virus-specific serum immunoglobulin G (IgG) antibodies in the same specimen or a later specimen by another serologic assay (e.g., neutralization or hemagglutination inhibition) [33].

Probable cases of infection have (1) a stable (twofold or smaller change) but elevated titer of virus-specific serum antibodies or (2) virus-specific serum IgM antibodies detected by antibody-capture EIA but with no available results of a confirmatory test for virus-specific serum IgG antibodies in the same or a later specimen [33].

On average IgM and IgG develop rapidly after WNV viremia (about 4–7 days respectively) and viral RNA persist for about 2 weeks before becoming undetectable [61]. IgM may persist for 3–6 months and IgG will persist for many years, likely conferring immunity against new WNV infections [62].

Some trends in basic laboratory tests, although non-specific, may point towards West Nile Virus infection. For example, elevated white blood cell count (greater than 10,800/mm^3, but rarely greater than 20,000/mm^3), mild decrease in hemoglobin (less than 13.5 g/dL in males and less than 12.0 g/dL in females), hyponatremia (less than 135 mm/L), elevated creatinine kinase, abnormal liver function tests (aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, bilirubin), or transiently elevated lipase (usually asymptomatic) [57].

Cerebrospinal fluid shows a moderate lymphocytic pleocytosis (greater than 5 cells/mm^3 or a mean cell count of approximately 225/mm^3), with up to half the patients have a neutrophil predominance and sometimes there are no cells [55, 57]. The protein is moderately elevated (greater than 40 mg/dL), and the glucose ratio is typically normal.

Radiologically, in most cases of WNND Magnetic resonance imaging shows high signal intensities in the deep gray matter of the nervous system. Such abnormal signals can be found in thalamus, basal ganglia, mesial temporal structures, brain stem, cerebellum and the spinal cord [63]. Patients with WNND may sometimes have normal neuroimaging studies [57, 63].

As mentioned above, studies that definitively diagnose or confirm WNV infection includes viral isolation, amplification of viral nucleic acid and antigens with polymerase chain reaction, or enzyme-linked immunosorbent assay to detect WNV IgM or IgG antibodies [10, 33, 54]. It is to be noted however that some WNV-infected patients have persistent WNV IgM serum and/or cerebrospinal fluid after recovery without ongoing disease and hence interpretation of serological tests needs to be done carefully in conjunction with the clinical syndrome and careful consideration of other deferential diagnosis especially in cases of atypical presentation [64].

Other diagnostic studies include electroencephalography for patients with seizures and electromyogram studies for nerve conduction abnormalities [57]. These studies, although sensitive, are less specific than the traditional serologic studies mentioned above. Electroencephalograms show diffuse slowing and, in some cases, focal seizure activity. Nerve conduction studies typically show the reduced motor axonal

Table 17.4 West Nile Neuroinvasive disease diagnosis

Clinical criteria	Laboratory criteria	Other tests
Fever and	– *Isolation of virus* (specific viral antigen or genomic sequences in tissue by PCR, blood, cerebrospinal fluid); or	– Brain MRI: abnormal signals in the basal ganglia, thalamus, cerebellum, and brainstem.
– Acute confusion, or Acute signs of central or peripheral neurologic dysfunction (e.g., paresis or paralysis, nerve palsies, convulsions, or abnormal movements).	– Virus-specific immunoglobulin M (IgM) antibodies demonstrated in CSF or blood, or	– Nerve conduction studies show reduced motor axonal amplitudes.
– CSF pleocytosis consistent with viral meningitis syndrome	– A fourfold or greater change in virus-specific serum immunoglobulin G antibody titer.	

Summarized from: CDC. Surveillance for human West Nile virus disease—United States, 1999–2008. Surveillance summaries. MMWR April 2, 2010;59(SS02);1–17

amplitudes consistent with anterior horn cell damage, although there may also be some slowing of conduction velocities and some changes to sensory nerves [57, 65]. Nerve conduction studies are helpful to differentiate WNND from Guillain-Barre syndrome (Table 17.4).

7 Treatment

There is no current approved specific treatment for West Nile Virus infection. The current recommendation is supportive treatment. Yet three major therapeutic approaches are leading the research in finding a definitive treatment for this disease. These are Interferons, Ribavirin and intravenous Immunoglobulin.

7.1 Interferons

Interferon Alpha has been shown in vitro to be efficient in inhibiting WNV replication. It protects, restricts and enhances cellular and neuronal response to WNV infection [37]. Although some studies have shown that virulent WNV of lineage one, exhibits inherent resistance to interferon Alpha and Beta [41], Kalil, et al reported two cases of successful neurologic improvement after treatment with interferon Alpha 2b when given within 72 h of presentation [66]. On the other hand, there have been case reports showing ineffectiveness and poor outcomes with patients treated with interferon during an outbreak in Israel [67]. The therapeutic role of interferons is yet to be established.

7.2 Ribavirin

Some in vitro studies indicated that high doses of Ribavirin in animal model can be protective against the effects on WNV on the animal cell [68]. Ribavirin is an antiviral agent that Inhibits replication of RNA and DNA in wide range of viruses (non-specific) and can be associated with serious side effects in high doses. Ribavirin in some animal studies was shown to be associated with increased mortality in the context of WNV infection and hence there is not yet a consensus of the efficacy and safety of this drug in WNV infection in humans [69].

7.3 Intravenous Immunoglobulins

The use of intravenous immunoglobulins for WNV infections is also under research with the rational that high titers of WNV antibodies can mount a protective effect against the WNV [70, 71]. Several clinical trials are underway for the treatment of WNV. Nevertheless, to date studies showing efficacy and responses to these different therapies in humans remain inconclusive and treatment remains mostly supportive.

8 Prognosis

The prognosis of WNV infection appears to be variable but favorable in general, depending mostly on the presentation ranging from West Nile fever to neuroinvasive disease. The most common symptoms post-infection were fever and fatigue lasting for several days. Aching, general malaise, and weakness lasting weeks to months were reported in some series [58, 64, 72]. The prognosis of patients with WNV meningitis and encephalitis, is also generally favorable but persistent headaches, fatigue, focal neurological deficits may persist for months to years [73]. Important predictive risk factors such as age, use of immunosuppressants, and persistent comorbidities play an important role in the overall outcome [29, 74, 76, 77].

Longitudinal cohort studies of long term outcome and prognosis of WNV infected individuals showed that on average physical and mental function as well as mood and fatigue, appear to return to normal within 1 year of symptom onset. Patients with WNND took slightly longer to recover, and the recovery rate of meningitis and encephalitis cases were about the same. Patients without preexisting comorbidities had faster recovery of physical function [76]. A slower recovery rate is associated with WNV paralysis (poliomyelitis-like syndrome) where recovery was found to be much slower and sometimes rare [78, 79].The overall case-fatality rate with WNND is about 9–10 %; highest in older individuals and those with comorbidities [76].

9 Vaccination and Prevention

WNV has caused, and certainty has the potential to cause, large epidemics of arboviral illness in the Western Hemisphere. Consequently, the need for efficient modes of prevention and the development of a human vaccine are mandatory. At this time the most effective prevention methods remain as mosquito avoidance, vector control and personal protection measures [79]

Mosquito repellants, elimination of breeding sites and barrier methods such as window screens are emphasized during epidemic seasons.

Previous efforts for the early detection and monitoring of WNV activity have used dead bird density or spatial scan statistic as a proxy for transmission risk for humans [80]. Another, perhaps more accurate, approach is the DYCAST system (The Dynamic Continuous-Area Space-Time system). This is a biologically based spatiotemporal model that uses statistical and geographical analysis of public reports of dead birds to identify areas at high risk for West Nile virus transmission to humans, implemented in New York City in 2001 and Chicago, IL in 2002 [81]. Results from prospective implementation of the DYCAST system in California showed that this model provided accurate and early identification of areas at high risk for human WNV transmission during an epidemic in 2005, and was used to assist public education campaigns, surveillance, and mosquito control programs [73, 82]. Early warning of high-risk areas for West Nile Virus activity allows preventative measures to be implemented in a timely and effective manner.

Public health education programs should target older adults, people who are immunosuppressed and those with co morbidities, because they are at increased risk for neuroinvasive disease and death. In the absence of an effective human vaccine, the cornerstones of WNV disease prevention will continue to be (1) community-level mosquito control (larviciding, adulticiding, and breeding-site reduction), (2) peridomestic measures (repairing and installing door and window screens, using air conditioning, and reducing breeding sites), and (3) personal protection measures (use of repellents, use of protective clothing, and avoidance of outdoor exposure when mosquitoes are most active). WNV surveillance continues to be important for monitoring seasonal WNV activity and targeting prevention and control activities [33].

The improved screening of banked blood with the use of Minipool nucleic-acid amplification testing (MP-NAT) and Individual Donation NAT (ID-NAT) significantly reduced WNV transmission via blood transfusion [83].

A major emphasis remains to produce a human vaccine. Although no human vaccine is available yet, the future remains optimistic, given the substantial impact made in veterinary public health with the currently licensed four equine WNV vaccines in the USA. These include a formalin-inactivated virus, a recombinant Canarypox virus expressing prM/E proteins of WNV [84]. A chimeric virus vaccine from an infectious clone of yellow fever 17D virus [85], and a DNA vaccine, the first DNA vaccine to be licensed in any country [86]. Promising data regarding a human vaccine is being analyzed since reports of results of a phase one human clinical trial in subjects receiving WNV DNA vaccine documented the development of

neutralizing antibodies to the WNV [87]. If a human vaccine becomes available and in conjunction with proper preventions methods, the risk of WNV infection and its complications can be substantially be reduced.

10 West Nile Virus and Bioterrorism

There has been growing concern about the use of microbes as weapons for a number of reasons [88, 89]. First, microbes have the potential of killing or harming a large number of people in a short period of time. Second, their deliberate spread can be hidden or go unnoticed until large numbers of people get sick and present to hospitals, emergent care facilities, and doctors' offices. Third, even the slightest suspicion of a bioterrorism attack, can cause significant panic and havoc among people and have significant financial consequences. Fourth, bioweapons are relatively inexpensive to create and sometimes referred to as the "poor man's atomic bomb." Finally yet importantly, as the power of biological sciences grow it seems inevitable that more potent and diverse bioweapons will be created. West Nile Virus can be targeted for such use; it is moderately easy to disseminate by infected mosquitoes and birds, has moderately high virulence with significant morbidity and mortality with CNS disease and has the potential of even higher virulence with genetic manipulation. As matter of fact, when the virus first appeared in the USA the US government and the CDC have considered the act of bioterrorism, but further investigations showed that the virus activity was consistent with its natural behavior. It is believed that the introduction of the virus was work of nature by bird migration or accidental by imported infected birds. It is important however to understand the potential of using WNV as a bioterrorism agent and physicians in the USA should be familiar with the various clinical presentation and means of diagnosing illnesses caused by West Nile Virus [90].

11 Global Warming and West Nile virus

There are several indications that the rising temperatures of the planet had aided the spread of vector borne infections, including WNV [22]. Similar to other arboviral diseases, the spread of the virus is influenced by vector spread. Climate, such as temperature and rain fall have significant impact on vectors' geographic habitats, life cycles, feeding behavior, and evolution. As mentioned above, nucleotide sequencing studies have showed that WNV introduced to New York in 1999 (NY99) was closely related to a strain of WNV from Israel (Isr98) at the time. The weather in New York during the spring and summer of 1999 had been particularly warm and humid which favored intensive mosquito breeding and efficient arbovirus transmission, resulting in the epidemic [22, 91, 92]. WNV then evolved and adapted to its

new environment and is now expected to continue to be an endemic virus with frequent outbreaks in the USA [92]. Climate change is a current global concern and its consequences on health and human diseases should be regarded with greater importance and urgency.

12 Conclusion

West Nile virus is one of the most widely distributed of all arboviruses in the world and has been reported in all continents except Antarctica. West Nile Virus belongs to the family Flaviviridae together with Japanese encephalitis viruses (JE), Saint Louis Encephalitis viruses (SLE), Dengue and Yellow fever viruses. The virus is maintained in nature in an enzootic cycle of mosquito–bird–mosquito with humans and other vertebrates as incidental dead end hosts. The journey of West Nile virus from Africa where it was first discovered, in Uganda west of the Nile, in 1937 till it reached the Western hemisphere in 1999, has been intriguing, interesting and exemplifies the evolution and migration of viruses. To date, globally, there are over 350,000 estimated human cases and in the USA, approximately 37,000 cases reported with 1,500 deaths. The incidence has since declined due to improved vector control efforts, but is still detectable, making WNV an endemic pathogen to the USA, with the potential of forming seasonal epidemics (July to September). Currently, WNV is the most common cause of arboviral CNS infection in the USA. Up to 80 % of human infections however are asymptomatic. Both of the humoral and cell-mediated immune systems are important in controlling the virus, and are usually efficient and terminate the virus in the peripheral organs in the majority of immune-competent individuals. The virus however is neurotropic and in certain high-risk individuals (elderly or immunocompromised) can cause devastating neurological disease with mortality approximately around 10 %. Other milder presentations include West Nile fever, which presents as a self-limited Flu-like illness. Diagnosis is confirmed serologically or by PCR technology and treatment is supportive at this time. Prevention is by vector control and personal protective measures against mosquitos bite. Vaccines for veterinarian use are available and has significantly reduced incidence in horses. Vaccines trials for human are under way and are promising. Although it is less likely that the introduction of the virus to the western hemisphere is a deliberate act of bioterrorism and more likely to be accidental or work of Nature, the potential for the West Nile Virus to be weaponized is there and should not be ignored by the health care establishment. The changing climate effect on the spread of arboviral viruses, exemplified by WNV, is evident, cannot be ignored and should be regarded with greater urgency.

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Chapter 18
Zika Virus

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Core Message Although Zika virus is generally considered a relatively benign flavivirus, it is hypothesized that the study of this virus is useful as an indicator of other more virulent viruses. The increased spread and prevalence of Zika virus thus may be indicative of similar changes in more virulent viruses. It is also hypothesized that Zika may mutate into a more virulent form than what has hitherto occurred.

1 Introduction

Among many public health alerts, the global spread of arboviruses is of concern and alarm. The hypothesis in this chapter is that the inclusion of Zika virus in arbovirus monitoring is a well-justified expense because its spread may be diagnostic for the spread of flaviviruses, its spread is largely unexplained, and the virus has the potential to mutate into strains that are more virulent. Moreover, such evolutionary studies are of importance in and of themselves for the same reasons.

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2 Epidemiology: Temporal and Geographical Associations

Zika virus is a flavivirus and is related to other arboviruses such as yellow fever virus, Japanese encephalitis virus, dengue virus, and West Nile virus [1]. The Eighth Report of the International Committee on Taxonomy of Viruses describes several neglected species of mosquito-borne flavivirus, some of which are without apparent pathogenicity in animals, but which are pathogenic in humans. This group includes Zika virus, lineage-2 West Nile virus, and Usutu virus [2].

In 1947, Zika virus was originally isolated from a febrile sentinel rhesus monkey and from a pool of *Aedes africanus* mosquitoes in the Zika forest in Uganda during a yellow fever study [3]. Zika virus was first detected in humans 5 years later in 1952 using neutralizing antibody testing in sera from East Africa [3–5]. Zika virus was first isolated from a human in Uganda [6].

In specimens from humans in Nigeria, from 1964 to 1970, there were 15 types of arboviruses among 171 isolations. The majority of isolations were from children below 4; however, isolations were made from all age groups. Zika virus was isolated at low frequency in comparison to yellow fever, Chikungunya, dengue types 1 and 3, and Tataguine (endemic in Ibadan). Additional viruses isolated were Bwamba, and Bunyamwera group viruses that were isolated from humans for the first time. Isolation rates varied from peaks in 1969 to lows in 1965 and 1967. Zika virus isolation rates also varied by season: peaks in rainy seasons (June to August) and lows in dry seasons (January to February). Zika virus was also detected in a study in Ibadan, Nigeria, in 1975 [5].

In 1954, Zika virus was detected during viral serological survey studies. In 38 localities in 6 states in India 15 arboviruses were studied including yellow fever, Bwamba fever, Bunyamwera Ilhéus Semliki Forest virus, St. Louis encephalitis virus, West Nile virus, dengue types 1 and 2, West Nile virus, Uganda S, Nitaya, Japanese B, Murray Valley virus, and Russian spring-summer encephalitis virus [7].

Between 1977 and 1978, in Malaysia and Indonesia, there were clusters of Zika virus infection towards the end of the rainy season when *Aedes aegypti* flourish. Thirty patients had serological tests for alpha and flavivirus infections including Zika—MR766, Japanese encephalitis—Nakayama, dengue type 2—New Guinea C, Tembusu—MM1775, and Murray Valley encephalitis—original [8].

Three strains of Zika virus were isolated as part of yellow fever studies in the Ivory Coast in 1999. Amaril, yellow fever, and dengue viruses were prevalent among vector and human populations in this study [9]. The first known isolation of yellow fever from *Aedes africanus* mosquitoes was in Africa, in Touba, the Ivory Coast.

In Sabah, Malaysia, Zika virus infection was shown in some of 60 semi-captive and 84 free-ranging orangutans (*Pongo pygmaeus pygmaeus*). Both groups showed evidence of exposure to 10 of 46 additional viruses including Japanese encephalitis virus [10].

This confirmed earlier studies in North Bornean forests where arbovirus transmission in wild orangutans was studied and included three virus families (Flaviviridae, Alphaviridae, and Bunyaviridae). Viruses detected included Zika, Japanese encephalitis, dengue 2, Langat, Sindbis, Tembusu, Batai, and Chikungunya viruses [11].

How far had the virus spread? Surprisingly, Zika virus spread further, beyond the confines of Africa and Asia as it was detected on Yap Island in Micronesia in 2007 in the southwestern Pacific Ocean [1]. In June 2007, Zika virus appeared in Southeast Asia and Federated States of Micronesia, in Yap state, more than 60 years since its detection in 1947 in Uganda [12]. The illness was characterized by conjunctivitis, arthralgia, and rash affecting 100 individuals whereas prior studies had detected only 14 infected individuals. No deaths, hospitalizations, or hemorrhagic manifestations were found. It was estimated that 74 % (95 % CI, 68–77 %) of residents of Yap, 3 years and older, were recently virus infected. The predominant mosquito species vector was *Aedes hensilli*. Public health officials and clinicians were alerted as Zika virus had expanded outside Africa and Asia. In this outbreak, no dengue virus or other arboviral RNAs were detected [13].

Zika virus is one of the arboviruses that have spread in Southeast Asia including dengue, Japanese encephalitis, and Kunjin as well as alpha viruses such as Chikungunya, Sindbis, and Getah. The increase and spread of these viruses has been overall exponential since 2009 and generally is linked to several complex factors (as discussed below) [12].

Zika virus infections were also detected among people in Nigeria, Uganda, Egypt, India, Pakistan, North Vietnam, Thailand, Malaysia, Indonesia, and the Philippines. Sentinel animal and mosquito studies also supported the endemic presence of Zika virus in Africa and Southeast Asia. In addition, Zika virus was detected north and west of the Wallace line (a biogeographical line situated between Borneo and Java demarcating a syzygy of species between Australia and South East Asia) [14].

Occupational infection of Zika virus was reported in a scientist who contracted the virus in the laboratory after having had yellow fever virus vaccination. This individual demonstrated an anamnestic response related to yellow fever virus that complicated virus identification. The problem was resolved after isolation of Zika virus during the acute phase of disease [15]. An Australian traveler upon returning from Indonesia had been infected with Zika virus [16]. Another publication reported that two American scientists contracted Zika virus when working in Senegal in 2008. One of them upon returning to the USA transmitted the arbovirus to his wife. Clinical and serologic evidence helped identify Zika virus. The route of transmission from the scientist to his wife was concluded to be sexual [17].

Table 18.1 provides a brief outline of the chronology and geography of Zika virus studies.

3 Vectors and Reservoirs

3.1 Mosquitoes

Aedes mosquitos play a significant role in Zika virus transmission. In 1972 in Sierra Leone during an entomological and serologic survey, sera from children up to 14 years were analyzed for 12 antigens from viruses including Zika, Chikungunya, West Nile, and yellow fever using HI and CF testing. The prevalence was much

Table 18.1 Chronology and geography of Zika virus studies

Study year	Study locations	References
1947	Zika forest, Uganda, Nigeria, and East Africa	[1, 3–5]
1954	India	[4]
1970	Nigeria	[5]
1978	Malaysia, Indonesia	[8]
1999	Ivory Coast	[9]
2001	Sabah, Malaysia	[10, 11]
2007	New World, Easter Islands, Nepal, Argentina, Hawaii, Scandinavia, Saudi Arabia	[18]
2007	Micronesia	[13]
2008	Southeast Asia, Australia	[19]
2009	Southeast Asia	[12]
2008–2011	Senegal, Nigeria, Uganda, Egypt, India, Pakistan, North Vietnam, Malaysia, Indonesia, the Philippines, Borneo/Java, Micronesia, USA	[1, 14, 17]
2012–2013	Indonesia, Singapore, Australia, Tahiti, Germany	[16, 20–24]

greater than the incidence for arboviruses but varied among different geographical sites. In most areas, Zika virus abounded and was active whereas, for example, Chikungunya virus was active in Northeast savannas and plateaus. The entomological survey indicated that pools of water and *Ae. aegypti* larvae were present in greater abundance in urban dwellings near mines than in rural areas. It was concluded that conditions were ripe for epidemic outbreaks within a few years [25].

There are potential additional mechanisms of spread of Zika virus because of its relatedness to other viruses. In Central and West Africa, arbovirus survival was studied during interepidemic periods. Several yellow fever strains were isolated from *Aedes africanus*, *Aedes furcifer-fylori*, *Aedes opol*, and *Aedes luteocephalus*. Savannas (including savannas without forest and differentiated savannas with forests) that have abundant *Isoberlinia doka* (a hardwood tree) were associated with sylvatic yellow fever circulation. The primary endemic areas of yellow fever include equatorial moist forests, termed the emergence zone. The sylvatic yellow fever circulation in this forest zone was concluded a major threat and source of yellow fever for humans via penetrating epizootics into the savannas. A case in point supporting this model was the 1978 Gambia outbreaks. Several additional observations further support this approach and model for the spread of such arboviruses. Transovarial transmission (TOT) was demonstrated for mosquitos including *Aedes aegypti* and *Aedes furcifer-fylori*, and explained an emergent zone for survival of virus in the dry season. The size of monkey populations appears to further influence the degree of virus propagation. In addition, tick eggs and adults were sources of yellow fever virus. This reservoir acts as a tributary adding to the vertebrate-mosquito cycle promoting arbovirus survival [26].

TOT of arboviruses is a serious concern and was demonstrated for Culex flavivirus (CxFV). CxFV was detected using reverse transcription-polymerase chain reaction

(RT-PCR) in *Culex pipiens* (L) mosquitos captured in the field. Their progeny were viral infected as well and viral RNA was detected in several progeny tissues (salivary glands, ovaries, testes, head, fat bodies, and midgut) [27]. Previously, TOT had been proposed as a mechanism contributing to the spread of Zika and yellow fever viruses in Uganda [28]. Thus, TOT should be studied in greater detail and therapies developed to combat the fail-safe mechanism that allows seasonal survival of arboviruses.

There was also an interesting dynamic of Zika virus spread by *Aedes* (*stegomyia*) *africanus* (*theobald*) arboreal mosquitoes in the Zika forest in Uganda. Between November 1961 and June 1963, on a 120-ft (36.5-m) tower, twelve Zika virus strains and one strain of a different Group B arbovirus were isolated. Pools of mosquitoes were used for the virus isolations. Serum antibodies from Zika virus forest small animals did not show any reactivity with Zika virus. It was stated that some other virus reservoir was responsible for the infected mosquitos and that convection currents above the forest canopy could spread virus-infected mosquitoes during the first few hours after sunset [29].

Yellow fever virus-resistant and susceptible phenotype inbred (isofemale) *Aedes aegypti* mosquito lines were produced. Resistance was due to a block in the virus life cycle that prevented virus passage beyond the mosquito midgut during its life cycle. In addition to yellow fever virus, other flaviviruses that were restricted included Zika, dengue 1–4, and Uganda S viruses. Further mosquito genetics studies indicated that the midgut resistance phenomenon is due to a group of genes that includes a major gene and several minor genes or several genes in a group that are linked [30].

A recent Chikungunya virus pandemic in the Singapore area prompted further study of mosquito vectors. More than 20 arboviruses are transmitted by sylvatic *Aedes albopictus* mosquitoes in and around Singapore. Further studies demonstrated that *Aedes albopictus* mosquitoes were capable of transmitting Zika virus. In Singapore and environs, the same *Aedes* mosquito vector is shared among dengue and Chikungunya viruses as well as Zika virus. Existing programs in Singapore to control dengue and Chikungunya viruses may help control Zika virus as well [20, 21, 31].

Several methods in use to control mosquito vectors that transmit arboviruses include insecticides, genetically modified sterile insects, and draining swamps [32]. A recent novel approach was described by Darbro et al. [33], which utilizes the fungus, *Beauveria bassiana*. In laboratory conditions, this fungus reduces *Aedes aegypti* longevity and fecundity, whereas egg batch size and viability were unaffected. In semi-field conditions in northern Queensland, Australia, mosquito survival was reduced in cages of various sizes and there was some reduction in blood feeding [33]. This approach requires some caveats as fungi can mutate, immune-compromised humans may be susceptible to fungal infections, and fungal infected mosquitos may develop resistance with unpredicted consequences. Identifying the effective molecules that affect the mosquito life cycle may be most specific and effective in applying this method of control towards arbovirus control.

3.2 Monkeys

In 1947 Zika virus was originally isolated from a febrile sentinel monkey in Uganda during a yellow fever study [3]. In Uganda, monkeys serve as two types of sylvan hosts for yellow fever (YF). (1) As an enzootic state in the Zika forest in Western Uganda (Bwamba County) and (2) as epizootics in central Uganda zone of forest savannas. However, an epizootic for Zika virus occurred in two episodes in the Zika forest near Entebbe: (1) in 1969, post-epizootic of 1962–1963, with consequent accumulation of nonimmune monkeys and (2) in 1970, when biting densities increased for *Aedes africanus*. Eighteen months after that, an intensive epizootic for YF developed. This contradicted the hypothesis that subsequent YF epizootics would be subdued by Zika virus infections in nature for red-tail monkeys. Two factors important for further study of flavivirus mosquito are transovarial and phlebotomine sand-fly transmission [28].

During the Zika forest yellow fever epizootic in 1972, several other arbovirus antibodies were discovered as well in monkeys near Entebbe, Uganda. The viruses in addition to yellow fever included Zika, West Nile (WN) O'nyong-nyong (ONN), Chikungunya (CHIK), and Wesselsbron (WESS). That these viruses are immunologically cross-reactive was known at the time of the study. In addition, it was found that although YF virus is deadly for humans it is mild in monkeys in their sylvan natural habitat [34, 35].

3.3 Additional Species

Many species have been under the radar with unsuspected potential flavivirus infections. However, several different species are implicated in their susceptibility to viruses related to Zika virus and function as potential reservoirs: West Nile virus—cat, dog, horse, alligator, deer, primate, rodent, rabbit, reptile, opossum, bird, and raccoon; Japanese encephalitis virus—bird, pig, cow, horse, monkey, and rodent; St. Louis encephalitis virus—bird, armadillo, rodent, opossum, raccoon, and squirrel; yellow fever virus—monkey, opossum, rodent, kinkajou (an arboreal raccoon-like mammal with a prehensile tail but NOT a primate), bat, hedgehog, wild dog, mongoose, wild bird, anteater, and squirrel; dengue virus—bat, chipmunk, rabbit, guinea pig, mouse, Yucatan miniature pig, and horse [36].

Recent work indicates that snakes are a reservoir for EEEV in North America. This adds to the list of animal reservoirs for EEEV and possibly related viruses and may have an impact on our understanding of additional reservoirs for flaviviruses and Toga viruses [37].

Thus, the jury is still out related to Zika virus reservoirs as well.

3.4 *Cell Culture Susceptibility Profile*

A study of several strains of dengue virus infectivity of cell cultures derived from various species demonstrated a wide ability of these strains to infect various cell types, although no correlation from in vitro and in vivo situations could be made. However, it was proposed that dengue virus strains might be more prevalent in bats than had been hitherto considered. The virus strains were DENV-1 Hawaii, DENV-2 New Guinea C, DENV-3H87, DENV-4H241, DENV-1 BC-89/94, DENV-2 BC-100/98, DENV-3 BC-14-97, DENV-1 WestPac-74, DENV-2s16803, DENV-3 CH5548904500, and DENV-4 341750. The species were free-tailed bat, chicken, cottontail rabbit, human, domestic cat, horse, grey fox, raccoon, North American mule deer, Virginia opossum, sheep, nine-banded armadillo, domestic pig, rhesus monkey, cow, domestic dog, and eastern woodchuck [38].

4 Conditions for Spread

4.1 *Ecology and Geography*

During the last 20 years, human actions have been pinpointed as profound influential variables in virology and vector-driven diseases of viral origin. This has become most evident in Oceania and in Southeast Asia. Viruses, their vectors, their geographical distributions, increased demographic and ecologic dysgenesis, and increased travel and trade are contributory factors. Barboza et al. [19] also review emergent viruses in the Pacific and Southeast Asia including Zika virus, dengue, Chikungunya, and Japanese encephalitis viruses. Likewise, the steady annual increase in Ross River and Barmah viruses in Australia, the Nipah virus deadly epidemics in Southeast Asia, and lyssavirus including Kunjin and Murray Valley viruses are examples of consequences of ecologic and geographic alterations [19].

Many flaviviruses cause diseases in humans, livestock, and wildlife. Vector-borne flaviviruses have spread globally at increased rate during the last two decades. This occurred outside the bounds of the traditional geographical ranges of these viruses. For example, there are increased cases of introduction of West Nile virus into the New World and Easter Islands in the Pacific; outbreaks of dengue in Nepal, Argentina, and Hawaii; Usutu virus into Europe; tick-borne encephalitis in Scandinavia; and tick-borne Alkhurma Kyasanur Forest disease virus in Saudi Arabia [18]. One may well ask whether these events are due to global warming. See below for a discussion in regard to the impact of global warming and viral spread.

4.2 Global Warming

Zika virus as an emergent virus is reflective of the emergence and spread of other viruses as mentioned. It is thus important to describe the global setting, as it exists for the spread of Zika and related viruses and this setting is global warming. The following is a brief description of the effects of global warming on the spread of infectious diseases.

The Copenhagen United Nations Climate Change Conference in 2009 produced important information related to global climate change. This conference took place to continue the work of the Kyoto protocol that went into effect in 2005 and expired in 2012. The Kyoto protocol, although ratified by 187 countries, was not ratified by the USA. Climate change is due to an imbalance of inbound vs. outbound terrestrial radiation energy. However, unfortunately, the conference ended without a resolution addressing global climate change and vector-borne and waterborne infectious diseases [39].

The spread of mosquito-borne viruses into geographical zones that have had temperate climates (e.g., Usutu virus in Central Europe) appears to be associated with global warming. Moreover, a rise in international trade and travel further facilitated permanent establishment of mosquito-borne viruses. This is occurring in industrialized countries worldwide and facilitates spread from competent mosquito vectors to less competent vectors [2].

4.3 Social Change and Urbanization

The main arbovirus vectors are *Aedes aegypti* and *Aedes albopictus* mosquitos. Their spread is due to human behaviors including the slave trade from the fifteenth to nineteenth centuries, economic enterprises and expansion, more recent globalization of trade and economics, urbanization of Latin America and Asia, increased concentrations of human populations, and concomitant sanitary issues that promote the spread of mosquito vectors. Arboviruses reflect this vector spread and across 100 countries, for example, there is a pandemic of 50 million dengue infections annually with spread continuing [40].

The term climate change is a euphemism for global warming. The damage that is occurring and projected to occur is actually due to the increased temperature. This process results in increased vector activity and disease transmission. For example, the Anopheles mosquito that transmits malaria needs temperatures of just 16 °C and above to complete its life cycle. As global warming increases, so do deleterious results increase as well, including vector-borne disease, diarrheal disease, malnutrition, and injury from natural disasters. A major consequence of these effects is an increase of premature deaths. Premature deaths and disability are measured in disability-adjusted life-years (DALYs) per million population as follows: Africa 3,071.5; Eastern Mediterranean 1,586.5; Latin America and Caribbean 188.5;

Southeast Asia 1,703.5; Western Pacific 111.4; developed countries 8.9; and global average 1,111.7 [39].

The golden toad (*Bufo periglenes*) and Monteverde harlequin frog (*Atelopus sp.*) became extinct 23 years ago in Costa Rica. In addition, it is estimated that 67 % of approximately 110 or so species of *Atelopus* that are endemic to the American tropics are also extinct. A pathogenic chytrid fungus (*Batrachochytrium dendrobatidis*) is primarily to blame. This ecologic "writing on the wall" is due to global warming [41]. Thus, the spread of infectious disease can have profound and unanticipated effects on ecology, biology, and evolution.

The distribution, transmission, and abundance of vectors that bear and transmit diseases are being enhanced by global warming. Encephalitic viruses as well as dengue, malaria, and plague are increasing due to the infestation of such vectors and their cognate arthropods into geographical regions that were hitherto too cool for their presence. Likewise, south of the Southwest US-Mexican border, Mexican states show a 500-fold increase in dengue disease. West Nile virus has taken up residence in the USA near stagnant water, golf courses, waterways, swamps, and ponds. St. Louis and Equine encephalitis viruses are spreading as well. The increased spread of typhoid and cholera into Zimbabwe and Ethiopia also exemplifies and supports the contention of such deterioration—due to hygiene and water quality deterioration in conjunction with global warming [42].

The mean global temperature increased approximately by 1° centigrade during the last several hundred years. However, during the next 20 years it is anticipated to increase by 2–3° centigrade. Consequently, it is expected, for example, that the global malaria risk population will increase from 3 to 5 % and diarrheal diseases will increase by 10 %. The increased prevalence of malaria will be due to the vector-borne spread of this disease into geographical areas including the East African highlands where it has not yet been endemic [39]. Thus, warnings are dire and expectations grim.

5 Clinical

5.1 Clinical Findings and Neurological Disease

Zika virus is not as benign as it is sometimes considered to be and may be one of the most commonly reported human illnesses where it occurs. Most of the cases are described with relatively mild disease. Initial clinical descriptions indicate fever, headache, body pains, and rash as the manifestations of Zika virus infection. It can present with a syndrome reminiscent of influenza infection and thus may be underreported. Symptoms include lymphadenopathy, edema, retro-orbital pain, and diarrhea. In addition, common presentations accompanying the febrile illness include maculopapular rash, arthralgia, and conjunctivitis and are frequently confused with dengue virus infection that may also result in underreporting Zika virus infection.

Other symptoms range from fever and headache to fatigue, malaise, stomachache, dizziness, anorexia, hematospermia, prostatitis, dizziness, and lightheadedness [1, 5, 6, 8, 18, 43–45]. Although severe neurologic manifestations have not as yet been reported, misdiagnosis or underdiagnosis cannot be excluded.

In a mouse model, newborn and 5-week-old mice were inoculated intracerebrally with Zika virus. Astrocytes became enlarged and there was some destruction of pyriform cells of the hippocampus. Virions were produced within the endoplasmic reticulum in both neurons and astrocytes [46].

There are indications that the Zika-related viruses do indeed cause invasive neurological infections and disease including encephalitis due to Japanese encephalitis virus and encephalitis/meningitis due to West Nile virus and Chikungunya virus [47]. Whether Zika virus causes or has the ability to cause neurologic disease in humans is still unclear. More work needs to be done to investigate the effect of Zika and other related viruses on the central nervous system (CNS).

5.2 Diagnosis

There is still some difficulty in clinically diagnosing Zika virus infection because it is easily mistaken for other arbovirus infections including Chikungunya and dengue fever [44].

Differential diagnosis of Zika virus infection includes other arboviral diseases causing fever, headache, rash, and arthralgia; in addition tick-borne encephalitis (TBE) flavivirus infection causes severe hemorrhagic fevers, meningitis, and encephalitis. Molecular and immunological methods are important concerning the question of specificity of diagnosis. PCR and serologic studies have been used to make diagnosis of Zika virus infection. For example, in 2010, Zika virus infection of a child was confirmed in Cambodia using PCR in addition to immunological methods in specimens taken in the field. Furthermore, dengue, West Nile virus, and yellow fever virus infections were excluded [44]. Zika virus infection has similar manifestations to other arboviral infections. Clinicians should be aware of this and utilize additional confirmatory tests to make the diagnosis in patients who live in or have recently visited Zika virus endemic areas. See also Sect. 5, below.

6 Molecular biology

6.1 Viral Molecular Pathogenesis

Complete genome sequences were produced for the first time for Zika virus (as well as Bagaza and Kedougou viruses). Open reading frames (ORFs) were characterized including protein cleavage sites, gene sizes, distribution of cysteine residues, potential glycosylation sites, and unique motifs. Genetic relatedness was studied

using alignment procedures for full-length ORFs of the viruses vs. selected reference viruses and other African flaviviruses. Specific conserved organizational patterns were found for 3′-terminal noncoding regions that correlated with virus grouping. Zika virus is representative of the Spondweni virus group. Kedougou virus is only slightly more distantly related to Zika and Spondweni. Bagaza virus is interrelated to West Nile virus in several segments of its genome and representative of another distinct group (Ntaya).

Of the sequenced mosquito-borne flaviviruses, Zika virus has a 3′-noncoding region (NCR) with conserved sequences (CS) organized in a CS1-CS2-CS3 pattern. This is new for the Spondweni virus group. Kedougou and Bagaza viruses have also been sequenced, with GenBank accession numbers AY632540 and AY632545, respectively. Based on the partial sequence of the NS5 protein, Bagaza virus is 98 % identical to Israel turkey virus (ITV) GenBank EU303198; both viruses show a high degree of immune (neutralization) similarity [18].

Highly conserved universal primers from sequences in the 3′-coding region of the NS5 gene were used in reverse transcription/polymerase chain reaction (RT/PCR) for the rapid detection of mosquito-borne flaviviruses (Zika, West Nile, Japanese encephalitis, yellow fever, dengue 1, dengue 2, dengue 3, and dengue 4 viruses). This region of the NS5 gene showed less amino acid identity (20–36 %) across viruses than sequences in the C-terminus of the NS5 gene (56–76 % amino acid identity). In addition, recombinant plasmids containing flavivirus cDNA (derived from RNA from experimentally infected mosquitoes) were used in dot-blot membrane and digoxigenin detection methods. Zika virus classification was confirmed using serology [48].

Zika virus is a single-stranded positive-sense RNA virus and is approximately 11,000 nucleotides in length. Its relationship to Spondweni, Kedougou, and Bagaza viruses is illustrated in the phylogenetic tree, Fig. 18.1. There are 5′- and 3′-untranslated regions on either side of one ORF encoding a polyprotein in the genome. Phylogenetic analysis places Zika virus in three groups, West African (three strains analyzed), East African (two strains analyzed), and Asian (three strains analyzed) [14, 18]. A PCR amplicon 100 bp fragment from a 2010 Cambodian child patient had 100 % identity to accession number EU545988 Zika virus NS5 gene. The phylogenetic position of the patient's Zika virus was not stated [44].

Severe hemorrhagic fevers, meningitis, and encephalitis can be caused by tick-borne encephalitis (TBE) flaviviruses. The viruses that cause these diseases are pathogenic with a high mortality rate and are pathogenic due to inhibition of the interferon (IFN) response in the infected individual. Langat virus (LGTV) is a member of this group and is highly sensitive to the effects of IFN. A luciferase reporter gene driven by each of IFN-α/β and -γ-responsive promoters was inhibited by this virus in infected cells via the IFN-mediated JAK-STAT (Janus kinase-signal transducer and activator of transcription) signal transduction pathway. Several mechanisms of inhibition were IFN-α signaling blocks of Jak1 and Tyk2 Janus kinases and IFN-γ stimulation-associated Jak1 phosphorylation. Of all viral nonstructural (NS) proteins, NS5 alone inhibited IFN-γ-induced STAT1 phosphorylation. Moreover, NS5 forms complexes with IFN-α/β and -γ receptors. These observations were

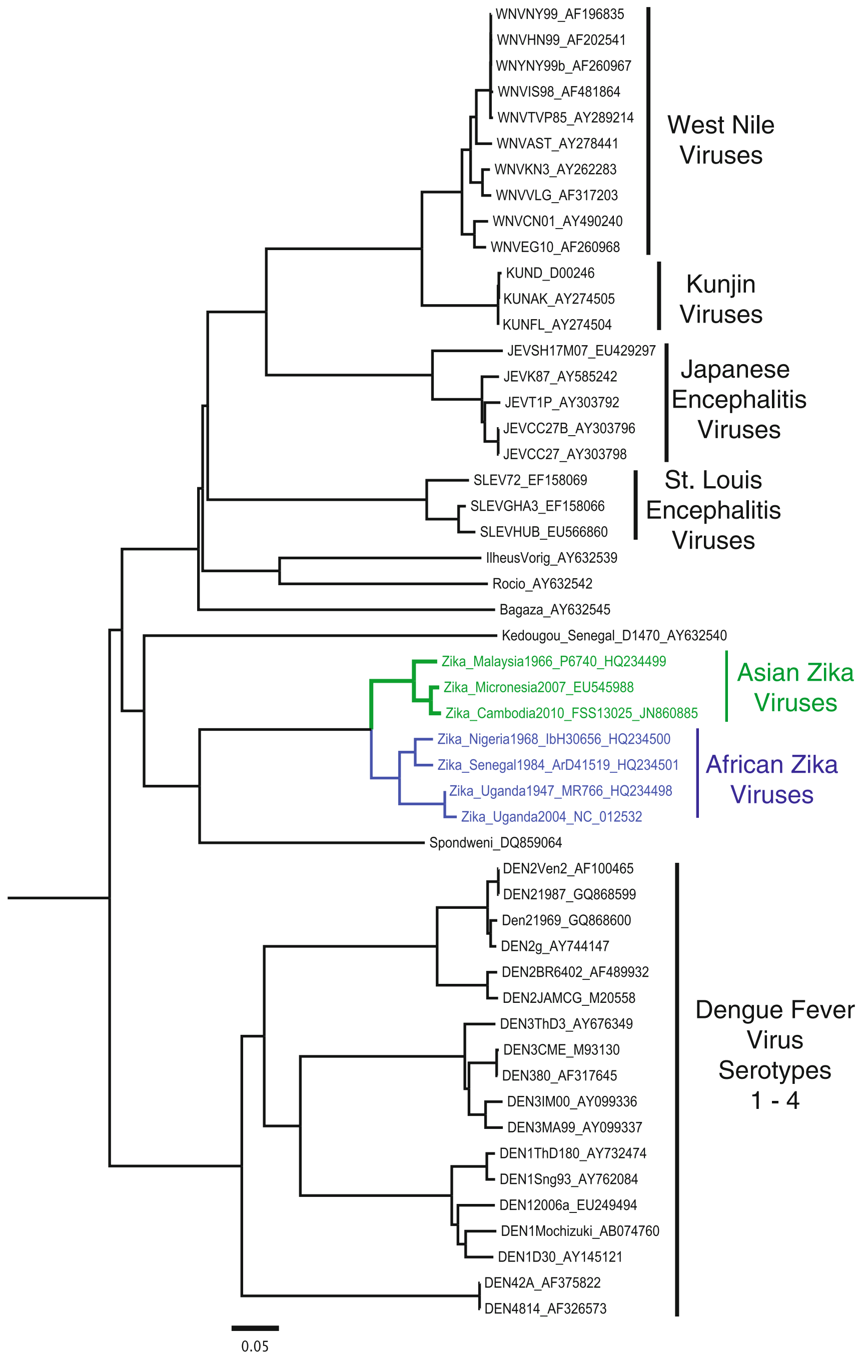

Fig. 18.1 Phylogenetic tree of Zika virus with related flaviviruses. Sequences were obtained from GenBank. Complete viral genome sequences were aligned with MAFFT, and a maximum likelihood phylogenetic tree was constructed from the DNA alignment using DNAML [49]

confirmed in LGTV-infected human monocyte-derived dendritic cells [50]. It should be noted that LGTV and Zika virus share additional molecular novelties in terms of their replication pathways.

6.2 Life-Cycle Observations

Unexpectedly, the detection of virus-specific antigen in the nuclei including nucleoli of Zika and Langat virus-infected cells brought into question flavivirus replication and the role of the nucleus. Overall, MAb 541 was specific for flavivirus ENV (envelope proteins) and MAB 109 specific for flavivirus NS1 (nonstructural glyco-proteins). The detection of Zika virus proteins with the nucleus and nucleolus o virus-infected cells may be due to early transient transport of the polyprotein from cytoplasm into the nucleus followed by protein processing and transport back into the cytoplasm where viral maturation occurs later. Very careful experiments were done to rule out artifacts and viral contamination in these studies, thus providing greater credence that Zika virus may have some different viral properties compared to other flavi-arboviruses including WNV (West Nile virus), YFV (yellow fever virus), Bussuquara virus, and Ntaya virus [51].

6.3 Molecular Epidemiology, Evolution, and Phylogenetic Analyses

Zika arbovirus has been known since the 1950s to be dispersed in Asia as well as Africa. Based on phylogenetic analysis of complete genomes, two genetic lineages exist for Zika virus that correspond to African and Asian geographical regions. Genetic relationships and sources of Zika strains that occurred in the Federated States of Micronesia (Yap Island) in 2007 and in Cambodia (a pediatric case) in 2010 were investigated. Between 1947 and 2010, isolates had been accumulated and stored from Nigeria, Senegal, Uganda, Cambodia, and Malaysia.

The complete genome sequences of these isolates and additional published sequences were used for phylogenetic analysis. Two main Zika virus lineages were identified, African and Asian. It was concluded that the Cambodian case and Yap outbreak were Southeast Asian in origin. The virus proteins appear to lose glycosyl-ation sites over time. It may be inferred that Zika virus made its way from Africa where it was first discovered to Southeast Asia whence it spread further including Yap [45].

Table 18.2 summarizes the Zika virus complete genomes that have been sequenced. The accession numbers are provided from GenBank. Figure 18.1 shows a phylogenetic tree of the complete Zika virus sequences and related flaviviruses. The phylogenetic tree in Fig. 18.1 indicates that the Zika viruses circulating in Asia (Malaysia, Micronesia, and Cambodia) are distinct from those circulating in Africa

Table 18.2 Genetic sequences of Zika viruses (as of July 2013) [49, 52]

[a]Country	Year	Isolate	GenBank accession number
Uganda	1947	MR766[a]	HQ234498
Nigeria	1968	IbH_30656	HQ234500
Senegal	1984	ArD_41519	HQ234501
Uganda	2004	–	NC_012532
Micronesia	2007	–	EU545988
Malaysia	1966	P6-740	HQ234499
Cambodia	2010	FSS13025	JN860885

[a]It should be noted that several additional viral passages of the same Ugandan isolate, MR766, were sequenced and produced identical sequences (AY632535, EU303241, EU074027, AY326412, and AF372422). – = no isolate name

(Nigeria, Senegal, and Uganda), but the two populations are no more diverse than those found within a single serotype of dengue fever viruses. The sequences (cf. Table 18.2) of the 1947 and 2004 Uganda isolates are 99.89 % identical to each other, indicating that Zika virus evolves rather slowly over time and that the African and Asian populations have been evolving for many decades. Similarity plots across the genomes (not shown) exhibited no evidence of recombination within or between isolate genotypes. However, recombination between dengue viruses has recently been detected [52].

Sequencing of many additional isolates of Zika virus would be needed, in order to estimate a true date of divergence between the African and Asian lineages, but a comparison can be made to dengue viruses, which have been more heavily sampled and sequenced.

6.4 Serology

Lanciotti et al. [14] describe genetic and serologic properties of Zika virus during the Yap state (Micronesia) epidemic.

The fundamental immunological finding in this study of the Yap state outbreak is that IgM antibodies had cross-relativities against other arbovirus flaviviruses. One interpretation of these findings is that Original Antigen Sin is being exhibited. This would suggest that the immune response is under some restriction and implies that there could be developing and spreading evolutionary changes of Zika virus that could lead to states of greater pathogenicity.

An epidemic of arthralgia, rash, and conjunctivitis was described by physicians in Yap state, Federated States of Micronesia in April 2007. Dengue virus was indicated as the cause using rapid ELISA. However, specimens sent to the Arbovirus Diagnostic Laboratory at the Centers for Disease Control and Prevention (CDC, Fort Collins, CO, USA) for confirmatory testing in June 2007, using IgM capture

dengue antigen ELISA, confirmed recent flavivirus infection. However, Zika virus reverse transcription-PCR (RT-PCR) assays followed by DNA sequencing supported 90 % nucleotide identity with Zika virus. Therefore, the Yap epidemic was due to Zika virus [14, 53, 54].

Zika, yellow fever, Chikungunya, and dengue type 2 viruses were identified as arboviruses with highest prevalence in humans in Nigeria. Antibodies to six arboviruses were surveyed in 267 human sera from the Kainji Lake region of Nigeria. One hundred and fifty-eight (59 %) had flavivirus hemagglutination-inhibiting (HI) antibody and 139 (52 %) had alphavirus HI antibody. The prevalence of antibodies was Zika—56 %, dengue type 2—46 %, yellow fever—31 %, Chikungunya—45 %, Semliki Forest—25 %, and Sindbis—33 % [55].

Sera randomly selected from 446 individuals across various age groups in Nigeria were tested for flavivirus IgM antibodies using hemagglutination inhibition (HI). Sixty-nine percent (314 sera) tested positive for three or more flaviviruses including Zika, West Nile, Potiskum, Uganda S, and yellow fever viruses. The prevalence was greater in younger than in older individuals [56].

Earlier studies using serological techniques (cross-hemagglutination inhibition and cross-complement fixation) and reactions did not find many antigenic differences distinguishing Zika viruses vs. flaviviruses such as Uganda S, Potiskum, Banzi, dengue type 1, and dengue type 2 viruses. However, differences were observed comparing Zika, Banzi, and Uganda S viruses vs. yellow fever, Wesselsbron, and Potiskum viruses in these studies [57].

The detection of serum IgM antibodies against Zika virus (using ELISA) is indicative of infection 2–5 months previously. This methodology was used in addition to viral isolation from mosquitoes for comparisons across several villages in southeastern Senegal 1988–1990. Human infections with Zika virus occurred in 1990 and epizootic outbreaks occurred annually. In addition, dengue 2 virus was isolated from mosquitoes and humans over the years of the study. However, other flaviviruses were isolated including Wesselsbron, Ked Kedougou, Westle, Chikungunya, Crimean-Congo hemorrhagic fever, and Rift Valley fever viruses as well as viruses that were not considered of public health concern during that period [58].

Serology studies (hemagglutination inhibition and immunofluorescence tests) in the Karamoja district, Uganda, using sera from 132 resident adults collected in 1984 detected 47 % positive for Chikungunya virus (and Semliki Forest alpha viruses (Togaviridae)) and 16 % positive for flaviviruses. It is stated that the latter were most likely mainly due to West Nile virus and included Zika and Wesselsbron viruses. A few individuals had antibodies against Marburg, Ebola-Zaïre, Ebola-Sudan viruses (*Filoviridae*), Lassa virus (Arenaviridae), and Crimean-Congo hemorrhagic fever virus. Yellow fever and dengue type 2 viruses were absent as were Ilesha, Tahyna, Sicilian sand fly fever phlebovirus, and Bunyamwera (Bunyaviridae) [59].

In Southeast Gabon, 197 adult human sera, 28 paired sera of mothers and their newborns, and 34 simian sera were surveyed for arbovirus HI and CF antibodies. Eighty-eight percent of the human sera had yellow fever virus due to vaccination,

58 % against Orungo virus, and 20 % against Chikungunya virus (that was a recent infection demonstrated by CF). Zika, Chikungunya, yellow fever, Uganda S, and Orungo viruses were transmitted transplacentally. Zika virus and Chikungunya viruses were detected as well in simian sera [60].

In April 1979, a survey of human sera was done in southeast Central African Republic. HI was studied in 459 sera and CF in 50 sera. Eighty-nine percent of the tested population had antibodies to Zika, yellow fever, West Nile, Chikungunya, Semliki forest, Sindbis, Uganda S, Bunyamwera, and Zinga viruses. Zika and Chikungunya viruses were active primarily in adults. CF assay detected Orungo virus antigens in 88 % sera. Ilesha, Bwamba, CHF-Congo, Dugbe, Bhanja, Tataguine, Nyando, and Bangui antigens were not detected. CF assay also detected antibodies for CHF-Congo and Bhanja viruses [61].

A study in Pakistan detected complement-fixation antigen for eight Toga viruses in 372 serum samples (43 humans, 172 domestic animals, and 157 rodents). The prevalence rates were Zika 2.4 %, West Nile (WN) 7.8 %, Japanese encephalitis (JE) 3.2 %, and Sindbis (SIN), Chikungunya (CHIK), Uganda S (UGS), and Royal Farm (RF) viruses 1.6–1.3 %. Dengue 1 (DEN) virus antigen was present in serum of one human patient. In human sera, antibodies were detected to all viruses except for RF that was detected in domestic animals and rodent sera. Studies in the epidemiology of Zika, JE, and WN viruses should include the role of rodents [62].

Between October 1977 and December 1977, in North-West Ivory Coast, at the end of the rainy season, an unexpected high number of deaths occurred among 100 patients with febrile hemorrhagic jaundice. This is an area with a high prevalence of yellow fever. Serological and epidemiological surveys indicated that during this period, vectors that could potentially carry yellow fever were detected. However, no viruses had been isolated and neutralization, complement fixation, and hemagglutination inhibition tests were then performed using antigens from six flaviviruses, i.e., Zika, yellow fever, West Nile, Uganda S, Wesselsbron, and Ntaya. These analyses were performed on two to three sera from 49 school children and 29 adults who had a recent history of jaundice, some with hemorrhagic symptoms. For comparison, sera were analyzed from 402 inhabitants of surrounding villages as well as 53 young rural workers. Twenty-one cases definitely had yellow fever, 20 cases probably had yellow fever, 15 cases were inconclusive, and 476 individuals definitely did not have yellow fever [63].

During 1985–1987, at the Dan refugee camp near Hargeysa, Somalia, malaria-like illness epidemics affected a few thousand residents. In some patients, headache, back and joint pains, fever, chills, and sweats were described, lasting up to 10 days. Malaria was not detected in blood smears from acutely ill patients. Zika, Chikungunya, Rift Valley fever, Crimean-Congo hemorrhagic fever, yellow fever, and Sindbis viruses were all absent in 10 convalescent and 28 acute sera using indirect fluorescent antibody (IFA) and hemagglutination inhibition (HI) tests. However, IFA and HI tests demonstrated dengue 2 antibody in 39 % (15/38) and 11 of 29 (38 %) sera, respectively. In 60 % (17/28) and 14 % (4/28) of the sera, using enzyme immunoassay (EIA), IgG and IgM antibody to dengue 2, respectively, was detected [64].

6.5 Antibody Enhancement of Viral Infection

In a serological study, suboptimal concentrations of several antibodies that did not neutralize their viral targets enhanced viral infection in murine macrophage cell culture. In addition, some heterologous combinations of antibodies and viruses showed such infection enhancement as well. Homologous enhancement was greater than heterologous enhancement. These effects were observed for Zika, West Nile, Wesselsbron, and Uganda S viruses, Dakar yellow fever, Potiskum, and dengue 2 viruses. Potiskum virus antibody showed the widest ability for heterologous virus infection enhancement [65, 66].

It was initially hypothesized that in regions of Nigeria where Zika virus is endemic other flaviviruses are less prevalent because of some serological cross-reactivity among these viruses, and thus some cross-resistance [67]. However, as the current hypothesis indicates, cross-reactive antibodies may enhance viral infection.

6.6 Multiple Viruses Present Contemporaneously

The presence of multiple related viruses with varying degrees of pathogenicity is more than a clinical diagnostic problem as this is also a problem in pathology and treatment. For example, many additional arboviruses have been found in conjunction with Zika virus in Lombok, Indonesia [68], adding to the danger of heterologous antibody-enhanced infections. The arboviruses that infect humans included in the study were Zika, Japanese encephalitis (JE), MVE, Tembusu (TMU), LGT, KUN, SEP, dengue type 2 (DEN-2), CHIK, RR, GET, SIN, BUN, BAT New Guinea C, Murray Valley encephalitis (MVE), MM 1775, and BAK. Testing was also done for infections in ducks, chickens, wild birds, bats, cattle, horses, goats, and rats. Infections of domestic animals included JE, MVE, KUN and SEP, BAT, and BUN [68]. Thus, there is a danger posed by the multiple virus infections, immunity, and cross-reactivity. Moreover, the utilization of vaccines in this context adds to the complexity. Due to cross-reactivity and antibody-virus infection enhancement, Zika virus vaccination would help or hinder a virus vaccination program.

Another study further supported the occurrence of contemporaneous multiple virus infections. Between 1971 and 1975, virology and seroepidemiology of Zika, yellow fever, dengue, West Nile, and Wesselsbron viruses were studied in four locales in Oyo State, Nigeria. Zika virus was isolated from two human cases with mild febrile illness. Percentage positive sera (measured by hemagglutination inhibition tests) were 31 % Zika, 50 % yellow fever, 46 % West Nile, and 59 % Wesselsbron. Forty percent Nigerians tested had neutralizing antipodes to Zika virus. Fifty percent individuals positive for Zika virus were positive for Zika virus alone or Zika virus and one other flavivirus, 40 % were positive for Zika virus and two other viruses, and 10 % were positive for Zika virus and at least three other flaviviruses. Of the other

viruses in these Zika antibody-positive individuals, 81 % were positive for dengue type 1, 58 % were positive for yellow fever, 7 % were positive for Wesselsbron, 6 % were positive for West Nile, and 3 % were positive for Uganda S [67].

Numerous additional studies support the occurrence of Zika virus with a high prevalence in several regions in Africa (approximately 40–50 %) contemporaneous among several additional flaviviruses. For example, in south-eastern Gabon these additional viruses have included yellow fever, Chikungunya, Koutango, and Wesselsbron viruses [69]; in Upper Casamance and in Eastern Senegal these included yellow fever, Chikungunya, West Nile, Bunyamwera, and Sindbis viruses (and related to where migrating birds rest) [70]; and in Igbo-Ora, Nigeria, dengue types 1 and 2, yellow fever, Chikungunya, West Nile, and Wesselsbron [71].

6.7 Vaccination and Superinfection, Multiple Infections

It had been hypothesized prior to 1980 that 17D yellow fever vaccine did not induce complement-fixing antibodies and that wild yellow fever virus infection did induce complement-fixing antibodies. However, the following studies demonstrated that this vaccine does produce such antibodies in specific situations and permitted distinction between natural yellow fever infection and vaccination. Yellow fever virus seroepidemiological studies were being done during a yellow fever vaccination campaign using 17D strain yellow fever in Gambia, West Africa, in 1979, during which a yellow fever epidemic ensued. Fifty-eight vaccinated participants were studied in three groups (see Table 18.3): group 1 had participants with pre-vaccination yellow fever-neutralizing antibodies; group 2 had participants without any pre-vaccination yellow fever-neutralizing antibody or hemagglutination-inhibiting antibodies to heterologous flaviviruses (including Zika, Uganda S, Ntaya, West Nile, dengue 1, or Spondweni); and group 3 had participants who lacked pre-vaccination yellow fever-neutralizing antibodies. However, group 3 participants

Table 18.3 Flavivirus vaccination outcomes [72]

Group	Prior exposure to wild yellow fever virus	Prior exposure to Zika, Uganda S, Ntaya, West Nile, dengue 1, or Spondweni viruses	Yellow fever vaccination outcome
Group 1	Pre-vaccination neutralizing antibodies	No prior exposure to HI antibodies	No response. But 24 % had complement-fixing (CF) antibodies
Group 2	No prior exposure to neutralizing antibodies	No prior exposure to HI antibodies	Seroconverted. Produced neutralizing antibodies and/or HIV but no CF antibodies
Group 3	No prior exposure to neutralizing antibodies	With prior exposure to HI antibodies	46 % produced homologous CF antibodies. Nine patterns of HI and homologous and heterologous CF antibodies

nonetheless had heterologous flaviviral hemagglutination-inhibiting antibodies. The findings were that group 1 participants sustained no vaccination response except for production of complement-fixing antibodies in 24 % of this group of vaccines. Group 2 participants upon vaccination seroconverted and sustained yellow fever-neutralizing and/or hemagglutination-inhibiting but no complement-fixing antibodies. Group 3 participants (46 %) produced homologous CF antibodies. There were nine patterns of HI and homologous and heterologous CF antibodies [72].

These studies demonstrate a high prevalence of flavivirus exposure (including Zika virus) that obscures what might have been expected to be a clear response to vaccinations on a wide scale. Moreover, the yellow fever vaccine anamnestic response did result in CF antibodies that were indistinguishable from natural yellow fever virus infection-induced CF antibodies [72].

6.8 RT-PCR

Zika fever diagnosis entails serology and virus isolation that are time-consuming procedures. In addition, serology frequently shows cross-reactivity and is not specific. Thus, tests that are more specific are required. A single-step reverse transcriptase polymerase chain reaction (RT-PCR) procedure was thus developed for the detection of Zika virus RNA. The assay targeted the envelope protein-coding region and was evaluated for sensitivity, specificity, and reproducibility. Additionally, the test was evaluated for its ability to detect Zika virus isolates preserved during the prior 40 years from a variety of hosts from several African countries. The RT-PCR test can be clinically helpful to detect Zika virus infection in regions where other clinically related arboviruses including dengue and Chikungunya viruses co-circulate. The first-generation RT-PCR test detected 7.7 pfus per reaction. The test was 100 % reproducible in patient serum and in cell cultures. Moreover, 19 other flaviviruses were undetected [43]. The same investigators developed a rapid single-step RT-PCR test that can be performed in less than 3 h using sequences from the NS5 protein-coding region of African Zika virus based on representative sequences from GenBank [49]. This assay is able to detect 37 Zika virus isolates from mosquitoes. It remains to validate the test for clinical use [73].

In Singapore, an initial analysis of 88 specimens of patient plasma from patients who had dengue-like disease but not Chikungunya did not show any Zika virus RNA. The newly devised PCR assay had a sensitivity of 140 copies of synthetic RNA per reaction. Further testing on known Zika virus plasma aliquots is needed. In addition, a wider and larger sample of patients needs to be studied in the community [31]. In this regard, it should be noted that Zika virus infection could be masked and not easily confirmed. Past circulation of arboviruses that had been silent (undetected) is the case in the Cameroons (Fako Division). In related studies, although virus isolation was not accomplished, serological studies detected Zika virus antibodies [74].

7 Conclusions

Prior to the 1980s, it was generally considered that the world had entered a new era of increased health and reduced infectious disease because of the defeat of smallpox, TB, and polio. What is the magnitude of the novel contemporaneous changes in arbovirus and vector evolution that appear to be occurring? Remarkable changes have occurred since the 1990s in arbovirus evolution (flaviviruses, mosquito-borne). Since 1999, West Nile virus completely occupied the Americas and since 1995, Japanese encephalitis virus extended its grip to Australia (northeastern). Subsequently, since 2001, Usutu virus occupied Europe; since 2010, Tembusu virus invaded duck farms in China; Bagaza virus caused encephalitis in India and reached Spain (southern). It is also remarked that new vertebrate hosts and mosquito species have become involved in the complex recent proliferation [75]. Similarly, Zika virus has been spreading as remarked in Table 18.1.

The hypothesis based on the data presented in this work is that detection and emergence of Zika virus has been associated with an upsurge of other more pathogenic flavivirus infections including for example dengue 1 and 2, JEV, and yellow fever. Some of these pathogenic viruses are proliferating and not yet fully controlled. The findings reported here of emergent viruses increasingly detected raise many health concerns as to what havoc emergent viruses may play in the future given what has happened with the spread of HIV and HCV, since the 1980s.

Furthermore, in Africa and tropical America, due to flavivirus superinfections including the high prevalence of Zika virus, with high backgrounds of flavivirus immune responses, seroepidemiological studies need to be conducted prior to institution of vaccination programs. An additional caveat is that such tropical risk regions are expanding due to global warming [72].

Clearly, much molecular and epidemiological work needs to be done. Unfortunately, contemporaneous economics and social structures are unable to support properly the work that is needed to understand and rectify current global health issues.

Postscript Consistent with the hypotheses presented in this chapter, as of 1-5-2014, Zika virus has demonstrated continuous spread. On 12-20-2013, the CDC issued a health advisory in regard to visiting French Polynesia (Tahiti) due to Zika virus. Zika virus spread to the islands of Arutua, Bora Bora, Fakarava, Hao, Hiva Oa, Huahine, Moorea, Nuku Hiva, Raiatea, Rangiroa, Tahaa, Tahiti, Takaroa Ahe, Tikehau, and Ua Pou. There were 35,000 suspected cases and 99 confirmed cases reported [22]. In addition, Zika virus was detected in an individual in Germany who had traveled in Thailand [23, 24]. Recent studies further support the need for continued surveillance due to the persistent spread of Zika virus under the radar and concomitant with other flaviviruses. Moreover, the possibility is raised that the prevalence of this virus is under-reported. Moreover, a range of symptoms is denoted, from mild to more severe disease including Guillain-Barre syndrome [76, 77].

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Chapter 19
Arenaviruses

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Core Message As research models, arenavirus infections such as those induced by Junín virus and lymphocytic choriomeningitis virus (LCMV) have been central to the discovery and characterization of many features of the immune system. In addition, these models have been used to study the establishment of persistent viral infections and relationships between viruses and rodent reservoirs. From the human perspective, several arenaviruses are important as zoonotic pathogens with significant consequences, causing viral encephalitis and meningitis and severe and often fatal hemorrhagic disease.

1 Introduction

In a general sense, geographic distribution may be used to separate the arenaviruses into Old World (OW) and New World (NW) viruses. With the exception of Tacaribe virus (TCRV), a NW arenavirus possibly associated with bats, all currently classified arenaviruses have a natural rodent reservoir ("mammarenaviruses"). The geographic distribution of these reservoirs generally correlates to a restriction of the distribution of the

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viruses and endemic disease. Recently, several novel arenaviruses were identified in snakes (“reptarenaviruses”), and whether an intermediate rodent or mite is involved in their transmission is not known (Tables 19.1, 19.2, and 19.3 and references within).

Arenaviruses were originally characterized ultrastructurally through electron micrographs of lymphocytic choriomeningitis virus (LCMV) particles and LCMV-infected cells about 45 years ago [53]. Virions were found to be of variable size and shape, budding from the plasma membranes with visible spikes and ribonuclease-resistant electron-dense bodies within the particles (also shown with Lassa particles in Fig. 19.1). On the basis of these morphological features, in 1969 researchers initially suggested that LCMV, Machupo virus (MACV), and TCRV should be reorganized in a single taxonomic group with LCMV as the prototype virus [54]. This was quickly followed by serological studies confirming cross-reactivity between LCMV, TCRV, MACV, Amaparí virus, Junín virus (JUNV), Paraná virus, Pichindé virus (PICHV), Tamiami virus, and Latino virus, but not other arthropod-borne viruses or mouse viruses [55]. Several biological properties of arenaviruses were also listed as evidence for their separation from arthropod-borne viruses: (1) arenaviruses are RNA and not DNA viruses, (2) rodent vectors play a role in arenaviral disease transmission, (3) arenaviruses produce persistent carrier state in rodents, and (4) they do not require arthropods in their life cycle. A more formal naming proposal was presented in 1970 [56] naming this virus group “Arenoviruses,” from the Latin word “arena” (= sand) based on the characteristic electron-dense granules in arenavirions. This name was later changed to Arenavirus (and later to *Arenavirus*), ostensibly to prevent confusion with “Adenovirus.” Lassa virus (LASV) was classified as an arenavirus in 1970 after in vitro characterization of several isolates [2, 57].

Smaller arenavirions tend toward being spherical, whereas larger particles are pleomorphic or “cup-shaped” [53, 54, 58]. The typical mean particle size is approximately 110–130 nm in diameter, although individual particles may range from 50 to over 350 nm in diameter. Particles are spotted with electron-dense granules of approximately 20 nm in diameter, later determined to be host ribosomes. Often, the formation of large intracytoplasmic inclusion bodies is observed in vitro in tissue culture and in vivo [53, 54, 56, 59–61]. These tubuloreticular inclusion structures (shown in Fig. 19.2) are also seen in cells infected with other viruses, such as Epstein Barr virus or Ebola virus [62], and have recently been the starting point of the discovery of a novel group of arenaviruses in snakes associated with inclusion body disease (IBD) [50, 51, 63–66].

2 Genome Organization, Viral Proteins, and Replication Strategy

2.1 Genome Structure

Arenaviruses have bisegmented, single-stranded ambisense RNA genomes. These segments are designated by their length: small, S (approximately 3.5 kb) and large, L (approximately 7.3 kb) [67, 68]. The L segment encodes a viral RNA-dependent

Table 19.1 Old World arenaviruses ("Old World Mammarenaviruses")

Virus	Abbreviation	Distribution	Reservoir species (reservoir(s))	Year identified	Human disease	Reference(s)
Classified Old World arenaviruses						
Ippy virus	IPPYV	South Africa	*Arvicanthis* sp. (unstriped grass rats)	1970		[1]
Lassa virus	LASV	Guinea, Liberia, Mali, Nigeria, Sierra Leone	*Mastomys natalensis* (Natal mastomys)	1969	Lassa fever	[2, 3]
Lujo virus	LUJV	South Africa, Zambia	Unknown (isolated from human)	2008	Viral hemorrhagic fever	[4]
Luna virus	LUNV	Zambia	*Mastomys natalensis* (*Natal mastomys*)	2009		[5]
lymphocytic choriomeningitis virus	LCMV	Worldwide?	*Mus musculus* (house mouse)	1933	lymphocytic choriomeningitis, aseptic meningitis	[6–10]
Mobala virus	MOBV	Central African Republic	*Praomys* sp. (soft-furred mice)	1983		[11]
Mopeia virus	MOPV	Mozambique, Zimbabwe	*Mastomys natalensis* (Natal mastomys)	1977		[12]
Morogoro[a]	MORV	Tanzania	*Mastomys natalensis* (Natal mastomys)	2004		[13]
Unclassified Old World arenaviruses						
Dandenong virus	DANV	Australia	Unknown (isolated from human)	2008	Possibly	[14]
Gbagroube[a]		Côte d'Ivoire	*Mus setulosus* (Peters's mouse)	2005		[15]
Jirandogo		Ghana	*Mus baoulei* (Baoule's mouse)	2011		[16]
Kodoko virus	KDKV	Guinea	*Mus minutoides* (African pigmy mouse)	2006		[17]
Lunk virus	LNKV					
Menekre virus[b]		Côte d'Ivoire	*Hylomyscus sp*. (African wood mice)	2005		[15]
Merino Walk Virus	MRWV	South Africa	*Myotomys unisulcatus* sp. (Busk Karoo rat)	1985		[18]

HF hemorrhagic fever, *sp.* species

[a] Only sequence and seroprevalence data available, not virus isolation

[b] Only sequence data available, not virus isolation

Table 19.2 New World arenaviruses ("New World Mammarenaviruses")

Virus	Abbreviation	Distribution	Reservoir species (reservoir(s))	Year identified	Human disease	Reference(s)
Classified New World arenaviruses, Clade A						
Allpahuayo virus	ALLV	Peru	*Oecomys bicolor* (white-bellied oecomys) and *Oecomys paricola* (Brazilian oecomys)	1997		[19]
Flexal virus	FLEV	Brazil	*Oryzomys* sp. (rice rats)	1975		[20]
Paraná virus	PRAV	Paraguay	*Oryzomys angouya* (Angouya oryzomys)	1965		[21]
Pichindé virus	PICHV	Colombia	*Oryzomys albigularis* (white-throated oryzomys)	1965		[22]
Pirital virus	PIRV	Venezuela	*Sigmodon alstoni* (Alston's cotton rat)	1997		[23, 24]
Classified new world arenaviruses, Clade A/B (aka. A/rec, or North American Tacaribe Serocomplex)						
Bear Canyon virus	BCNV	USA: California	*Peromyscus californicus* (California deermouse) *Neotoma macrotis* (big-eared woodrats)	1998		[25]
Big Brushy Tank virus	BBTV	USA: Arizona	*Neotoma albigula* (white-throated woodrat)	2002		[26]
Catarina virus	CTNV	USA: Texas	*Neotoma micropus* (southern plains woodrat)	2007		[27]
Skinner Tank virus	SKTV	USA: Arizona	*Neotoma mexicana* (Mexican woodrat)	2002		[28]
Tamiami virus	TMMV	USA: Florida	*Sigmodon alstoni* (Alston's cotton rat)	1963		[29, 30]
Tonto Creek virus	TTCV	North America (USA: Arizona)	*Neotoma albigula* (white-throated woodrat)	2001		[26]
Whitewater Arroyo virus	WWAV	USA: New Mexico, Oklahoma, California, Colorado, Utah	*Neotoma albigula* (white-throated wood rats)	1993	Controversial	[31–33]
Classified New World arenaviruses, Clade B						
Amaparí virus	AMAV	Brazil	*Neacomys guianae* (Guianan neacomys)	1964		[20, 34]
Chapare virus	CHAPV	Bolivia	Unknown (isolated from human)	2004	Viral hemorrhagic fever	[35]

Classified New World arenaviruses, Clade A						
Cupixi virus	CUPXV	Brazil	*Oryzomys megacephalus* (Azara's broad-headed oryzomys)	1970		[36]
Guanarito virus	GTOV	Venezuela	*Zygodontomys brevicauda* (short-tailed zygodont)	1990	"Venezuelan hemorrhagic fever"	[37]
Junín virus	JUNV	Argentina	*Calomys musculinus* (drylands laucha)	1958	Junín/Argentinian hemorrhagic fever	[38, 39]
Machupo virus	MACV	Bolivia	*Calomys callosus* (big laucha)	1963	Machupo/Bolivian hemorrhagic fever	[40, 41]
Sabiá virus	SABV	Brazil	Unknown (isolated from human)	1990	"Brazilian hemorrhagic fever"	[42]
Tacaribe virus	TCRV	Trinidad, West Indies	*Artibeus jamaicensis trinitatis* (Jamaican fruit-eating bat)	1956		[43]
Classified New World arenaviruses, Clade C						
Latino virus	LATV	Bolivia	*Calomys callosus* (big laucha)	1973		[44, 45]
Oliveros virus	OLVV	Argentina	*Necromys benefactus* (Argentine akodont)	1990		[46, 47]
Unclassified new world arenaviruses						
Ocozocoautla de Espinosa[a]	OCEV	Mexico	*Peromyscus mexicanus* (Mexican deermouse)	2000		[48]
Real de Catorce[a]	RCTV	Mexico	*Neotoma leucodon* (White-toothed woodrat)	2005		[49]

HF hemorrhagic fever, *sp.* species

[a]Only sequence data available, not virus isolation

Table 19.3 Newly detected or isolated arenaviruses ("Reptarenaviruses") from snakes

Unclassified arenaviruses from snakes					
Virus	Abbreviation	Distribution	Reservoir species (reservoir(s))	Year identified	Reference(s)
CAS virus[a]	CASV	USA: California	*Corallus annulatus* (annulated tree boa)	2012	[50]
Collierville virus[a]	CVV	USA: California	*Boa constrictor* (boa constrictor)	2012	[50]
Golden Gate virus	GOGV	USA: California	*Boa constrictor* (boa constrictor)	2012	[50]
ROUT virus[a]	ROUTV	Netherlands	*Boa constrictor* (boa constrictor), *Corallus caninus* (emerald tree boa)	2013	[51]
University of Helsinki virus	UHV	Germany, UK, Costa Rica	*Corallus annulatus* (annulated tree boa), *Corallus hortulanus* (common tree boa), *Boa constrictor* (boa constrictor)	2012	[52]

ROUTV was previously known as Boa Av NL B3
[a]Only sequence data available (no virus isolate)

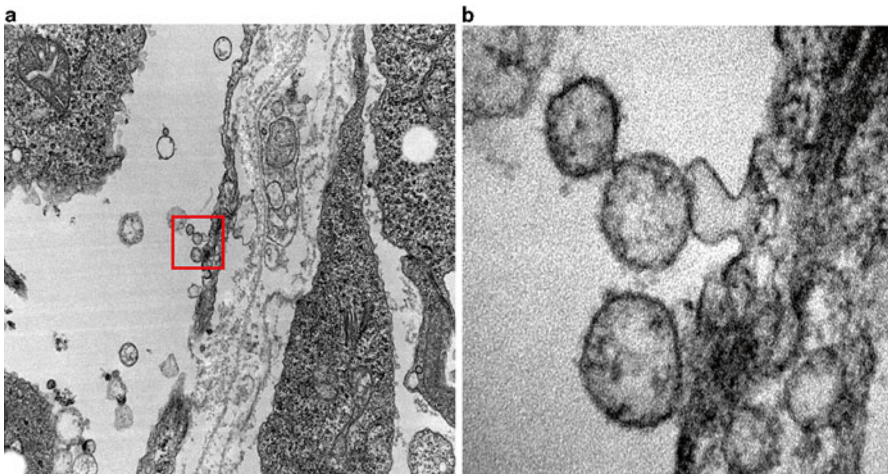

Fig. 19.1 Lassa virus particles budding from a stellate cell of a crab-eating macaque. Electron micrographs of virions (90–100 nm in diameter) budding from a presumed dendritic cell from an inguinal lymph node of a crab-eating macaque. Tissue was harvested 10 days following aerosol exposure to Lassa virus, Josiah strain. (**a**) Low magnification at 25,000× and (**b**) *inset*, shown at high magnification at 150,000×

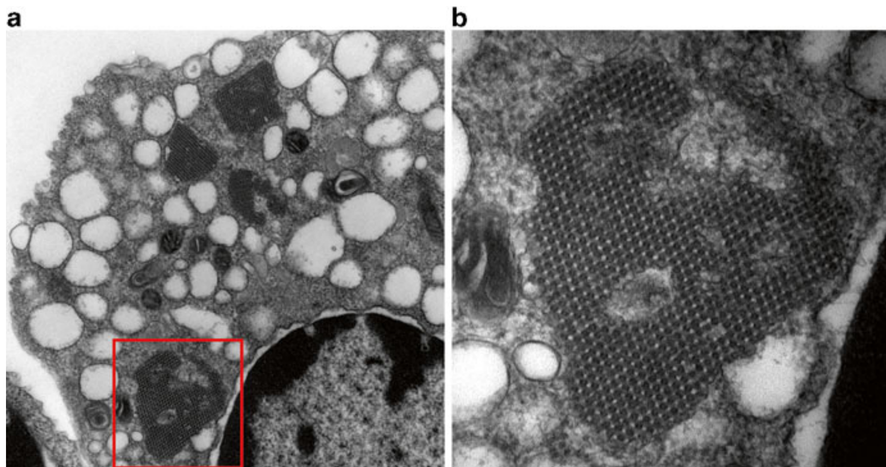

Fig. 19.2 Tubuloreticular structures in Lassa virus-infected circulating lymphocytes from a crab-eating macaque. Electron microscopy reveals burlap-like tubuloreticular structures (TRS) in a circulating lymphocyte collected 8 days following aerosol exposure to Lassa virus, Josiah strain. (**a**) Low-magnification (30,000×) shows multiple, highly ordered TRS in the cytoplasm. (**b**) Higher magnification (80,000×) of *boxed area*, showing cross-sectional detail of a single TRS

RNA polymerase (L), as well as the matrix protein Z. The S segment encodes the nucleoprotein (NP) and the glycoprotein precursor (GPC) [69]. With such limited genome coding capacity, each expressed viral protein must play more than one role in the virus life cycle and suppression of the host immune response.

2.2 *Role of Viral Proteins*

2.2.1 NP

During viral infection, NP is the most abundantly produced viral protein. NP is involved in genomic RNA encapsidation and formation of viral ribonucleoprotein complexes (RNPs). NP binds to both genomic and antigenomic RNA and has immunosuppressive effects via the C-terminal domain [70, 71] that contains 3′–5′ exoribonuclease activity [72]. NP is encoded by the S segment, and translated from the subgenomic viral complementary mRNA [73]. The interaction of NP and L may be involved in the transient release of the RNA template from the nucleocapsid and in the movement of L during transcription [74].

2.2.2 L

Based on sequence [75] and mass (greater than 200 kDa), researchers presumed that the L protein was an RNA-dependent RNA polymerase consisting of multiple domains, which was later confirmed by mutational analysis and crystallographic

studies [76–80]. L has at least four conserved regions with separate transcription, cap-snatching, and genome replication functions [76, 78, 80, 81]. L also interacts with Z and NP. The interaction of NP and L may be required for the release of template RNA during transcription [74].

2.2.3 Z

Z is a self-associating protein forming dimers that can form virion-like particles (VLPs) with myristoylation sites for membrane targeting. The primary function of Z is to serve as a viral matrix protein, recruiting NP and the GP to the site of budding particles at the plasma membrane [71, 82, 83]. The release of viral particles from the cell requires the intracellular cargo receptor ERGIC-53 and its associated machinery [84], and the budding process has been modeled in vitro [85]. Also, Z appears to have an immune-modulatory role, as a domain was identified in NW arenaviruses (but not LASV or LCMV) that inhibited type I interferon (IFN) induction of the retinoic acid-inducible gene 1 (RIG-I) signaling pathway [86]. Z inhibits viral RNA synthesis by directly binding to L [71] and exerts inhibitory effects on polymerase activity.

2.2.4 GPC

GPC is expressed as a single polypeptide precursor that is cleaved in the lumen of the endoplasmic reticulum. The cleaved stable signal peptide (SSP) remains stably associated with the GP spike complex. SSP plays essential roles in endosomal trafficking and pH-mediated fusion and interacts with Z [87–89]. Further proteolytic processing cleaves GP to separate GP1 and GP2, producing a globular head domain, a transmembrane region, and spontaneous trimer formation [90, 91].

The trimeric GP spike complex on the virion surface mediates cell entry of arenavirions; GP1 mediates cell attachment and receptor binding, whereas GP2 mediates membrane fusion within the endosome [92, 93]. GP2 is typical class I fusion protein and, during fusion, undergoes a conformational change involving a characteristic six-helix bundle [94]. The association of GP with membrane microdomains and Z promotes efficient budding at the plasma membrane [95].

2.3 Receptor Usage, Attachment, Entry, and Uncoating

Cellular entry of arenavirions is mediated by at least two defined receptors. The primary OW cellular receptor is the highly conserved cell surface protein α-dystroglycan (α-DG). This receptor is the entry receptor for LCMV, LASV, Mobala virus, Mopeia virus, Ippy virus, Oliveros virus, and Latino virus [92, 96]. Transferrin receptor 1 (TfR1) was first identified as the cellular receptor for the pathogenic NW arenaviruses JUNV, MACV, Guanarito virus, and Sabiá virus (SABV) [93]. Later studies

examined and compared arenavirus usage of TfR1 from hosts of different species [74, 97, 98], and preference of virion binding to human TfR1 correlated directly with pathogenicity. Lujo virus (LUJV) appears to enter cells via both α-DG- and TfR1-independent mechanisms, suggesting the existence of a third arenavirus receptor [99].

Following attachment, virion internalization occurs via clathrin-dependent or clathrin-independent mechanisms depending on receptor usage and virus. Similarly, differences in endosomal trafficking are also observed. However, a pH-dependent fusion step of the viral and cellular membrane is required [100–102]. Once virions are internalized and uncoated, virus replication is restricted to the cytoplasm where L initiates transcription at the 3′ end of each genomic RNA segment.

2.4 Ambisense Coding Strategy and Replication

Arenaviruses use an ambisense coding strategy, whereby each single-stranded RNA genome segment has two open reading frames in opposite orientation (viral genomic sense versus the viral complementary sense). The noncoding intergenic regions (IGR) between the two open reading frames of each segment of most arenaviruses are predicted to form one- or two-stem-loop hairpin structures (SABV segments are predicted to have three-stem loop structures [103]). This G:C rich hairpin configuration was first identified in the S segment of PICHV [104, 105], and its role as a putative terminator of L was suggested [105]. Both the L and S segments also have terminal noncoding untranslated regions (UTRs) at their extremities; these conserved regions of reverse complementary sequence promote the circularization of each genome segment into "panhandle" structures via base pairing [106]. The coiled, circular filaments of viral RNA genome have been made visible by electron microscopy using purified TCRV nucleocapsid [107]. The 3′ UTR of each segment also serves as a conserved promoter for L.

Arenavirus RNA synthesis is initiated after delivery of each of the two genomic segments, each associated with L, into the cytosol. Primary transcription from the 3′ end of each genomic template results in mRNA transcribed from the NP and L genes in antigenomic orientation, terminating at nonspecific sites within the distal end of the stem loop in the IGR. As an example of the ambisense strategy for the S segment, NP mRNA would be transcribed directly in this fashion from the viral genome. However, transcription of GPC gene would not occur until the replication intermediate step of viral complementary RNA has been completed. Regulation of the switch from transcription to replication is controlled by the local abundance of particular viral proteins. At early times after uncoating, gene expression of NP and L is favored as the limiting amounts of NP reduce the read-through capability of L. Viral RNA synthesis is also promoted at this time, when low concentrations of Z protein are present. As the intracellular concentrations of Z increase following transcription and translation, the functions of Z might be modulated to increase the inhibition of viral RNA synthesis by directly interacting with L [71]. Z directly binds to L and exerts inhibitory effects on the polymerase activity in a dose-dependent

manner, potentially driving the shift from viral replication to virus assembly and budding. This interaction of Z with L would also ensure that L is packaged into virions prior to release.

The arenavirus ambisense coding strategy is hypothesized to play a role in the establishment of persistence in the rodent host, as well as immune evasion by limiting and regulating transcription and replication at critical times during the arenavirus replication cycle.

3 Human Disease

3.1 Transmission

Humans usually become infected via direct contact with rodents by inhaling dried excreta (feces, urine) during occupational exposure (laboratory workers, rodent sellers, farm workers) or from keeping rodents as pets [108]. Destruction of natural habitat due to human expansion increases the potential for human contact with infected rodents and may be a factor in zoonotic transmission.

3.2 Clinical Presentation and Pathogenesis

The incubation period for human arenavirus infections ranges from 7 to 21 days followed by onset of influenza-like clinical signs and symptoms, including general malaise, sore throat, high fever, headache, myalgia, and lymphadenopathy. Progression of disease typically includes gastrointestinal symptoms such as nausea, vomiting, and diarrhea [109–113]. Disease presentation may range widely, from very mild to severe disease. More severe disease and poorer prognosis is generally associated with higher viral loads [114].

In cases that resolve, recovery typically occurs within 8–10 days of disease onset and is usually concomitant with appearance of circulating antibody and measurable cellular responses [112]. Severe disease is characterized by deterioration in the patient's condition that includes facial edema, severe pulmonary effusion, and bleeding from mucosal surfaces. Neurological signs, including tremors, disorientation, hyporeflexia, and ataxia may also present. Patients who succumb to disease (approximately 15–30 % of cases of viral hemorrhagic fever-causing arenaviruses) may experience respiratory distress, as a result of pulmonary edema, and/or encephalopathy, which sometimes results in seizures and coma, followed by shock [115]. In the case of Lassa fever, nosocomial outbreaks are sometimes associated with higher incidence of fatality, ranging from 36 to 65 % [116]. Survivors of Lassa fever may experience diffuse hair loss and changes in nail beds. Sensorineural deafness, a common clinical feature that occurs during convalescence and late stage of disease, is noted in approximately 15 % of cases [117].

Unlike other highly virulent hemorrhagic fever viruses, such as Ebola virus, arenaviruses are not distinguished by causing prominent hemorrhagic features or

disseminated intravascular coagulation (DIC) [111]. However, viral infection of endothelia and disruption of vascular function plays a prominent role in pathogenesis caused by hemorrhagic fever-causing arenaviruses, particularly in the case of LASV. Impaired vascular regulation is the causative underlying mechanism of facial erythema or edema, conjunctivitis, hypotension, pulmonary and pericardial edemas, and shock. In some cases, petechial or macular rash likely results from increased vascular permeability [116].

LASV and JUNV are perhaps the best characterized of the OW and NW hemorrhagic fever viruses, respectively, and diverge in their histological and pathological features of disease. Lassa fever is characterized by a viral hepatitis [118] that is not as prominent in patients with Junín hemorrhagic fever. Renal necrosis is more pronounced in patients with Junín hemorrhagic fever than in patients with Lassa fever, and these necrotic sites correspond to presence of high viral replication [113]. Other OW arenaviruses, such as LUJV, and NW arenaviruses, such as Chapare virus, MACV, GTOV, and SABV, cause diseases with very similar presentation.

Prominent differences in OW and NW arenavirus infections become more readily apparent in regard to the immune response. Lassa fever results in generalized immune suppression [119, 120], whereas Junín hemorrhagic fever promotes development of a deregulated systemic inflammation resulting from uncontrolled cytokine production [121–123]. Survival from Lassa fever is dependent on a strong cellular response whereas humoral immunity is less important [114]. Conversely, neutralizing antibodies are much more important for controlling NW arenavirus disease. Results from animal modeling of arenavirus infection suggest that complement fixation is a critical component of the effectiveness of the humoral immune response, although cellular immunity is important [124].

Pathogenesis is thought to partially result from virus damage to the endothelial system. Endothelial cells support high levels of virus replication without causing cell death, as arenaviruses do not undergo lytic cell replication. This replication initiates release of inflammatory mediators such as prostaglandins and nitric oxide, which promote vascular permeability [125]. Additionally, arenaviruses are known to cause thrombocytopenia as a result of abnormal platelet aggregation [126] and reduced complement activity [127], both of which contribute to coagulopathy and tissue edema. Generally, severity of arenaviral disease is proportional to concentrations of IFN-α, tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6), particularly in Junín hemorrhagic fever [121–123].

4 Animal Models of Highly Pathogenic Arenaviruses

4.1 Rodents

4.1.1 Laboratory Mice

Since the almost simultaneous discovery of LCMV by three groups [6–10], the use of LCMV in vitro and in laboratory mice as a research model [128–133] has been critical to the fundamental understanding of the immune system, particularly in

regard to cellular immunity. LCMV models have allowed investigators to study all aspects of the T lymphocyte response. These aspects include early interactions of T cells with dendritic cells in the context of major histocompatibility complex (MHC) restriction, the determination of immunodominant peptides and development of tetramer reagent systems, the phases of T cell expansion and contraction, and establishment of memory cells that occur following infection. Most of this research compared the dynamics of the murine immune response induced by the LCMV Armstrong isolate, which results in an acute infection of 7–10 days, to the clone 13 isolate, which establishes a chronic infection (≥3 months) of laboratory mice.

For the pathogenic arenaviruses, rodent models of disease provide an economical way to characterize pathogenesis, vaccine immunogenicity, host-range restriction, and therapeutic drug evaluation. Infection of laboratory mice with arenaviruses generally leads to a transient or persistent infection without characteristic pathogenesis seen in primates and requires extensive virus adaptation to promote virulence. As a result, most mouse models of highly pathogenic arenavirus infections typically rely on gene-knockout variants that produce mice with an immune-compromised status rendering them more susceptible to viral infections in general.

Two gene knockout models utilize either signal transducer and activator of transcription 1 (STAT1) or type I interferon (IFNαβR) receptor knockout mice to cripple the IFN response and establish a pathogenic model without the requirement for virus adaptation to the host. The STAT1 gene family is activated in response to type I IFN triggered by viral infection and regulates expression of a variety of genes important for cell viability and immune function regulation.

STAT1 knockout mice have previously been utilized for both wild-type LASV [134] and MACV [135] exposures resulting in lethal disease characterized by weight loss, disseminated infection, high serum and tissue viral titers, and death. Additional models have also been developed with similar results by eliminating the gene for IFN-α- and IFN-β-receptors, effectively disabling the IFN response. IFNαβR knockout mice have been used for a variety of both OW and NW arenaviruses with success [136, 137].

Laboratory mice that are typically not susceptible to LASV infection become unable to control viral replication and present with severe Lassa fever-like disease when murine MHC class I is replaced with a humanized ortholog. Depletion of T cells revokes the conferred lethality and development of significant disease, despite the ability of the virus to maintain high-level replication, suggesting an important role for T-cells in LASV pathogenesis. The absence of T cells may lead to an abolition of appropriate activation of antigen-presenting cells, i.e., T cells may be contributing to deleterious inflammatory responses mediated by monocytes/macrophages [138].

T cells are also important for JUNV pathogenesis. Murine models that make use of athymic mice persistently infected with JUNV have been described. The neurovirulence of JUNV in laboratory mice has been previously hypothesized to depend on the presence of T lymphocytes [139]. To achieve virulence in suckling mice, splenocytes from persistently infected athymic animals were passively transferred

via the intracranial route. Transfer of virus-infected cells results in brain lesions and establishment of acute disease, followed by death within 25 days [140]. Normal splenocytes did not affect viral burden in the brain nor result in pathology. Results of these studies highlight the role for T cells in neurovirulence and pathogenesis, at least in the murine model. The establishment of persistent infection is also critical for pathogenesis as splenocytes taken from athymic mice just after infection were unable to confer disease, whereas those harvested 30–45 days postinoculation produced a lethal outcome [141].

4.1.2 Guinea Pigs

Guinea pig models of arenavirus infections have been widely used to study pathogenesis and to evaluate the efficacy of potential vaccines and therapeutics. Current guinea pig models of arenavirus infection caused by both NW and OW hemorrhagic fever-causing arenaviruses appear to closely resemble human disease, but do not epitomize its neurological aspects. Strain 13 guinea pigs have been the primary animal model to date, presumably because they are more susceptible to arenavirus infections than Hartley guinea pigs [142]. Both LUJV and LASV infection of strain 13 guinea pigs results in uniform susceptibility and high lethality with similar pathological features [142, 143]. Animals rapidly develop high fever and weight loss progressing to lethargy, reduced grooming, and death. Viremia and tissue titers are consistent with disseminated viral disease involving most visceral and lymphatic organs. Histologic findings from animals infected with LUJV include hepatic infarction with associated necrosis and fibrin deposition, whereas the most prominent histologic feature in LASV infection is interstitial pneumonia.

JUNV infection models utilizing strain 13 guinea pigs are characterized by prominent hematologic and lymphatic involvement including necrosis and cellular depletion and hemorrhage [144, 145]. Further study of the hematological changes of bone marrow during the course of infection revealed a significant increase in cells with abnormal morphology [145, 146].

4.2 Nonhuman Primates

4.2.1 Common Squirrel Monkey

Walker et al. first described a nonhuman primate model of Lassa fever in the common squirrel monkey (*Saimiri sciureus*) [147]. Four monkeys were inoculated intramuscularly and serially sampled post-exposure on days 7, 12, 14, and 28 to both evaluate the clinical course and characterize progression of disease pathology. Animals exhibited a variable clinical course with an incubation period between 8 and 18 days. Early clinical signs included development of anorexia, polydipsia, and lassitude. Early presence of detectable virus in the tissues involved lymph nodes,

liver, and kidneys followed by dissemination through various other organs in a pantropic manner. Histopathological findings suggested similarities between the common squirrel monkey model and human disease pathology that included germinal necrosis in lymphoid organs, myocarditis, acute arteritis, renal tubular necrosis, hepatocytic regeneration, and chronic inflammation of the choroid plexus, ependymal, and meninges with cerebral perivascular cuffing.

4.2.2 Tufted Capuchins

Intracerebral JUNV infection of tufted capuchins (*Cebus apella*) [148] results in clinical signs after a 2-week incubation period, including weight loss and mild-to-moderate central nervous system involvement that resolves in most animals. Despite resolution, some animals still have detectable viral antigen in the brain as long as 5 months post exposure. Hemorrhagic manifestations do not develop. The clinical response to infection is not uniform, though all animals develop high antibody responses. Although the model does not reproduce the human disease faithfully, it may have utility to study effects of the virus on the central nervous system or to evaluate viral persistence.

4.2.3 Common Marmoset

Both JUNV and LASV infection models utilizing the common marmoset (*Callithrix jacchus*) have been described [149–154]. Except for microscopic neurological irregularities [155], JUNV infection in common marmosets shares pathological and hematological characteristics with human disease. Common marmosets infected with JUNV intramuscularly developed characteristic disease [156]. Animals initially presented with anorexia, lassitude, weight loss, thrombocytopenia, and leukocytopenia, followed by progression to severe fatal neurological and hemorrhagic disease approximately 3 weeks after exposure. Histologically, development of multifocal hemorrhage, microscopic lesions of the central nervous system, interstitial pneumonia, lymphocytic depletion, hepatocytic necrosis, and loss of bone marrow cellularity correlate with high virus concentrations [155].

Further evaluation of the hematological values of JUNV-infected marmosets revealed anemia and alteration of blood coagulation as evidenced by reduction of platelets and disruption of enzymatic activation of thrombin. These alterations ultimately led to a state of DIC [157, 158]. Complement activation was independent of clotting abnormalities, though this finding is inconsistent with what is known about human disease and remains to be further evaluated in nonhuman primate models.

A later study also described LASV infection in experimentally infected common marmosets that echoed human disease [149]. Following subcutaneous inoculation with LASV strain Josiah, common marmosets developed a systemic illness including fever, weight loss, high viremia and viral tissue loads, liver damage, and substantial morbidity. Virus tissue tropism was extensive as indicated by extremely

high viral titers in the spleen, lymph nodes, lung, liver, kidney brain, and adrenal glands. The most prominent microscopic features included hepatic necrosis, interstitial nephritis, and depletion of lymphoid cells. Additionally, these histologic findings suggested impairment of adaptive immune responses by depletion of T and B cells and ablation of macrophage expression of MHC class II. The common marmoset therefore appears to be a suitable model for further characterization of Lassa fever pathogenesis.

4.2.4 Rhesus Monkey

The disease caused by LASV in rhesus monkeys (*Macaca mulatta*) shares many striking similarities with human Lassa fever, including onset of high fever, general weakness and malaise, pleural and pericardial effusion, hemorrhagic manifestations (e.g., bleeding from mucosal surfaces), shock, and death [116]. Several authors reported on LASV Josiah exposure of rhesus monkeys via the subcutaneous route of exposure with very similar findings [159–162]. Animals developed clinical signs (high fever, anorexia, reduced responsiveness) 4–12 days post exposure. This model was not uniformly lethal, and survivors tended to present with signs of disease later than their moribund counterparts. As disease progressed, animals became increasingly lethargic and presented with petechial skin rash, recumbency, elevated liver enzyme concentrations, and weakness. Although not ubiquitously reported, some cases involved aphagia, constipation, conjunctivitis, and hiccups. End-stage disease involved hypotension and hypothermia just prior to death.

Gross pathology and histological studies of LASV-infected rhesus monkeys resembled human disease, including pulmonary congestion, pleural effusion, pericardial edema, fibrin deposition, and gross visceral hemorrhage. The most prominent histological findings included necrotizing hepatitis and interstitial pneumonia [161]. While coagulopathy consistent with DIC was not observed, increased time for sample clotting was observed occasionally, suggesting a clotting abnormality consistent with viral hemorrhagic diseases with associated platelet aggregation [160, 162]. High virus titers in tissues were consistently reported in excess of serum viremia and included liver, lung, adrenal glands, pancreas, spleen, kidneys, lymph nodes and neurological tissues, with liver, spleen, and lungs generally yielding the highest virus titers. With the exception of a single animal that developed hind leg paralysis following apparent recovery from clinical signs at day 58 [162], no other neurological findings were reported. This finding is in contrast to the smaller primate models described previously (such as common marmosets and common squirrel monkeys). Intravenous inoculation of LASV strain Josiah into rhesus monkeys led to similar clinical presentation and pathological findings as those recorded after subcutaneous inoculation [163].

JUNV infection in rhesus monkeys can be established by the intramuscular and aerosol routes of exposure. McKee et al. compared several strains of JUNV (Romero, Espindola, Ledesma, and P-3551) in rhesus monkeys to characterize differences in disease course and outcome [164, 165]. Animals initially presented with similar

onset independent of strain, including progressive anorexia, lassitude, and diarrhea or constipation. JUNV infection in macaques infected with the Romero strain spontaneously resolved without developing more substantial illness.

In macaques infected with the other three strains (i.e., Espindola, Ledesma, P-3551), JUNV infection progressed to debilitating illness and, in most cases, death. These strains induced a pronounced loss of body weight, facial erythema developing into macular rash, conjunctivitis, oral ulcerations, and in some cases hypothermia precluding death by 24–48 h.

All three strains evolved into distinct disease phenotypes. Espindola strain infection induced a primarily hemorrhagic disease, including widespread petechial rash, mucous membrane and/or nasal bleeding prior to death, and was associated with severe bacteremia [164–166]. In contrast, animals infected with the Ledesma strain developed early bacteremia and a prominent neurological disease, including encephalopathy, tremors, spontaneous and isolated limb paralysis, and balance disturbances. Animals infected with the P-3551 strain presented with a disease that shared components of both JUNV Espindola and Ledesma strain infections, but disease was generally milder (all animals infected with the Espindola strain succumbed to disease, whereas infection with the other two strains did not necessarily have a lethal outcome).

Investigators of a study assessing the aerosol route of exposure used the Espindola strain of JUNV but induced disease was similar to disease seen in intramuscularly inoculated animals [167]. All macaques developed acute signs 2–3 weeks post exposure, including anorexia, malaise, and weight loss, followed by development of rash, thrombocytopenia, lymphadenopathy, oral hemorrhage, and mucosal bleeding. Animals surviving beyond 3 weeks experienced a wasting illness prior to death. Interestingly, no distinct neurological signs were noted following aerosol exposure in rhesus monkeys.

MACV, the causative agent of Machupo/Bolivian hemorrhagic fever in humans, was also studied in rhesus monkeys. Initials signs were present within a week of subcutaneous inoculation and included depression, progressive anorexia followed by constipation, and intermittent diarrhea [168, 169]. Animals generally either succumbed to disease in this initial phase or progressed to develop neurological manifestations (tremors, nystagmus, lack of coordination, paresis, coma). Most animals succumbed during this neurological phase of disease, but some recovered. Animals that survived the first phase of disease typically developed neutralizing antibodies [168]. The mean time to death was also partially dependent on age and weight, with younger animals succumbing earlier. The mean time to death for smaller and larger rhesus monkeys was 19.3 days and 30.5 days, respectively.

Viremia in animals exposed to MACV was highest during the initial 2 weeks of infection but was still present in animals that had neurological signs. Interestingly, in an experiment in which complement was selectively depleted, viremia increased overall, highlighting the importance of complement fixation for clearance of the virus by antibodies [168]. These findings indicate that clinicians should exercise caution when passive transfer of convalescent serum is considered to treat human disease.

Gross and microscopic lesions included lymphocytic infiltrates in brain, spinal cord, pancreas, intestine, liver, kidneys, adrenals, heart, and skeletal muscle. Additional lymphocytic inflammation was noted in the nervous system [170].

Disease in rhesus monkeys, unlike NW monkeys, appears to correlate well with human disease induced by both the OW and NW arenaviruses (specifically LASV, JUNV, and MACV). Progression of the clinical phase for the rhesus monkey model is well mirrored in human case reports, making these models particularly well suited for studies exploring pathogenesis or evaluating medical countermeasures, including both vaccine and therapeutic approaches.

4.2.5 Crab-Eating (Cynomolgus) Macaque

Crab-eating macaques (*Macaca fascicularis*) have been used as models for infection caused by highly virulent arenaviruses, including LASV and MACV. As with rhesus monkeys, arenavirus disease in crab-eating macaques caused by LASV and MACV share major defining characteristics with human disease.

Following intramuscular inoculation of LASV, animals develop high fever, anorexia, mild-to-moderate depression, and dehydration between days 3 and 10. Facial edema occurs in some animals. Progressive anorexia and severe dehydration are followed by development of neurological signs, including convulsions and seizures, which rapidly increase in duration and severity until death [171].

Significant clinical parameters of LASV infection included increases in D-dimer and protein C plasma concentrations followed by elevation of liver enzyme and blood urea nitrogen concentrations in late stages of disease. Viremia occurred early in disease, starting as early as day 3, and peaked at approximately 2 weeks prior to death.

Increases in peripheral cytokine concentrations were significant for IL-6, IL-1β, eotaxin, and monocyte chemoattractant protein-1 (MCP-1) [171]. Baize et al. demonstrated that production of large quantities of IL-6 was correlative with fatal outcome. Survivors tended to have early and robust cell-mediated immune responses, further supporting the pivotal role of T cells over humoral responses in survival of Lassa fever [120]. Other studies supported these findings by demonstrating substantial increases in chemokines and cytokines in crab-eating macaques following inoculation with LASV, including those associated with immunosuppressive activities [172, 173].

Gross necropsy findings of LASV infection revealed lymphadenopathy with associated congestion, pale and friable livers, enlargement of the adrenal glands and pancreas, renal congestion, and pericardial effusion. Focal, petechial hemorrhage was noted on the mucosal surface of the urinary bladder, and congestion of the ileocecal junction suggested gastrointestinal involvement.

Histology supported gross pathological findings with antigen staining primarily associated with antigen-presenting cells in lymph nodes, spleen, and thymus. Hepatic and renal changes included lymphoplasmacytic and neutrophilic inflammation with substantial immunostaining in animals sacrificed during late-stage disease.

Fibrin deposition was also noted in both tissues. Mild interstitial pneumonia occurred in a single animal, and cardiac involvement was evident by neutrophilic inflammation of the pericardium. LASV antigen staining was present in all tissues evaluated, indicating systemic dissemination of virus. Microscopic examination of neurological tissue indicated meningoencephalitis in the cerebrum, cerebellum and brain stem with neuronal necrosis and gliosis. Endothelial and histiocytic cells were antigen positive in terminal cases [171].

Crab-eating macaques inoculated subcutaneously with MACV (Carvallo strain) exhibited clinical progression and pathogenesis similar to rhesus macaques with a biphasic disease character consisting of initial fever, anorexia, and depression followed by development of neurological symptoms often leading to death. Unlike rhesus monkeys, however, crab-eating macaques succumbed to disease without development of signs equal in severity to those in rhesus monkeys inoculated with an equivalent dose of virus. The mean time to death for MACV-infected crab-eating macaques was 17 days post-exposure [168].

Aerosol and intramuscular exposure of macaques with the Chicava strain of MACV caused a similar disease course as seen with the Carvallo strain in crab-eating macaques [174]. Animals exhibited similar biphasic disease, and death occurred within 3 weeks of exposure. Similar to previous studies, lymphadenopathy with associated congestion, viral hepatitis, and gastrointestinal hemorrhage were present. Histologic findings consisted of necrosis and apoptosis of cells of affected tissues, including liver, pancreas, adrenal glands, lymph nodes, stomach, and intestines. Interstitial pneumonia was also present in some cases. As expected, inflammation within the central nervous system was also histologically confirmed.

4.3 Use of Surrogate Models of Highly Virulent Arenaviruses

Work with OW and NW arenaviruses that cause viral hemorrhagic fevers in humans (LASV, Lujo virus, MACV, JUNV, SABV, GTOV, and Chapare virus), is restricted to biosafety level 4 conditions, limiting the work to a few specialized facilities. As a result, surrogate models utilizing related viruses in both rodent and primates have been developed for disease modeling purposes [175–186]. Several arenaviruses (e.g., TCRV, PICHV, MOPV, LCMV) or attenuated varieties of parental viruses that do not cause substantial disease in humans (except immunocompromised individuals) have been used in the development of both rodent and primate models with less inherent risk to researchers.

While these surrogate models can and have provided a wealth of information in advancing understanding of their highly pathogenic relatives, caution should be exercised with the extent to which these models can be used to identify pathogenic mechanisms and correlates of human disease. Most rodent models are based on gene knockouts that fundamentally alter the immune response, and nonhuman primate models rarely completely recapitulate the disease resulting from more virulent arenavirus members. These models are best suited to be used to specifically explore

pointed questions about aspects of these diseases that the models can faithfully reproduce. Alternatively, surrogate models can be used to ask more general questions about arenavirus replication applicable to all family members.

5 Vaccines and Therapeutics

5.1 Vaccines

5.1.1 Live Attenuated or Nonpathogenic Viruses

Currently, the only licensed, yet not FDA-approved, vaccine for use in the prevention of disease caused by an arenavirus is Candid#1. This vaccine has been clinically demonstrated to be save and efficacious against JUNV infection [187]. Using recombinant viruses in a laboratory mouse model of JUNV infection, the parental JUNV XJ44 strain was shown to be attenuated via a single amino acid change in GPC at position 427 (phenylalanine to isoleucine) [188, 189]. Vaccine safety and immunogenicity were demonstrated in rabbits, guinea pigs, and rhesus monkeys, and finally in randomized clinical trials in humans [190]. The vaccine has been successful in reducing both disease magnitude and severity of Junín hemorrhagic fever and is licensed in Argentina for vaccination of people living in high-risk areas where JUNV virus is endemic [187].

Another live attenuated vaccine candidate with substantial promise is the chimeric virus ML-29 containing the LASV S segment and the MOPV L segment. This recombinant virus was generated by coinfection of Vero cells with both viruses followed by plaque purification of the ML-29 virus clone [191]. In guinea pigs vaccinated with ML-29 and inoculated with LASV, disease did not develop. Immunogenicity was then evaluated in rhesus monkeys, and virus-specific cellular immunity to LASV and MOPV antigens, as well as LCMV, was demonstrated. The rhesus monkeys did not develop overt disease, nor were there histological lesions following vaccination, suggesting that ML-29 could be used for prevention of Lassa fever [192].

Nonpathogenic arenaviruses have also been evaluated as vaccine candidates against disease caused by more virulent arenaviruses. Early studies using MOPV indicated cross-protection against LASV infection in rhesus monkeys, as the monkeys had no signs of disease and survived otherwise fatal infection [193]. However, liver and kidney histological alterations were noted in rhesus monkeys infected with MOPV in the absence of overt clinical signs of disease, indicating that arenaviruses thought to be apathogenic may not be entirely safe [163]. Thus, caution should be exercised when evaluating the safety of closely related viruses thought not to cause disease in humans.

Similar approaches with TCRV have also been used successfully in the common marmoset primate model of JUNV disease [153, 154, 194–196]. Intramuscular or intranasal inoculation of marmosets with TCRV prior to injection with a lethal dose of JUNV provided protection from disease development and death.

Additionally, intrathalamic inoculation of animals with TCRV caused no clinical signs of disease, histopathologic changes, or viremia up to 480 days post-inoculation. Common marmosets developed measurable, protective immune responses as early as 3 weeks following exposure to TCRV. Results of these studies suggest TCRV may be a viable and safe candidate for vaccination against the pathogenic JUNV.

XJC13, an attenuated variant of JUNV derived from the parental XJ strain, was tested for efficacy as a vaccine candidate in common marmosets [151]. Following intramuscular inoculation of XJC13, no fatality or signs of overt illness were observed in animals up to 420 days post-inoculation. The only evidence of pathogenicity was slight weight loss between days 18 and 40 post-inoculation, after which animals' weight rapidly normalized. Viremia was detectable between day 6 and 22 post-inoculation with virus spread limited to lungs, spleen, lymph nodes, and bone marrow. Ganglionic hypertrophy with immunoblast proliferation was detected in animals sampled approximately 3 weeks after inoculation without recovery of virus. Measureable infectious virus could not be isolated at sampling time points greater than 1 year post-inoculation, although viral antigen staining was present in some organs.

All animals developed neutralizing antibody responses from week 3 onward. At days 60 or 380 following XJC13 inoculation, animals were inoculated with a lethal dose of the parental JUNV strain. XJC13 exposure conferred protection to all animals, whereas all control animals died. This study provides evidence that common marmosets may be useful in evaluating attenuated vaccines for JUNV infection.

5.1.2 Recombinant Vaccine Vector Approaches

More targeted approaches for the development of recombinant vaccine virus vectors have also been used. Vaccinia virus vectors modified to express LASV NP or GPC successfully protected guinea pigs against lethal LASV infection [197, 198]. Multiple vaccinia virus vaccines expressing different LASV antigens were tested in nonhuman primates, including vectors expressing only N-terminal (GP1) or C-terminal (GP2) parts of GPC, whole GPC or NP. Only whole GPC or administration of both GP1 and GP2 provided significant protection against disease and death in both rhesus monkey and crab-eating macaque models [199]. All animals receiving either GP1 or GP2 vaccines succumbed to disease, and 80 % of NP-vaccinated animals died despite development of high antibody titers. In comparison, all animals receiving both the GP1 and GP2 vaccines simultaneously survived, and 90 % of the animals receiving whole GPC survived even in the absence of significant antibody responses. The results of these studies suggest that a predominant cellular response is important in conferring protection and that whole GPC of LASV is necessary in eliciting a protective outcome.

A similar strategy was used for the development of a candidate vaccine against JUNV infection. A recombinant vaccinia virus expressing either GPC or NP of TCRV or GPC of JUNV was used to vaccinate guinea pigs. This approach resulted in partial protection of guinea pigs following lethal JUNV injection in both groups (50 % for TCRV GPC and 72 % for JUNV GPC) [200]. Interestingly, while recombinant

vaccinia virus expressing NP protein elicited a neutralizing antibody response, the vaccine was not protective. Conversely, both GPC vaccines were protective in the presence of low or undetectable neutralizing antibodies. Protection with recombinant vector vaccines against LASV and JUNV infection without appreciable antibody responses suggest that cell-mediated immunity (e.g., T cell responses) may play a prominent role in protection of animals from arenavirus infection.

Vesicular stomatitis Indiana virus (VSV) has also been used as a recombinant vaccine vector. Replication-competent VSV expressing LASV GPC protected nonhuman primates from lethal LASV infection. Transient viremia developed following inoculation, but no outward clinical signs of disease were noted [201, 202]. As was seen with the vaccinia virus vector, the VSV vaccine elicited strong cellular immune responses in vaccinated monkeys. In contrast to other vaccines, however, rVSV expressing LASV GP also induced a humoral response, although the contribution of this response to the positive outcome was impossible to determine.

Vaccination with the well-described yellow fever virus 17D backbone modified to express LASV GP1 and GP2 has resulted in partial protective efficacy in guinea pigs. Approximately 6 weeks post vaccination, five of six guinea pigs inoculated subcutaneously with 1,000 PFU of LASV survived; however, all animals developed clinical signs of disease (e.g., fever, loss of body weight and viremia) [203]. The vaccine also successfully elicited CD8+ T-cell responses in both CBA/J+ mice and strain 13 guinea pigs. As the vaccine failed to protect common marmosets from lethal LASV infection, the likelihood of efficacy in humans may be questionable [204].

A Venezuelan equine encephalitis virus replicon particles (VRP)-based vaccine has also been tested and found effective in protecting guinea pigs from lethal LASV infection [205]. Both individual vaccine strategies, VRPs expressing LASV GP or NP, were protective, as was vaccination with both vaccines simultaneously. None of the vaccinated animals developed signs of disease, and the majority of guinea pigs did not develop viremia as a consequence of LASV inoculation. Unlike previous vaccine strategies in which the use of NP did not lead to protection, results of this study provide evidence that an NP vaccine strategy may be viable. None of the vaccinated animals developed significant neutralizing antibody responses following vaccination, again suggesting a central role for cellular immunity in prevention of arenavirus disease.

Perhaps one of the most interesting approaches to development of a vaccine against LASV infection has been the expression of LASV NP in *Salmonella* Typhimurium. Mucosal immunization of mice elicited both virus NP-specific humoral and T cell responses [206]. Further evaluation of efficacy in an LCMV laboratory mouse model suggested that protection against LCMV infection could be achieved with the strategy. Experiments using this strategy with LASV, both in rodents and nonhuman primates, remain to be performed [207].

5.1.3 Inactivated and Virion-Like Particle Vaccines

Inactivated vaccine strategies for the prevention of arenavirus disease are underexplored. Virion-like particles (VLPs) containing LASV GP1, GP2, Z, and NP have been evaluated for their ability to induce antibody responses [208]; however, they have

yet to be evaluated for efficacy. LASV particles inactivated by gamma-irradiation failed to protect rhesus monkeys from lethal infection with live LASV, despite development of a humoral antibody response. This failure is attributed to a lack of an adequately induced cellular immunity following vaccination [209]. Likewise, guinea pigs vaccinated with formalin-inactivated JUNV developed neutralizing antibodies, but these animals were not protected from lethal disease [210]. Taken together, results of these studies suggest that non-replicating approaches are unlikely to provide protective immunity against arenaviral infections.

5.1.4 DNA Vaccines

Electroporation of DNA plasmids encoding viral genes and uptake by host cells can induce immunity to targets by promoting host cell expression of viral proteins. Cross-presentation of these antigens by antigen-presenting cells thus may elicit a potentially protective immune response. To evaluate this approach for vaccination against LASV infection, both the immunogenicity and efficacy of electroporation of DNA plasmid vaccine expressing LASV NP was evaluated in mice using LCMV or PICHV inoculant. A single inoculation induced cellular CD8+ immune responses and resulted in lower viral titers in vaccinated mice euthanized 4 days post-virus inoculation as compared to non-vaccinated controls [211]. While these results are encouraging, it remains to be demonstrated that these vaccines can provide protection against LASV infection. Furthermore, DNA vaccines are known to elicit rather weak immune responses and often require multiple dosing in prime-boost strategies or additional adjuvants to provide both protection and durability. As mice were inoculated with virus 3 weeks post-vaccination, the duration of protection with this DNA vaccine approach is unclear.

A DNA plasmid expressing LASV GPC was efficacious in protecting both guinea pigs [212] and nonhuman primates [213] from otherwise lethal LASV infection. In initial studies, 5/6 guinea pigs were protected, although the vaccine did not provide sterilizing immunity. Subsequent improvements in delivery and codon optimization of the GPC gene resulted in complete protection, and no viremia developed in vaccinated animals. Similarly, this strategy also completely protected crab-eating macaques.

5.2 Therapeutics

5.2.1 Passive Transfer Using Immune Sera

Multiple studies have highlighted the protective value of immune sera treatment to counter JUNV infection in both common marmoset and guinea pig models. Guinea pigs were protected from illness as many as 6 days post-challenge, though development of viremia and neurological complications (encephalitis, meningitis detected

at necropsy) did occur [214, 215]. Similar results were seen in common marmosets inoculated with JUNV—a 75 % survival rate following treatment with immune sera 6 days post-inoculation [152]. All animals developed clinical signs. Some survivors also developed neutralizing antibody titers following convalescence. Collectively, these studies suggest that passive immune therapy may be a promising approach for treatment of NW arenavirus infections.

The effectiveness of passive immune treatment has also been shown in nonhuman primate and guinea pig models of LASV infection. Multiple methods were used to characterize the neutralizing antibody components of animal or human convalescent serum, including immunofluorescent and standard plaque reduction neutralization titer techniques. The quality and concentration of neutralizing antibodies was clearly correlated with favorable outcome [216–218], and therapeutic cut-off values predictive of a favorable outcome were established. Treatment with neutralizing antibodies coupled with ribavirin therapy resulted in enhanced protection in the crab-eating macaque models of LASV and JUNV infections, underlining the advantages of combinational therapy approaches [219, 220]. A single study assessed the role of complement in neutralization of JUNV [221]. Presence of complement was critical for neutralization of virulent JUNV strains, but not for attenuated strains, suggesting that complement activation may play an important role in the quality of the neutralizing antibody response.

Passive transfer of immune sera has also been tested experimentally in rhesus monkeys or crab-eating macaques inoculated with MACV [222]. Immunoglobulin of human origin was given either pre- or post-virus inoculation. Animals receiving sera were protected from developing initial clinical illness; however, some survivors later developed neurological signs and subsequently succumbed to disease. Neurological development may have had a greater association with high doses of immunoglobulin, suggesting that neurological pathology may be at least in part mediated by delivery of treatment.

5.2.2 Drugs Targeting Viral Entry

Preventing virion cell entry in theory prevents a virus from establishing infection and therefore subsequent replication. Cell entry begins with engagement of attachment factors present on the target cell surface by arenaviral GP1, leading to internalization, endosomal trafficking, and virus uncoating. Thus, targeting cell-surface receptors involved in engagement of arenaviral glycoproteins and host pathways involved in permitting access of virus to the cell following attachment is an attractive therapeutic strategy.

Virulent NW arenaviruses (all of which belong to clade B) utilize human hTfR1 by recognition of structures distinct from the transferrin-binding site [74, 93, 223]. Understanding the binding site necessary for arenavirion attachment presents the possibility of targeting the site for therapeutic intervention. Using a monoclonal antibody to hTfR1 targeting the region necessary for arenavirus GP1 binding, but dispensable for transferrin binding, Helguera et al. successfully blocked infection of

HEK293 cells by all NW arenaviruses. The antibody may be promising for studies in nonhuman primates as the antibody is cross-reactive with transferrin receptor orthologs of primates belonging to several species.

OW arenaviruses are thought to utilize extracellular matrix ligands for attachment and entry, presenting a more difficult challenge for inhibiting entry at the cell surface. Despite this possible hurdle, phosphorothioate DNA oligonucleotides can potently inhibit LCMV infection by interfering with the virus–αDG interaction, thus preventing viral entry by steric blockade [224].

Small molecule inhibitors are capable of blocking entry by preventing pH-mediated fusion of the arenaviral GP1 with cellular entry receptors that are relatively specific to arenaviruses in multiple cell types [225]. High-throughput screening of various compounds yielded lead candidate small molecule inhibitors, ST-193 and ST-294, which are effective at blocking LASV, JUNV, MACV, and GTOV GP-mediated entry by inhibiting membrane fusion [226, 227]. ST-193 tested in the guinea pig model of LASV infection significantly reduced fatality [228].

Lassa virus GPC is proteolytically cleaved by cellular site 1 protease (S1P) to generate the attachment protein GP1 and the fusion-active transmembrane protein GP2. PF-42942, a small molecule inhibitor of S1P, had no impact on transcription, translation, or budding of LCMV and LASV, but had a modest effect on virus cell entry [229]. Thus, the anti-arenavirus activity of PF-42942 is primarily related to inhibition of S1P-mediated processing of GPC. More recent studies indicate that PF-42942 may work against NW arenaviruses as well [230]. Using small molecule inhibitors of S1P may therefore hold promise as a novel antiviral strategy in preventing arenavirus infection.

Imidazothiazole carbohydrate derivatives also have potential utility in blockade of JUNV at the point of infection [231]. Cells were preincubated with varying concentrations of these compounds, compounds were premixed and incubated with virions prior to cell infection, or cells were treated at time of infection. Preincubation with virions yielded little reduction in infectivity, but both pretreatment of cells or simultaneous addition of drug and virions reduced infection.

Trifluoperazine and chlorpromazine, both drugs in the phenothiazine class, proved efficacious in vitro against JUNV, TCRV, and PICHV. These effects were achieved at IC_{50} concentrations ranging from 7.7 to 23 μM. Time-of-addition experiments revealed that the drugs acted early in the replicative cycle, likely by modulating actin microfilaments and affecting viral entry [232].

5.2.3 Drugs Targeting Viral Replication

Ribavirin, the only off-label drug for treatment of arenavirus infections, is a nucleoside analogue and still remains the treatment drug of choice, despite its well-known toxicity [233–237]. Ribavirin reduces morbidity and fatality in both clinical and experimental conditions of Old and New World arenavirus infections when provided early in course of clinical disease [161, 219, 238–242]. Ribavirin is thought to exert its antiviral activity by negatively regulating RNA synthesis. While the

precise mechanism remains elusive, ribavirin may inhibit inosine monophosphate dehydrogenase activity, leading to depletion of intracellular GTP pools [243], although results of some studies have challenged this idea [244]. Another possible explanation for ribavirin's antiviral effect may be direct mutagenesis of viral RNA [245]. Other drugs targeting inosine monophosphate dehydrogenase, which may be as efficacious as ribavirin but be less toxic, may be worth investigating.

T705, also known as favipiravir, is a pyrazine derivative that is effective in vitro against arenavirus infections. The mechanism of action involves disruption of the early intermediate phase of virus replication by inhibition of L activity [246, 247]. In vivo studies using PICHV rodent models yielded promising results. Twenty and seventy eight percent of guinea pigs treated with favipiravir by the oral or peritoneal routes, respectively, survived inoculation with PICHV when treatment occurred with 48 h [248]. Those animals who succumbed to disease experienced a prolonged disease course, and surviving animals presented with less severe disease overall. In hamsters, initiating treatment during the most severe stage of disease still altered disease outcome. These results suggest that T705 is an exceptional candidate for further preclinical development to treat arenavirus disease [249, 250]. Most of the studies described above incorporated ribavirin as a comparative treatment control. Not only did T705 outperform ribavirin in direct studies, but it also was significantly less toxic [246, 248, 250].

The antibiotic pyrazofurin was tested both in vitro and in guinea pigs inoculated with PICHV [251]. Results in cell culture were promising as relatively low concentrations of the drug, 2 μg/ml, markedly inhibited plaque formation of multiple arenaviruses. The mechanism of action is attributed to inhibition of de novo synthesis of nucleotides by blocking the activity of orotic acid monophosphate decarboxylase and preventing formation of uridine. Unfortunately, results in guinea pig studies were disappointing as treatment did not prevent lethal outcome or alter viral loads.

A few studies have also evaluated the use of type I IFNs, specifically IFN-α, as a treatment for arenavirus infection. Generally speaking, results of these studies indicated arenavirus infections to be relatively insensitive to IFN treatment [252–255]; however, at least one study suggested that treatment with type I IFNs can reduce LASV replication in HuH7 and Vero cells [256]. Additionally, therapeutic benefit has also been achieved by treating hamsters with IFN alfacon-1 immediately following and up to 2 days after exposure to PICHV [257]. A protective effect in the same hamster model was also achieved using the non-replicating recombinant adenovirus platform DEF201 encoding consensus IFN alfacon-1 in pre- and post-prophylaxis approaches. These results suggest that IFN treatment may be at least partially beneficial to controlling arenavirus infections [258].

Several other compounds, including *S*-adenosyl-L-homocysteine (SAH) hydrolase inhibitors [259–262], brassinosteroids [263], myristic acid [264], carboxamide derivatives [265], and zinc-finger-reactive compounds [266], have anti-arenaviral activity. Zinc-finger-reactive compounds are thought to act via inhibition of Z, presenting yet another viral replication cycle target. To date, none of these compounds have been evaluated in animals or demonstrated to have significant advantages over ribavirin as a therapeutic alternative.

Kinase inhibitors have also been evaluated for efficacy in treating both NW and OW arenavirus infections. Genistein is a general tyrosine kinase inhibitor that blocks infection of cells by PICHV, likely at the step of entry. Activation of transcription factor-2 protein (ATF-2) and cyclic adenosine monophosphate response element binding protein (CREB) in Vero cells by PICHV was inhibited following treatment with genistein, and this inhibition correlated with decreased viral entry [267]. A similar suppression of infection was observed when genistein was paired with tyrphostin, another kinase inhibitor. The drugs both demonstrated individual efficacy and a synergistic effect when combined [268]. Genistein was also tested in the Syrian golden hamster model of PICHV infection with successful reduction in fatality and improved clinical profile [269].

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Chapter 20
Ebola Virus Disease

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Core Message Ebola virus disease (EVD) is caused by four ebolaviruses: Bundibugyo virus (BDBV), Ebola virus (EBOV), Sudan virus (SUDV), and Taï Forest virus (TAFV). Contrary to oft-repeated messages in the public press and some scientific review articles, ebolaviruses are naturally not airborne, and mutations are unlikely to result in airborne mutants. Although bats are repeatedly brought up in discussions as possible natural host reservoir(s) of ebolaviruses, scientific evidence for any bat association is currently lacking for BDBV, SUDV, and TAFV. In the case of EBOV, antibody and genomic fragment detection thus far, at best, indicate exposure of certain bats to the virus, thus emphasizing the gaps in knowledge regarding ebolavirus reservoirs. Incorrect beliefs about ebolaviruses both in the Western world ("massive blood loss", "liquefying organs") and African countries ("witchcraft", "sorcery") hinder EVD outbreak control efforts and often lead to stigmatization of infected individuals and their relatives. While the development of efficacious medical countermeasures against EVD is of paramount importance for future preparedness, spread of EVD could be prevented by avoidance of direct person-to-person contact, proper use of personal protective equipment, and improved education of government officials, public and health-care professionals, and religious leaders about the disease.

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1 General Information

The mononegaviral family *Filoviridae* includes three genera: *Cuevavirus*, *Ebolavirus*, and *Marburgvirus* [1, 2]. Infections with ebolaviruses and marburgviruses cause Ebola virus disease (EVD) and Marburg virus disease (MVD), respectively [3], both of which are frequently lethal in humans. EVD and MVD outbreaks have previously been rare, sporadic, and limited (reviewed in ref. [4]), but a massive EVD outbreak in heavily populated areas of West Africa beginning in late 2013 [5], accompanied with exported cases to multiple countries, has highlighted filovirus infections as a global concern [6]. This chapter focuses on EVD.

There are five ebolaviruses: Bundibugyo virus (BDBV), Ebola virus (EBOV), Reston virus (RESTV), Sudan virus (SUDV), and Taï Forest (TAFV) (Fig. 20.1) [2]. EBOV and SUDV were discovered in 1976, whereas RESTV, TAFV, and BDBV were discovered in 1989, 1994, and 2002, respectively (reviewed in ref. [4]). With the exception of RESTV, all ebolaviruses cause EVD in humans (11 presumable human infections with RESTV were shown by post-hoc antibody responses, but clinical signs were not apparent). The lethality of EVD tends to vary by geographic location and the involved ebolavirus, but little statistical evidence supports the idea that one ebolavirus, excluding RESTV, is more virulent than another [7]. The natural host reservoirs of ebolaviruses are unclear, and, therefore, how ebolaviruses are introduced into the human population is not known. EBOV is speculated to circulate in certain healthy pteropid bat populations, but live EBOV has not yet been isolated from a bat,

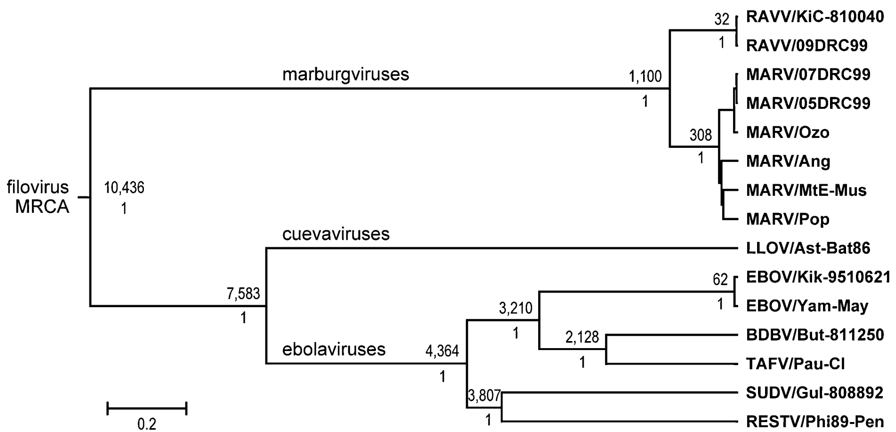

Fig. 20.1 Bayesian coalescent analysis of representative isolates of all currently known filovirus clades (i.e., cuevaviruses, marburgviruses, ebolaviruses). Maximum clade credibility tree is shown with the MRCA number at each node. Posterior probability values are shown beneath MRCA estimates in years. Scale is in substitutions/site based on an analysis performed by Dr. Serena Carroll, Centers for Disease Control and Prevention (CDC). *BDBV* Bundibugyo virus, *EBOV* Ebola virus, *LLOV* Lloviu virus, *MARV* Marburg virus, *MRCA* most recent common ancestor, *RAVV* Ravn virus, *RESTV* Reston virus, *SUDV* Sudan virus, *TAFV* Taï Forest virus

nor have full-length EBOV genomes been detected in any of these animals (reviewed in ref [8]). Accidental hosts of ebolaviruses include nonhuman primates (in the case of TAFV and possibly in the case of EBOV) and humans [7].

2 Molecular Biology

Ebolaviruses, like other filoviruses, produce enveloped virions that assume pleomorphic and often filamentous shapes (width ≈ 80 nm; length ≈ 800–19,000 nm [9]). An ebolavirion contains one or possibly several copies of a linear, monopartite, single-stranded RNA genome of negative polarity. This genome is, on average, ≈19 kb long and contains seven genes in the order 3′-*NP-VP35-VP40-GP-VP30-VP24-L*-5′ that encode the seven structural proteins NP, VP35, VP40, $GP_{1,2}$, VP30, VP24, and L, respectively [10, 11]. In addition, the *GP* gene encodes three additional, soluble proteins that are produced via cotranscriptional editing [12, 13]. The function of these soluble factors, sGP [14], ssGP [15], and Δ-peptide [16, 17] has yet to be determined.

Together with the genome, the nucleoprotein (NP), polymerase cofactor (VP35), transcriptional activator (VP30), and RNA-dependent RNA polymerase (L) form the ribonucleoprotein complex. In these complexes, the genome wraps around a helix of self-associated NP [18]. Polymerase complexes of VP35, VP30, and L move along the genome (or are fixed at particular location in virions). While NP provides structural integrity, the polymerase complexes are responsible for transcribing genes into mRNAs or replicating the entire genome [19, 20]. The matrix protein (VP40) is, next to NP, another major structural protein. VP40 recruits the ribonucleoprotein complexes and drives virion morphogenesis and budding from the host cell [21–24]. Both VP40 and the secondary matrix protein (VP24) regulate mRNA transcription and genome replication [25]. The glycoprotein ($GP_{1,2}$) is integrated into the virion envelope. $GP_{1,2}$ is a trimer of GP_1–GP_2 heterodimers and functions as a typical class I membrane fusion protein [26]. $GP_{1,2}$ determines ebolavirus cell, tissue, and host tropism by engaging with specific ebolavirus receptors on and inside of host cells [27, 28].

VP35, $GP_{1,2}$, and VP24 also mediate immune suppression. For instance, VP35 inhibits production of interferon (IFN) α/β by binding double-stranded RNA (synthesized as an intermediary product during ebolavirus infection) and shielding this RNA from recognition by pattern recognition receptors, such as retinoblastoma inhibiting gene 1 protein (RIG-1) and melanoma differentiation associated protein-5 (MDA-5) [29, 30]. In addition, VP35 inhibits phosphorylation of IFN response factors 3 and 7 [31] and functions as a host-cell RNA silencing suppressor [32]. VP24 compliments VP35 by inhibiting host-cell signaling downstream of IFN-α/β/γ via karyopherin trapping in the cytoplasm [33–35]. Finally, $GP_{1,2}$ has been identified as a viral factor that antagonizes the antiviral effects of the cellular restriction factor tetherin [36].

Ebolavirus life cycle. The ebolavirus life cycle begins with attachment of the ebolavirion to cognate receptors on host-cell surfaces. Mediated by $GP_{1,2}$, this attachment triggers endocytosis of the virion [37, 38]. Subsequently, $GP_{1,2}$ binds to Niemann-Pick C1 in the endo/lysosome, which results in fusion of the virion envelope with the endo/lysosomal membrane. After fusion, the ribonucleoprotein complex is released into the cytosol, where ebolavirus replication occurs exclusively [27, 28]. The polymerase complex synthesizes nine distinct mRNAs that encode the ten nonstructural and structural ebolavirus proteins [19, 20]. Following mRNA translation, the intracellular concentration of these proteins (in particular NP), determines when transcription is switched to genome replication, which occurs via intermediate synthesis of fully encapsidated antigenomes. Genomes, NP, VP35, VP30, and L assemble into progeny ribonucleocapsids [19, 20] that are then recruited by VP40 and possibly VP24 for assembly of progeny virions [21–24]. $GP_{1,2}$ synthesis occurs in the endoplasmic reticulum. New synthesized and correctly processed $GP_{1,2}$ trimers are then transported to and inserted into host-cell membranes [39]. Progeny virions then bud from the host-cell surface, thereby acquiring their envelopes and $GP_{1,2}$ [22].

3 Pathogenesis

The current understanding of EVD pathogenesis is based largely on in vitro experiments and the use of in vivo models of EBOV infection (reviewed in ref. [4]). EBOV can infect a wide variety of mammalian cell types of vastly divergent species.

EBOV infects macrophages early in the disease course, as evidenced by the presence of inclusion bodies in these cells in infected nonhuman primates [40, 41]. In vitro experiments with human macrophages suggest that after EBOV infection, macrophages support viral replication and produce large amounts of pro-inflammatory cytokines and chemokines [42]. Dendritic cells are also infected in vitro, but do not appear to produce cytokines or become activated, although they do support virion production [43, 44]. In vivo data on dendritic cells as early targets of infection are suggestive but not conclusive. Regardless, EBOV infection results in rapid replication, spreads via the blood stream and lymphatic system, and infects multiple other cell types, including hepatocytes, fibroblasts, epithelial cells, adrenal cortical cells, endothelial cells, and reproductive cells [45].

EBOV replication is aided by its ability to inhibit type I IFN production and signaling by the action of VP24 and VP35 [46]. This inhibition abrogates an important early anti-viral arm of the immune response and results in enhanced viral replication and spread throughout multiple tissues.

Additionally, inhibition of type I IFN likely results in hyper-inflammatory cytokine responses [47]. Systemic production of large amounts of interleukin (IL)-1, IL-6, IL-8, tumor necrosis factor (TNF)-α, monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, and other pro-inflammatory mediators, along with increased production of tissue factor, likely leads to

widespread coagulation [48–52]. Disseminated intravascular coagulation is induced, as evidenced by generation of fibrin degradation byproducts (D-dimers), thrombocytopenia, depletion of clotting factors, and declining protein C concentrations [52]. As a result, widespread deposition of microthrombi blocks small blood vessels, leading to extensive hypoxemic infarcts in affected tissues (e.g., liver, spleen, kidneys, reproductive organs). Other signs of coagulopathy include petechial rash, easy bruising, bleeding from mucous membranes and other internal and external sites, and extensive visceral effusions in internal organs [45]. Note, however, that external blood loss rarely reaches volumes that are life-threatening. Furthermore, EBOV-induced increase in endothelial permeability occurs later in infection. This increase then leads to (probably extensive) third spacing, resulting in interstitial and myocardial edema.

B and T cell responses are not sufficient to control infection. Originally, such insufficient responses were thought to possibly be due to severe bystander lymphocyte apoptosis that is induced in lethal EBOV infection However, later animal experiments showed that inhibition of lymphocyte apoptosis did not affect animal survival, at least in a mouse model [53]. Furthermore, activated T cells are found in late stages of lethal EBOV infection in mice and nonhuman primates. Mouse studies showed that this cell population contains EBOV-specific CD8+ T cells that confer protection after adoptive transfer [54]. Therefore, impaired innate immune responses combined with hyper-inflammatory cytokine production are likely to dampen B and T cell responses that develop too late to control infection.

Together, disseminated intravascular coagulation, hypovolemic shock, and multiple organ failure are thought to be the main causes of death in ebolavirus-infected experimental animals and, by extrapolation, in infected humans.

Clinical presentation. EVD cannot be diagnosed merely by observation of clinical manifestations, as they are unspecific and common to many infections and, therefore, easily confused with those of numerous other more prevalent diseases. The incubation period of EVD is thought to vary from 2 to 21 days, after which a biphasic syndrome develops. The first phase, lasting 5–7 days, is similar to common viral infections or falciparum malaria, including sudden onset of fever and chills, malaise, joint pain and muscle aches, headache, cough, and development of a maculopapular rash. The second phase (sometimes following a 1–2 day period of relative remission after the first phase) is characterized by gastrointestinal tract abnormalities (e.g., abdominal pain, vomiting, diarrhea), vascular system involvement (e.g., edema, orthostatic hypotension), and central nervous system signs (confusion, tremors, coma). Importantly, clinical presentation varies and while typical, many of the above-mentioned clinical signs may not develop. Hemorrhagic manifestations include conjunctivitis, bleeding from mucosal surfaces and venipuncture sites, and blood in vomit, urine, and feces. Clinical laboratory signs of EVD include leukopenia (typically due to loss of lymphocytes) with a left shift followed by leukocytosis (caused by an increase in granulocytes). Platelet loss also does occur, as do signs of liver and pancreas dysfunction (elevated serum enzyme concentrations of AST, ALT, GGT, and amylase). Other clinical indicators in the blood include low potassium concentrations and elevated protein and creatinine concentrations.

Coagulopathy findings are frequent, and include thrombocytopenia and prolonged prothrombin and partial thromboplastin times [55–58].

Patients who succumb to disease usually do so 4–14 days after onset of clinical signs. Those who survive often undergo prolonged and sometimes incapacitating sequelae characterized by a wide range of clinical signs, including hearing loss, joint pain, fatigue, psychosis, hair loss, skin peeling, and inflammation of the eyes, testes, salivary glands, liver, or spinal cord [59]. In rare cases, EBOV can persist in the testes of survivors, possibly transmitting EVD via sexual intercourse to the partner long after the original patient's recovery [59–61]. Whether such persistence is also possible in infections with the other ebolaviruses remains to be determined.

4 Diagnosis

Due to the nonspecific clinical signs, EVD is difficult to diagnose based on clinical presentation alone. Multiple diseases with similar signs are more prevalent in ebolavirus-endemic regions, including falciparum malaria, typhoid fever, viral hepatitis, measles, certain systemic bacterial infections, and even other hemorrhagic fevers such as Lassa fever and yellow fever. Thus, these diseases are more frequently included in a differential diagnosis for a febrile patient (reviewed in ref. [4]). For instance, at the beginning of the ongoing 2014 EVD epidemic in West Africa, EVD was not at the top of the list of possible causes of the observed viral hemorrhagic fever. Ebolaviruses were only theorized to be endemic in West Africa but, with the exception of TAFV, had never been encountered there. However, epidemiological anamnesis can be helpful in establishing a preliminary diagnosis. For example, those with a history of direct contact with nonhuman primates or bush meat or extended excursion into tropical rain forests are at a heightened risk for ebolavirus infection, as are those with contact with known ebolavirus-infected patients.

Confirmatory diagnosis of active ebolavirus infection is primarily achieved through the use of reverse transcriptase polymerase chain reaction (RT-PCR) [62], which has a detection limit of 1,000–2,000 virus genome copies per ml of serum. An alternative method is the use of an antigen capture enzyme-linked immunosorbent assay (ELISA) for the detection of ebolavirus proteins [63]. Detection of previous ebolavirus infections can be accomplished using IgG (or IgM, for recent infections) ELISA for detection of antibodies against ebolavirions. This method can also be used to detect infection in actively infected patients, but is less sensitive than RT-PCR, since antibody concentrations may be variable in patients and may develop days after onset of clinical signs. All these assays can be performed on sterilized samples to render ebolaviruses noninfectious, thereby diagnostic assays can be performed in regular laboratories without the risk of infecting laboratory workers. Ebolaviruses can also be isolated in cell culture using untreated clinical samples and plaque assays [64, 65], which are relatively simple to perform for virus quantification. However, these types of assays must be conducted in maximum-containment (biosafety level 4) laboratories.

5 EVD Outbreak History

Overviews of all recorded human EVD outbreaks and total human case numbers are presented in Tables 20.1 and 20.2. Almost all outbreaks began with a single ebolavirus introduction. Direct contact with the index case then led to unrecognized

Table 20.1 Overview of recorded Ebola virus disease (EVD) outbreaks

Virus	Country	Year(s)	Number of deaths	Number of infections	Case-fatality rate (%)
BDBV	Uganda	2007–2009	39	116	33.62
	Democratic Republic of the Congo	2012	13	36	36.11
EBOV	Zaire	1976	280	318	88.05
	Zaire	1977	1	1	100.00
	Gabon	1994–1995	32	52	61.54
	Zaire	1995	245	317	77.29
	Russia	1996	1	1	100.00
	Gabon	1996	21	31	67.74
	Gabon→South Africa	1996–1997	46	62	74.19
	Gabon, Republic of the Congo	2001–2002	97	124	78.23
	Gabon, Republic of the Congo	2002	10	11	90.91
	Republic of the Congo	2002–2003	128	143	89.51
	Republic of the Congo	2003–2004	29	35	82.86
	Russia	2004	1	1	100.00
	Republic of the Congo	2005	9	11	81.82
	Democratic Republic of the Congo	2007	186	264	70.45
	Democratic Republic of the Congo	2008–2009	15	32	46.88
	Guinea→Liberia, Nigeria, Sierra Leone, Senegal, Mali, Spain, USA, UK[a]	2013–2015	10,460	25,213	41.49
	Democratic Republic of the Congo	2014	49	66	74.24
SUDV	Sudan	1976	151	284	53.17
	UK	1976	0	1	0
	Sudan	1979	22	34	64.71
	Uganda	2000–2001	224	425	52.71
	Sudan	2004	7	17	41.18
	Uganda	2011	1	1	100.00
	Uganda	2012	3	6	50.00
	Uganda	2012	4	11	36.36
TAFV	Côte d'Ivoire→Switzerland	1994	0	1	0

Arrows (→) indicate international case exportation
[a]As of January 21, 2015

Table 20.2 Total case numbers of recorded human ebolavirus infections

Virus	Total number of deaths (1976 to present)	Total number of infections (1976 to present)	Case-fatality rate (%)
BDBV	52	152	34.21
EBOV[a]	11,610	26,682	43.51
SUDV	412	779	52.89
TAFV	0	1	0
All ebolaviruses combined[a]	12,074	27,614	43.72

[a]As of March 29, 2015

secondary and tertiary infections. Outbreaks were usually terminated by outside intervention once ebolavirus infection was recognized and the transmission chains were interrupted. Such interruption was accomplished by preventing direct (skin-to-skin) contact between people in affected areas; introducing proper personal protective equipment for health-care workers; introducing proper disinfection methods for bodily fluids, tissues, and clinical materials [66]; and ensuring that syringes/needles were not used multiple times in the often underequipped hospitals of Equatorial Africa. Until 2014, outbreak termination was aided by the often (but not always) rural location of EVD outbreaks as the mobility of EBOV-infected people was overall limited [4]. The current EVD outbreak in West Africa, by far the largest filovirus disease outbreak ever observed, has reached tens of thousands of EVD cases because of the urban distribution of the infection, de facto absent border control between the affected countries (mainly Guinea, Liberia, and Sierra Leone), and better roads that increased mobility of their inhabitants. Yet unpublished laboratory experiments suggest that the EBOV variant responsible for the 2014 EVD outbreak does not differ in any significant way from previous variants.

6 Animal Models

Nonhuman primates seem to be generally susceptible to ebolavirus infection and are considered the gold standard for evaluation of medical countermeasures. EBOV infects and causes severe disease in rhesus monkeys (*Macaca mulatta*), crab-eating macaques (*Macaca fascicularis*), grivets (*Chlorocebus aethiops*), hamadryas baboons (*Papio hamadryas*), and common marmosets (*Callithrix jacchus*) [41, 67–70]. Data for the other ebolaviruses are more scant as baboon and marmoset experiments have not yet been undertaken or reported, although SUDV is pathogenic in rhesus monkeys and crab-eating macaques [69]. Widely used laboratory mouse and guinea pig (*Cavia porcellus*) models for EBOV infection are available using laboratory mouse-adapted and guinea pig-adapted EBOV, respectively [71, 72]. These models are used for early evaluations of candidate countermeasures and

understanding of molecular pathogenesis. Both adapted viruses are characterized by a few genomic mutations compared to wild-type EBOV. More recently, laboratory mice lacking the type I IFN receptor are being used more frequently for wild-type EBOV, SUDV, and RESTV infection [73, 74]. Golden hamsters (*Mesocricetus auratus*) infected with laboratory mouse-adapted EBOV develop substantial coagulopathy [75].

7 Outbreak Control

It is extremely important to remember that ebolaviruses are not naturally airborne pathogens. Under certain artificial conditions, however, generation of airborne particles may occur, such as during centrifugation of samples and clinical or pathological procedures involving suctioning, drilling, or sawing. Ebolaviruses are transmitted almost exclusively through direct person-to-person contact. A number of measures have proven effective in containing the spread of EVD in previous outbreaks, which have mostly occurred in sparsely populated areas. Such measures include: isolation of patients; patient contact tracing with subsequent isolation; appropriate use of simple personal protective equipment (PPE), such as gloves, gowns, and mouth protection by health-care workers; non-reuse of syringes/needles and other possible contaminated equipment; and modified patient burial practices. During the current outbreak in heavily populated areas such as the spillover of cases from Liberia into Nigeria, containment of EVD was successful using patient isolation and patient contact tracing [76].

8 Vaccines

Several candidate vaccines are efficacious against ebolavirus infection in nonhuman primate animal models (reviewed in ref. [77, 78]). Protection from EBOV-induced disease or death has been described using vesicular stomatitis Indiana virus (VSV) [79], adenovirus (AdV) [80], virus-like particles (VLP) [81], Venezuelan equine encephalitis virus-like replicon particles (VRP) [82], and human parainfluenza virus vaccine platforms [83], along with prime-boost platforms. In addition, VSV, AdV, and VRP-based vaccines are protective against SUDV [82, 84, 85], whereas VSV-based vaccine is effective against TAFV [84]. Both VSV and DNA prime/AdV boost vaccines are protective against BDBV infection [86, 87]. Human clinical trials have begun with VSV-based vaccines against EBOV and AdV-based vaccines against EBOV and SUDV. In addition, plasmid DNA vaccine trials against EBOV and SUDV are also in progress.

9 Therapeutics

Many therapeutics have been tested against ebolaviruses, and some have shown encouraging efficacy in animal models.

Antisense therapies. Two types of antisense therapies have been studied in ebolavirus infections. These therapies are based on targeting and degrading or inhibiting translation of ebolaviral mRNAs. small interfering RNAs (siRNAs) formulated in liposomes and directed against EBOV protect guinea pigs and macaques from EBOV infection [88, 89].

Phosphorodiamidate morpholino oligomers (PMOs) are synthetic, single-stranded RNA antisense molecules that are remarkably stable and can be formulated in saline. PMOs function by binding to target mRNAs and inhibiting translation at the ribosome. PMOs have shown efficacy when targeting EBOV genes in laboratory mice, guinea pigs, and macaques [90, 91].

Small molecule inhibitors. Results of mouse studies show the efficacy of small molecules against EBOV and confirm the efficacy of in vitro screening using eGPF-expressing EBOV as a starting point for drug discovery. The antioxidant compound NSC 62914 is protective against EBOV infection [92], as are the small molecules FG-103 and FG-106 [92, 93]. A synthetic adenosine analog, BCX4430, shows remarkable efficacy against Marburg virus infection in nonhuman primates and also protects mice from EBOV infection [94]. The adenosine analog 3-deazaneplanocin A is effective in mice against EBOV infection in a type I-IFN-dependent manner [95, 96]. Favipiravir (T-705), a pyrazinecarboxamide derivative that has broad-spectrum antiviral activity, is protective against EBOV infection in two different laboratory mouse models [97, 98].

Anti-clotting factors. Anti-clotting factors have been tested for controlling EBOV-induced coagulopathy. The recombinant nematode anticoagulant protein c2 (rNAPc2), which inhibits coagulation initiated by tissue factor, showed modest protection in EBOV-infected rhesus monkeys [99]. Administration of the human anticoagulant activated protein C (APC) was protective in 2 out of 11 EBOV-infected rhesus monkeys [100].

Vaccines as post-infection therapy. Vaccines that are protective when given pre-exposure have also been tested as post exposure therapeutics against ebolaviruses. When given 20–30 min after challenge, the VSV SUDV vaccine protected 4/4 SUDV-infected rhesus monkeys [101], and the VSV EBOV vaccine protected 4/8 EBOV-infected rhesus monkeys [102]. An AdV vaccine protected mice from EBOV when administered 30 min after challenge [103]. Furthermore, a VLP EBOV vaccine rescued mice when given 24 h after challenge [104, 105]. Together, these data suggest vaccines may be useful as post-exposure therapeutics.

Antibody therapy. Results from an uncontrolled experiment performed in 1995 suggested that passive transfer of whole blood (used because serum isolation equipment was unavailable) protected seven of eight patients suffering of EVD from a lethal outcome, while non-treated patients died at an 80 % rate [106, 107]. In 1995,

Russian researchers reported that passive transfer of hyperimmune equine serum from EBOV-vaccinated horses was able to protect hamadryas baboons from EBOV infection [108, 109]. A different batch of hyperimmune equine serum was sent to researchers in the US, who were unable to confirm these findings in EBOV-infected macaques receiving a higher dose of a different variant of EBOV [110]. Additionally, monoclonal antibody therapies that were effective in mouse or guinea pig models of EBOV did not show success in initial macaque studies. This led many investigators to question the validity of using antibodies in EVD treatment. However, in 2012, Dye et al. demonstrated that passive transfer of concentrated IgG from immune macaques rescued naïve EBOV-infected macaques from high-dose EBOV infection, even when treated 48 h after challenge [111]. Following this study, results of several studies confirmed protection against EBOV infection in macaques receiving two- or three-component monoclonal antibody cocktail preparations. Animals treated with two monoclonal antibodies resulted in limited protection before and after EBOV challenge [112], whereas those receiving three monoclonal antibodies after EBOV challenge were moderately protected [113, 114]. Different combinations of three-monoclonal cocktails resulted in complete protection in macaques after EBOV challenge when given 1–5 days after infection, depending on the cocktails used [115, 116]. Antibody preparations based on these latter studies have been used experimentally in human EVD patients, but, at this time, the efficacy of these preparations in controlling EVD is unknown. In addition, convalescent serum transfers are currently being tested in the current EVD outbreak in humans.

10 Future Directions

The very large number of cases and case contacts (at the time of writing more than 25,000) could lead to the identification of humans that are relatively or even absolutely resistant to ebolavirus infection. Study of these individuals could thereby provide valuable hints in regard to key events in EVD pathogenesis. A more complete understanding of EVD pathogenesis, in turn, could lead to the development of novel antiviral agents. In addition, the clinical evaluation in humans of already promising candidate vaccines or therapeutics, previously deemed unethical, is now not only possible but by many deemed obligatory to contain the outbreak and possibly save countless lives. The “export” of EVD from West Africa into North America and Europe via air travel also emphasizes the potential of EVD to develop into a pandemic. This threat in turn may move filovirus research from a niche specialty into the virology mainstream, thereby increasing the likelihood of discoveries that may rapidly and effectively counter filovirus infections.

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Chapter 21
XMRV: Emerging Human Infection or False Alarm

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1 Background

XMRV is closely genetically related to xenotropic murine leukemia virus (MLV or MuLV), a ubiquitous rodent gamma-retrovirus. XMRV was discovered in 2006 during analysis of the association of gene single nucleotide polymorphisms (SNPs) in the 2′,5′-A-dependent Ribonuclease L gene (RNaseL) with prostate cancer [1]. It was proposed that XMRV was the first gamma-retrovirus that might be related to human disease. Later, it was claimed that XMRV might be associated with chronic fatigue syndrome (CFS) as well. The prevalence of XMRV in the general population was unknown; however, its putative prevalence was reported in specific disease populations [2]. Research on the epidemiology, risk, and pathogenesis of some prostate cancers supported the involvement of XMRV in association with allelic variants of the RNaseL locus [3]. These early reputed discoveries of XMRV were from prostate secretions as well as prostate tissues [1, 4–6]. XMRV infection was linked to high-grade prostate cancer with putative evidence for the presence of XMRV DNA and protein expression. Immunohistochemistry (IHC) showed XMRV

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proteins in malignant prostate epithelial cells but were not correlated with XMRV infection and RNaseL SNPs. It was proposed that there might be a more widespread population at risk for XMRV infection and prostate cancer [7]. In addition, in a serum-based assay to detect neutralizing antibodies to XMRV in prostate cancer patients, neutralizing antibodies against XMRV were found that correlated with PCR and fluorescence in situ virus detection in prostate tissue [8].

XMRV was also implicated in chronic fatigue syndrome (CFS). Many studies had searched for viral causes of CFS that had remained unconfirmed and the supposed linkage of CFS with XMRV took the field by surprise [9]. In the new studies, XMRV was putatively detected by culture from blood and blood component of patients with CFS and in their respiratory secretions [10, 11]. In addition, MLV-like *gag* sequences were detected in 86.5 % of PBMCs from CFS patients vs. 6.8 % from healthy volunteers [12]. These studies also suggested XMRV infections could be transmitted to permissive cell lines from CFS patient plasma [13]. However, additional studies failed to show any correlation of XMRV and CFS using quantitative PCR and neutralizing antibodies [14–17]. Indeed, many XMRV studies showed cumulative evidence for laboratory contamination as discussed later in this chapter [18]. In this regard, Table 21.1 summarizes literature that dealt with XMRV detection and Table 21.2 summarizes literature indicating that purported detection of XMRV was due to laboratory contamination.

Table 21.1 XMRV association with disease

Disease	XMRV associated	XMRV unassociated
Prostate cancer	Urisman (2006) [5] Dong (2007) [4] Kim (2008) [71] Fischer (2008) [32] Hong (2009) [6] Knouf (2009) [69] Arnold (2010) [8] Schlaberg (2009) [7] Bhosle (2010) [72]	Sfanos (2008) [73] D'Arcy (2008) [74] Hohn (2009) [36] Martinez-Fierro (2010) [75] Cornelissen (2010) [76] Verhaegh (2010) [77] Switzer (2011) [39] Sakuma (2011) [40] Khodabendehloo (2013) [43] Rezai (2013) [44]
Chronic fatigue syndrome	Lombardi (2009) [10]	Erlwein (2010) [47] Groom (2010) [17] Van Kuppeveld (2010) [49] Hong (2010) [78] Switzer (2010) [16] Henrich (2010) [48] Hohn (2011) [15] Shin (2011) [27] Satterfield (2011) [50] Schutzer (2011) [68]
ALS		McCormick (2008) [62]
Lymphoid malignancies		Waugh (2011) [63]
SLE		Balada (2011) [64]
Autistic disorders		Lintas (2011) [65]
Fibromyalgia		Luczkowiak (2011) [67]

Table 21.2 XMRV laboratory contamination

Study	Technique
Hue (2010) [36]	Taqman qPCR for XMRV *pol* sequences
Oakes (2010) [46]	Taqman qPCR for XMRV *pol* sequences
Robinson (2010) [41]	PCR assays for mitochondrial DNA (mtDNA) or intracisternal A particle (IAP) long terminal repeat DNA
Sato (2010) [15]	One-step RT-PCR kits amplifying the partial gag of XMRV or other MLV-related viruses(primer sets are 419F and 1154R, and GAG-I-F and GAG-I-R)
Sakuma (2011) [40]	Real-time PCR of XMRV gag sequences and nested PCR for XMRV/MLV gag sequences
Yang (2011) [37]	PCR with XMRV specific primers
Garson (2011) [42]	Nucleotide BLAST searches using each of the 14 integration site sequences against the GenBank nr database
Sfanos (2011) [79]	XMRV/MLV IHC; PCR using genomic GAPDH PCR primers; full-length or near full-length viral genomes prepared from the LAPC4, VCaP and EKVX cell lines; Vectorette PCR using a virus-specific forward primer and a vectorette-specific reverse primer; phylogenetic analysis and infectivity assay
Tuke (2011) [45]	Lo et al.'s modified XMRV gag TaqMan assay, using probe P2 and primers F3 and R4, which is able to co-detect the pMLVs [12]
Knox (2011) [52]	PCR and RT-PCR in specimens from CFS patients and those previously positive for XMRV
Katzourakis (2011) [80]	PCR using MLV-like sequences in longitudinal specimens from patients 15 years apart

2 General Virology

XMRV is a gamma-retrovirus with a positive single-stranded RNA genome, 95 % sequence similarity to endogenous MLV, and 93–94 % similarity to several exogenous murine viruses [2, 5, 19]. Using RNA from prostate tissues, an XMRV molecular clone was constructed that was replication-competent in a few PC cell lines, e.g. DU145 and LNCaP. The XMRV-long terminal repeat (LTR) was expressed as well [4, 20]. Purported XMRV infection of primary prostatic epithelial and stromal cells was enhanced by acid phosphatase semen-derived enhancer of virus infection (SEVI). In addition, XMRV RNA was detected in prostatic secretions of some men with PC. It was thus proposed that XMRV might be spreading in the human population [6]. However, many subsequent studies failed to detect XMRV and to verify these disease associations. Moreover, evidence of contamination of specimens, which will be discussed later in this chapter, led Stürzel et al. to study further. They generated replication competent XMRV reporter viruses encoding a green fluorescent protein or a secretable luciferase as tools to analyze virus infection of human cell lines or primary human cells. Transfection of proviral DNAs into LNCaP prostate cancer cells resulted in readily detectably reporter gene expression and production

of progeny virus. Inoculation of known XMRV susceptible target cells revealed that these virions were infectious and expressed the reporter gene, allowing for a fast and highly sensitive quantification of XMRV infection. This technique showed that both reporter viruses were capable of establishing a spreading infection in LNCaP and Raji B cells and could be easily transmitted. However, after inoculation of primary human blood cells such as CD4 T cells, macrophages or dendritic cells, infection rates were very low, and a spreading infection was never established. Moreover, XMRV-infected primary cells-derived supernatants did not contain infectious virus. This evidence suggested that even though XMRV can replicate in some human cell lines, all tested primary cells were largely refractory to XMRV infection and did not support viral spread. Therefore, these results indicated that XMRV is not a human pathogen [21]. The concern became uppermost, that there has been widespread laboratory viral and reagent contamination with murine retroviruses.

3 Antiretroviral Inhibitors

Antiretroviral therapeutic (ART) drugs marketed for treatment of HIV infection had activity against XMRV, in vitro. The retroviral integrase inhibitors, raltegravir and L-000870812, two nucleoside reverse transcriptase inhibitors, zidovudine (ZDV), and tenofovir disoproxil fumarate (TDF) inhibited XMRV replication. It was proposed that using these drugs in combination would delay or prevent the selection of drug-resistant viruses that occurs with HIV treatment [22, 23]. However, XMRV is highly resistant to nonnucleoside reverse transcriptase inhibitors [24]. Additional studies were done advocating the use of ARTs to inhibit the replication of XMRV in PC and CFS. This was, however, at a point in time when there was little information yet about contamination [25]. Nonetheless, APOBEC3 as well as ARTs inhibited XMRV in another study [26]. However, antiretroviral off-label treatment of CFS was proposed as unjustified [27]. This caveat definitely should be applied to possible ART treatments of any postulated XMRV-associated disease.

4 Molecular Biology

The PC 1 (HPC-1) gene is also known as RNaseL (2′,5′-oligoisoadenylate synthetase dependent) and is located at 1q24-q25 in the human genome [28, 29]. Patients with prostate cancer carrying a mutation in the HPC-1 gene locus had stromal cells surrounding prostate tumors that purportedly contained XMRV [30]. The RNaseL innate immunity pathway has been studied since its discovery in 1978 and shown to be a primary line of defense against viruses by cleaving viral RNA [31]. Some RNaseL SNP variants with lower activity were considered permissive for XMRV growth [1]. Specifically, the RNaseL variant SNP, R462Q is associated with prostate cancer and is a prostate cancer susceptibility factor. The homozygous state of

RNaseL allele R462Q (QQ) is underrepresented and XMRV was rarely detected in nonfamilial prostate in Northern European cancer patients. In those patients with the RNaseL allele R462Q (QQ) genotype, 40 % was XMRV positive. In familial prostate cancer patients, XMRV was detected rarely (less than 1 %) [32]. Coevolution of XMRV and RNaseL was hypothesized to result in the spread and pathogenesis due to XMRV [3]. Questions of laboratory viral and reagent contamination have underscored the more recent interpretation of the results. Be that as it may, many additional genes have been implicated in prostate cancer including the following genes (with abbreviations): CTBP2 (C-terminal binding protein 2, active gene expression in prostate tissue), MSMB (microseminoprotein, beta, produces a semen protein, decreased in prostate cancer), LMTK2 (lemur tyrosine kinase 2, cyclin-dependent kinase 5/p35-regulated kinase involved in spermatogenesis), KLK3 (kallikrein-related peptidase 3, prostatic specific antigen [PSA], serum protease, elevated in prostate cancer), JAZF1 (JAZF zinc finger1, normally transcription repressor), CPNE3 (copine 3, mediate membrane–cytoplasm interaction), IL-16 (interleukin-16, lymphocyte chemoattractant factor, anchors ion channels), CDH13 (cadherin 13 (truncated), calcium-dependent cell–cell adhesion glycoprotein), EHBP1 (EH domain-binding protein 1, Links clathrin-mediated endocytosis to the actin cytoskeleton), NUDT10 (nudix-type motif 10, nucleoside diphosphate linked moiety X, inositol phosphatase, signal transduction), and NUDT11 (nudix-type motif 11, nucleoside diphosphate linked moiety X, inositol phosphatase, signal transduction) [28].

5 Detection Methods

As described in the previous sections, detection of XMRV was done in several methods. In the research setting, XMRV was detected using nested and real-time PCR and immune assays included serologic assays, flow cytometry, Western blot, and enzyme-linked immunosorbent assay (ELISA). Initially, SNPs in the RNaseL were used during the initial discovery of XMRV-associated diseases by being identified as the hereditary prostate cancer 1 gene [1]. Further investigations of XMRV in prostate tissues, Dong et al. constructed a Full-Length, Replication-Competent XMRV Clone by using two overlapping partial cDNAs of XMRV strain VP62 and validated by the complete sequencing of the full-length XMRV VP62 (GenBank accession no. EF185282) [4]. Other methods in the early XMRV discoveries utilized microarray-based screening. This method was designed to screen for viruses from all known viral families. The amplified and labeled fragments contained amplified and labeled host and potential viral sequences, then hybridized to a DNA microarray (Virochip, University of California San Francisco, San Francisco, United States). The researchers recovered the entire XMRV genome from the tumor and further examined the association of the virus and the RNASEL genotype by using nested RT-PCR [5]. Moreover, quantitative PCR was used to amplify XMRV proviral DNA from formalin-fixed, paraffin-embedded tissues; and IHC using XMRV-specific antibody detecting XMRV in prostate tissues [7]. In one study, several methods were

used in different type of specimens. Genotyping of RNASEL variant using TaqMan genotyping assay (Applied Biosystems, Foster City, CA). Nested PCR analysis byAmpliTaq gold Kit (Applied Biosystems) is used for detection of XMRV in tissues; and Qiagen QIAquick gel Extraction Kit was used in sequencing DNA bands. Fluorescence in situ hybridization (FISH) assay, which was generated by excision of the full-length XMRV cDNA from the pXMRV plasmid1 using NotI and HindIII restriction enzymes (New England Biolabs, Ipswich, MA) has also been used [8]. Serum-based assay was used to detect neutralizing antibodies against XMRV proteins [7, 8]. Nested PCR or real-time PCR in blood samples or PBMCs were also used in early investigation of XMRV in correlation with CFS [10, 11]. The U.S. Food and Drug Administration (FDA) has not approved any of these methods for testing in the clinic. Moreover, donated blood is not screened for XMRV [33–35].

6 Evidence for Contamination

Many publications confirm continued lack of detection of XMRV. Contradictory and irreproducible results of recent research on the possibility that XMRV may be a human pathogen and a cause of prostate cancer and CFS support questions of contamination. The detection of laboratory reagent and tissue viral contamination is of central concern and vitiated the basis for the original findings related to XMRV. XMRV had been found in healthy controls and the XMRV-specific PCR primers later were found to amplify common murine endogenous viral sequences. Mouse DNA-contaminated patient specimens and nonspecific PCR reactions confounded XMRV detection. XMRV that was isolated from the tumor cell line 22Rv1 was similar to unlinked patient-derived XMRV. The *pol* sequences from these PC patients were possibly derived from XMRV and Maloney MLV. The original findings were made further questionable because the Maloney MLV envelope showed a lack of tropism for human cells [36]. Based on the analysis of the DNA from CWR22 and 22Rv1, the presence of XMRV in 22Rv1 was likely an artifact [37].

Many studies did not support the correlation between XMRV and prostate cancer. Studies in Germany showed a prevalence of 12.9 % for the homozygous SNP R462Q mutation in prostate tumor specimens but failed to show either antibodies for the XMRV gag and envelope proteins or XMRV-specific RNA or DNA in these tissues [38]. XMRV was rarely detected in nonfamilial prostate cancer specimens with homozygous mutation R462Q (QQ) [32]. In addition, detection of XMRV DNA by PCR in PC patient tissue revealed no correlation between XMRV serology and had very low (1.9 %) detectable XMRV DNA, undetectable mouse DNA, and was negative for viral RNA [39]. More recently, the lack of XMRV sequences and of strong anti-XMRV neutralizing antibodies indicated no or very low prevalence of XMRV in a cohort of 110 PC patients and 40 benign prostrate specimens. The prior positive real-time PCR results were due to laboratory and reagent contamination and positive IHC-specimens were due to nonspecific immune reactions [40].

Robinson et al. examined XMRV sequences in DNA purified from prostate cancer tissues. There were only 4.8 % positive for XMRV-like sequences whereas 21.5 % positive for XMRV-negative cases. These findings supported the interpretation that there were mouse DNA contaminants; in addition, intracisternal A particle (IAP) long terminal repeat DNA sequences were detected as well further supporting contamination [41].

Another study using BLAST searches for XMRV integration site sequences in prostatic tissues demonstrated that two of 14 integration sites were identical to sites that had been cloned in the same laboratory using the human prostate DU145 cell line that had been experimentally infected. Retrovirus infections had not previously exhibited identical integration sites. Therefore, it was suggested that PCR contamination had occurred and further weakened the view that XMRV was a human pathogen [42].

Another study of correlation between XMRV and prostate cancers was conducted in Iran. The investigator performed a case–control study with genomic DNA extracted from formalin-fixed and paraffin-embedded prostate tissues of 163 Iranian patients (63 prostate cancers and 100 benign prostate hyperplasias). They used a conventional and a nested PCR assay using primers targeting to an env specific sequence of XMRV. They did not detect XMRV in samples either from prostate cancers or benign prostate hyperplasias using XMRV specific primers [43].

A study failed to illustrate association between prostate cancer and XMRV in matched prostate and normal tissue from Australian patients. Purified genomic DNA (gDNA) matched from normal and cancer formalin-fixed paraffin-embedded (FFPE) prostate tissue from 35 Australian prostate cancer patients. RNase L polymorphism R462Q was determined by allele-specific PCR and contaminating mouse DNA was detected using qPCR targeting mouse intracisternal A particle long terminal repeat DNA. The gDNA was successfully purified from 94 % (66/70) of normal and cancer FFPE prostate tissues. RNase L typing revealed 8 % was homozygous (QQ), 60 % was heterozygous (RQ) and 32 % was wild type (RR) for the RNase L mutation. None of the 66 samples tested were positive for XMRV. The findings were consistent with other studies demonstrating that XMRV is a laboratory contaminant that has no role in the etiology of prostate cancer [44].

To support the evidence of contamination, cDNA from the whole blood of patients with CFS were tested using Invitrogen Platinum Taq (IPT) and Applied Biosystems Taq Gold LD (ABTG) with four gag sequences, followed by further sequencing by ABTG reamplification. Sequence comparisons showed similarity among these sequences, endogenous MLVs, and pMLV. Reagents were contaminated with pMLV sequences [45]. Furthermore, a study using Taqman qPCR failed to detect XMRV pol sequences in any of 112 peripheral blood specimens from CFS patients or 36 healthy controls. Moreover, there were specimens positive for XMRV DNA by a less sensitive PCR assay detecting a different portion of the XMRV genome, and were positive for highly abundant intracisternal A-type particle (IAP) long terminal repeat and murine mitochondrial cytochrome oxidase sequences. This study indicated extensive contamination of human specimens with murine sequences [46].

Several studies that had negative outcomes were originally conducted to demonstrate an XMRV association with disease. A large US study to elucidate the relationship between XMRV and CFS was done in blood specimens from 200 self-reported healthy volunteers and 100 CFS patients included patient specimens from the original study that had reported XMRV in CFS patients. XMRV and related MLVs viral sequences, virus growth, and antibodies to these viruses were not detected in any of the patient specimens, including those from the original study. The authors report that at least some of the discrepancies in previous studies were most likely due to the presence of trace amounts of mouse DNA in the Taq polymerase enzymes used in earlier studies. Virus growth in cell culture in prior studies was considered to be due to contamination as well [21]. The US studies on XMRV and CFS including a study in Kansas and Georgia using multiple molecular and serologic assays showed no evidence of XMRV infection [16]. Moreover, another study failed to show correlation of XMRV in various diseases such as CFS, HIV infection, rheumatoid arthritis and patients who received either organ or hematopoietic stem cell transplants using Lombardi et al. (PCR outer) primer set [10], Urisman et al. (PCR inner) primer set [5], and the Erlwein et al. primer set [14, 47, 48]. There were similar findings for a Dutch cohort between December 1991 and April 1992, using PCR targeting XMRV integrase and *gag* genes. XMRV sequences were not detected in specimens from any of the patients or controls. This study also demonstrated that it is possible to obtain and utilize uncontaminated reagents [49].

A study from 20 states in the US used blood from 45 CFS patients and 42 controls for both XMRV and MLV. Using the same CFS key clinical characteristics as in the Lombardi et al. study [10] highly sensitive and generic DNA and RNA PCR, as well as a new Western blot assay employing purified whole XMRV as antigen, there was no evidence of XMRV or MLV in the CFS patients or controls [50].

A Japanese study used one-step commercial RT-PCR kits, which detected XMRV gag sequences in CSF patient sera. The PCR primer sets were 419F, 1154R, GAG-I-R, and GAG-I-F. The sequences detected were compared with sequences of polytrophic endogenous MLV, XMRV, and endogenous MLV-related viruses derived from CFS patients. The result showed that the *gag*-related sequences were identical (99.4 %) and the *env*-related sequences were identical (99.6 %) to the polytrophic endogenous MLV. The kits were concluded to have been contaminated with MLV genome sequences [51].

Another study of CSF blood specimens from 43 of 61 patients that had previously been identified as XMRV-positive used PCR and reverse transcription-PCR to detect viral DNA and RNA. ELISA was used to detect virus-specific antibodies. There was no evidence of XMRV or other MLVs in any of these specimens. Further analysis in this study of commercial laboratory reagents detected MLV sequences. Thus, previous evidence linking XMRV and/or MLVs to CFS was likely due to laboratory specimen and/or reagent contamination [52].

There was no detection of XMRV DNA by PCR in PBMCs or RNA in plasma from discordant twins for CFS [53]. In addition, there was no detection of XMRV in HIV+ patients with immunosuppression [54]. Another serological studies in Japan found no association between XMRV infection of patients with prostate

cancer or with CFS [55]. Use of combined PCR and immunological techniques indicated and confirmed no role for XMRV in human disease [56].

XMRV-derived plasmids continue to be developed for in vitro and in vivo use for gene transfer. Thus, the danger of contamination is current and continues [57]. In vitro XMRV *env* vaccine studies in mice elicited immunity. Antibodies were detected by ELISA and by virus neutralization. However, immunity only lasted for 3 weeks [58]. Interestingly, Miyazawa pointed out that vaccines themselves are often produced using rodent (xenospecies) retroviral plasmid systems, and may transmit endogenous retroviruses (ERVs) to humans. This is an additional potential source of contamination [59]. In addition, the spectrum of tissue, cell, and nucleic acid preparations as well as patients potentially contaminated by murine viruses may be widening. A related caution is that since porcine tissue is often used for xeno-transplantation in humans, it was proposed that testing should be done for XMRV prior to transplantation. Retroviruses can recombine to produce new strains of virus, and porcine endogenous retroviruses (PERVs) are present in porcine tissues [60]. All these results point to caveats to guard against virus and nucleic acid potential contamination and escape from the laboratory, the dangers of their use in the clinic, as well as contamination of laboratories and reagents. Due to the evidence of contaminations, previously published literature were retracted by the authors including Lombardi et al. (Science 2009), Lo et al. (Science 2010), and Urisman et al. (PLoS One 2006) [61].

7 Clinical Studies

The involvement of XMRV in diseases in addition to prostate cancer and CFS has been investigated. These analyses did not demonstrate any association of XMRV with diseases including amyotrophic lateral sclerosis (ALS) [62], lymphoid malignancies [63], systemic lupus erythematosus (SLE) [64], and autism [65].

Since XMRV is closely related to murine leukemia viruses, the possible association to human lymphoid malignancies was analyzed. Waugh et al. studied DNA specimens from patients in the UK with lymphoid malignancies and benign lymphadenopathy quantitative PCR assays for XMRV. XMRV was not detected in any of the specimens [63]. However, XMRV had been suspected to circulate in the general population. Using TaqMan PCR in peripheral blood DNA, a London cohort of 540 HIV-1-positive patients was analyzed for the presence of XMRV and related viruses. There were no positive specimens in this patient cohort; it was concluded that XMRV or related viruses were not circulating at a detectable level in HIV-1-positive patients in London or in the general population [66]. Blood specimens from 95 SLE patients and 50 healthy controls were also analyzed by PCR. No XMRV was detected by PCR [64]. Moreover, using five sets of nested PCR primer of XMRV *gag* and *env* regions, with confirmation by using full-length molecular viral clone VP62, there was no evidence of MLV-related sequences in the specimens from 15 patients diagnosed with fibromyalgia [67].

In addition, an investigation of XMRV in cerebrospinal fluid did not find any correlation of CFS and XMRV or other common viruses including human adenoviruses, alpha-viruses, herpes viruses (HHV 1, 2, 3, 4, 5, 8), human parvovirus B19, dengue viruses 1, 2, 3, and 4, West Nile Virus, Japanese Encephalitis Virus, St. Louis Encephalitis Virus, enteroviruses A-D, and any coxsackieviruses [68].

In the controversial area of autistic disorder, correlation with XMRV or MLV-related viruses was studied using nested PCR targeted to gag DNA in specimens from blood, postmortem brain tissue, and semen. Once again, no XMRV gag DNA sequences were detected [65].

8 Conclusions

Initial research proposed a causal link for XMRV infection with prostate cancer and CFS. Issues addressed included the origin of the virus, its mode of transmission, its role in disease pathogenesis, and the possible use of chemotherapy and vaccines [1, 31]. However, subsequent work revealed that research laboratory and clinical laboratory contamination were central issues and the data no longer supported the initial claims. Nonetheless, it is important to note the issues raised by the initial findings and the careful and detailed laboratory follow-up. Contamination of tissue and reagents are key issues [69]. It is not the first time that rodents were putatively associated with a human disease. Indeed, rodents have been proposed as the cause of at least 35 human diseases. Many of these findings are now in question because of the potential for rodent contaminants in the reagents and tissues and this needs further follow-up [70]. The initial detection of XMRV and its putative association with human diseases was vitiated by later findings of contamination. This demonstrates that clean reagents and quality control are of crucial importance as well as proper design for controls.

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Chapter 22
Prion Diseases, HIV-1 Associated Neurocognitive Disorders, and Alzheimer's Disease: Implications for Protein Misfolding

Brian Giunta, Alireza Minagar, and Francisco Fernandez

Core Message This chapter discusses the topic of prion, tau, and Aβ proteins. All serve physiologic functions but become neurotoxic when aggregation and subsequent protein misfolding ensue. These three proteins all have the ability to aggregate into misfolded structures. In addition, they have the possibility to be used as a set of biomarkers for neurodegenerative clinical assessment.

1 Introduction

1.1 Classical Prion Diseases

Prion diseases, also known as transmissible spongiform encephalopathies (TSEs), are progressive neurodegenerative disorders that affect an array of mammals. In humans, they comprise Creutzfeldt–Jakob disease (CJD), variant Creutzfeldt–Jakob disease (vCJD), fatal familial insomnia (FFI), Kuru, Gerstmann–Sträussler–Scheinker disease (GSS), and variably protease-sensitive prionopathy (VPSPr).

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Natural TSEs in animals include scrapie seen in sheep and goats, bovine spongiform encephalopathy (BSE), and chronic wasting disease (CWD) of deer and elk [1].

Prion diseases are part of a group of disorders that are attributed to misfolding and ordered aggregation of proteins, which include Alzheimer's disease (AD), Parkinson's disease (PD), systemic amyloidosis, and some cases of human immunodeficiency virus (HIV) neurocognitive disorders (HAND). In classical prion disease, the cellular prion protein PrP^{C}, after partial misfolding, converts into a somewhat protease-resistant disease-associated isoform, PrP^{Sc} which aggregates in the brain and forms deposits which promote neurodegeneration [1].

Notable features of prion diseases among these disorders are their wide phenotypic presentations as well as the various etiologies (including sporadic, genetic, and acquired) and the transmissibility between individuals. Prions occur, like normal infectious agents, in various different strains known as natural isolates of infectious prions. They are each marked by unique clinical and neuropathological features, which are reproducible upon serial passage within the same host genotype. The different strains of prion are believed to be the primary cause of TSE phenotypic variation. Further, the host variability in the gene encoding PrP^{C} (*PRNP*), as defined by polymorphisms or mutations, also affects the disease phenotype [1].

Clinically, CJD is the most common prion disease of in humans and presents with subacute but progressive dementia, myoclonus, and electroencephalography (EEG) abnormalities. Neuropathologically, CJD manifests with loss of neurons, spongiform changes in the brain and development of astrocytosis. A variant form of CJD presents at younger ages with behavioral and psychiatric abnormalities early in its course, few EEG abnormalities, and typical magnetic resonance imaging (MRI) alterations characterized by the presence of hyperintense pulvinar.

1.2 HAND

Cognitive impairment occurs in 15–50 % of HIV infected patients with [2–4] HIV-associated dementia (HAD) presenting as the most severe form [5]. With the introduction of combined antiretroviral therapy (cART), the incidence of HAD has dramatically decreased. Recently, patients—both long-term infected and treated—including those with systemically well-controlled infection, started to present with milder memory problems and slowness, difficulties in concentration, planning, and multitasking; collectively termed HIV-associated neurocognitive disorders (HAND; [3]).

Although the pathophysiologic mechanisms underlying HAND are not fully elucidated, an abundance of clinical and laboratory investigations suggest that HIV proteins, advanced age, protein misfolding, and co-morbid neurodegenerative disease(s) may interact resulting in the clinical presentation of this disorder [6, 7]. This is concerning as there are some 60,000 HIV-infected individuals over the age of 50 and 10,000 over the age of 65. Furthermore, it has been predicted that 50 % of prevalent acquired immunodeficiency syndrome (AIDS) cases in the USA will fall into this older age group by the year 2015 [8].

As noted above, in prion disease the cellular prion protein, PrP^{C}, after partial misfolding converts into a somewhat protease-resistant disease-associated isoform,

PrP^{Sc}, which aggregates in the brain and forms deposits that are associated with the neurodegeneration. Although the phenomenon of PrP^{C} to PrP^{Sc} conversion does not occur in HIV infection, protein misfolding has been noted in several preclinical and clinical reports in the form of self-assembling misfolded tau and amyloid-beta (Aβ) proteins. Biomarkers as indicators for the progression of HAND remain elusive [9]. Aβ and/or tau, in addition to the normal prion protein, PrP^{C}, may be able to be used a biomarker of the disease which is elaborated upon in the rest of this chapter.

2 Aβ, AD, and HAND

Amyloid precursor protein (APP) "amyloidogenic" proteolysis by β- and γ-secretases yields β-amyloid (Aβ) peptides implicated in AD [10, 11] and to some degree, HAND [12–14]. In the "nonamyloidogenic" pathway, APP is cleaved at the α-secretase site, yielding soluble APP-α (sAPP-α); and precluding Aβ generation [15]. Aβ plaques are composed of a tangle of regularly ordered fibrillar aggregates called amyloid fibrils [16] a protein misfolding shared by other peptides such as prions. Several lines of epidemiological evidence signal a role for Aβ in HAND development while some studies have not yet fully implicated over production of the protein as a contributor to HAND. First, it is known that pathological similarities exist between HAND and neurodegenerative disorders such as AD [12–14] (Fig. 22.1). The former is more so

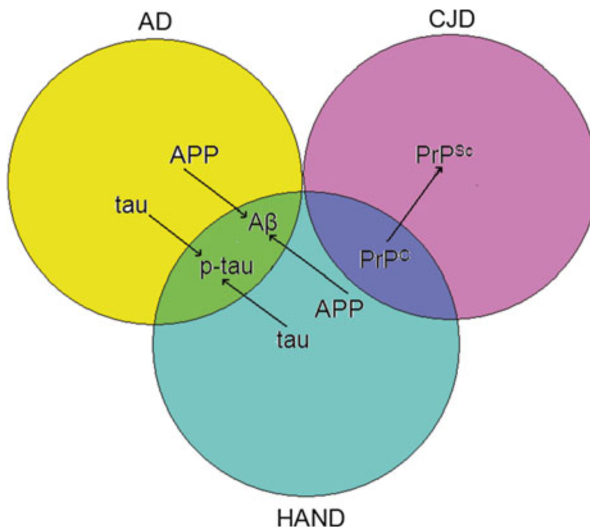

Fig. 22.1 Protein misfolding across three diseases: CJD, AD, and HAND. Protein misfolding has been implicated in the pathogenesis of several neurodegenerative diseases. Here, the overlap in misfolded proteins is illustrated. In regard to AD and HAND, there are reports of increased phospho-tau (p-tau) which predisposes to misfolded aggregates of p-tau (NFT). Moreover for both AD and HAND, epidemiological reports indicated increase in CNS Aβ. In regard to HAND and the protypical prion disease, CJD, a common link is PrP^{c}. The soluble form may serve as a biomarker for HAND, while the misfolded form (PrP^{Sc}) is the central disease causing agent of CJD

characterized by extracellular deposits of $A\beta_{1-42}$ in the form of plaques and aggregations of microtubule-associated tau yielding neurofibrillary tangles (NFT). In contrast, with HIV infection, the plaques are more diffuse [17] rather than neuritic [18].

Cerebrospinal fluid (CSF) biomarkers may mirror pathogenic cerebral amyloid deposition. Decreased CSF $A\beta_{1-42}$ and increased CSF tau can differentiate symptomatic AD individuals and cognitively normal individuals at high risk for symptomatic AD from cognitively normal individuals at low risk for symptomatic AD [19, 20]. In that regard, at least some HAND patients have CSF $A\beta_{1-42}$ values comparable to symptomatic AD individuals, that is, reduced [12, 21] (Fig. 22.1). This is salient because reductions in CSF $A\beta_{1-42}$ have been found in almost all individuals with increased fibrillar amyloid deposition within the brain as assessed with positron emission tomography (PET) amyloid binding of *N*-methyl-[11C]2-(4-methylaminophenyl)-6-hydroxybenzothiazole (^{11}C-PiB) [22–25]. Likewise, AIDS dementia complex (ADC) patients had significantly decreased CSF $A\beta_{1-42}$ and increased total and phospho (t-tau and p-tau respectively) concentrations similar to AD [12].

Achim and colleagues [14] reported increased Aβ by both autopsy examination and PET imaging of HIV patients' frontal cortex. Specifically, cases with HIV encephalitis (HIV-E) were about twice as likely to have amyloid detected (72 %) than HIV+ patients without HIV-E (38 %; [14]). In the same year Clifford and colleagues reported $A\beta_{1-42}$ measurements in CSF of cognitively impaired patients with HIV were similarly reduced as in patients with mild AD. Normal or slightly depressed CSF tau and phospho-tau measurements distinguished these patients with HAND from patients with AD [21].

Further analysis as to why low CSF $A\beta_{1-42}$ is observed is needed however there are several reasons which may explain altered Aβ metabolism in HIV disease. First, HIV-1 transactivator of transcription (Tat) protein may compete with APP and/or apolipoprotein E (an Aβ chaperone) for binding to the low density lipoprotein receptor related protein (LRP), thus inhibiting LRP mediated clearance of Aβ from brain interstitial fluid to periphery [26]. Second, APP cleavage products (sAPPα and sAPPβ) have been reported to be reduced in the CSF of patients with HAND versus those AD or HIV-negative controls, with sAPPα (a neurotrophic protein) showing a slight decline in the asymptomatic HIV state [27, 28].

In 2010 Ances and colleagues reported cognitively unimpaired HIV+ participants, even with low CSF $A\beta_{1-42}$ (<500 pg/mL), did not have (11)C-PiB parameters suggesting brain fibrillar amyloid deposition. This dissimilarity between cognitively unimpaired HIV+ and preclinical AD may reflect differences in $A\beta_{1-42}$ production and/or formation of diffuse plaques [29]. This same group, in 2012, reported symptomatic AD patients were significantly older, had significantly lower CSF $A\beta_{1-42}$, and had sign*i*ficantly higher CSF tau levels than other groups. Regardless of degree of impairment, HIV patients did not have increased ^{11}C-PiB [30]. Possible reasons for the absence of ^{11}C-PiB in HIV patients are: (1) decreased $A\beta_{1-42}$ production secondary to decreased synaptic activity; (2) increased intraneuronal $A\beta_{1-42}$ deposition yielding a reduction in overall extracellular concentrations making them

undetectable by ^{11}C-PiB [14]; and/or (3) increased $A\beta_{1-42}$ brain deposition but in a more diffuse, non-fibrillar form that is undetectable by ^{11}C-PiB [13, 31]. Future longitudinal examinations within older HIV+ participants are required to determine if diffuse, or oligomeric forms could with time subsequently become fibrillar (^{11}C-PiB positive) deposits [12, 21].

3 Tau and HAND

Tau protein is a highly soluble microtubule-associated protein (MAP) found mostly in neurons. One of tau's primary functions is to modulate the stability of axonal microtubules. Tau is active primarily in the distal portions of axons where it provides microtubule stabilization and/or flexibility as needed. Tau interacts with tubulin to stabilize microtubules and promote tubulin assembly into microtubules. Tau has dual mechanisms of modulating microtubule stability: isoforms and phosphorylation. Hyperphosphorylation of tau protein promotes self-assembly of tangles of neurotoxic paired helical filaments and straight filaments [32].

There have been several reports regarding tau pathology in HIV in addition to AD (Fig. 22.1). One of the first reports investigating the HIV infection on hyperphosphorylated tau deposition was reported in 2006 by Anthony and colleagues who examined HIV-infected subjects before and after the advent of cART. They reported elevated levels of hyperphosphorylated tau in the hippocampus of many HIV-infected subjects, compared with age-matched controls. Moreover, the greatest levels of hyperphosphorylated tau were noted in cART-treated subjects [31]. It is hypothesized by the authors that HIV infection and/or the use of cART therapy may predispose to accelerated aging of neurons in the form of hyperphosphorylated tau deposition in the hippocampus. Within the age groups studied it should be noted that the significant neuropathological changes remained subclinical and were not yet associated with cognitive impairment. Clifford and colleagues (2009) measured total and phospho-tau in 49 HAND subjects compared to 50 controls of similar age or 21 HIV+ subjects without cognitive impairment. Results indicated that HAND subjects had slightly lower CSF total and phospho-tau compared to both control HIV+ groups [21]. It has also been reported that in the frontal cortex of patients with HIV encephalitis (HIVE), increased levels of cylin-dependent kinase 5 (CDK5) and p35 expression were associated with abnormal tau phosphorylation. In addition, transgenic mice expressing the HIV protein gp120 exhibited increased brain levels of CDK5 and p35, alterations in tau phosphorylation, and dendritic degeneration [33]. Steinbrink and colleauges examined 94 patients (82 men and 12 women; mean age 45 ± 10 years) with HIV infection, but without opportunistic infections of the central nervous system (CNS) using MRI and CSF analysis. They reported cognitive impairment was significantly correlated with total tau increases, but not with phospho-tau [9]. Likewise Smith and colleagues found HIV positive subjects expressed more total tau protein than HIV negative controls (highest in a 58 year old), as did injection drug users, but brain viral loads showed no relation to tau and amyloid [34].

4 PrP^c and HAND

Although the phenomenon of PrP^C to PrP^{Sc} conversion does not occur in HIV infection, there is some evidence that PrPc could be used as a biomarker of the disease. As an adhesion and signaling molecule PrP^c plays a role in several processes including transmigration of leukocytes across the endothelium [35]. The transmigration is disrupted during HIV infection of the CNS [35–38]. It has been recently reported that CSF soluble (s)PrP^c was elevated in HAND patients as compared to uninfected individuals and HIV infected individuals without HAND [35, 37]. The significant increase of sPrP^c in individuals with HAND was independent of viral load or CD4$^+$T cells counts but correlated with increased CSF chemokine (C-C motif) ligand (CCL2). The authors concluded that increased sPrP^c was not due to immune suppression but was mediated by HIV infection and CCL2, showing the importance of this chemokine in PrP^c release (Fig. 22.1). Interestingly, elevated CCL2 level in the CSF of humans is an indicator of neurocognitive impairment [39]. Further, in pigtail macaques it is predictive of severity of encephalitis [40]. In vitro studies examining the effect of CCL2 on BMVEC (bovine brain microvascular cells), neurons, and astrocytes also showed that this chemokine induced increased PrP^c release between 30 min and 24 h [37]. Thus high CCL2 levels combined with HIV infection induced shedding of PrP^c in the CSF may be one of the pathogenic processes leading to HAND.

The finding of Roberts and colleagues indicated that astrocytes, BMVEC, and neurons are the main source of sPrP^c in HAND patients. In addition, HIV infected peripheral blood monocytes showed increased release of PrP^c followed by a sudden decrease 4 days post-infection which was maintained for up to 7 days [37]. This shed PrP^c may promote monocyte entry into the CNS by dysregulating the normal PrP^c–PrP^c interactions which control baseline transmigration for surveillance [41].

To study the release of PrP^c during the time course of HIV CNS pathogenesis, the pigtail macaque model of NeuroAIDS, in which 90 % of animals develop SIV encephalitis (SIVE) within 3 months of infection [42], was used. CSF samples from different stages of SIV infection indicated animals presenting with severe encephalitis had elevated sPrP^c levels during early and late stages of infection as compared to uninfected animals or those with mild SIVE. As these stages are characterized by elevated CCL2 in the CNS it is likely CCL2 and/HIV infection modulate sPrP^c secretion as noted earlier [41].

It has also been found that PrP^c was selectively increased in the brains HAND patients as compared to uninfected individuals and HIV infected patients without HAND. Neuronal and astrocytic PrP^c was significantly increased in patients with both minor motor cognitive disorders (MCMD) and HIVE [35, 37]. Astrocytes in patients with MCMD displayed hypertrophy, proliferation, and extensive process formation along with elevated PrP^c expression [35, 37].

5 Protein Misfolding as a Therapeutic Target

Currently there is no disease modifying or curative therapy for classical prion disease, AD, or HAND. What follows is a brief review of current potential therapies. For prion diseases in which PrP is misfolded into PrP^{S}, drugs from various molecular families (such as polyanionic, tetrapyrrolic, or tricyclic compounds, polyene antibiotics, tetracyclins, β-sheet breaker peptides, Congo red, and others) are reported to attenuate prion replication, but none are of practical for clinical translation secondary to efficacy, pharmacology, or toxicity problems [43–46]. Using PrP^{Sc}-based assays, screening of compound libraries for antiprion therapeutics is ongoing [47–52]. Most recently, Karapetyan and colleagues screened an array of pharmaceuticals approved for human use using a PrP–FRET-enabled high throughput assay (PrP–FEHTA) [43]. They found that astemizole and tacrolimus each lowered cell-surface PrP and inhibited prion replication in neuroblastoma cells. Specifically, tacrolimus reduced total cellular PrP by a nontranscriptional mechanism. Astemizole prolonged the survival time of prion-infected mice while tacrolimus did not. Astemizole is used in humans to treat chronic allergic rhinitis and also crosses the blood–brain barrier, making it a potential treatment for CJD patients and for prophylactic use in familial prion diseases [43].

For HAND and AD, the misfolding and aggregation of Tau and Aβ (Fig. 22.1) may be possible therapeutic targets. In regard to both, therapeutic strategies consist of mechanism-based disease-modifying therapies such as vitamin E, mechanism based therapies which attempt to compensate for neurotransmitter deficits, and psychotropics administered for treatment of behavioral symptoms including psychosis and depression. Several other agents have been used including ginko biloba, statins, and nonsteroidal anti-inflammatory drugs (NSAIDS). Efficacy has not been clearly shown for most of these treatments although binding of naproxen and ibuprofen to NFT's and plaques occurs in human tissue [53].

Five drugs have been approved by the FDA for treatment of AD symptoms. Galantime, rivastigmine, donepezil, and tacrine are cholinesterase inhibitors [54, 55]. Although reduced symptoms have been shown [56], benefits to cognition are unconfirmed. Tacrine is rarely prescribed due to serious side effects, including possible liver damage [55, 57]. All have been shown to modestly slow progression of cognitive symptoms and reduce problematic behaviors in a subset of patients but at least half who take these medications fail to respond to them [54, 57]. Side effects associated with acetylcholinesterase inhibitors include upset stomach, nausea, vomiting, diarrhea, headache, dizziness, changes in appetite, sleep disturbance, muscle weakness, and weight loss. These medications also may increase the risk of stomach irritation and ulcers. The *N*-methyl-D-aspartate (NMDA) receptor antagonist, memantine, is targeted against glutamate excitotoxic neuronal damage [54, 58] in moderate-to-severe cases of AD [54, 59]. Side effects of this medication include headache, dizziness, vomiting, cough, back pain, confusion, constipation, and sleepiness [60–62].

Gamma (γ)-secretase inhibitors modulate an enzyme involved in Aβ and tau production. Those tested clinically include LY450139 (phase II; [63, 64]) and

R-flurbiprofen (phase III; [65]). Early agents produced toxicity related to other γ-secretase substrates, especially Notch protein. Proteolytic processing of Notch by γ-secretase is an essential step after activation of the pathway. Consequently γ-secretase inhibitors (GSI) block Notch pathway activation [66, 67]. Newer agents are more specific inhibitors. LY450139, in normal human volunteers, given doses ranging from 5 to 50 mg/day over 14 days, decreased plasma Aβ concentrations up to 40 % in a dose-dependent manner. Unfortunately the critical CSF Aβ concentrations were unchanged. Furthermore at the 50 mg/day dose, adverse events that were possibly drug-related were noted. R-flurbiprofen is currently in phase III testing ($N = 2,400$). In a 1-year phase II study 207 subjects with mild-to-moderate AD receiving 400 mg twice daily, 800 mg twice daily, or placebo revealed no statistical significance in any memory measures. A subset of mild patients on the 800 mg twice daily dose, however, who developed high blood levels of the drug, demonstrated significant benefits in ADLs and overall function [65].

Due to side effects related to Notch, there is a newer emphasis on γ-secretase modulators (GSM) rather than pure inhibitors. Chronic treatments with two GSM's, ibuprofen and CHF5074, showed higher activity of CHF5074 in reducing brain plaque deposition and spatial memory deficits in transgenic mice expressing human APP with Swedish and London mutations (APP_{SL} mice). Further CHF5074 was found to be more effective than ibuprofen in reducing tau pathology [68].

6 Conclusion

Future randomized control trials will be necessary to determine if PrP^c, Aβ, phospho- and/or total tau may be used as reliable biomarkers for the progression of HAND and possibly other neurodegenerative disorders. Treatments which could ameliorate protein misfolding in the CNS which address multiple targets across multiple neurodegenerative disease models may have the highest potential to advance the clinical treatment of patients suffering such disorders as HAND classical prion disease, and AD and could be disease modifying.

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Chapter 23
Origin and Evolution of Human Immunodeficiency Viruses

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1 Origins

1.1 Primate Lentiviruses

Human immunodeficiency virus (HIV) is a retrovirus that is classified in the genus *Lentivirus*. Lentiviruses are uniquely distinguished from other retroviruses by having a long latency period between infection and the manifestation of symptoms, a characteristic that confers the genus its Latin etymology (*lentus* being the adjective for "slow") [1]. Moreover, lentiviruses are transmitted between hosts without the need for an intermediate vector, infect a broad range of mammalian hosts, and have a worldwide distribution. There are presently seven recognized major lentivirus lineages reflecting the known mammalian host range (lagomorph, equine, small ruminant, bovine, feline, prosimian, and simian [2, 3]). The virus genomes representing these lineages share a common genomic structure comprising three major genes (*gag*, *pol*, and *env*) and two regulatory genes (*tat* and *rev*); however, there are also a number of accessory genes that vary in number, type and relative location.

African nonhuman primate species are the natural hosts of simian immunodeficiency viruses (SIV), although SIV has also been isolated from Asian nonhuman primate species as well [7]. Lentiviruses have not yet been isolated in New World monkeys [8]. Over 40 SIVs have been characterized, each specific to a certain primate host [9]. In their respective natural hosts, infection by SIV generally does not lead to the depletion of CD4+ T-cell populations typically seen in HIV-1 infection

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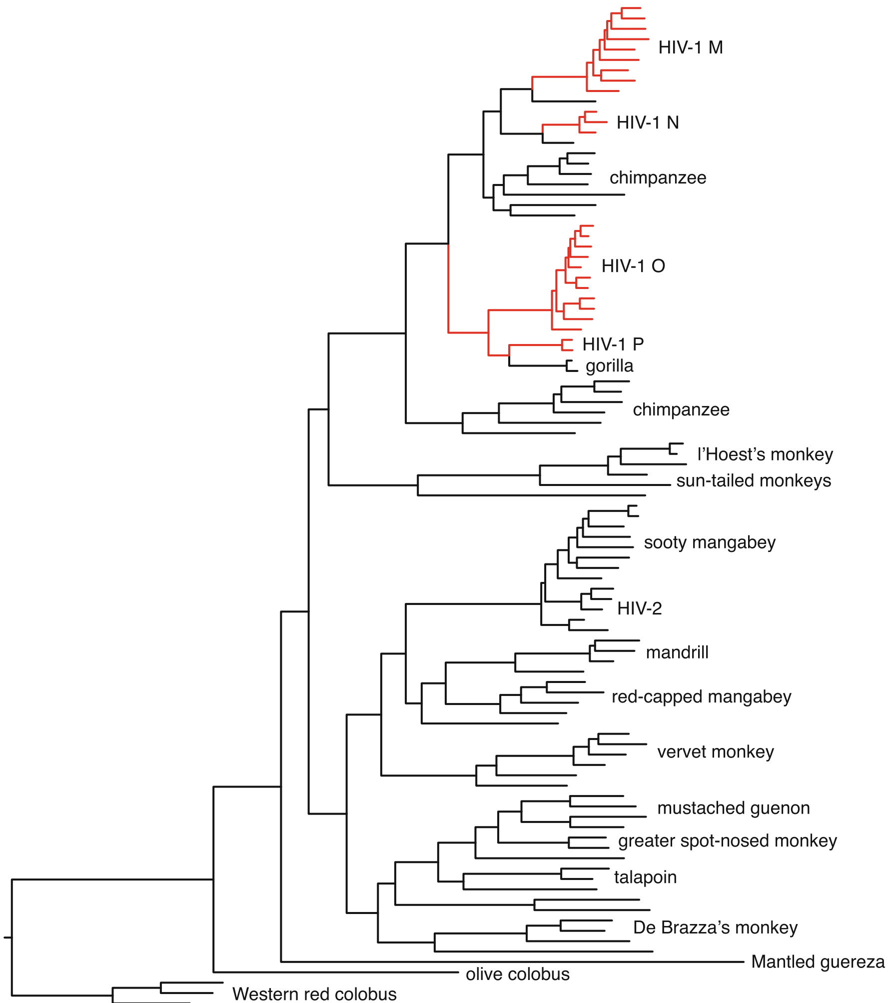

Fig. 23.1 A phylogeny reconstructed from SIV and HIV *gag* sequences. Sequences were obtained from the 2012 Sequence Compendium published by the Los Alamos National Laboratory HIV Sequence database (http://www.hiv.lanl.gov) and realigned using the program MUSCLE [4] under default settings. This alignment was used to reconstruct a phylogeny using the maximum likelihood heuristic implemented in RAxML [5]. The tree was rooted at the longest internal branch within the SIV clade representing the *Colobus* clade (western red colobus, olive colobus, and mantled guereza). For reference, the long terminal branch leading to SIV_{col} (mantled guereza) has a length of 1.14 expected nucleotide substitutions per site, consistent with previous work [6]

despite the proliferation of the virus to large numbers within the host (high viral load). However, the experimental infection of a nonhuman primate host by an SIV strain from a host belonging to a different species results in CD4 cell depletion similar to that seen among AIDS patients [10].

Viral and immunological mechanisms that prevent pathogenicity and vertical transmission in natural SIV hosts have illuminated potential targets for HIV treatment and vaccine development. The initial phases of infection unfold similarly in both natural and nonnatural hosts. The first few weeks of infection are marked by high levels of viral replication, followed by viral spread to peripheral lymphoid tissues and rapid depletion of mucosal CD4+ T-cells [11], notably in the gut-associated lymphoid tissue (GALT). A major determinant of HIV-1 disease progression is chronic immune activation. One source of chronic immune activation has been pinpointed to the evolutionary loss in HIV-1 and its precursor SIV strains of the role of the accessory gene nef in dampening the responsiveness of infected cells to further antigenic stimulation [12].

1.2 HIV Zoonoses

1.2.1 Emergence of the Virus

The global HIV pandemic originated from multiple transmissions of SIV from nonhuman primate into human hosts (Fig. 23.1). SIV found in chimpanzees (*Pan troglodytes*, SIZ_{cpz}) is the closest relative of HIV-1, and SIV circulating within sooty mangabeys (*Cercocebus atys atys*, SIV_{sm}) is the closest relative of HIV-2 [13, 14]. More than 11 zoonotic events have occurred between other primates and humans transmitting SIV and leading to the HIV/AIDS pandemic [15]. Three such transmissions involved SIV_{cpz} from common chimpanzees (*Pan troglodytes troglodytes*) generating HIV-1 groups M, N, and O [15][1], while eight other known transmissions of SIV_{sm} from sooty mangabeys produced HIV-2 groups A through H [15].

1.2.2 HIV-1

The earliest confirmed HIV-1 infection (ZR59) was identified from an archived blood plasma sample collected during 1959 in the city of Kinshasa in what was then the Belgian Congo (today the Democratic Republic of Congo) [16]. The second oldest known HIV-1 infection (DRC60) was sampled from a paraffin-embedded lymph node sample archived during 1960 also in Kinshasa [17]. ZR59 was a HIV-1 subtype D infection, whereas DRC60 was classified as HIV-1 subtype A. The considerable genetic distance between these earliest available HIV-1 sequences, sampled just months apart (ZR59 and DRC60) from the same region of Africa, clearly indicates that diversification of HIV-1 occurred decades before the sample

[1] At the production stage of this book chapter, a paper from D'arc et al. was published online in the Proceedings of the National Academy of Sciences (USA) presenting new evidence that HIV-1 group O was most likely introduced into the human population from gorillas (SIVgor) which in turn derived from SIV in chimpanzees (SIVcpz).

collection dates. Analysis of the ZR59 and DRC60 sequences with other historical and contemporaneous sequences revealed that HIV-1 group M was most likely transferred into humans from common chimpanzees (*Pan troglodytes*) in approximately 1908 (Bayesian skyline 95 % credible interval 1884–1924; [17]). Phylogeographic analyses have reconstructed the location of this transmission event to Northern Cameroon [18]. Using similar techniques, cross-species transmission of HIV-1 group N was estimated to have occurred in approximately 1963 (1948–1977) [15, 19] in South Central Cameroon [15]. HIV-1 group O is estimated to have crossed the species barrier in approximately 1920 (1890–1940). Intriguingly, a sister-group SIV lineage to HIV-1 group P was discovered in gorillas (*Gorilla gorilla*) suggesting a role for gorillas as intermediate hosts between chimpanzees and humans. However, the geographic location of this transmission event is unknown [15].

1.2.3 HIV-2

The earliest confirmed HIV-2 infection was discovered retrospectively from a sample collected in 1978 [20]. This infection was hypothesized to have occurred in Portuguese Guinea (today Guinea-Bissau) at some time between 1956 and 1966. HIV-2 subtypes are mainly restricted to western Africa and classified as epidemic (A and B) and nonepidemic subtypes (C–H) [21]. Molecular clock studies estimated that the cross-species transmission of the HIV-2 subtype A clade occurred in approximately 1940 (1924–1956) [15, 21]. Using similar techniques, the cross-species transmission of HIV-2 subtype B clade has been estimated to have occurred approximately in 1945 (1931–1959) [15, 21], and that both HIV-2 clade A and B cross-species transmission events occurred in Côte d'Ivoire [15]. The six nonepidemic HIV-2 lineages do not appear to be transmissible among humans [15, 22].

1.2.4 Controversy and Resolution

An erroneous hypothesis, the "oral polio vaccine (OPV)" hypothesis of the origin of HIV, was advanced in 1987 in Rolling Stone magazine by journalist Tom Curtis and later expounded upon by journalist Edward Hooper, igniting public controversy. In essence, the OPV hypothesis posited that the HIV pandemic began as a result of distribution of oral poliomyelitis vaccine contaminated with SIV from Kisangani in the Belgian Congo during the late 1950s and early 1960s. Evidence from phylogenetic and population genetic studies [15, 23, 24] incontrovertibly shows the OPV hypothesis to be wrong. In particular, the SIV variant infecting chimpanzees from the Kisangani region proved to be phylogenetically distinct from all HIV strains, which is clear evidence that chimpanzees from this region could not have been the origin of the HIV pandemic [23].

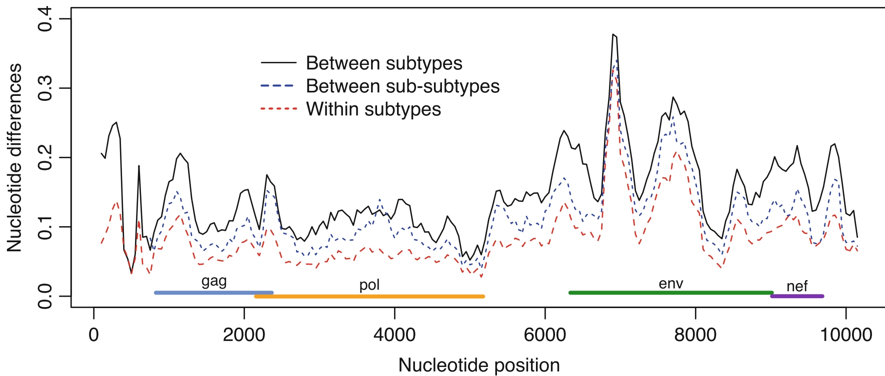

Fig. 23.2 Mean nucleotide differences at different levels of HIV diversity. Each *line* represents a moving average of the frequency of nucleotide differences between sequences in a window of 200 bases. Gaps due to insertions or deletions were omitted. The analysis was performed on 39 full-length HIV genome sequences comprising the current non-recombinant HIV-1 group M subtype reference set curated by the Los Alamos National Laboratory HIV Sequence Database (http://www.hiv.lanl.gov/). Note that our calculations do not adjust for multiple hits

1.3 Subtypes and Recombinant Forms

Molecular evidence (see Sect. 1.4) suggests that highly diverse strains of HIV-1 group M, which would eventually cause the global pandemic, were already circulating in human populations in central Africa well before the 1950s [17, 25]. The extensive diversity of the M group is structured into nine highly distinct subtypes, denoted as subtypes A–D, F–H, J, and K [26]. Viruses that were initially categorized as subtype E form a distinct group in molecular phylogenies[2] reconstructed from *env* sequences; however, the rest of the genome sequence closely resembles subtype A. Consequently, these viruses are treated as a recombinant of subtype A and a putative nonrecombinant subtype E lineage that has yet to be discovered. This A/E recombinant variant has expanded into its own regional epidemic in Southeast Asia and, as a result, was recognized as the first "circulating recombinant form" (CRF01AE).

Figure 23.2 illustrates the extent of nucleotide divergence within and between the HIV-1 subtype reference sequences curated by the Los Alamos National Laboratory HIV Sequence Database. The mean difference between within- and among-subtype divergence is about 6.3 % (about six expected nucleotide differences for every 100 bases) and is significantly greater in the genomic regions encoding *env* and *nef* (Wilcoxon test, $p = 1.4 \times 10^{-8}$). The difference of these quantities provides a better sense of the evolutionary divergence among subtypes. We also plotted the divergence between the "sub-subtypes": of A and F (denoted as A1 and A2, and F1 and F2, respectively)—clades within established subtypes that are not sufficiently diverged to be considered subtypes themselves.

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[2] A molecular phylogeny is a tree that represents how genetic sequences are related by common ancestors.

1.4 Timing the Ancestor—Molecular Clocks

Mutations in the HIV-1 genome are estimated to occur at a rate between 3.4×10^{-5} and 4×10^{-5} mutations per base, per generation [27, 28] (see Sect. 2.1). Generations are estimated to last between 1.2 and 2.6 days [29–31] (see Sect. 4.2). Assuming that most mutations are neutral or nearly neutral, the rate at which they accumulate in diverging lineages roughly corresponds to this mutation rate (the molecular clock [32]). Thus, we can estimate the clock rate to be somewhere between 1.3×10^{-5} and 3.3×10^{-5} mutations per base, per day, though in the less variable p17 region it may be as low as 7.4×10^{-6} mutations per base per day [33].

The dynamics of HIV evolution do not always agree with the assumption of a single rate of evolution across all branches of a phylogenetic tree. Within a host, events such as the initiation of the host immune response can cause a change in the number of accumulated substitutions in response to selection [34, 35]. Between hosts, varying levels of immune response can influence the substitution rate [35, 36]. However, it is also unreasonable to assume that the clock rate along each branch is totally independent, since the biological mechanisms of HIV mutation are the same regardless of the host environment. The relaxed molecular clock [37] was proposed to allow for such variation. Under this model, rates for different branches are drawn independently at random from a continuous probability distribution. In studies of HIV, this distribution has often been assumed to be lognormal [15, 17, 38–40], though analyses have also been done assuming exponentially [38] or normally [41] distributed rates.

Both strict and relaxed molecular clocks have been used to date the common ancestor to the modern HIV-1 group M pandemic to the 1930s [17, 25]. More generally, the relaxed clock has typically been better supported than the strict clock in studies of HIV [17, 42], which is unsurprising given that the strict clock does not allow for the empirically demonstrated variability in HIV's rate of evolution both within and among hosts. However, there are also caveats to the relaxed clock, namely that the improved fit may simply be a result of overparameterization, and that variance in the estimated time to most recent common ancestor may be large [43].

The relaxed clock, as described, is also known as the uncorrelated relaxed clock, since the rates along different branches are independent. A family of alternatives to this model is autocorrelated molecular clocks, in which rates are dependent on the rates of other, nearby branches [44, 45]. Though they have shown promise in application to other organisms [46], these models have not yet been extensively tested on HIV data.

2 Mechanisms of HIV Genetic Variation

2.1 Mutation

Although the term "mutation" can refer to any spontaneous genetic change, we use this term here to refer specifically to single nucleotide mismatch errors that can arise during replication. Like many retroviruses, the HIV genome undergoes a high rate

of mutation because the virus-encoded reverse transcriptase (RT), which converts the single-stranded virus RNA into double-stranded DNA, lacks the capability for proof-reading. Current estimates of the HIV mutation rate in cell culture range from 3.4×10^{-5} to 4×10^{-5} per base per replication cycle [27, 28]. Because the genome is about 9,700 base pairs in length (with substantial variation among viruses; e.g., ranging from 8,349 to 9,719 bases among the HIV reference genomes), these estimates suggest that about one of every three genome replications results in a new mutation. In addition, the HIV genome can be directly modified by the innate antiviral immune response mediated by a human-encoded enzyme (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G, or APOBEC3G) which deaminates cytidine to uracil in the negative strand of HIV DNA, thereby resulting in the conversion of guanine (G, the original complement of cytosine) to adenine (A, the complement of uracil) on the positive strand [47]. This G-to-A hypermutation, which is characteristic of lentiviruses infecting primates [48], has exerted sufficient selective pressure for the virus populations to have evolved a counterdefense in the form of an accessory gene (*vif*) encoding a protein that induces the degradation of APOBEC3G [49].

The impact of APOBEC-induced hypermutation for inhibiting the growth of HIV, particularly on *vif*-deficient variants [50], lends credence to the concept of using other mechanisms to artificially accelerate the mutation rate of HIV. The underlying premise is that the mutation rate of HIV is so high that any further increase would result in an unsustainable accumulation of deleterious mutations in the virus population (a model-predicted phenomenon known as an "error catastrophe") [51, 52]. An older example of this concept is ribavirin, a nucleoside analogue that has antiviral activity against a broad spectrum of RNA and DNA viruses, and has also been found to be a potent mutagen [53]. Other nucleoside analogues, such as 5-OH-dCTP [54] and KP-1212/1461 [55], induce hypermutation and a loss of replicative fitness of HIV in vitro. More recently, in a phase II clinical trial KP1212/1461 was reported to have the predicted effect on the genetic composition of virus populations within subjects, but without a significant impact on viral load relative to the control population [56, 57].

2.2 Insertion and Deletion

An insertion is the addition of nonhomologous nucleotides into a genome copy; conversely, a deletion is the loss or omission of nucleotides during replication. When we encounter two sequences of different lengths due to a stretch of nucleotides without a homolog in the other sequence, we can infer that either an insertion or deletion has occurred. Because it is not possible to discriminate between these two possibilities without knowing the ancestral sequence, the polymorphism is known as an "indel". The HIV genome undergoes extensive sequence insertions and deletions that tend to accumulate in regions of the genome that can tolerate length variation. For example, the HIV envelope glycoprotein contains several disulphide

loops that extend outward from the surface of the virus particle to interact with host cells and the extracellular environment [58]. These loops can vary substantially in length, and the incorporation of functional motifs, such as N-linked glycosylation sites, can play a significant role in mediating escape from the neutralizing antibody response [59]. Longer glycosylated loops block access to highly immunogenic neutralization epitopes on the surface of the virus envelope [34, 60].

There are several insertions and deletions in the HIV genome occurring at reproducible locations, which can also play an important role in the adaptation of the virus population. For example, insertions between codons 69 and 70 of HIV RT, in conjunction with an amino acid substitution at codon 69, are associated with resistance to all FDA-approved nucleoside RT inhibitors [61]. In addition, insertions near HIV Gag cleavage sites can compensate for mutations in HIV protease that confer resistance to multiple protease inhibitors at the cost of reduced enzymatic activity [62].

2.3 Recombination

Recombination is the exchange of genetic material between genomes. While it does not create genetic variation like mutation, recombination may restore genetic combinations that have been removed from the population through the action of selection and genetic drift [63]. The conversion of the single-stranded viral RNA genome into double-stranded DNA is not only a source of genetic variation through mutation, but it is also the mechanism of recombination. Each HIV particle carries two positive-sense RNA genomes that are compactly packaged as a dimer that is the substrate of the HIV reverse transcriptase. This dimerization is initiated by a structure within the 5′ untranslated region (UTR) of each genome copy that folds into a complex secondary structure, and is further mediated by the matured products of the cleaved HIV Gag and Gag-Pol polyproteins [64]. Recombination occurs when the HIV RT switches templates during reverse transcription of the dimer. In fact, two template switching events are requisite for replication of the 5′ and 3′ ends of the genome [65]. The rate of recombination appears to be restricted by successful dimerization of the HIV genomic strands; for instance, accumulation of mutations within the 5′ UTR between HIV subtypes accounts for a reduced rate of dimerization and consequently lower rates of recombination in vitro [66].

While HIV RT can undergo multiple template switches along the length of the genome, not all of these events result in recombination. For example, the exchange of genetic material between genomes that are identical on one side of the breakpoint does not create any new combinations of alleles. Notwithstanding the accumulation of mutations within a single lineage, recombination is limited by the probability that a cell becomes multiply infected by genetically distinct lineages of HIV. Thus, while the in vitro rate of recombination between single copies of HIV carrying markers in linkage disequilibrium has been estimated at about 3×10^{-4} per base per generation [67], the "effective" rate of recombination that accounts for the frequency of multiple-infected cells has been estimated to be over an order of

magnitude lower (1.4×10^{-5} per base per generation [68]). Even so, the rate of recombination is comparable to the considerable mutation rate of HIV, and one would expect about one in every seven replication events to produce a recombinant genome.

3 HIV Evolution Among Hosts

3.1 Variability of the HIV Genome

As the first genetic sequences of HIV began to accumulate in research centers around the world, investigators immediately became aware of the extensive sequence diversity of this virus [69]. The study of this variability drove the development of new techniques in the field of molecular evolution for analyzing rates of protein evolution. At the time, the established practice for quantifying these rates was by counting differences in protein-coding (codon) sequences that had diverged along related lineages. Mutations within a codon either result in a different amino acid upon translation (nonsynonymous) or leave the original amino acid intact (synonymous). The null hypothesis is that protein evolution is neutral, such that natural selection is “indifferent” to changes in the amino acid sequence. If there is no significant difference between the number of nonsynonymous and synonymous changes, then we would be unable to reject this null hypothesis [70].[3] In fact, nonsynonymous differences are generally observed less often than synonymous differences [72]. The overwhelming majority of nonsynonymous differences are culled by purifying selection; there is generally an evolutionary disadvantage to modifying a protein that is the product of a long history of adaptation to its environment. However, in some cases natural selection causes variants carrying a novel amino acid to proliferate. If a variant has the same selective advantage in all environments, or if the environment remains constant, then the elevated rate of nonsynonymous evolution will be a transient phenomenon as the favored variant takes over the population, at which point selection will resume a purifying mode of action. However, many genes are exposed to perpetually changing selective environments. For example, HIV and other pathogens are constantly moving between different host environments that exert different selective pressures on the virus. In these circumstances, elevated rates of nonsynonymous evolution can be sustained as different amino acids gain and lose selective advantages in different host environments, causing an accumulation of nonsynonymous variation among diverging lineages over time (diversifying selection). (For a more comprehensive review of interpreting signatures of natural selection from molecular variation, see [73].)

[3] Note that we do not assume that synonymous mutations evolve neutrally in an absolute sense; they are only being used as a frame of reference to measure the evolutionary consequences of changes in the amino acid sequence. Without a doubt, selection operates on the HIV genome at the level of the nucleotide sequence. For example, the HIV RNA genome folds into a complex and functionally significant secondary structure due to Watson-Crick base pairing interactions [71].

Applying these methods to HIV sequence data has revealed substantial variation in the nonsynonymous rates of evolution among its protein-coding genes. For example, HIV *env* gene sequences encoding the virus envelope glycoproteins are substantially more variable than the other major genes, *gag* and *pol* [74, 75]. Diversifying selection plays an important role in driving high rates of nonsynonymous substitutions in HIV *env* because the envelope glycoproteins are exposed on the surface of the virus particle and present an important target for the immune system. To illustrate these patterns with modern data, we carried out an analysis of selection for all full-length HIV genome sequences that have been published since 2010 ($n = 95$), excluding sequences from HIV subtypes N and O. These data included a wide variety of subtypes, including A1, B, C, D and F1, and circulating recombinant forms CRF01-AE, CRF35-AD, and CRF53, isolated from individuals in China [76–81], Gabon [82], Malaysia [83], Iran [84], Russia [85], Cameroon [86], the United States [87], Singapore [88], and France [89], reflecting a renewed focus on characterizing the genomic variation of HIV on a global scope. For each of seven reading frames in the HIV genome (*gag*, *pol*, *env*, *vif*, *vpr*, *vpu*, and *nef*), we removed regions with high levels of insertion/deletion (indel) polymorphisms. Furthermore, we removed regions with overlapping reading frames in which a mutation that is synonymous in one reading frame may be nonsynonymous in another, making these sites difficult to analyze (but see [90]).

We quantified patterns of selection using a fast Bayesian approach [91] that estimates the difference between mean posterior rates of nonsynonymous and synonymous mutations, denoted by the symbols β and α, respectively.[4] Where $\beta > \alpha$, there is evidence of diversifying selection driving the accumulation of different amino acids at that site. Conversely, $\beta < \alpha$ indicates purifying selection preventing any amino acids other than the current residue from accumulating in the population. As expected, we found that selection was predominantly in a purifying mode, acting against the accumulation of codon substitutions that would result in amino acid replacements (Fig. 23.3). Overall, purifying selection was strongest in the HIV *pol* gene. What was surprising, however, was that the median value of $\beta - \alpha$ for the *env* gene (−0.27) was not substantially greater than the other genes with the exception of *pol*. Moreover, the *env* gene as a whole would not be considered to be under significant diversifying selection because its mean value of $\beta - \alpha$ is well below zero.

This result highlights one of the major limitations of characterizing selection at the level of genes—it is unreasonable to expect the majority of codons to be uniformly exposed to the same magnitude of diversifying selection, given that they assume a variety of structural and functional roles in the protein [93]. For instance, the surface glycoprotein encoded by HIV *env* (gp120) comprises both conserved and

[4] In the original frequentist framework, the nonsynonymous and synonymous rates are usually denoted by d_N and d_S, respectively. By convention, selection is measured by the ratio $\omega = d_N/d_S$, such that the null hypothesis of neutral protein evolution is represented by $\omega = 1$. One drawback of using this ratio is that it becomes numerically unstable when the number of synonymous substitutions is low. More generally, the ratio of two random variables has problematic statistical properties which can lead to severe biases under some conditions [92].

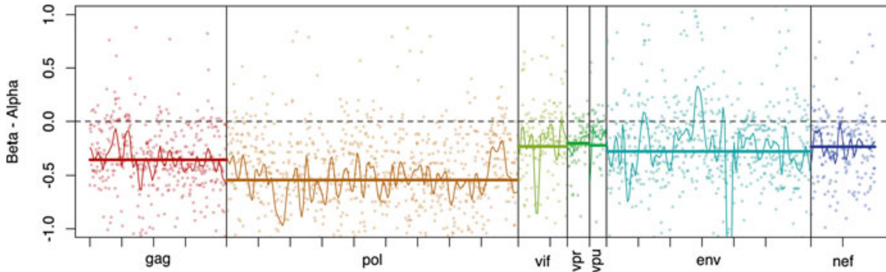

Fig. 23.3 Patterns of selection across protein-coding regions of the HIV genome. Selection is quantified by the difference between the non-synonymous (β) and synonymous (α) rates of codon substitutions. These estimates were generated using a fast, unconstrained Bayesian algorithm for inferring selection [91]. For each gene, the median $\beta-\alpha$ value is indicated by a *bold line* segment, and a *curved line* represents a smoothed spline of the moving average over ten codons in steps of 5. A *dashed line* at $\beta=\alpha$ indicates the expected value for a neutrally-evolving codon

"hypervariable" regions, the latter originally defined by having fewer than 25 % of residues conserved in a comparison of seven HIV isolates [94]. These regions roughly correspond to buried residues and surface-exposed loops of the gp120 tertiary structure, respectively. The hypervariable regions are referred to as V1 through V5; the third region, V3, is a key genetic determinant of the tropism and immunological phenotypes of the virus. In 1998, Nielsen and Yang published a landmark paper [95] in which they extended comparative methods for quantifying diversifying and purifying selection on amino acid sequences, allowing one to directly measure the heterogeneity of selection pressures among codons. In their paper, they applied their method to analyze partial HIV *env* sequences from a single patient to find evidence of significant heterogeneity in the strength of diversifying selection among sites.

Our analysis of HIV genome sequences employs a modern descendant of Nielsen and Yang's method [91]. As shown in Fig. 23.3, we found evidence of extensive heterogeneity in the strength and mode of selection among codons within protein-coding regions of the HIV genome, including contiguous sets of codons in the *env* gene under diversifying selection. These codons correspond roughly to the hypervariable regions V1 through V5 that play important roles in allowing the virus to escape neutralizing antibodies (see Sect. 3.2). Furthermore, there is also a conspicuous dip in the moving average of $\beta-\alpha$ near the middle of *env* that indicates strong purifying selection. This interval corresponds to a highly conserved motif encoding the residues FNCGGEFF (Env amino acids 376–383) [96] and is a potential target of the cellular immune response [97] (see Sect. 3.2).

3.2 Adaptation to the Immune Response

Variation in rates of evolution across the HIV genome is largely shaped by the host-specific adaptive immune response [98]. The immune response mounted by each host exerts a different selective pressure on HIV genomic variation; thus, HIV

lineages are exposed to varying host environments as they spread. Both the humoral and cellular arms of the immune system play important roles in shaping the evolution of HIV within the host. The cellular response against an HIV infection is mediated by the lysis of infected host cells by CD8+ cytotoxic T lymphocytes (CTLs). Peptides that originate from HIV-encoded proteins are bound in a specific manner by human leukocyte antigen molecules (which are encoded by some of the most variable loci in the human genome) and presented on the surface of the infected cell to stimulate a CTL response. CTLs against antigenic HIV-derived peptides (epitopes) are detectable about a month into infection, in association with the decline of the virus population following peak viremia [99].

The humoral response against viruses in the extracellular environment is mediated by neutralizing antibodies that bind onto the exposed surface of the virus particle in a way that prevents the particle from proceeding through the infection cycle. Neutralizing antibodies tend to appear later in the course of an HIV infection than CTLs, although they can be detected as early as 2 months postinfection [34]. As they are the most exposed component of the virus particle outside of the cell, the HIV envelope glycoproteins are the major targets of the humoral response. For example, the third variable loop of HIV gp120 was quickly identified as an important target of the neutralizing antibody response [100].

The great challenge of HIV evolution is that these components of the adaptive immune response tend to be specific to the transmitted variant and its descendants. For example, a host can eventually develop a vigorous neutralizing antibody response that is highly effective at inhibiting that host's own virus population (although the rapid evolution of HIV enables the virus to stay one step ahead of this response [101]). These antibodies, however, are seldom able to inhibit HIV populations from another host (cross-neutralization). Nevertheless, a highly active area of research in HIV is seeking out and characterizing broadly neutralizing monoclonal antibodies that may confer protection against a large number of HIV variants and subtypes [102, 103].

4 HIV Evolution Within Hosts

4.1 The Transmission Bottleneck

In the case of HIV, the virus population within a host is often descended from a single variant (founder virus) among a small number of viruses that were transmitted from the previous host. This results in a transient, drastic reduction in population size that is referred to as a "transmission bottleneck". In order to establish an infection in a new host, HIV must overcome the natural defenses of the host. Intestinal and genital surfaces of the human body, where exposure to HIV is most likely to result in a productive infection, tend to be protected by a mucosal barrier to infection. Mucosal surfaces harbor large persistent numbers of activated lymphocytes and immunoglobulin A antibodies. These defenses have a strongly limiting effect

on the number of virus particles that manage to establish productive infections within host cells. However, when viruses are introduced directly into the blood stream, as in the case for transmission from a contaminated blood product (transfusion) or injection drug use, then the viruses bypass this mucosal barrier. When this occurs, it is likely that the virus population experiences less of a transmission bottleneck.

Preliminary evidence of the HIV transmission bottleneck stemmed from the relative lack of genetic sequence variation in acute infections, e.g., [104]. As sequencing technologies became more sophisticated (see Sect. 4.3), investigators have been able to characterize levels of genetic variation at early stages of infection in greater detail. For example, Keele et al. [105] fit a Poisson model of sequence diversification [106] to next-generation sequence data sets derived from early samples from 102 subjects infected by HIV subtype B to estimate that roughly 75 % of the infections descended from single founder variants. Among $n=20$ male subjects in this study who reported having sex with men, the prevalence of having a single founder virus was relatively greater (50 %) but this difference was not statistically significant. The remainder of the study population reported as heterosexual with only one subject reporting the use of injection drugs. Similar studies of populations with a greater prevalence of injection drug use (IDU) have reported that IDU subjects were more likely to harbor infections descended from multiple variants [107].

The transmission bottleneck has important consequences for the evolution of HIV within hosts. For instance, the virus population must reaccumulate genetic variation that becomes shaped by the host environment, including the host-specific immune response. The prevalence of severe transmission bottlenecks also suggests that there may be a nonarbitrary subset of HIV genetic variants that tend to establish infections. In other words, transmission may select for genotypes with specific characteristics. For example, there is some evidence that transmitted genotypes tend to encode shorter variable loops in *Env*, although this pattern appears to be largely confined to HIV subtype C [108]. Such characteristics, if they generally exist, may present important targets for the development of anti-HIV vaccines, which must be the most effective on the transmitted variants that establish infections.

4.2 Population Genetics of HIV

Even within a single host, HIV has displayed one of the fastest rates of adaptation ever measured in the natural world [36]. The high mutation rate and high turnover of HIV populations makes this virus a ready subject for population genetic analysis. While most population genetic research concerns organisms that accumulate genetic diversity much more slowly than is observable during a typical study (e.g., over the course of centuries), an HIV infection creates a population where change in genetic diversity can be easily observed over the course of months, weeks, or even days.

If one wishes to directly simulate the natural history of a population evolving under, say, a Wright–Fisher model [109, 110] (or, more generally, an exchangeable

Cannings model [111]) or a Moran model [112], one has to keep track of the state of every individual of the population; one may also need to track each individual's state during all time steps. Suppose that we want to track the history of a population over a number of generations. In a Wright–Fisher model, a generation corresponds to a single time step. Thus, simulating the natural history of this population requires computational resources (time and memory) that scale with both the number of generations and the size of the population. The Moran model has overlapping generations, so many time steps are required for each generation. Consequently, the computational resources required may be greater orders of magnitude. This quickly becomes computationally expensive. Because the memory required balloons so quickly, it can become outright prohibitive.

Fortunately, many population analyses can be performed in terms of the *ancestral process* of a sample from the population. This process describes the *backwards-in-time* history of a small sample of the population. If a small sample is drawn from the population in the present day, the ancestral process describes the genealogy, or "family tree", of the individuals in the sample.

Kingman's *coalescent* [113, 114] provides a valuable means for modeling the ancestral process ([115] is a good reference on the subject). It is widely applicable, being an appropriate model for the genealogies of samples from a large population under a Wright–Fisher model (or a Cannings model under certain restrictions), as well as the Moran model. The primary advantage to using a coalescent model is computational tractability. The computational resources used to simulate a coalescent history for a small sample from a large population scale with the size of the sample rather than the size of the population, and do not scale with the number of generations.

4.2.1 Effective Population Size

Key to any population genetics analysis is the concept of *effective population size*, often denoted N_e. The classical definition of N_e is the number of individuals in the population that are responsible for the progeny comprising the next generation. Consider a very large population, of fixed size 1,000,000, where only 1,000 of them actually reproduce in each generation; in this case, $N_e = 1{,}000$.

In practice, N_e is used to calibrate a theoretical model so that it appropriately represents the real-world data one wishes to study. Kouyos [116] provides a good description of how N_e is chosen, which we recapitulate here. One has data from a given real population; from these data, an estimate of a *quantity of interest* is desired. In order to make such an estimate, a mathematical population model must be used; such models are necessarily idealized in some way or another to make them tractable. This mathematical population model has population size as a parameter. In order to better relate this idealized model to the data at hand, another *calibration quantity* that is easily measured for the real population (such as pairwise genetic diversity) is obtained: the plan now is to choose N_e so that if the idealized theoretical population has size N_e, the same quantity for the theoretical population is equal to

the real. Now that the model has been adjusted to (hopefully) better reflect the real population, we calculate the quantity of interest for the model population and use that as an estimate of the real quantity of interest.

The idealized model is typically a stochastic model, such as the aforementioned Wright–Fisher model. Such models allow for the effects of mutation, some more specialized ones allow for selective effects, and crucially they all allow for *drift*, or changes to the genetic makeup of a population from stochastic variation in transmission between generations. If the effective population size is very large, these random effects can "wash out" via averaging so that when viewed with the appropriate scaling the population evolves *deterministically*: selectively favored alleles appear immediately and proceed to fixation in a well-determined manner described by ordinary differential equations. This averaging does not occur sufficiently when N_e is small, however, and in such a setting, random effects are too significant to be ignored.

Debate over the value of N_e for HIV started toward the end of the 1990s and persisted through the start of the twenty-first century. Several authors [117–122] argued for an effective population size on the order of 1,000 (the stochastic evolution regime), while Rouzine and Coffin [123] found evidence for an effective population size one or two orders of magnitude larger (in the deterministic evolution regime). Kouyos [116] argued that different values for N_e are likely necessary for different analyses, as different features of the virus may be evolving under different processes: the *env* gene, for example, is a target of immune response and therefore likely experiences selective pressure, whereas other portions of the viral genome may be evolving neutrally. Thus [116] stresses the importance of choosing a calibration quantity that is a result of the same evolutionary process as the quantity of interest, and of choosing a model which properly captures this evolutionary process.

4.2.2 Generation Time

One component of HIV's high turnover rate is its short generation time. The serial coalescent model, discussed below, was used in [31] to estimate the generation time of HIV-1 in vivo at 1.2 days. Previously, Coffin [29] estimated that HIV underwent 300 replication cycles per year, and Perelson et al. [30] estimated a viral generation time of 2.6 days using a deterministic model of viral reproduction.

Using the second of these estimates, it is argued in [29] that, under a simple dynamical model using estimates for the mutation rate derived from other retroviruses, an established HIV infection likely develops every possible single-point mutation between 10^4 and 10^5 times per day.

4.2.3 The Serial Coalescent

Kingman's coalescent assumes a sample drawn in the present day and models its ancestry. This makes it a reasonable choice for analyses where the individual viruses in the sample are all drawn at the same time, such as the viruses in a single sample

of blood plasma. This is not the case for the study of HIV within the host, in which sequence data from infected individuals are typically generated from samples soon after diagnosis, as well as at follow-up appointments. Combine this with the fast evolution of HIV—significant genetic diversity can develop in the time between samples, meaning that it is inappropriate to simply consider all samples as contemporaneous—and it becomes evident that the original coalescent is not appropriate for the analysis of HIV sequence data.

This is a difficulty, but it also suggests potential for deeper analyses. One limitation of the coalescent is that it is impossible, using genetic data from a single time point, to tease apart the effects of time and mutation rate. Genetic diversity in a population may have accrued quickly due to a high mutation rate, slowly due to a low mutation rate, or somewhere in the middle. The coalescent cannot differentiate between these alternative explanations, as it models time in units of *generations* rather than real time units such as years, months, or days. Thus external information must be used in order to estimate the actual time scale that a coalescent tree exists on. However, if one has serial samples, there is now a means to calibrate the timing in our model based on the times between samples and the amount of diversity developed between them.

Rodrigo and Felsenstein [119] developed the *serial coalescent* for this setting. The serial coalescent no longer assumes that all of the individuals in the sample are sampled at the same time. Moreover, it offers the ability to use the serial sample data to estimate the lengths in the tree in real time units, and therefore also the mutation rate per unit of real time rather than per generation. This therefore lends itself well to the application of models incorporating a molecular clock.

Both Kingman's coalescent and the serial coalescent are implemented in the software package BEAST (Bayesian Evolutionary Analysis by Sampling Trees [124]). The Kingman coalescent is the typical prior used for data sampled at a single time point; the serial coalescent is the typical prior for a tree generated from sample data where the samples come from two or more time points. BEAST also allows the inference of parameters of molecular clock models in the latter case.

4.3 Next-Generation Sequencing

Next-generation sequencing has been a boon to HIV clinical analysis. In a matter of days, the viral population in a patient sample can be prepared into a library and sequenced at high coverage. Targeting specific portions of the genome for sequencing with primers yields ultradeep coverage at thousands of reads per locus [125]. This ultradeep coverage is ideal for phylogenetic reconstruction, detecting even minor variants and increasing phylogenetic signal [126, 127]. The high-throughput nature of next-generation sequencing demands automated bioinformatic tools for efficient analysis. Since all sequencing technologies introduce sequence errors, the start of every phylogenetic reconstruction pipeline starts with read cleaning. Once reads have been cleaned and aligned, they can be fed into other programs to

measure the prevalence of specific mutations [125, 128], reconstruct the evolution of the virus within a host [127, 129], or to estimate dates of infection [130].

There are several algorithms for reconstructing a phylogeny and timing the most recent common ancestor. The most popular software use maximum likelihood or Bayesian inference. In a phylogenetic tree generated from sequence data, each sequence forms a leaf. The internal nodes of the tree correspond to ancestral sequences that must be inferred from the data. Each sequence character substitution between branches is assigned a probability. Maximum likelihood selects the tree with the highest likelihood. Each tree likelihood is calculated as the product of the probabilities of the substitutions in each of its branches [131]. Bayesian inference samples trees at random in proportion to their posterior probability given the data and a prior distribution. Typically, the posterior distribution is approximated using some form of Markov Chain Monte Carlo that samples trees by a random walk over the set of all possible trees [132].

The molecular clock previously described in Sect. 1.4 calculates the amount of time passed in each tree branch. Since the majority of infections originate from a single founder virus, the timing of the most recent common ancestor can approximate the date of infection [133]. When longitudinally sampled sequence data are available, for which sampling dates are known, the molecular clock can be calibrated with greater precision using the known time between nodes [124].

The Roche 454 next-generation sequencing platform was the first such platform to be widely adopted for HIV research due to its longer read lengths (over 500 bp in practice) [134]. However, this platform and others based on similar chemistries have been plagued by high rates of error, particularly within single nucleotide repeats that are common in the A-rich HIV genome sequence [135]. This high error rate is especially problematic for HIV because it is exceedingly difficult to differentiate sequencing errors from the extensive genetic variation of a virus population. Presently, the Illumina Miseq is rapidly overtaking the Roche 454 platform in the field of HIV research, owing to its substantially lower error rates [136] and competitive read lengths of up to 300 bp, which can be paired to span greater lengths. Once mature, "third-generation sequencing" has the potential to widespread adoption in the clinical arena. Third-generation sequencing refers to single molecule sequencing techniques that can produce extremely long reads up to 15 kbp [137] that would completely encompass a full HIV genome. Reconstruction of full-length HIV genomic variants [138] and examination of fitness effects of multiple loci acting simultaneously are some examples of analysis that may be enabled through third-generation sequencing. Comparisons against next-generation and third-generation sequencing have found that all technologies are capable of identifying variants within a population and forming the same conclusions in receptor tropism genotypic assays [139]. For example, Pacific Bioscience Single Molecule Real Time (SMRT) reads have already been used to characterize the diversity of splice variants in HIV [140]. In addition to the long reads that third-generation sequencing provides, no PCR amplification is required and it uses smaller sample amounts of DNA. Unfortunately, the longest reads have error rates of around 15 % [137]. Regardless of the specific technology, high-throughput sequencing is rapidly expanding the frontiers of studying the evolution of HIV within hosts.

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Chapter 24
Global Protein Sequence Variation in HIV-1-B Isolates Derived from Human Blood and Brain

Seetharaman Balaji, Patil Sneha, Murugappan Rama, and Paul Shapshak

Core Message This chapter discusses the proteins encoded by HIV-1 and their sequence variation in blood and brain isolates. Sequence analysis based on their phylogeny is also addressed. The analysis of HIV-1 sequence variation not only helps in tracking the epidemic spread of HIV-1 but also to understand its transmission, pathogenicity, antiretroviral therapy, or development of novel vaccine.

1 Introduction

Human Immunodeficiency Virus (HIV) is the subject of extensive research since the discovery of the virus in 1983 [1, 2], after the first reported cases of acquired immunodeficiency syndrome (AIDS) in 1981 [3, 4]. Since its recognition, more than 60 million people have been infected with HIV around the world, and approximately 25 million people have died of AIDS. Currently, more than 33.3 million (range: 31.4–35.3 million), worldwide, are living with HIV [5–7]. In the mid-1980s,

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it was evident that two types of HIV with slightly different genome structures, HIV-1 and HIV-2 were circulating in human populations. Both viruses are characterized by extensive genetic diversity. However, HIV type 1 (HIV-1) is predominant. HIV-1 infection not only spreads to various lymphoid organs [8] but also to the central nervous system, CNS [9, 10] and causes a slowly progressive dementia in at least 30–60 % of AIDS patients [11–13]. The blood–brain barrier that protects the brain from infectious diseases and harmful chemicals from entering and damaging the brain is weakened by HIV-1. This marks the brain as an important reservoir for HIV-1, possibly an "immunological sanctuary" though the genital tract is a primary source of new HIV infection transmission [14].

There are numerous research articles that address early penetration of HIV into the brain, compartmentalization, evolution, molecular diversity, and functional consequences [15–18] including HIV-associated dementia (HAD). The progression of such diseases associated with HIV-1 infection may also due to increase in viral sequence diversity. HIV-1 infections become highly compartmentalized in the patient, with each lymphoid compartment seeded by a distinct viral lineage [19]. For instance, HIV-1 in the CNS was recognized to be distinct from the circulating virus in the blood of the same patient [20]. HIV-1 may reside in several cell types besides macrophages. As a result, diverse genotypes or quasispecies are produced at all stages of the disease. The molecular determinants for these differences are unknown.

Retroviruses have similar genomic organization; however, the structure of HIV-1 is different from other retroviruses. HIV-1 is roughly spherical and approximately 120 nm in diameter. It is a complex retrovirus composed of two strands of RNA encoding at least 15 distinct proteins [21]. In HIV-1, the major genes encoding structural proteins are found in the other retroviruses as well. Nevertheless non-structural ("accessory") genes are unique to HIV-1 and are enveloped by a lipid bilayer. The proteins in the viral machinery play an important role in its life cycle, from the viral attachment to the final process of virus budding [21, 22]. Gag polyprotein contains structural proteins including Matrix (MA), capsid (CA) and nucleocapsid (NC) generates the virion's internal structure. Envelope (Env) glycoprotein contains gp160 (the precursor to gp120 and gp41), gp120 is located at the surface (SU), and gp41 is found in the transmembrane (TM). They are embedded in the viral envelope and are involved in the infection process. The polypeptide glycosaminoglycan-polymerase (Gag-Pol) contains the Gag encoded proteins as well as functional proteins (enzymes), i.e., protease (PR), reverse transcriptase (RT), and integrase (IN). Besides these, regulatory proteins such as regulator of virion (Rev) and trans-activator of transcription (Tat); and other accessory proteins including negative regulatory factor (Nef), virion infectivity factor (Vif), viral protein r (Vpr), and viral protein u (Vpu) are also present. The Gag gene provides the basic physical structure of the virus, and pol provides the basic mechanism by which retroviruses reproduce their RNA via a DNA intermediate [23], while the other proteins are involved in the infection process as well as multiplication. The role of these proteins is listed in Table 24.1.

Table 24.1 The role of structural and functional proteins in HIV-1 life cycle

Viral protein classes	Role	Protein name
Structural proteins	Entry	Env polyprotein
		gp120 surface
		gp41 transmembrane
Enzymatic proteins	Reverse transcription	Pol polyprotein
		p51 reverse transcriptase
		p15 RNase H
		p66 RT+ RNase H
		Vif protein (p23)
Regulatory proteins	Transcription	Rev protein (p19)
		Tat protein (p16/p14)
Accessory proteins	Integration	Vpr protein (p12/p10)
		p31 Integrase
	Maturation	p10 Protease
	Assembly	Gag polyprotein
		p17 matrix
		p24 capsid
		p2
		p7 nucleocapsid
		p1
		p6 Vpr binding
		Nef protein (p27/p25)
		Vpu protein (p16)

2 Structural Proteins

Extensive research on the structural biology of HIV for more than 25 years has revealed the atomic details of the HIV proteins, which are publicly available in the Protein Data Bank (PDB) [24] some of them are depicted in Fig. 24.1. Using these structural data, researchers have rationally designed inhibitors and also trying to develop vaccines against HIV [25]. Protein sequences of HIV-1 B isolates of blood and brain belonging to different geographical locations were retrieved between 20th July, 2013 and 6th Jan, 2014 from NCBI (http://www.ncbi.nlm.nih.gov/) and LANL (http://www.hiv.lanl.gov/) public resources. The total number of retrieved HIV-1 B sequences derived from blood and brain are showed in Tables 24.2 and 24.3, their corresponding data (geographical regions and accession numbers) can be found in Tables 24.4 and 24.5. From these, representative sequences were selected to reflect geographical diversity and were analyzed for their sequence conservation, conserved and semi-conserved substitutions, as well as their variation using ClustalW (http://www.clustal.org/clustal2/) and Weblogo (http://weblogo.berkeley.edu/logo.cgi).

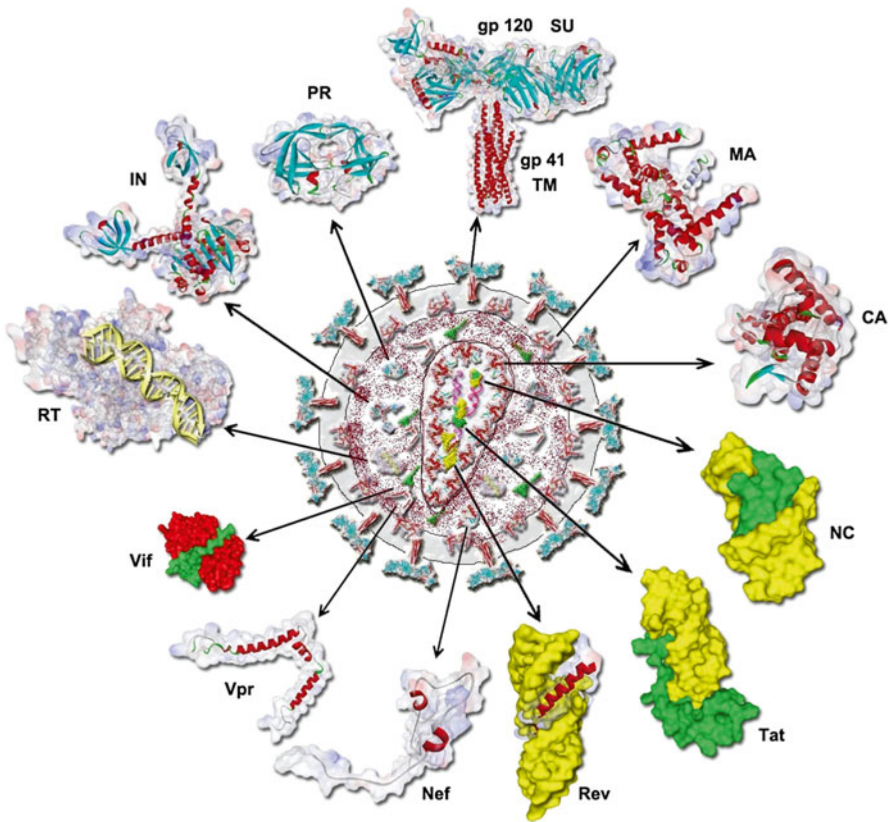

Fig. 24.1 The structural proteins of HIV, adapted from educational resources of PDB (poster: URL http://www.pdb.org/pdb/education_discussion/educational_resources/)

Table 24.2 Protein sequences of HIV-1B brain isolates

	Protein	Brain	Frontal lobe	Basal ganglia	Parietal region of the brain	Occipital region of the brain	Caudate region	Deep white matter	Menin-geal tissue	Total
1	Env	85	89	04	–	07	04	05	18	212
2	Gag	–	01	–	–	–	–	–	–	01
3	Nef	42	35	–	04	04	–	–	–	85
4	Pol	–	01	–	–	–	–	–	–	01
5	Rev	09	25	04	–	–	–	–	–	38
6	Tat	09	25	03			–	–	–	37
7	Vif	–	01	–	–	–	–	–	–	01
8	Vpu	12	23	04	–	–	–	–	–	39
9	Vpr	–	01	–	–	–	–	–	–	01

Table 24.3 Protein sequences of HIV-1B blood isolates

	Protein	Blood	PBMC	Plasma	Total
1	Env	126	331	388	845
2	Gag	52	132	298	482
3	Nef	42	271	232	545
4	Pol	34	140	167	341
5	Rev	30	134	141	305
6	Tat	47	127	136	310
7	Vif	56	176	265	497
8	Vpu	46	135	223	404
9	Vpr	48	191	232	471

2.1 Gag Proteins

The Gag polyprotein is the precursor (p55) to the internal structural protein. During translation, the N-terminus of p55 is myristoylated [26] activating its association with cell membranes. Gag proteins are organized with domains that may be cleaved into one or more small proteins or peptides. The cleavage pattern is represented as X and Y in the following pattern MA-X-CA-NC-Y [27]. The virus encoded protease (a product of the pol gene) cleave [28] the Gag polyprotein into four smaller proteins designated MA (matrix, p17), CA (capsid, p24), NC (nucleocapsid, p7), and p6 during the process of viral maturation [29].

2.1.1 Matrix Protein (MA)

The N-terminal domain of Gag upon myristoylation (at the end of p55) gives rise to membrane- or matrix-associated (MA) protein, p17 [30]. Myristoylation is essential for retroviral assembly. The consensus sequence for myristoylation is M-G-X--X.S/T. Mutation of glycine block the budding of virions, which in turn accumulate Gag inside the cell [28]. MA proteins assemble into trimers and remain attached to the inner surface of the viral membrane. This is vital for new viruses to bud from the surface of infected cells. During budding MA may also interact with the Env proteins. The three-dimensional crystal structure of HIV-1 MA protein is available in PDB; entry 1hiw [31]. In the analyzed sequences, the start of p17, membrane binding region and nuclear localization are annotated. The amino acid residues followed by the phosphorylation site of p17 shows more variation towards the end of p17 in brain isolate. The same trend is observed in p2 and also in Vpr binding residues of p6 of blood derived HIV-1 sequences. These variations could not be confirmed presently due to lack of brain derived sequences.

Table 24.4 HIV-1 B protein sequences of brain

Name	Subtype	Location	Source	Accession numbers
GP120-41	B	USA	Frontal lobe	AAB05604.1
	B	USA	Brain	ABB03894.1, ABB03893.1, ABB03890.1, ABB03889.1, ABB03892.1, ABB03891.1
	B	USA	Caudate region of brain	AAF75506.1, AAF75505.1, AAF75504.1, AAF75503.1
	B	USA	Deep white matter of brain	AAF75502.1, AAF75501.1, AAF75500.1, AAF75499.1, AAF75498.1
	B	UK	Frontal lobe	ADH93666.1
	B	USA	Uncultured brain tissue from mid frontal gyrus	ABT17547.1, ABT17546.1, ABT17545.1, ABT17544.1, ABT17540.1, ABT17539.1, ABT17538.1, ABT17537.1, ABT17536.1, ABT17535.1
	B	USA	Uncultured meningeal tissue from brain	ABT17491.1, ABT17490.1, ABT17489.1, ABT17488.1, ABT17487.1, ABT17486.1, ABT17479.1, ABT17478.1, ABT17477.1, ABT17476.1, ABT17475.1, ABT17474.1, ABT17473.1, ABT17472.1, ABT17471.1, ABT17470.1, ABT17469.1, ABT17468.1
	B	USA	Brain tissue of a neurological AIDS patient	AAA44221.1
	B	UK	Frontal lobe	ABK33518.1, ABK33517.1, ABK33516.1, ABK33515.1, ABK33514.1, ABK33513.1, ABK33512.1, ABK33511.1, ABK33500.1, ABK33499.1, ABK33498.1, ABK33497.1, ABK33496.1, ABK33495.1, ABK33494.1
	B	UK	Basal ganglia	ABK33503.1, ABK33504.1, ABK33502.1, ABK33501.1 ABK33471.1, ABK33470.1, ABK33468.1, ABK33467.1, ABK33466.1, ABK33469.1
	B	USA	Occipital lobe	AER22574.1, AER22573.1, AER22572.1, AER22571.1, AER22570.1, AER22569.1, AER22568.1
	B	USA	Frontal lobe	AER22567.1, AER22566.1, AER22565.1, AER22564.1, AER22563.1, AER22562.1, AER22561.1, AER22560.1, AER22559.1, AER22558.1
	B	USA	Frontal lobe	AER22502.1, AER22501.1, AER22500.1, AER22499.1, AER22498.1, AER22497.1, AER22496.1, AER22495.1, AER22494.1, AER22493.1, AER22492.1, AER22491.1, AER22490.1, AER22489.1, AER22488.1, AER22487.1

	B	USA	Frontal lobe	AER22468.1, AER22467.1, AER22466.1, AER22465.1, AER22464.1, AER22463.1, AER22462.1, AER22461.1, AER22460.1, AER22459.1, AER22458.1, AER22457.1, AER22456.1, AER22455.1, AER22454.1
	B	USA	Frontal lobe	AER22425.1, AER22424.1, AER22423.1, AER22422.1
	B	UK	Brain	AEH41916.1, AEH41914.1, AEH41912.1, AEH41905.1, AEH41904.1, AEH41903.1, AEH41900.1, AEH41898.1, AEH41892.1, AEH41888.1, AEH41885.1, AEH41882.1, AEH41893.1, AEH41898.1
	B	USA	Brain	AEH41887.1, AEH41884.1, AEH41883.1, AEH41895.1, AEH41906.1, AEH41890.1, AEH41907.1, AEH41909.1, AEH41910.1, AEH41911.1, AEH41913.1, AEH41915.1
	B	Australia	Brain	AEH41851.1, AEH41850.1, AEH41849.1, AEH41848.1, AEH41847.1, AEH41846.1
	B	USA	Brain	AAA44331.1, AAN39738.1, AAN39737.1, AAN39735.1, AAN39732.1, AAN39730.1, AAN39729.1, AAN39723.1, AAN39721.1, AAN39720.1, AAN39719.1
	B	USA	Brain	ACN58204.1, ACN58203.1, ACN58202.1, ACN58201.1, ACN58200.1, ACN58199.1, ACF75471.1, ACF75470.1, ACF75469.1
	B	USA	Brain	AAM09790.1, AAM09791.1, AAM09792.1
	B	UK	Brain	AAM09793.1, AAM09794.1, AAM09795.1
	B	USA	Frontal lobe	ADU15852.1
	B	UK	Brain	AEB21943.1, AEB21944.1, AEB21945.1, AEB21946.1, AEB21947.1, AEB21948.1, AEB21949.1, AEB21950.1, AEB21951.1, AEB21952.1, AEB21953.1, AEB21954.1, AEB21955.1, AEB21956.1, AEB21957.1, AEB21958.1, AEB21959.1
Nef	B	USA	Brain	UniProtKB: P12479.3
	B	Canada	Brain	AAU90015.1, AAU90014.1, AAU90013.1, AAU90012.1, AAU90011.1, AAU90010.1, AAU90009.1, AAU90008.1, AAU90007.1, AAU90006.1, AAU90005.1,
	B	USA	Brain	ADV02750.1, ADV02735.1, ADV02733.1, ADV02732.1, ADV02713.1, ADV02709.1, ADV02700.1, ADV02685.1, ADV02684.1, ADV02683.1, ADV02680.1, ADV02674.1,
	B	UK	Brain	ADV02749.1, ADV02738.1, ADV02724.1, ADV02723.1, ADV02722.1, ADV02721.1, ADV02712.1, ADV02696.1, ADV02695.1, ADV02679.1, ADV02673.1
	B	Australia	Brain	ADV02748.1, ADV02734.1, ADV02715.1, ADV02705.1, ADV02686.1
	B	USA	Autopsied frontal lobe	ACZ27767.1

(continued)

Table 24.4 (continued)

Name	Subtype	Location	Source	Accession numbers
	B	USA	Frontal lobe from AIDS patient	ABI50763.1, ABI50762.1, ABI50761.1, ABI50760.1, ABI50759.1, ABI50758.1, ABI50757.1, ABI50756.1, ABI50755.1, ABI50754.1, ABI50753.1, ABI50752.1, ABI50751.1, ABI50750.1, ABI50749.1, ABI50748.1, ABI50747.1, ABI50746.1, ABI50745.1, ABI50744.1, ABI50743.1, ABI50742.1, ABI50741.1, ABI50740.1, ABI50739.1, ABI50738.1, ABI50737.1, ABI50736.1, ABI50735.1, ABI50734.1
	B	Australia	Derived from right parietal region of the brain	AAC56429.1, AAC56428.1, AAC56427.1, AAC56426.1
	B	Australia	Derived from left occipital region of the brain	AAC56423.1, AAC56424.1, AAC56425.1, AAC56422.1
	B	Australia	Right frontal region of the brain	AAC56421.1, AAC56420.1,
	B	Australia	Left frontal region of the brain	AAC56419.1, AAC56418.1
	B	USA	Brain	AAA44222.1
Rev	B	USA	Frontal lobe	AAB05603.1
	B	UK	Frontal lobe	ABL10724.1, ABL10723.1, ABL10709.1, ABL10708.1, ABL10707.1, ABL10706.1, ABL10705.1, ABL10704.1, ABL10703.1
	B	UK	Basal ganglia	ABL10713.1, ABL10712.1, ABL10711.1, ABL10710.1
	B	USA	Frontal lobe	ABL10696.1, ABL10695.1, ABL10694.1, ABL10693.1, ABL10692.1, ABL10691.1, ABL10690.1, ABL10680.1, ABL10679.1, ABL10678.1, ABL10677.1, ABL10676.1, ABL10675.1, ABL10674.1, ABL10673.1

Tat	B	USA	Frontal lobe	AAB05602.1
	B	UK	Frontal lobe	ABL10669.1, ABL10668.1, ABL10667.1, ABL10666.1, ABL10665.1, ABL10664.1, ABL10663.1, ABL10662.1, ABL10661.1, ABL10660.1, ABL10648.1, ABL10647.1, ABL10646.1, ABL10645.1, ABL10644.1, ABL10643.1
	B	UK	Basal ganglia	ABL10651.1, ABL10650.1, ABL10649.1
	B	USA	Frontal lobe	ABL10627.1, ABL10626.1, ABL10625.1, ABL10624.1, ABL10623.1, ABL10622.1, ABL10621.1, ABL10620.1
Vpr	B	USA	Frontal lobe	AAB05601.1
Vif	B	USA	Frontal lobe	AAB05600.1
Vpu	B	USA	Brain isolate	AAA44220.1
	B	USA	Brain isolate	UniProtKB: P12516.1
	B	UK	Brain-derived virus isolated from PBMC	ABL10619.1, ABL10618.1, ABL10617.1, ABL10613.1, ABL10612.1, ABL10612.1, ABL10611.1
	B	USA	Brain-derived virus isolated from PBMC	ABL10582.1, ABL10581.1, ABL10580.1
	B	UK	Frontal lobe	ABL10616.1, ABL10615.1, ABL10614.1, ABL10600.1, ABL10599.1, ABL10598.1, ABL10597.1
	B	UK	Basal ganglia	ABL10604.1, ABL10603.1, ABL10602.1, ABL10601.1
	B	USA	Frontal lobe	ABL10590.1, ABL10589.1, ABL10588.1, ABL10587.1, ABL10586.1, ABL10585.1, ABL10584.1, ABL10583.1, ABL10573.1, ABL10572.1, ABL10571.1, ABL10570.1, ABL10569.1, ABL10568.1, ABL10567.1, ABL10566.1
Gag	B	USA	Frontal lobe	AAB05598.1
Pol partial cds	B	USA	Frontal lobe	AAB05599.1
Gag-pol fusion polyprotein precursor	B	USA	Brain	CAI64014.1, CAI64013.1, CAI64012.1

Table 24.5 HIV-1 B protein sequences of blood

Name	Subtype	Location	Source	Accession numbers
Env	HIV 1-B	Netherlands	Peripheral blood mononuclear cells (PBMC)	AAA64870.1, AAX86720.1, AAX86729.1, AAX86738.1, ABA06604.1, ACE76311.1, ACE76318.1, ACE76326.1, ACE76336.1, ACE76345.1, ACE76356.1, ACE76366.1, ACE76383.1, ACE76402.1, ACE76445.1, AAC54640.1, ADD12330.1, ADD12335.1, ADD12388.1, ADD12400.1, ABA06602.1, ABA06603.1
	B	Trinidad and Tobago	PBMC	AAW64254.1, AAW64255.1
	B	Trinidad and Tobago	Human peripheral blood lymphocytes	AAA75182.1
	B	UK	Peripheral blood mononuclear cells	CAD10950.1, CAD10949.1, CAD10894.1, CAD10948.1, CAD10947.1, CAD10946.1, CAD10945.1, CAD10944.1, CAD10943.1, CAD10942.1, CAD10941.1, CAD10940.1, CAD10939.1, CAD10938.1, CAD10937.1, CAD10936.1, CAD10935.1, CAD10934.1, CAD10933.1, CAD10932.1, CAD10931.1, CAD10930.1, CAD10929.1, CAD10928.1, CAD10927.1, CAD10926.1, CAD10925.1, CAD10924.1, CAD10923.1, CAD10922.1, CAD10921.1, CAD10920.1, CAD10919.1, CAD10918.1, CAD10917.1, CAD10916.1, CAD10915.1, CAD10914.1, CAD10913.1, CAD10912.1, CAD10911.1, CAD10910.1, CAD10909.1, CAD10908.1, CAD10907.1, CAD10906.1, CAD10905.1, CAD10904.1, CAD10903.1, CAD10902.1, CAD10901.1, CAD10900.1, CAD10899.1, CAD10898.1, CAD10897.1, CAD10896.1, CAD10895.1, CAD10893.1, CAD10892.1, CAD10891.1, CAD10890.1, CAD10889.1, CAD10888.1
	B	Myanmar	Peripheral blood mononuclear cell	BAC77450.1, BAC77466.1, BAC77493.1
	B	China	PBMC	AAN83918.1, ABV72401.1, ABV72405.1, ABV72410.1, ABV72417.1, ACZ57355.1, ADE20148.1, ADE20157.1, ADI62504.1, ADI62512.1, ADI62626.1, AAW88562.1, AAW88564.1, AAW88566.1, AAX45239.1, AAX45241.1, ABK42019.1, AEF33730.1, AEF33760.1, AAC05236.1, ABL63504.1, AAY57434.1, AAY57425.1, AAY57416.1, AFN62012.1
Env	B	UK	Peripheral blood of infected patients T-cells (CD3+)	CAD59665.1, CAD59666.1, CAD59662.1, CAD59661.1, CAD59658.1, CAD59657.1, CAD59654.1, CAD59653.1, CAD59652.1, CAD59651.1, CAD59646.1, CAD59645.1, CAD59644.1, CAD59643.1, CAD59642.1

Env	B	UK	Peripheral blood of infected patients dendritic cells	CAD59664.1, CAD59663.1, CAD59660.1, CAD59659.1, CAD59656.1, CAD59655.1, CAD59650.1, CAD59649.1, CAD59648.1, CAD59647.1, CAD59641.1, CAD59640.1, CAD59639.1, CAD59638.1, CAD59637.1
Env	B	UK	PBMC	AEB22035.1, AEB22037.1, AEB22045.1, AEB22042.1
Env	B	France	PBMC	ABK32878.1, ABK32877.1, AAB60578.1, AAA76690.1, AAC02516.1, AAC02518.1, AAC02520.1, AAC02522.1, AAC02526.1, AFN61962.1
Env	B	Australia	PBMC	AAG36996.1, AAG37010.1, AAV67932.1, AAV67925.1, AAV67939.1, AEH41858.1, AEH41861.1, ABI20201.1, ABI20237.1, ABI20264.1, ABI20354.1, ABI20327.1, AAD03197.1, AAD03215.1, AAD03223.1, AAD03231.1, AAD03247.1
Env	B	USA	PBMC	AAX33125.1, AAX33116.1, AAX33107.1, AAX33098.1, AAX33089.1, AAX33081.1, AAX33072.1, AAX33063.1, AAX33054.1, AAX33045.1, AAX33036.1, AAX33027.1, AAX33018.1, AAX33009.1, AAX33000.1, AAX32992.1, AAX32983.1, AAX32974.1, AAX32965.1, AAX32956.1, AAX32947.1, AAX32939.1, AAX32930.1, AAX33202.1, AAX33195.1, AAX33186.1, AAX33177.1, AAX33168.1, AAX33159.1, AAX33150.1, AAX33134.1, AGV32982.1, AGV33399.1, AAD40637.3, AEK77314.1, AEK77319.1, AEK77368.1, AEK77330.1, ABC47954.1
Env	B	USA	Cell type: monocyte Tissue type: blood	AAF75522.1, AAF75521.1, AAF75520.1, AAF75519.1
Env	B	USA	PBMC	ABJ99510.1, ABJ99536.1, ABJ99563.1, ACM49912.1, ACM49916.1, ACM49920.1, ACM49928.1, ACM50268.1, ACM50252.1, ACM50015.1, AFE02250.1, AFE02277.1, AAT38214.1, AAT38215.1, AEX89554.1, AEX89719.1, AEX90296.1, ACE78465.1, ACE78483.1, ACE78540.1, AAC40587.1, AAC40589.1, AAC40591.1, AAC40593.1, AAC40597.1, AAW64250.1, AAW64251.1
Env	B	Italy	PBMC	AAW64259.1, AAW64260.1
Env	B	Thailand	PBMC	ADI62638.1, ADI62653.1, AAB37195.1, AAY25588.1, AAY25597.1
Env	B	Georgia	PBMC	ABB29353.1, ABB29369.1, ABB29378.1
Env	B	Belgium	PBMC	ABY26915.1, ABC47957.1, ABC47958.1, ABC47960.1, ABC47961.1, AAA45075.1
Env	B	South Korea	PBMC	AAX83059.1, ABB96421.1, ABB96430.1, ABB96439.1, ABB96455.1, AAF35356.1

(continued)

Table 24.5 (continued)

Name	Subtype	Location	Source	Accession numbers
Env	B	Brazil	PBMC	ABV28254.1, ABV28263.1, ABV28349.1, ABV28324.1, ABC88307.1, ABC88334.1, ABC88343.1, ABC88352.1
Env	B	Argentina	PBMC	ABD39388.1, ABD39406.1, ABD39417.1, ABD39444.1, ABD39435.1, ABD39426.1
Env	B	Ukraine	PBMC	ABI15281.1, ABI15290.1, ABI15301.1
Env	B	Spain	PBMC	ACJ37064.1, ACJ37077.1, ACJ37109.1, ACJ37100.1
Env	B	Spain	PBMC MT4 cells	ADK12733.1, ADK12791.1, ADK12836.1, ADK12912.1, ADV58152.1, ADV58175.1, ADV58245.1
Env	B	Spain	PBMC	AAG16791.1, AAG16800.1, AAG16817.1, AAG16849.1, AFN61971.1
Env	B	Denmark	PBMC	ABV00728.1, ABV00763.1, ABV00798.1, ABV00831.1, ABV00781.1
Env	B	South Africa	PBMC	ACO50040.1
Env	B	Jamaica	PBMC	ADG57018.1, ADG57029.1, ADG57074.1, ADG57057.1, ADG57040.1
Env	B	Uruguay	PBMC	AEK79425.1, AEK79434.1
Env	B	Paraguay	PBMC	AEK79352.1, AEK79381.1
Env	B	Bolivia	PBMC	AFN61991.1
Env	B	Poland	PBMC	AGF33018.1
Env	B	Germany	PBMC	AGF33008.1, AAA86252.1, AAA85237.1

Env	B	USA	Plasma	AAQ97567.1, AAQ97576.1, AAQ97540.1, AAQ97531.1, AAQ97513.1, AAQ97504.1, AAQ97495.1, AAQ97485.1, AAQ97477.1, AAQ97459.1, AAQ97450.1, AAQ97467.1, AAQ97441.1, AAQ97648.1, AAW64262.1, AAW64263.1, AAW64267.1, ABF47478.1, ABF47505.1, ACE63752.1, ACE63929.1, ACE66756.1, ACE64483.1, ACE65702.1, ACE66931.1, ACE65537.1, ACE69129.1, ACE69214.1, ACE67679.1, ACE67779.1, ACE67810.1, ACE68293.1, ACE68494.1, ACE68729.1, ACE70410.1, ACE70548.1, ACE70693.1, ACE71417.1, ACE71647.1, ACE71663.1, ACE71940.1, ACE72120.1, ACE72454.1, ACD42129.1, ACE73048.1, ACD42019.1, ACE67155.1, ACE67303.1, ACE67073.1, ACE67010.1, ACE66958.1, ACE66300.1, ACE66041.1, ACE66020.1, ACE65814.1, ACE64259.1, ACD31881.1, ACD31920.1, ACD31970.1, ACD32023.1, ACD32124.1, ACD32176.1, ACD32393.1, ACD32499.1, ACD32531.1, ACD32575.1, ACD32599.1, ACD32687.1, ACD33004.1, ACD33133.1, ACD33153.1, ACD33231.1, ACD33363.1, ACD34960.1, ACD40358.1, ACD41317.1, ACD41353.1, ABI79732.1, ABI80034.1, ABI31623.1, ABI31642.1, ABO52893.1, ABN42294.1, AAX12631.1, AAX12635.1, AAX12678.1, AAX12636.1, AAW58935.1, ABA01387.1, ABA01405.1, AAP57319.1, AAP57322.1, AAP57343.1, AAP57347.1, AAP57356.1, AAP57385.1, ACF16077.1, ACF16155.1, ACR51129.1, ACR52504.1, ACN86716.1, ACN86735.1, ACN86818.1, ACN86859.1, ACI04514.1, ACN86932.1, ACN86943.1, ACN86966.1, ACN86980.1, ACN87169.1, ACN87215.1, ACS91558.1, ACS91559.1, AEO80324.1, AEO81003.1, AEO81174.1, AEO81400.1, AEO82168.1, AEO81790.1, AEO82944.1, AEO83342.1, AEO83589.1, AEO84282.1, AEO86038.1, AEO86575.1, ADC81227.1, ADC81639.1, ADC81647.1, ADC81938.1, ADC55396.1, ADC55400.1, AAD10899.1, AAD10947.1, AGG78174.1, AGG76739.1, AGG77670.1, AGH02328.1, AGH01883.1, AGG92739.1, AGG92032.1, AFZ63360.1, AFZ62972.1, AFE02722.1, AFE02618.1, AFE02372.1, AFE02278.1, AFH01332.1, AET76364.1, AFM95447.1, AFM95447.1, AEM53591.1, AFB40306.1, AFB39731.1, AFB37908.1, AFB36905.1, AFB36451.1, AEQ75941.1, ADZ36181.1, ADZ35142.1, ADZ33480.1, ADZ32567.1, AEG76891.1, AEG76850.1, AEG76786.1, AEG76775.1, AEG76746.1, AEW28182.1, AEW28121.1, AFM44212.1, AFM44212.1, AFK88479.1, AFK88068.1, ADN34414.1, AEN22031.1, AEN21674.1, AEN21070.1, AEN20588.1, AEN20272.1, AEO23959.1, AEO23453.1
Env	B	Thailand	Plasma	ABD04233.1, ABD04251.1, ABD04269.1, ABD04278.1, AGK29356.1, AGK29559.1, AGK29322.1, AGK29352.1, AGK29337.1, AFU33289.1, AFU26520.1, AEY78533.1, ADY68402.1, ADY68376.1, AFK29877.1, AEO17374.1, AEO17651.1, ADI62500.1
Env	B	Japan	Plasma	BAG31022.1, BAG31040.1, BAG31067.1, BAG31121.1, BAG31112.1

(continued)

Table 24.5 (continued)

Name	Subtype	Location	Source	Accession numbers
Env	B	Trinidad and Tobago	Plasma	AAW64256.1, ACE69334.1, ACE69508.1, ACE69538.1, ACE69730.1, ACE69869.1, ACE69911.1, ACE70009.1, ACE70123.1, ACE70230.1, ACE70398.1, ACE70724.1, ACE70977.1, ACE71060.1, ACE71299.1, ACD33501.1, ACD33872.1, ACD34727.1, AEN24995.1, AEN24721.1, AEN24566.1, AEN24547.1, AEN24405.1
Env	B	Switzerland	Plasma	ABS59248.1, ABS59251.1, ABS59253.1, ABS59275.1, ABS59258.1, ABS59290.1, ABS59282.1, ABS59272.1, ABS59265.1, ABS59250.1, AFM44083.1, AFM43892.1, AGL45765.1, AGL45956.1, AGL45921.1, AGL45730.1
Env	B	China	Plasma	ABY71518.1, ABY71519.1, ABY71520.1, ABY71521.1, ABY71523.1, AGO06382.1, AFV34102.1, AFV34119.1, AEI88157.1, AEI88175.1, ADI62389.1, ADI62456.1
Env	B	Spain	Plasma	ACJ37064.1, ACJ37077.1, ACJ37109.1, ACJ37100.1, ACY70072.1, ACY70131.1, ACY70081.1, ACZ82306.1, ACZ82324.1, ADO32845.1, ADO32878.1, ADN52559.1
Env	B	Belgium	Plasma	ACN86860.1, ACN86870.1, ACN86875.1, ACN86891.1, ACN86895.1
Env	B	UK	Plasma	ACN87027.1, ACN87039.1, ACN87042.1, ACN87038.1, ACN87035.1, AEB22051.1, AEB22046.1, AER38397.1, AER38386.1, AER38367.1, AER38328.1, AER38279.1, ADR66765.1, ADR66619.1, ADI33149.1, ADI33077.1, ADI33073.1, ADI33093.1,
Env	B	Canada	Plasma	ACN87098.1, ACN87101.1, ACN87110.1, ACN87120.1, ADZ34937.1, ADZ34913.1, ADZ34979.1, ADZ34858.1
Env	B	Italy	Plasma	ADC43053.1, ADC43056.1, ADC43064.1, ADC43062.1, ADC43104.1, AER38393.1, AER38371.1
Env	B	Germany	Plasma	ADC43065.1, ADC43068.1, ADC43075.1, ADC43076.1, ADC43115.1, AGK62361.1, AGK62443.1, AGK62431.1, AGK62388.1, AFD62918.1, AFD62954.1, AFM44051.1, AFM44194.1,
Env	B	Australia	Plasma	ADC55384.1, ADC55385.1, ADC55386.1, ADC55387.1, ADC55393.1, AGK37838.1, AGK37841.1
Env	B	France	Plasma	AFM44078.1
Env	B	Poland	Plasma	AEW28310.1
Env	B	Brazil	Plasma	AEW28047.1, AEW28055.1, AEW28063.1, AER38283.1, ADV17588.1, ADV17578.1, ADV17552.1, ADV17525.1, ADV17502.1,
Env	B	Sweden	Plasma	AEO22540.1, AEO22465.1, AEO22348.1, AEO22198.1, AEO22303.1
Env	B	Jamaica	Plasma	AER38352.1

Env	B	Peru	Plasma	ADZ33975.1, ADZ33996.1, ADZ33835.1, ADZ33619.1, ADZ33870.1, ADZ32588.1
Env	B	South Africa	Plasma	ADR66569.1
Env	B	Netherlands	Plasma	AEB39872.1, AEB39859.1, AEB39793.1, AEB39824.1, AEB39846.1
Env	B	Gambia	Plasma	ADP55178.1
Env	B	Columbia	Plasma	ADV17501.1, ADV17497.1, ADV17492.1, ADV17466.1, ADV17471.1
Env	B	South Korea	Plasma	AAX83059.1, ABB96421.1, AFA42878.2, AFI39037.1, AFI39055.1
Env	B	USA	Blood	ADZ39048.1, ADZ39047.1, ADZ39045.1, ADZ39044.1, ADZ39043.1, ADZ39042.1, ADZ39041.1, AAQ86673.1, AAQ86665.1, AAQ86657.1, AAQ86649.1, AAQ86641.1, AAQ86633.1, AAQ86625.1, AAQ86617.1, AAQ86609.1, AAQ86601.1, ACO91388.1, ACO91387.1, ACO91386.1, ACO91385.1, ACO91384.1, ACO91383.1, ACO91382.1, ACO91381.1, ACO91380.1, ACO91379.1, ACO91378.1
Env	B	Trinidad and Tobago	Blood	AAG22517.1, AAG22516.1, AAG22515.1, AAG22514.1, AAG22518.1, AAG22513.1, AAG22512.1, AAG22511.1, AAG22510.1, AAG22509.1, AAG22508.1, AAG22506.1, AAG22504.1, AAG22503.1, AAG22502.1, AAG22501.1, AAG22500.1
Env	B	Japan	Blood	BAJ41167.1, BAJ41158.1, BAJ41149.1, BAJ41140.1, BAJ41131.1, BAJ41122.1, BAJ41113.1, BAK41860.1, BAK41851.1, BAK48599.1, BAK48600.1, BAK48601.1, BAK48602.1, BAK48603.1, BAK48604.1, BAK48605.1, BAK48606.1, BAK48607.1, BAK48608.1, BAK48609.1, BAK48610.1, BAK48611.1, BAK48612.1, BAK48613.1, BAK48614.1, BAK48615.1, BAK48616.1, BAK48617.1, BAK48618.1, BAK48619.1, BAK48620.1, BAK48621.1, BAK48622.1, BAK48623.1, BAK48624.1, BAK48625.1, BAK48626.1, BAK48627.1, BAK48628.1, BAK48629.1, BAK48630.1, BAK48631.1, BAK48632.1, BAK48633.1, BAK48634.1, BAK48635.1, BAK48636.1, BAK48637.1, BAK48638.1, BAK48639.1, BAK48641.1, BAK48642.1, BAK48640.1, BAK48643.1, BAK48644.1, BAK48645.1, BAK48646.1, BAK48647.1, BAK48648.1, BAK48649.1, BAK48650.1, BAK48651.1, BAK48652.1, BAK48653.1, BAK48654.1, BAK48655.1, BAK48656.1, BAK48657.1, BAK48658.1, BAK48698.1, BAK48697.1, BAK48696.1, BAK48695.1, BAK48694.1
Env	B	Russia	Blood	AAV90745.1, AAV30106.1, AAV30097.1, AAT76857.1,
Env	B	Cyprus	Blood	AEB52441.1, AEB52480.1, AEB52527.1, AEB52553.1, AEB52578.1, AEB52703.1, AEB52748.1, AEB52855.1
Env	B	Netherlands	Blood	AAC54649.1
Gag	B	China	PBMC	AAN83911.1, AAQ64621.1, AAQ64618.1, ABL63498.1, ACZ57348.1, ADE20141.1, ADE20159.1, ADE20150.1, AAT09643.1, AAC05234.1, AFN62006.1

(continued)

Table 24.5 (continued)

Name	Subtype	Location	Source	Accession numbers
Gag	B	USA	PBMC	AAX33197.1, AAX33179.1, AAX33161.1, AAX33152.1, AAX33118.1, AAX33074.1, AAX33047.1, AAX33011.1, AAX32985.1, AAX32923.1, ABJ99503.1, ABJ99521.1, AAA44853.1, AAZ14761.1, AAZ14767.1, AAZ14763.1, ACM49910.1, ACM49914.1, ACM50080.1, ACM50266.1, ADF45365.1, ADF45387.1, ADF45405.1, ADF45403.1, ACE78542.1, ACE78458.1, AAB59875.1, AAO63143.1, AAP12627.1, AAO63149.1, AAP12627.1, AAO63134.1, AAC32293.1, AAA86730.1, AGV32830.1, AGV32975.1, AFN61997.1
Gag	B	Australia	PBMC	AAD39400.1, AAD03190.1, AAD03232.1, AAF35354.1, ADF45364.1, ADF45389.1, ABI20194.1, ABI20230.1, ABI20257.1, ABI20347.1, ABI20194.1
Gag	B	Indonesia	PBMC	BAH09835.1, BAH09837.1, BAH09839.1, BAI77504.1, BAI77513.1
		Spain	PBMC	ACJ37059.1, ACJ37072.1, ACJ37104.1, ACJ37079.1, ACJ37088.1, ADK12726.1, ADK12784.1, ADK12829.1, ADK12905.1, ADV58153.1, ADV58176.1, ADV58246.1, AFN61964.1, AGF32907.1, AGF32923.1, AAG16784.1, AAG16793.1, AAG16810.1, AAG16843.1
		Argentina	PBMC	AAL07549.1, AAL07691.1
		South Korea	PBMC	AF224507.1, ABU42061.1, AAX83057.1
		Netherlands	PBMC	AAC54633.1, AAX86731.1, AAX86749.1, AAX86713.1
		Brazil	PBMC	ABC88300.1, ABC88327.1, ABC88336.1, ABC88345.1, ABV28247.1, ABV28256.1, ABV28317.1, ABV28344.1
		Denmark	PBMC	ABV00696.1, ABV00721.1, ABV00704.1, ABV00747.1
		Germany	PBMC	AAA86246.1, AAA85230.1, ADF45347.1, ADF45356.1, ADF45349.1, ADF45354.1, ADF45351.1, ADF45363.1, ADF45361.1, ADF45358.1, ADF45353.1, ADF45359.1
		France	PBMC	AAB59747.1, AAA76686.1, AAB60571.1, AFN61956.1, AFN61973.1
		Bolivia	PBMC	AFN61989.1
		Poland	PBMC	AGF33018.1
		Japan	PBMC	AAB03522.1
		Gabon	PBMC	AAA83391.1

Gag	B	USA	Plasma	AAQ97569.1, AAQ97560.1, AAQ97470.1, AAQ97461.1, AAQ97443.1, ADW27064.1, ADW27063.1, ADW27048.1, ADW27045.1, ADW27040.1, ADW27023.1, ADW27005.1, ADW26991.1, ADW26968.1, ADW26877.1, ACS91542.1, ACS91541.1, ABO52882.1, ABO52880.1, ABO52878.1, ABO52876.1, ACR53117.1, ACR53110.1, ACR53101.1, ACR53084.1, ACR52079.1, ACR52048.1, ACR52601.1, ACR52593.1, ABI80036.1, ABI79878.1, ABI79851.1, ABI80010.1, AAD10935.1, AAD10871.1, ACB36742.1, ACB36739.1, ACB36733.1, ACB36732.1, ACB36723.1, ACB36712.1, ACB36710.1, ACB36700.1, ACB36692.1, ACB36744.1, ADG34803.1, ADG34802.1, ADG34637.1, ADG34651.1, ACN94827.1, ACN94826.1, ACN94804.1, ACN94785.1, ADE45315.1, ADE45275.1, AFH37836.1, AFH37833.1, AFH37829.1, AFH37816.1, AFH37814.1, AFH37810.1, AFH37803.1, AFH37796.1, AFH37780.1, AFH37838.1, ABA01380.1, ABA01389.1, ABA01398.1, AAV53166.1, AAV53187.1, AAV53236.1, AAV53327.1, AAV53251.1, ABM05974.1, ABM05980.1, ABM05985.1, ABF47471.1, ABF47498.1, ACU50315.1, ACU50556.1, ACU50508.1, ACU50659.1, ACU50854.1, ACG55504.1, ACG55531.1, ACG55541.1, ACG55543.1, ACF93239.1, AEO84972.1, AEO85041.1, AEO85113.1, AEO85247.1, AEO85211.1, ADC55407.1, ADC55431.1, ADZ32733.1, ADZ32856.1, ADZ32987.1, ADZ34021.1, ADZ35566.1, AFB36444.1, AFB37384.1, AFB38607.1, AFB39647.1, AFB40575.1, AEW27564.1, AEW27584.1, AEM53683.1, AEM53701.1, AFM43999.1, AFM44595.1, AFZ62941.1, AGH00213.1, AGH00343.1, AGH02358.1, AGG77087.1, AGG77830.1, AGG77893.1, AET75990.1, AET76115.1, AET76357.1
	B	Canada	Plasma	ACN94821.1, ACN94820.1, ACN94819.1, AAQ86596.1, AAQ86612.1, AAQ86636.1, AAQ86652.1, AAQ86668.1, ADZ34853.1, ADZ34908.1, ADZ34932.1, ADZ34974.1, ADZ34988.1, ABY78606.1, ABY78604.1, ABY78602.1, ABY78600.1, ABY78596.1, ABY78594.1, ABY78588.1, ABY78579.1, ABY78576.1, ABY78575.1, ABY78565.1, ABY78559.1, ABY78553.1, ABY78536.1, ABY78294.1, ABY78287.1, ABY78268.1, ABY78256.1, ABY78040.1, ABY78041.1,
	B	Australia	Blood	AAD03241.1, AAD03225.1
	B	Germany	Plasma	ACN94816.1, ACN94809.1, ACN94818.1, ACN94814.1, AFM44044.1, AFM44053.1, AFM44062.1, AFM44151.1, AFM44196.1, CAD26945.1, CAD26940.1, CAD26927.1, CAD26921.1, CAD26947.1, CAD26920.1, CAD26919.1, CAD26938.1, CAD26931.1, CAD26932.1
	B	Thailand	Plasma	AFK29870.1, AEO17367.1, AEO17482.1, AEO17558.1, AEO17592.1, AEO17465.1, AEP14038.1, AFU26494.1, AFU26515.1, AFU26582.1, AFU28482.1, AFU33245.1

(continued)

Table 24.5 (continued)

Name	Subtype	Location	Source	Accession numbers
	B	China	Plasma	AEZ35420.1, AEY77155.1, AFV39343.1, AEY77182.1, AEY77170.1, AGO06375.1, AGO06300.1, AFV34095.1, AFV34104.1, AFV34112.1, AFZ77628.1, AFZ77630.1, AFZ77690.1, AFZ77714.1, AFZ77722.1, ACT76434.1, ACT76496.1, ACT76444.1, ACT76440.1, ACT76470.1
	B	Australia	Plasma	ACN94806.1, ACN94807.1, ACN94805.1, ACN94808.1, ADC55402.1, ADC55403.1, ADC55404.1, ADC55405.1, ADC55406.1
	B	South Korea	Plasma	AAF35354.1, AFA42871.1, ABB96415.1, ABB96432.1, AFI38977.1, AFI39021.1,
	B	Malaysia	Plasma	AEA77092.1, AEA77092.1
	B	Spain	Plasma	ACY70076.1, ADE34334.1, ADO32883.1, ACZ82301.1, ACZ82319.1, ADE34316.1, ADO32856.1, ADO32873.1, ACJ37119.1, ACJ37104.1, AFH76559.1, AFH76600.1, AFH76844.1, AFH76753.1, ACY70126.1, ACY70076.1
	B	Japan	Plasma	BAG31015.1, BAG31033.1, BAG31060.1, BAG31105.1, BAG31114.1, AFR66788.1, AFR66842.1, AFR66868.1, AFR66854.1, AFR66862.1
	B	South Africa	Plasma	ABF00910.1
	B	Argentina	Plasma	ACI05325.1, ACI05375.1, ACI05413.1, ACI05435.1, ACI05447.1
	B	Jamaica	Plasma	ACN80826.1, ACN80939.1, ACN80937.1, ACN80948.1, ACN80940.1
	B	UK	Plasma	ACO53447.1, ACO53449.1, ADK75302.1, ADK75277.1, ADK75280.1, ADK75287.1, ADK75290.1
	B	France	Plasma	ACN29603.1, ACN29605.1, ACN29607.1, ACN29609.1, ACN29611.1, AER35428.1, AER35430.1, AER35432.1
	B	Peru	Plasma	ADZ32542.1, ADZ32583.1, ADZ32637.1, ADZ32671.1, ADZ32726.1, ADZ32713.1
	B	Brazil	Plasma	AEW27527.1, AEW27532.1
	B	Poland	Plasma	AEW27694.1
	B	Switzerland	Plasma	AFM43885.1, AFM43909.1, AFM43972.1, AFM44085.1, AFM44109.1, AGL45725.1, AGL45760.1, AGL45881.1, AGL45951.1, AGL45867.1
	B	Uganda	Blood	AAA45091.1
	B	Philippines	Blood	AAA45003.1, AAA45002.1
	B	Brazil	Blood	AAA44225.1, AAA44224.1, AAA44226.1

	B	USA	Blood	AAA81036.1, AAQ86668.1, AAQ86660.1, AAQ86652.1, AAQ86644.1, AAQ86636.1, AAQ86628.1, AAQ86620.1, AAQ86612.1, AAQ86604.1, AAQ86596.1
	B	Australia	Blood	AAD03240.1, AAD03224.1, AAC54543.1
	B	Russia	Blood	AAV90743.1, AAV30099.1, AAV30090.1, AAT76855.1
	B	China	blood	AAY57427.1, AAY57409.1, AAY57418.1, ADU52603.1, ADU52605.1, ADU52607.1, ADU52613.1, ADU52611.1,
		Japan	Blood	BAK41853.1, BAK41844.1, BAJ41178.1, BAJ41169.1, BAJ41151.1, BAJ41133.1, BAJ41097.1
		Belarus	Blood	AAL78489.1
		Cyprus	Blood	AEB52522.1, AEB52599.1, AEB52698.1, AEB53021.1, AEB52915.1, AEB52988.1, AEB52818.1, AEB52875.1, AEB53003.1, AEB52892.1, AEB52452.1, AEB52698.1, AEB53003.1
		Netherlands	Blood	AAC54642.1
Pol	B	China	PBMC	AAQ64619.1, AAQ64622.1, ABK42013.1, ABL63499.1, AAN83912.1, ACZ57349.1, ADE20142.1, ADE20151.1, ADE20160.1, AAY57428.1, AAY57419.1, AFN62007.1, AAC05235.1
Pol	B	USA	Peripheral blood mononuclear cells (PBMC)	AAP12628.1, AAB59876.1, ABJ99504.1, ABJ99522.1, ABJ99530.1, ABJ99557.1, ABJ99548.1, ACM49911.1, ACM49927.1, ACM49998.1, ACM50200.1, ACM50267.1, ACE78459.1, ACE78534.1, AAX33180.1, AAX33144.1, AAX33128.1, AAX33075.1, AAX33057.1, AAX33057.1, AAX33039.1, AAX33021.1, AAX32994.1, AAX32924.1, AAX33198.1, AFN61998.1, AAA45053.1, AAC32294.1, AGV32976.1, AGV32831.1
Pol	B	South Korea	PBMC	AAX83058.1 ABB96424.1, ABB96433.1, ABB96442.2, AEX59156.1, AFI38978.1, AFI39022.1, AAF35355.1
Pol	B	Spain	PBMC	ACJ37060.1, ACJ37080.1, ACJ37105.1, ACJ37096.1, ADK12727.1, ADK12785.1, ADK12830.1, ADK12906.1, ADK12856.1, ADV58154.1, ADV58247.1, AAG16785.1, AAG16794.1, AAG16811.1, AAG16828.1, AAG16844.1, AFN61965.1
Pol	B	Thailand	PBMC	ABD04210.1, ABD04227.1, ABD04245.1, ABD04272.1, ABD04263.1, AAY25582.1, AAY25591.1
Pol	B	Netherlands	PBMC	AAC54634.1, AAX86714.1, AAX86723.1, AAX86732.1, AAX86741.1, AAX86750.1
Pol	B	Georgia	PBMC	ABB29349.1, ABB29365.1, ABB29374.1
Pol	B	Brazil	PBMC	ABV28248.1, ABV28257.1, ABV28318.1, ABV28345.1, ABC88301.1, ABC88328.1, ABC88337.1, ABC88346.1
Pol	B	Argentina	PBMC	ABD39402.1, ABD39384.1, ABD39411.1, ABD39420.1, ABD39438.1

(continued)

Table 24.5 (continued)

Name	Subtype	Location	Source	Accession numbers
Pol	B	Australia	PBMC	ABI20195.1, ABI20231.1, ABI20258.1, ABI20321.1, ABI20348.1
Pol	B	Ukraine	PBMC	ABI15277.1, ABI15286.1, ABI15295.1
Pol	B	Denmark	PBMC	ABV00697.1, ABV00705.1, ABV00722.1, ABV00748.1, ABV00757.1, ABV00775.1, ABV00793.1, ABV00739.1
Pol	B	South Africa	PBMC	ACO50034.1
Pol	B	Jamaica	PBMC	ADG57019.1, ADG57023.1, ADG57033.1, ADG57052.1, ADG57068.1
Pol	B	Uruguay	PBMC	AFN61982.1, AEK79419.1, AEK79428.1, AEK79466.1
Pol	B	Paraguay	PBMC	AEK79312.1, AEK79346.1, AEK79375.1
Pol	B	France	PBMC	AAB60572.1, AFN61957.1, AFN61974.1
Pol	B	Bolivia	PBMC	AFN61990.1
Pol	B	Venezuela	PBMC	AFN62014.1
Pol	B	Germany	PBMC	AAA86247.1, AAA85231.1, AGF33002.1, CAA49935.1, CAA51061.1, CAA49941.1, CAA50426.1, CAA49984.1
Pol	B	Poland	PBMC	AGF33019.1
Pol	B	Gabon	PBMC	AAA83392.1
Pol	B	USA	Plasma	AAQ97570.1, AAQ97498.1, AAQ97471.1, AAQ97453.1, AAQ97435.1, ABN42296.1, ABN42269.1, ABI80037.1, ABI80011.1, ABI79879.1, ABI79852.1, ABI79809.1, AAD10936.1, AAD10872.1, ACS91546.1, ACS91547.1, ADE45316.1, ADE45307.1, ADE45294.1, ADE45285.1, ADE45276.1, AGG77088.1, AGG77831.1, AGG77894.1, AGG76690.1, ACR53118.1, ACR53111.1, ACR52913.1, ACR52080.1, ACR52064.1, ACR52041.1, ACR52602.1, ACR52594.1, ACR52540.1, ABA01381.1, ABA01390.1, ABA01399.1, ABF47472.1, ABF47499.1, ACU55196.1, ACU55196.1, ACU55279.1, ACU55377.1, ACU55516.1, ACU55684.1, ACU55747.1, ADC67088.1, ADC67094.1, ADC67105.1, AFB36445.1, AFB36899.1, AFB37385.1, AFB38608.1, AFB39648.1, AFB40576.1, ADZ32734.1, ADZ32857.1, ADZ32988.1, ADZ34022.1, ADZ35138.1, AEW27565.1, AEW27585.1, AEW27690.1, AEW27620.1, AET75991.1, AET76116.1, AET76342.1, AET76358.1, AET76205.1, AFM44000.1, AFM44206.1, AFM44596.1, AGH01878.1, AFZ62942.1, AFZ62959.1
Pol	B	Canada	Plasma	AAQ86603.1, AAQ86619.1, AAQ86643.1, AAQ86659.1, AAQ86675.1, ADZ34854.1, ADZ34854.1, ADZ34933.1, ADZ34975.1, ADZ34989.1

Pol	B	Thailand	Plasma	AFK29871.1, AEO17368.1, AEO17466.1, AEO17645.1, AEO17593.1, AEO17568.1, AFU26495.1, AFU26583.1, AFU28483.1, AFU33246.1, AFU31744.1
Pol	B	South Korea	Plasma	AAF35355.1, ABB96424.1, AEX59156.1, AFI39031.1, AFA42872.1, AAX83058.1
Pol	B	Malaysia	Plasma	AEA77093.1
Pol	B/C	China	Plasma	ADJ19222.1, AFV34096.1, AFV34105.1, AFV34113.1, AGO06376.1, AEZ51381.1, AFV39561.1, AFV39528.1, AEY77189.1, AEY77193.1, AFZ77809.1, AFZ77877.1, AFZ77864.1, AFZ77826.1, AFZ77842.1, ADE34335.1, ADE34326.1, ADE34317.1,
Pol	B	Spain	Plasma	ACZ82320.1, ACZ82302.1, ADO32839.1, ADO32857.1, ADO32874.1, ADO32882.1, ACY70127.1, ACY70077.1, ACY70068.1, ACJ37120.1, AAQ97570.1, AAQ97498.1, AAQ97471.1, AAQ97453.1, AAQ97435.1
Pol	B	Japan	Plasma	ABN42296.1, ABN42269.1
Pol	B	South Africa	Plasma	ABI80037.1, ABI80011.1, ABI79879.1, ABI79852.1, ABI79809.1
Pol	B	Australia	Plasma	AAD10936.1, AAD10872.1
Pol	B	Peru	Plasma	ACS91546.1, ACS91547.1
Pol	B	Brazil	Plasma	ADE45316.1, ADE45307.1, ADE45294.1, ADE45285.1, ADE45276.1
Pol	B	Poland	Plasma	AGG77088.1, AGG77831.1, AGG77894.1, AGG76690.1
Pol	B	Switzerland	Plasma	ACR53118.1, ACR53111.1, ACR52913.1, ACR52080.1, ACR52064.1, ACR52041.1
Pol	B	Germany	Plasma	ACR52602.1, ACR52594.1, ACR52540.1
Pol	B	USA	Blood	AAA81037.1, AAQ86667.1, AAQ86659.1, AAQ86651.1, AAQ86643.1, AAQ86635.1, AAQ86627.1, AAQ86619.1, AAQ86611.1, AAQ86603.1
Pol	B	Russia	Blood	AAV90744.1, AAV30100.1, AAV30091.1, AAT76856.1
Pol	B	Belarus	Blood	AAL78496.1
Pol	B	Japan	Blood	BAK41854.1, BAK41845.1, BAJ41161.1, BAJ41152.1, BAJ41143.1, BAJ41134.1, BAJ41125.1, BAJ41107.1, BAJ41098.1
Pol	B	Cyprus	Blood	AEB52437.1, AEB52476.1, AEB52574.1, AEB52699.1, AEB52819.1, AEB52851.1, AEB52916.1, AEB52827.1, AEB52767.1, AEB52669.1 (partial)
Pol	B	Netherlands	Blood	AAC54643.1

(continued)

Table 24.5 (continued)

Name	Subtype	Location	Source	Accession numbers
Nef	B	USA	PBMC	AAA58303.1, AAA58305.1, AAA58307.1, AAA58320.1, AAA58319.1, AAA58283.1, AAA58291.1, AAA58263.1, AAA58258.1, AAA58262.1, AAG34569.1, AAG34582.1, AAG34593.1, AAG34634.1, AAG34626.1, ABJ99511.1, ABJ99537.1, ABJ99564.1, ACM49917.1, ACM49941.1, ACM50103.1, ACM50269.1, ACM50202.1, ABZ10801.1, AAA63848.1, AAA63804.1, AAA63815.1, AAA63838.1, AAA02634.1, AAA02668.1, AAA02672.1, AAA02657.1, AAA02665.1, AAX33203.1, AAX33178.1, AAX33001.1, AAX32931.1, AAX32957.1, ABY81169.1, ABY81172.1, ABY81214.1, ABY81200.1, AEW09745.1, AEW09773.1, AEW10195.1, AEW10136.1, AAF25230.1, AAF25234.1, AAF25303.1, AAF25318.1, AAF25268.1, AAB59883.1, AAA45058.1, AAA86737.1, AAA81044.1, AAB04700.1, AAB04753.1, AGV32847.1, AGV33413.1, AFN62005.1,
Nef	B	South Korea	PBMC	AAC17894.1, AAC17899.1, AAF35361.1, AAX83060.1, ABB96422.1, ABB96431.1, ABB96440.1, ABB96449.1, ABB96456.1, ACI24893.1, ACI24906.1, ACI24913.1, ACI24913.1, ACI25075.1, ACI25039.1
Nef	B	Korea	PBMC	AAC17902.1, AAC17898.1, AAC17891.1, AAC17895.1, AAC17896.1
Nef	B	Canada	Peripheral blood lymphocytes	ABV24187.1, ABV24186.1, ABV24184.1, ABV24183.1, ABV24153.1, ABV24100.1, ABV24098.1, ABV24085.1, ABV24087.1, ABV24084.1, AAU89984.1, AAU89986.1, AAU89993.1, AAU89996.1, AAU89987.1, AAU89985.1, AAU89994.1, AAU90004.1, AAU90000.1, AAU90001.1
Nef	B	Australia	PBMC	AAD03198.1, AAV67933.1, AAV67926.1, AAV67940.1, ABI20283.1, AAB36900.1
Nef	B	China	PBMC	AAT09648.1, ABL63505.1, ACZ57356.1, ADE20149.1, ADE20167.1, ACR27119.1, ACR27124.1, ACR27130.1, ABO44022.1, ABO44024.1, ABO44026.1, ABO44031.1, ABO44029.1, AAN83919.1, AAC05238.1, AFN62013.1
Nef	B	Russia: Mosow	PBMC	AAS01347.1
Nef	B	Myanmar	PBMC	BAC77519.1, BAC77519.1, BAC77519.1, BAC77467.1, BAC77476.1
Nef	B	Japan	PBMC	BAA93721.1, BAA93723.1, BAA93725.1, BAA93728.1, BAA93732.1, BAA93772.1, BAA93770.1, BAA93768.1, BAA93765.1, BAA93740.1
Nef	B	UK	PBMC	AAD31203.1, AAD31205.1, AAD31207.1, AAD31210.1, AAD31219.1, AAD31248.1, AAD31246.1, AAD31243.1, AAD31243.1, AAD31264.1
Nef	B	Italy	PBMC	AAD39184.1, AAD39186.1, AAD39193.1, AAD39191.1, AAD39189.1, AAM93757.1, AAM93761.1, AAM93769.1, AAM93779.1, AAM93796.1, AAM93802.1, AAM93820.1, AAM93852.1, AAM93852.1, AAM93895.1

Nef	B	Spain	PBMC	AAG16809.1, ACJ37112.1, ACJ37095.1, ACJ37103.1, ACJ37087.1, ADK12801.1, ADK12913.1, ADK12896.1, ADK12828.1, AAR20502.1, AAR20507.1, AAR20510.1, AAR20515.1, AAR20513.1, ADV58177.1, ADV58230.1, AFN61972.1
Nef	B	Netherlands	PBMC	AAX86721.1, AAX86730.1, AAX86739.1, AAX86748.1, AAX86757.1, AAC54641.1
Nef	B	Georgia	PBMC	ABB29354.1, ABB29370.1, ABB29379.1
Nef	B	Thailand	PBMC	ABD04216.1, ABD04252.1, ABD04270.1, ABD04279.1
Nef	B	Brazil	PBMC	ABC88308.1, ABC88335.1, ABC88344.1, ABC88353.1, ABV28255.1, ABV28282.1, ABV28316.1, ABV28325.1, ABV28264.1, ACY73298.1, ACY73311.1, ACY73322.1, ACY73345.1
Nef	B	Argentina	PBMC	ABD39389.1, ABD39418.1, ABD39427.1, ABD39436.1, ABD39445.1, ABY49071.1, ABY49073.1, ABY49076.1, ABY49087.1, ABY49088.1
Nef	B	Ukraine	PBMC	ABI15282.1, ABI15291.1, ABI15302.1
Nef	B	Denmark	PBMC	ABV00703.1, ABV00720.1, ABV00737.1, ABV00782.1, ABV00799.1, ABV00832.1
Nef	B	South Africa	PBMC	ACO50041.1
Nef	B	France	PBMC	ADL27730.1, ADL27726.1, ADL27690.1, ADL27688.1, ADL27692.1, AEW10324.1, AEW10328.1, AEW10331.1, AEW10340.1, AEW10335.1, AAA44875.1, AAA44880.1, AAA44890.1, AAA44908.1, AAA44943.1, AAA44969.1, AAB59752.1, AAA76691.1, AFN61963.1
Nef	B	Jamaica	PBMC	ADG57022.1, ADG57039.1, ADG57048.1, ADG57058.1, ADG57075.1
Nef	B	Switzerland	PBMC	ADJ17512.1, ADJ17539.1, ADJ17516.1, ADJ17550.1, ADJ17551.1, ADJ17555.1, ADJ17566.1, ADJ17574.1, ADJ17623.1, ADJ17689.1
Nef	B	Uruguay	PBMC	AEK79426.1, AEK79435.1, AFN61988.1
Nef	B	Paraguay	PBMC	AEK79317.1, AEK79353.1
Nef	B	Venezuela	PBMC	AFN62020.1
Nef	B	Germany	PBMC	AGF33009.1, AAA86253.1, AAA85238.1
Nef	B	Poland	PBMC	AGF33025.1
Nef	B	Gabon	PBMC	AAA83398.1
Nef	B	China	Plasma	AAC40691.1, AAC40690.1, AAC40689.1, AAC40688.1, AAC40687.1, AEI88158.1, AEI88176.1, AGO06383.1
Nef	B	Thailand	Plasma	AFK29878.1, AFU26500.1, AFU26521.1, AFU26623.1, AFU28488.1, AFU33290.1

(continued)

Table 24.5 (continued)

Name	Subtype	Location	Source	Accession numbers
Nef	B	USA	Plasma	AAQ97577.1, AAQ97568.1, AAQ97478.1, AAQ97541.1, AAQ97532.1, AAQ86674.1, AAQ86658.1, AAQ86650.1, AAQ86602.1, AAQ86610.1
Nef	B	USA	Plasma	AAR37066.1, AAR37199.1, AAR37143.1, AAV52971.1, AAV52989.1, AAV53165.1, AAV53148.1, AAV53143.1, ABA01397.1, ABA01406.1, AAP74168.1, AAP74178.1, AAP74188.1, ABM05986.1, ABL67008.1, ABM05989.1, ABM05995.1, ABO63965.1, ABN42303.1, ABO63973.1, ABO63970.1, ABY53234.1, ABY53276.1, ABY53277.1, ABY53278.1, ABY53289.1, ACO90706.1, ACO90752.1, ACO90807.1, ACO91136.1, ACO91362.1, ABI79733.1, ABI80035.1, ABI81124.1, ABI81048.1, ABI81169.1, ACS91544.1, ACS91545.1, ACU55757.1, ACU55915.1, ACU55971.1, ACU56114.1, ACU56252.1, AEO83605.1, AEO83920.1, AEO84143.1, AEO83962.1, AEO84971.1, AEO84289.1, ADB03653.1, ADB03691.1, ADB03683.1, ADB03665.1, ADE45283.1, ADZ32515.1, ADZ32862.1, ADZ32568.1, ADZ32964.1, ADZ32904.1, ADZ35572.1, ADQ53551.1, ADQ53622.1, ADQ53591.1, ADQ53555.1, AFB36452.1, AFB37392.1, AFB39732.1, AFB40583.1, AEM98393.1, AEM98406.1, AEW28492.1, AEW28287.1, AFM44603.1, AFM44213.1, AFQ32965.1, AGD79201.1, AGD79234.1, AGG77317.1, AGG77424.1, AGG77671.1, AGG78175.1, AGG76941.1, AGG91784.1, AGG92740.1, AGG93282.1, AGH01884.1, AGH02329.1, AAD10948.1, AAD10900.1, ADC81414.1, ADC81648.1, ADC82833.1, ADC83285.1, ADD66007.1, ADC81585.1, ADC67111.1, ADC67122.1, AET76185.1, AET76365.1, AET75998.1, ACR51130.1, ACR51199.1, ACR51496.1, ACR52505.1, ACR52554.1, ACR52562.1, ACR52836.1, ACR52950.1, ACR53124.1
Nef	B	Canada	Plasma	AAQ86602.1, AAQ86618.1, AAQ86642.1, AAQ86658.1, AAQ86674.1, ADZ34859.1, ADZ34914.1, ADZ34938.1, ADZ34994.1, ADZ34980.1
Nef	B	Japan	Plasma	BAG31023.1, BAG31041.1, BAG31068.1, BAG31095.1, BAG31122.1
Nef	B	Germany	Plasma	AAX08125.1, AAX08126.1, AAX08136.1, AAX08132.1, AAX08130.1, AFM44052.1, AFM44061.1, AFM44070.1, AFM44204.1, AFM44159.1, AFD62920.1
Nef	B	South Africa	Plasma	ABF00918.1
Nef	B	Venezuela	Plasma	ACC96153.1, ACC96156.1, ACC96158.1, ACC96162.1, ACC96166.1
Nef	B	Spain	Plasma	ACJ37118.1, ACZ82309.1, ACZ82327.1, ADE34324.1, ADE34342.1, ADO32846.1, ADO32864.1, ADO32881.1, ADO32890.1
Nef	B	Australia	Plasma	ADC67107.1, ADC67108.1, ADC67109.1, ADC67110.1, ADC67118.1

Nef	B	UK	Plasma	ADG86643.1, ADG86671.1, ADG86647.1, ADG86657.1, ADG86666.1, ADK75356.1, ADK75565.1, ADK75890.1, ADK75357.1, ADK75361.1
Nef	B	Switzerland	Plasma	ADJ17491.1, ADJ17494.1, ADJ17504.1, ADJ17508.1, ADJ17511.1, AGL45731.1, AGL45766.1, AGL45887.1, AGL45873.1, AGL45957.1, AFM43893.1, AFM43917.1, AFM43980.1, AFM44093.1, AFM44117.1
Nef	B	Peru	Plasma	ADZ32589.1, ADZ32548.1, ADZ32643.1, ADZ32677.1, ADZ32732.1
Nef	B	South Korea	Plasma	AEV66317.1, AEV66334.1, AEV66348.1, AEV66368.1, AEV66373.1, AEV66378.1, AEV66381.1, AEV66385.1, AEV66388.1, AEV66396.1, AAF35361.1, ABB96431.1, ABB96456.1, AFI39038.1, AFA42879.1
Nef	B	Brazil: Sao Paulo	Plasma	AEW28048.1, AEW28056.1, AEW28064.1
Nef	B	Poland	Plasma	AEW28311.1
Nef	B	France	Plasma	AEW10375.1, AEW10461.1, AEW10454.1, AEW10390.1, AEW10449.1, AFM44079.1
Nef	B	USA	Blood	AAA81044.1, AAA79606.1, AAA79602.1, AAA79603.1, AAA79604.1, AAA79601.1, AAA79599.1, AAA79605.1, AAA79600.1, AAA79607.1, AAQ86674.1, AAQ86658.1, AAQ86650.1, AAQ86602.1, AAQ86610.1
Nef	B	China	Blood	ABO44031.1, ABO44030.1, ABO44029.1, ABO44022.1, ABO44023.1, ABO44025.1, ABO44024.1, ABO44026.1, AAY57433.1, AAY57426.1, AAY57417.1
Nef	B	Russia	Blood	AAV90750.1, AAV30107.1, AAV30098.1, AAT76863.1
Nef	B	Australia	Blood	AAD03198.1, AAC56417.1
Nef	B	Japan	Blood	BAK41861.1, BAJ41168.1, BAJ41159.1, BAJ41150.1, BAJ41105.1, BAJ41114.1, BAJ41123.1, BAJ41132.1, BAJ41141.1
Nef	B	Netherlands	Blood	AAC54650.1
Rev	B	Australia	PBMC	AAD03195.1, AAD03204.1, AAD03213.1, AAD03221.1, AAD03229.1, AAD03237.1, AAD03245.1, AAV67930.1, AAV67923.1, AAV67937.1, ABI20199.1, ABI20280.1, ABI20316.1, ABI20253.1, ABI20352.1
Rev	B	China	PBMC	AAT09647.1, ABL63503.1, ABK42017.1, ACZ57353.1, ADE20146.1, ADE20155.1, ADE20164.1, AAN83916.1, AFN62010.1, AAC05242.1
Rev	B	Myanmar	Peripheral blood mononuclear cell	BAC77516.1, BAC77507.1, BAC77491.1, BAC77482.1, BAC77473.1, BAC77464.1

(continued)

Table 24.5 (continued)

Name	Subtype	Location	Source	Accession numbers
Rev	B	USA	PBMC	AAX33205.1, AAX33193.1, AAX33184.1, AAX33061.1, AAX32928.1, ACE78463.1, ACE78489.1, ACE78538.1, ABJ99508.1, ABJ99534.1, ABJ99561.1, ABJ99526.1, ABJ99552.1, AAB59880.1, AGV32844.1, AGV33064.1, AGV33410.1, AAA44310.1, AAA44852.1, AFN62002.1, AAA81041.1, AAB05048.1
Rev	B	South Korea	PBMC	AAF35358.1, ABB96419.1, ABB96428.1, ABB96437.1, ABB96453.1, ABB96446.1
Rev	B	Spain	PBMC	AAG16789.1, AAG16798.1, AAG16806.1, AAG16850.1, AAG16832.1, AAG16840.1, ACJ37065.1, ACJ37093.1, ACJ37101.1, ACJ37110.1, ADK12731.1, ADK12755.1, ADK12884.1, ADK12860, ADV58163.1, ADV58179.1, ADV58214.1, ADV58248.1, AFN61969.1
Rev	B	Thailand	PBMC	AAY25586.1, AAY25595.1
Rev	B	Netherlands	PBMC	AAC54638.1, AAX86718.1, AAX86727.1, AAX86736.1, AAX86745.1, AAX86754.1
Rev	B	Georgia	PBMC	ABB29356.1, ABB29372.1, ABB29381.1
Rev	B	Brazil	PBMC	ABC88305.1, ABC88332.1, ABC88341.1, ABC88350.1, ABV28252.1, ABV28261.1, ABV28279.1, ABV28314.1, ABV28322.1
Rev	B	Argentina	PBMC	ABD39391.1, ABD39415.1, ABD39424.1, ABD39433.1, ABD39442.1
Rev	B	Ukraine	PBMC	ABI15284.1, ABI15293.1, ABI15299.1
Rev	B	Denmark	PBMC	ABV00701.1, ABV00726.1, ABV00752.1, ABV00829.1, ABV00761.1
Rev	B	South Africa	PBMC	ACO50038.1
Rev	B	Jamaica	PBMC	ADG57017.1, ADG57037.1, ADG57046.1, ADG57055.1, ADG57072.1
Rev	B	Uruguay	PBMC	AEK79423.1, AEK79432.1, AEK79470.1, AFN61986.1
Rev	B	Paraguay	PBMC	AEK79316.1, AEK79350.1, AEK79379.1
Rev	B	France	PBMC	AAB59746.1, AAA76688.1, AFN61960.1, AFN61978.1
Rev	B	Bolivia	PBMC	AFN61994.1
Rev	B	Venezuela	PBMC	AFN62018.1
Rev	B	Germany	PBMC	AGF33006.1, AAA85235.1, AAA86251.1
Rev	B	Poland	PBMC	AGF33023.1
Rev	B	Gabon	PBMC	AAA83396.1
Rev	B	Thailand	Plasma	AFK29875.1, ABD04213.1, ABD04231.1, ABD04249.1, ABD04267.1, ABD04276.1, AEO17372.1, AEO17487.1, AEO17572.1, AEO17597.1, AEO17649.1

Rev	B	USA	Plasma	AAQ97574.1, AAQ97565.1, AAQ97439.1, AAQ97448.1, AAQ97457.1, ABA01385.1, ABA01394.1, ABA01403.1, ABF47476.1, ABF47503.1, ABI79730.1, ABI79822.1, ABI79883.1, ABI80032.1, ABI79970.1, AEO83673.1, AEO83917.1, AEO83973.1, AEO84140.1, AEO83856.1, AEO84286.1, AEO84595.1, AEO84968.1, ADE45298.1, AEM53589.1, AEM53680.1, AEW28119.1, AEW28146.1, AEW28300.1, AEW28284.1, AFM44210.1, AFM44004.1, AFZ62929.1, AFZ62946.1, AFZ62963.1, AFZ62970.1, AGG91781.1, AGG92737.1, AGG93279.1, AGH01881.1, AGH02326.1, AGG76737.1, AGG77315.1, AGG77421.1, AGG77668.1, AGG78172.1, AAD10894.1, AAD10942.1, AET75995.1, AET76294.1, AET76182.1, AET76346.1, AET76362.1, ADC81225.1, ADC81411.1, ADC81645.1, ADC81819.1, ADC82830.1, ADC83282.1, ACR52502.1, ACR52552.1, ACR52560.1, ACR52606.1, ACR51196.1, ACR51205.1, ACR51493.1, ACR51796.1, ACR52834.1, ACR52917.1, ACR52947.1, ACR53122.1, ABN42292.1, ABN42299.1
Rev	B	Canada	Plasma	AAQ86600.1, AAQ86616.1, AAQ86640.1, AAQ86656.1, AAQ86672.1
Rev	B	Japan	Plasma	BAG31020.1, BAG31038.1, BAG31065.1, BAG31092.1, BAG31119.1
Rev	B	South Africa	Plasma	ABF00915.1
Rev	B	Spain	Plasma	ACJ37125.1, ACY70073.1, ACY70082.1, ACY70132.1, ACZ82307.1, ACZ82325.1, ADO32844.1, ADO32862.1, ADO32879.1, ADO32888.1
Rev	B	UK	Plasma	ADK75272.1, ADK75285.1, ADK75312.1, ADK75330.1, ADK75336.1
Rev	B	China	Plasma	AEI88155.1, AEI88173.1, AFV34100.1, AFV34109.1, AFV34117.1, AGO06380.1
Rev	B	Brazil	Plasma	AEW28045.1, AEW28053.1, AEW28061.1
Rev	B	Poland	Plasma	AEW28308.1
Rev	B	Switzerland	Plasma	AFM43890.1, AFM43914.1, AFM43977.1, AFM44090.1, AFM44132.1
Rev	B	Germany	Plasma	AFM44049.1, AFM44067.1, AFM44156.1, AFM44192.1, AFM44140.1
Rev	B	France	Plasma	AFM44076.1
Rev	B	South Korea	Plasma	AFI39061.1, AAF35358.1, ABB96428.1, ABB96453.1, AFI39035.1
Rev	B	USA	Blood	AAA81041.1, AAQ86672.1, AAQ86664.1, AAQ86632.1, AAQ86600.1, AAQ86608.1
Rev	B	Australia	Peripheral blood	AAD03245.1
Rev	B	Russia	Blood	AAV90748.1, AAV30104.1, AAV30095.1, AAT76858.1
Rev	B	China	Blood	AAY57432.1, AAY57423.1, AAY57414.1
Rev	B	Japan	Blood	BAK41858.1, BAK41849.1, BAJ41156.1, BAJ41129.1, BAJ41102.1
Rev	B	Netherlands	Blood	AAC54647.1

(continued)

Table 24.5 (continued)

Name	Subtype	Location	Source	Accession numbers
Rev	B	Cyprus	Blood	AEB52443.1, AEB52482.1, AEB52499.1, AEB52530.1, AEB52605.1, AEB52898.1, AEB52733.1, AEB52799.1, AEB52850.1, AEB53020.1
Vif	B	Japan	PBMC	BAA20329.1, BAA20328.1, BAA20327.1, BAA20326.1, BAA20324.1, BAA20330.1, BAA20323.1, BAA20321.1, BAA20320.1, BAA20319.1, BAA20318.1, BAA20317.1, BAA20316.1, BAA20315.1, BAA20314.1, BAA20313.1, BAA20312.1, BAA20311.1, BAA20310.1, BAA20309.1, BAA20308.1, BAA20307.1, BAA20306.1, BAA20305.1, BAA20304.1, BAA20303.1, BAA20302.1, BAA20301.1, BAA20300.1, BAA20299.1, BAA93913.1, BAA93922.1, BAA93932.1, BAA93952.1, BAA93962.1, BAA93957.1
Vif	B	USA	PBMC	AAZ14768.1, AAZ14776.1, AAA81038.1, ACE78486.1, ABJ99505.1, ABJ99558.1, AAC02301.1, AAC02304.1, AAC02311.1, AAC02322.1, AAC02340.1, AAC02424.1, AAP12629.1, AAL65581.1, AGV32841.1, AGF32804.1, AAX33199.1, AAX33190.1, AAX33181.1, AAX33172.1, AAX33163.1, AAX33154.1, AAX33145.1, AAX33137.1, AAX33129.1, AAX33120.1, AAX33111.1, AAX33102.1, AAX33093.1, AAX33085.1, AAX33076.1, AAX33067.1, AAX33063.1, AAX33058.1, AAX33049.1, AAX33040.1, AAX33031.1, AAX33022.1, AAX33013.1, AAX33004.1, AAX32995.1, AAX32978.1, AAX32987.1, AAX32969.1, AAX32960.1, AAX32951.1, AAX32942.1, AAX32934.1, AAX32925.1
Vif	B	Australia	PBMC	AAG41565.1, AAG41567.1, AAD03192.1, AAD03226.1, AAD03242.1, ABI20196.1, ABI20313.1, AAV67934.1, AAV67920.1, AAV67927.1
Vif	B	China	PBMC	AAN83913.1, ABK42014.1, ACZ57350.1, ADE20143.1, ABL63500.1, AAC05239.1, AAY57429.1, AAY57420.1, AAY57411.1
Vif	B	Myanmar	PBMC	BAC77445.1, BAC77461.1, BAC77488.1, BAC77454.1, BAC77470.1, BAC77479.1, BAC77513.1
Vif	B	France	PBMC	ADL27536.1, ADL27580.1, AAB60573.1, AAD37871.1, AAD37910.1, AAD37902.1, AAD37885.1
Vif	B	South Korea	PBMC	AAX83061.1, AAF35357.1
Vif	B	Spain	PBMC	ACJ37074.1, ACJ37106.1, ADK12752.1, ADK12857.1, ADV58181.1, AAG16786.1, AAG16803.1, AAG16845.1
Vif	B	Thailand	PBMC	ABD04211.1, ABD04264.1, AAY25583.1, AAY25592.1
Vif	B	Netherlands	PBMC	AAC54635.1, AAX86715.1, AAX86733.1, AAX86751.1
Vif	B	Georgia	PBMC	ABB29350.1, ABB29366.1, ABB29375.1
Vif	B	Brazil	PBMC	ABV28249.1, ABV28311.1, ABC88302.1, ABC88329.1, ABC88338.1, ABC88347.1
Vif	B	Argentina	PBMC	AEG75748.1, AEG75755.1, ABD39385.1, ABD39412.1, ABD39421.1, ABD39439.1

Vif	B	Ukraine	PBMC	ABI15278.1, ABI15287.1, ABI15296.1
Vif	B	Denmark	PBMC	ABV00698.1, ABV00731.1, ABV00794.1, ABV00809.1
Vif	B	South Africa	PBMC	ACO50035.1
Vif	B	Jamaica	PBMC	ADG57015.1, ADG57034.1, ADG57053.1, ADG57069.1
Vif	B	Uruguay	PBMC	AFN61983.1, AEK79420.1, AEK79429.1, AEK79467.1
Vif	B	Paraguay	PBMC	AEK79313.1, AEK79347.1, AEK79376.1
Vif	B	Bolivia	PBMC	AFN61991.1
Vif	B	Venezuela	PBMC	AFN62015.1
Vif	B	Germany	PBMC	AAA86248.1, AGF33003.1
Vif	B	Poland	PBMC	AGF33020.1
Vif	B	Gabon	PBMC	AAA83393.1
Vif	B	USA	Plasma	ADZ32504.1, AAQ97571.1, AAQ97562.1, AAQ97535.1, AAQ97526.1, AAQ97508.1, AAQ97499.1, AAQ97490.1, AAQ97481.1, AAQ97472.1, AAQ97463.1, AAQ97454.1, AAQ97445.1, AAQ97436.1, AAQ97643.1, AFI49426.1, AFI49427.1, AFI49425.1, AFI49424.1, AFI49423.1, AFI49422.1, AFI49421.1, AFI49420.1, AFI49419.1, AFI49418.1, ACS91553.1, ACS91552.1, ACB21010.1, ACB21008.1, ACB21006.1, ACB21004.1, ACB21012.1, ACB20983.1, ACB20981.1, ACB20979.1, ACB20977.1, ACB20971.1, ADE45317.1, ADE45308.1, ADE45295.1, ADE45286.1, ADE45277.1, ABI80038.1, ABI80029.1, ABI80012.1, ABI79994.1, ABI79976.1, ABI79784.1, ABI79801.1, ABI79819.1, ABI79844.1, ABI79950.1, ACR53119.1, ACR53103.1, ACR53086.1, ACR53070.1, ACR53045.1, ACR52929.1, ACR52848.1, ACR52595.1, ACR52564.1, ACR52499.1, ACR52515.1, ACR52524.1, ACR52081.1, ACR52065.1, ACR52049.1, ACR52034.1, ACR51806.1, AET76359.1, AET76343.1, AET75992.1, AFZ62967.1, AAD10889.1, AAD10905.1, AAD10921.1, AAD10945.1, AAD10873.1, AAP74163.1, AAP74172.1, AAP74182.1, AAR37062.1, AAR37195.1, AAR37153.1, AAR37104.1, ABA01382.1, ABA01400.1, ABN42289.1, ABN42280.1, ABN42270.1, ABN42297.1, ACZ63322.1, ACZ63399.1, AEO83671.1, AEO84138.1, AEO84284.1, AEO84586.1, AEO84966.1, ADC55458.1, ADC55476.1, ADC81222.1, ADC81408.1, ADC81642.1, ADC81816.1, ADC82827.1, ADC83279.1, ADZ32517.1, ADZ32989.1, ADZ33593.1, ADZ33160.1, ADZ34023.1, ADZ36022.1, ADZ36178.1, ADF86598.1, ADF86692.1, ADF86937.1, ADF87034.1, ADF87137.1, AFB36900.1, AFB37386.1, AFB38609.1, AFB39649.1, AFB40301.1, AEM53586.1, AEM53677.1, AFM44001.1, AFM44597.1, AGG76588.1, AGG77312.1, AGG77418.1, AGG77832.1, AGG78169.1, AGG92353.1, AGG93011.1, AGH02323.1, ABF47473.1, ABF47500.1, AEW27566.1, AEW28116.1

(continued)

Table 24.5 (continued)

Name	Subtype	Location	Source	Accession numbers
Vif	B	Thailand	Plasma	AFK29872.1, AEO17369.1, AEO17646.1, AEO17569.1, AEO17484.1, AEO17560.1, AFU26517.1, AFU33286.1, AFU33247.1, AFU26584.1, AFU26619.1
Vif	B	Netherlands	Plasma	AGO97645.1, AGO97643.1, AGO97625.1, AGO97623.1, AGO97621.1, AGO97607.1
Vif	B	France	Plasma	ADD39714.1, ADD39713.1, ADD39715.1, ADD39712.1, ADD39711.1, ADD39710.1, ADD39709.1, ADD39708.1, ADD39707.1, ADD39706.1, ADD39624.1, ADD39625.1, ADD39622.1, ADD39615.1, ADD39614.1, ADD39608.1, ADD39623.1, ADD39567.1, ADD39565.1, ADD39563.1, ADD39561.1, ADD39559.1, AAD37864.1, AAD37913.1, AAD37906.1, AAD37869.1, AAD37889.1
Vif	B	Malaysia	Plasma	AEA77094.1
Vif	B	China	Plasma	AEI88153.1, AEI88170.1, AFV34097.1, AFV34106.1, AFV34114.1, AFM37030.1, AFM37042.1, AGO06377.1
Vif	B	Spain	Plasma	ADE34336.1, ADE34318.1, ACZ82303.1, ACZ82321.1, ADO32840.1, ADO32884.1, ACY70128.1, ACY70069.1, ACY70078.1, ACJ37121.1, ACJ37113.1, ACJ37106.1
Vif	B	South Korea	Plasma	ABB96416.1, ABB96443.1, AFA42873.1, AFA42863.1, AAK65987.1
Vif	B	Japan: Aichi, Nagoya	Plasma	BAG31017.1, BAG31035.1, BAG31062.1, BAG31107.1, BAG31116.1
Vif	B	Canada	Plasma	AAQ86597.1, AAQ86669.1, AAQ86653.1, AAQ86613.1, AAQ86637.1
Vif	B	Canada	Plasma	ADZ34855.1, ADZ34910.1, ADZ34934.1, ADZ34976.1, ADZ34990.1
Vif	B	South Africa	Plasma	ABF00912.1
Vif	B	Australia	Plasma	ADC55454.1, ADC55455.1, ADC55457.1, ADC55468.1, ADC55466.1
Vif	B	UK	Plasma	ADK75269.1, ADK75352.1, ADK76169.1, ADK76066.1, ADK75985.1
Vif	B	Peru	Plasma	ADZ32544.1, ADZ32585.1, ADZ32639.1, ADZ32728.1, ADZ32673.1
Vif	B	Brazil	Plasma	AEW27534.1, AEW27529.1, AEW28042.1, AEW28050.1, AEW28058.1
Vif	B	Poland	Plasma	AEW27696.1, AEW28305.1
Vif	B	Switzerland	Plasma	AFM43887.1, AFM44129.1, AFM44087.1, AFM43974.1, AFM43911.1
Vif	B	Switzerland	Plasma	AGL45727.1, AGL45762.1, AGL45918.1, AGL45953.1, AGL45939.1
Vif	B	Germany	Plasma	AFM44046.1, AFM44055.1, AFM44064.1, AFM44189.1, AFM44153.1
Vif	B	UK	Plasma	ADK75269.1, ADK75352.1, ADK76169.1, ADK76066.1, ADK75985.1
Vif	B	Peru	Plasma	ADZ32544.1, ADZ32585.1, ADZ32639.1, ADZ32728.1, ADZ32673.1

Vif	B	Brazil	Plasma	AEW27534.1, AEW27529.1, AEW28042.1, AEW28050.1, AEW28058.1
Vif	B	Poland	Plasma	AEW27696.1, AEW28305.1
Vif	B	Switzerland	Plasma	AFM43887.1, AFM44129.1, AFM44087.1, AFM43974.1, AFM43911.1
Vif	B	Switzerland	Plasma	AGL45727.1, AGL45762.1, AGL45918.1, AGL45953.1, AGL45939.1
Vif	B	Germany	Plasma	AFM44046.1, AFM44055.1, AFM44064.1, AFM44189.1, AFM44153.1
Vif	B	USA	Blood	AAA79672.1, AAA79666.1, AAA79660.1, AAA79678.1, AAA79653.1, AAA79646.1, AAA79631.1, AAA79624.1, AAA79593.1, AAA79587.1, AAA79580.1, AAA79574.1, AAA79567.1, AAA79560.1, AAA79553.1, AAA79546.1, AAA79539.1, AAA79533.1, AAA79527.1, AAA79520.1, AAQ86669.1, AAQ86661.1, AAQ86653.1, AAQ86645.1, AAQ86637.1, AAQ86629.1, AAQ86621.1, AAQ86613.1, AAQ86605.1, AAQ86597.1
Vif	B	Russia	Blood	AAV30101.1, AAV30092.1, AAV90746.1, AAT76860.1
Vif	B	Japan	Blood	BAK41846.1, BAK41855.1, BAJ41162.1, BAJ41144.1, BAJ41135.1, BAJ41126.1, BAJ41117.1, BAJ41153.1, BAJ41108.1, BAJ41099.1
Vif	B	Cyprus	Blood	AEB52438.1, AEB52477.1, AEB52494.1, AEB52524.1, AEB52601.1, AEB52729.1, AEB52793.1, AEB52844.1, AEB52877.1, AEB53023.1
Vif	B	Netherlands	Blood	AAC54644.1
Vpr	B	Australia	PBMC	AAB70162.1, AB70161.1, AAB70157.1, AAB70163.1, AAB70130.1, AAB70132.1, AAB70135.1, AAB70142.1, AAB70149.1, AAB70122.1, AAG32142.1, AAG38456.1, AAG38458.1, AAG32144.1, AAD37206.1, AAD37206.1, AAD37219.1, AAD37229.1, AAD37229.1, ABI20197.1, ABI20314.1, AAD03243.1
Vpr	B	Australia	Blood	AAD03227.1, AAD03227.1, AAD03243.1, AAD03219.1, AAD03235.1, AAV67935.1, AAV67928.1, AAV67921.1
Vpr	B	China	PBMC	AAT09644.1, ABK42015.1, ABL63501.1, ABX57673.1, ABX57678.1, ABX57686.1, ABX57682.1, ACZ57351.1, ADE20144.1, AAN83914.1, AGF32997.1, AAC05237.1,
Vpr	B	USA	PBMC	ACX34482.1, ACX34473.1, AFJ32702.1, AFJ32689.1, ABJ99506.1, ABJ99559.1, ACE78461.1, ACE78487.1, AAX33200.1, AAX33182.1, AAX33138.1, AAX33086.1, AAX33059.1, AAX33050.1, AAX33032.1, AAX33005.1, AAX32961.1, AAX32926.1, AAP12630.1, AAA44855.1, AGF32781.1, AGV32842.1, AGV33421.1, AAA81039.1

(continued)

Table 24.5 (continued)

Name	Subtype	Location	Source	Accession numbers
Vpr	B	Canada	PBMC	AAW88726.1, AAW88729.1, AAW88732.1, AAW88737.1, AAW88742.1, AAW88752.1, AAW88757.1, AAW88760.1
Vpr	B	Myanmar	PBMC	BAC77446.1, BAC77462.1, BAC77489.1, BAC77455.1, BAC77471.1, BAC77480.1, BAC77514.1
Vpr	B	Japan	PBMC	BAA93963.1, BAA93965.1, BAA93967.1, BAA93971.1, BAA93974.1, BAA93976.1, BAA93980.1, BAA93982.1, BAA93987.1, BAA93995.1
Vpr	B	South Korea	PBMC	AAF35360.1, AAX83062.1, ABB96417.1, ABB96426.1, ABB96435.1, ABB96444.1, ABB96451.1
Vpr	B	Spain	PBMC	ADV58182.1, ADV58251.1, AAR24626.1, AAR24629.1, AAR24633.1, AAR24633.1, AAR24643.1, ACJ37075.1, ACJ37107.1, ACJ37098.1, ADK12753.1, ADK12908.1, AAG16787.1, AAG16804.1, AAG16830.1, AAG16846.1, AAG16821.1, AFN61967.1
Vpr	B	India	PBMC	AAK11283.1, AAK11284.1, AAK11285.1, AAK11286.1, AAK11287.1, AAK11288.1, AAK11289.1, AAK11295.1, AAK11294.1, AAK11292.1
Vpr	B	Thailand	PBMC	AAY25584.1, ABD04212.1, ABD04229.1, ABD04247.1, ABD04265.1, ABD04274.1
Vpr	B	Netherlands	PBMC	AAX86716.1, AAX86734.1, AAX86734.1, AAX86743.1, AAX86752.1, AAC54636.1,
Vpr	B	Georgia	PBMC	ABB29351.1, ABB29367.1, ABB29376.1
Vpr	B	Brazil	PBMC	ABV28250.1, ABV28312.1, ABV28320.1, ABV28277.1, ABC88303.1, ABC88330.1, ABC88339.1, ABC88348.1
Vpr	B	Argentina	PBMC	ABD39386.1, ABD39413.1, ABD39422.1, ABD39440.1, ABD39431.1
Vpr	B	Ukraine	PBMC	ABI15279.1, ABI15288.1, ABI15297.1
Vpr	B	Denmark	PBMC	ABV00699.1, ABV00715.1, ABV00732.1, ABV00750.1, ABV00795.1, ABV00827.1
Vpr	B	South Africa	PBMC	ACO50036.1
Vpr	B	France	PBMC	AFN61959.1, AFN61976.1, AAB59749.1, ACX54861.1, ACX54866.1, ACX54863.1, ACX54868.1, ACX54869.1, AAB60574.1
Vpr	B	Jamaica	PBMC	ADG57020.1, ADG57035.1, ADG57054.1, ADG57070.1, ADG57044.1
Vpr	B	Uruguay	PBMC	AEK79421.1, AEK79430.1, AEK79468.1, AFN61984.1
Vpr	B	Paraguay	PBMC	AEK79314.1, AEK79348.1, AEK79377.1
Vpr	B	Bolivia	PBMC	AFN61992.1
Vpr	B	Venezuela	PBMC	AFN62016.1

Vpr	B	Germany	PBMC	AGF33004.1, AAA85233.1, AAA86249.1
Vpr	B	Poland	PBMC	AGF33021.1
Vpr	B	Gabon	PBMC	AAA83394.1
Vpr	B	China	Blood	ABX57685.1, ABX57685.1, ABX57684.1, ABX57679.1, ABX57686.1, ABX57678.1, ABX57677.1, ABX57676.1, ABX57675.1, ABX57672.1, ABX57673.1, AFM37031.1, AFM37043.1, AEI88154.1, AEI88171.1, AFV34098.1, AFV34107.1, AFV34115.1, AGO06378.1,
Vpr	B	Australia	Plasma	AAB70162.1, AB70161.1, AAB70157.1, AAB70163.1, AAB70130.1, AAB70132.1, AAB70135.1, AAB70142.1, AAB70149.1, AAB70122.1, ADC55477.1, ADC55478.1, ADC55479.1, ADC55480.1, ADC55491.1
Vpr	B	Thailand	Plasma	AFK29873.1, AEO17370.1, AEO17485.1, AEO17561.1, AEO17570.1, AEO17647.1, AEO17595.1, AFU26518.1, AFU26585.1, AFU26620.1, AFU33248.1, AFU33287.1
Vpr	B	France	Plasma	AET05977.1, AET05976.1, AET05993.1, AET05974.1, AET05970.1, AET05966.1, AET05956.1, AET05851.1, AET05859.1, AET05866.1, AET05878.1, AET05894.1, AET05974.1
Vpr	B	South Africa	Plasma	ACH89969.1, ACH89970.1, ACH89971.1, ABF00913.1
Vpr	B	Netherlands	Plasma	AGO97646.1, AGO97644.1, AGO97626.1, AGO97624.1, AGO97622.1, AGO97608.1
Vpr	B	USA	Plasma	ACS91555.1, ACS91554.1, AAQ97572.1, AAQ97536.1, AAQ97563.1, AAQ97509.1, ACB36868.1, ACB36871.1, ACB36878.1, ACB36885.1, ACB36894.1, ACB36908.1, ADE45318.1, ADE45309.1, ADE45296.1, ADE45287.1, ADE45278.1, ABI80030.1, ABI79995.1, ABI79951.1, ABI79820.1, AAD10922.1, AAD10906.1, ABN42302.1, ABN42290.1, ABN42281.1, ABN42271.1, AAR37063.1, AAR37105.1, AAR37154.1, AAR37196.1, ABA01383.1, ABA01401.1, ACZ63323.1, ACZ63400.1, ADG34206.1, ADG34220.1, AEO83672.1, AEO84139.1, AEO84285.1, AEO84594.1, AEO84967.1, ABF47474.1, ABF47501.1, ADF85023.1, ADF85193.1, ADF85088.1, ADF85288.1, ADF85416.1, ADF85528.1, ADZ32518.1, ADZ32859.1, ADZ33131.1, ADZ33617.1, ADZ34384.1, ADZ35140.1, ADZ35460.1, ADZ35734.1, AFB36447.1, AFB37387.1, AFB38506.1, AFB39650.1, AFB40302.1, AEM53587.1, AEM53678.1, AFM44220.1, AFM44138.1, AFM44002.1, AFM44598.1, AFJ32685.1, AFJ32705.1, AAD10898.1, AAD10946.1, ADC81223.1, ADC81409.1, ADC81643.1, ADC81817.1, ADC82828.1, ADC83280.1, AGG92028.1, AGG92354.1, AGG93012.1, AGH02120.1, AGH02324.1, AGG76735.1, AGG77313.1, AGG77419.1, AGG77666.1, AGG78170.1, ADC55482.1, ADC55488.1, ADC55499.1, ADC55492.1, AET75993.1, AET76118.1, AET76180.1, AET76344.1, AET76360.1, AEW27587.1, AEW28145.1, AEW28179.1, ACR53120.1, ACR53087.1, ACR52604.1, ACR52596.1, ACR52050.1, ACR52066.1

(continued)

Table 24.5 (continued)

Name	Subtype	Location	Source	Accession numbers
Vpr	B	South Korea	Plasma	AFA42874.1, AFI39059.1, AAF35360.1, ABB96426.1, ABB96451.1, AFI39033.1
Vpr	B	Malaysia	Plasma	AEA77095.1
Vpr	B	Spain	Plasma	ADE34328.1, ADE34337.1, ADE34319.1, ACZ82322.1, ACZ82313.1, ADO32841.1, ADO32859.1, ADO32876.1, ADO32885.1, ACY70079.1, ACY70070.1, ACY70129.1, ACJ37122.1, ACJ37114.1, ACJ37107.1
Vpr	B	Japan	Plasma	BAG31018.1, BAG31036.1, BAG31063.1, BAG31117.1, BAG31108.1
Vpr	B	Canada	Plasma	AAQ86598.1, AAQ86614.1, AAQ86638.1, AAQ86654.1, AAQ86670.1, ADZ34856.1, ADZ34911.1, ADZ34935.1, ADZ34977.1, ADZ34991.1
Vpr	B	UK	Plasma	ADK75270.1, ADK75303.1, ADK75329.1, ADK75353.1, ADK75907.1, ADK75922.1, ADK75818.1, ADK75830.1
Vpr	B	Peru	Plasma	ADZ32545.1, ADZ32586.1, ADZ32640.1, ADZ32674.1, ADZ32729.1, ADZ32716.1, ADZ33833.1, ADZ33868.1, ADZ33919.1, ADZ33973.1, ADZ33994.1
Vpr	B	Brazil	Plasma	AEW27530.1, AEW27535.1, AEW28043.1, AEW28051.1, AEW28059.1
Vpr	B	Poland	Plasma	AEW27697.1, AEW28306.1
Vpr	B	Switzerland	Plasma	AFM43888.1, AFM43912.1, AFM43975.1, AFM44088.1, AFM44130.1, AGL45728.1, AGL45763.1, AGL45919.1, AGL45954.1, AGL45884.1
Vpr	B	Germany	Plasma	AFM44047.1, AFM44056.1, AFM44065.1, AFM44138.1, AFM44199.1
Vpr	B	USA	Blood	AAA79632.1, AAA79594.1, AAA79540.1, AAA79679.1, AAA79618.1, AAA79528.1, AAA79521.1, AAA79534.1, AAQ86670.1, AAQ86654.1, AAQ86614.1, AAQ97644.1, AAQ86662.1
Vpr	B	Russia	Blood	AAV30102.1, ABD61772.1, AAV30093.1, AAV30093.1
Vpr	B	China	Blood	AAY57430.1, AAY57421.1, AAY57412.1, ABX57685.1, ABX57685.1, ABX57684.1, ABX57679.1, ABX57686.1, ABX57678.1, ABX57677.1, ABX57676.1, ABX57675.1, ABX57672.1, ABX57673.1
Vpr	B	Japan	Blood	BAK41856.1, BAK41847.1, BAJ41163.1, BAJ41154.1, BAJ41145.1, BAJ41136.1, BAJ41127.1, BAJ41100.1
Vpr	B	Cyprus	Blood	AEB52439.1, AEB52478.1, AEB52495.1, AEB52525.1, AEB52602.1, AEB53024.1, AEB52961.1, AEB52878.1, AEB52845.1, AEB52794.1
Vpu	B	Japan	PBMC	BAA94002.1, BAA94004.1, BAA94008.1, BAA94019.1, BAA94014.1, BAD20651.1, BAD20653.1, BAD20655.1
Vpu	B	Myanmar	PBMC	BAC77492.1, BAC77517.1, BAC77492.1, BAC77474.1, BAC77465.1, BAC77457.1

Vpu	B	Australia	PBMC	AAG32133.1, AAG32134.1, AAG32136.1, AAG32137.1, ABI20200.1, ABI20317.1, AAD03196.1, AAD03222.1, AAD03230.1, AAD03238.1, AAC54549.1, AAV67938.1, AAV67931.1, AAV67924.1
Vpu	B	South Korea	PBMC	ABB96420.1, ABB96454.1, ABB96438.1, ABB96429.1, ABB96447.1, AAF35359.1, AAX83063.1
Vpu	B	Spain	PBMC	ACJ37076.1, ACJ37099.1, ACJ37108.1, ADK12911.1, ADK12885.1, ADV58183.1, ADV58252.1, AAG16790.1, AAG16807.1, AAG16824.1, AAG16833.1, AAG16847.1, AFN61970.1
Vpu	B	Argentina	PBMC	ABD39387.1, ABD39443.1, ABD39425.1, ABY49095.1, AAN77658.1, AAN77660.1, AAN77688.1, AAN77686.1, AAN77673.1
Vpu	B	China	PBMC	ACZ57354.1, ADE20147.1, ADE20156.1, ADE20165.1
Vpu	B	China	PBMC	AAN83917.1, ABK42018.1, AFN62011.1, AAC05240.1, AAT09646.1, AAN83917.1
Vpu	B	USA	PBMC	AFN62003.1, AAP12633.1, ACE78464.1, ACE78490.1, ACX34391.1, ACX34426.1, ACX34456.1, ACX34483.1, ABJ99509.1, ABJ99562.1, ABJ99535.1, AAA79524.1, AAC32299.1, AGV32845.1, AGV33424.1, AAX33201.1, AAX33194.1, AAX33176.1, AAX33149.1, AAX32982.1, AAX32929.1
Vpu	B	Thailand	PBMC	ABD04215.1, ABD04277.1, ABD04250.1, AAY25587.1, AAY25596.1
Vpu	B	Netherlands	PBMC	AAX86719.1, AAX86728.1, AAX86737.1, AAX86746.1, AAX86746.1
Vpu	B	Georgia	PBMC	ABB29352.1, ABB29368.1, ABB29377.1
Vpu	B	Brazil	PBMC	ABC88306.1, ABC88333.1, ABC88342.1, ABC88351.1, ABV28253.1, ABV28280.1, ABV28315.1, ABV28323.1
Vpu	B	Ukraine	PBMC	ABI15280.1, ABI15289.1, ABI15300.1
Vpu	B	Denmark	PBMC	ABV00702.1, ABV00718.1, ABV00797.1, ABV00830.1, ABV00813.1, ABV00780.1
Vpu	B	South Africa	PBMC	ACO50039.1
Vpu	B	Jamaica	PBMC	ADG57021.1, ADG57038.1, ADG57038.1, ADG57056.1, ADG57073.1
Vpu	B	Uruguay	PBMC	AEK79424.1, AEK79433.1, AEK79471.1, AFN61987.1
Vpu	B	Paraguay	PBMC	AEK79351.1, AEK79380.1
Vpu	B	France	PBMC	AAB59750.1, AFN61961.1, AFN61979.1, AAB60577.1, AAA76689.1
Vpu	B	Bolivia	PBMC	AFN61995.1
Vpu	B	Venezuela	PBMC	AFN62019.1
Vpu	B	Germany	PBMC	AGF33007.1
Vpu	B	Germany	PBMC	AAA85236.1

(continued)

Table 24.5 (continued)

Name	Subtype	Location	Source	Accession numbers
Vpu	B	Thailand	Plasma	AFK29876.1, AFU33288.1, AFU26621.1, AFU28486.1, AFU26519.1, AFU26498.1, AEO17650.1, AEO17573.1, AEO17564.1, AEO17488.1, AEO17373.1,
Vpu	B	USA	Plasma	AEM53681.1, AEM53590.1, AAQ97575.1, AAQ97566.1, AAQ97539.1, AAQ97512.1, AAQ97469.1, AFM44601.1, AFM44223.1, AFM44211.1, AFM44005.1, AFZ63359.1, AFZ63359.1, AGH02327.1, AGH01882.1, AGG93015.1, AGG92031.1, AAP74166.1, AAP74176.1, AAP74186.1, AAR37064.1, AAR37106.1, AAR37155.1, AAR37197.1, AAR37127.1
Vpu	B	USA	Plasma	ABA01386.1, ABA01404.1, ABA01395.1, ABF47477.1, ABF47504.1, ABI79731.1, ABI79823.1, ABI79971.1, ABI80033.1, ABI79463.1, ABI79640.1, ABI79722.1, ABI79648.1, ABN42293.1, ABN42300.1, ACZ63403.1, ACZ63326.1, ACS91556.1, ACS91557.1, ACB29452.1, ACB29483.1, ACB29485.1, ACB29491.1, ACB29477.1, AFB40305.1, AFB38613.1, AFB37907.1, AFB36904.1, AFB36450.1, ADC55437.1, ADC55453.1, ADC81226.1, ADC81412.1, ADC81646.1, ADC81820.1, ADC82831.1, ADC83283.1, ADZ36180.1, ADZ36098.1, ADZ36024.1, ADZ35002.1, ADZ35141.1, ADE45299.1, AEW28181.1, AEW28120.1, ADF84500.1, ADF84634.1, ADF84534.1, ADF84534.1, ADF84886.1, ADF85016.1, AGG78173.1, AGG77669.1, AGG77422.1, AGG77316.1, AGG76932.1, AET76363.1, AET76347.1, AET76121.1, AEO83603.1, AEO84287.1, AEO84141.1, AEO84526.1, AEO84969.1, ACE65684.1, ACE66019.1, ACE66040.1, ACE66265.1, ACE66299.1, ACE66365.1, ACE66717.1, ACE66848.1, ACE66897.1, ACE67009.1, ACE67072.1, ACE67288.1, ACE67302.1, ACD31788.1, ACD31880.1, ACD31919.1, ACD31969.1, ACD32347.1,
Vpu	B	USA: Wilmington, NC	Plasma	ACD32458.1, ACD32530.1, ACD41535.2, ACE65536.1, ACE68292.2, ACR51128.1, ACR51206.1, ACR52246.1, ACR52503.1, ACR52607.1, ACR52835.1, ACR52948.1, ACR53123.1
Vpu	B	South Korea	PBMC	ABB96420.1, ABB96454.1, ABB96438.1, ABB96429.1, ABB96447.1
Vpu	B	Japan	Plasma	BAD20652.1, BAD20654.1, BAD20656.1, BAD20658.1, BAD20662.1, BAG31021.1, BAG31039.1, BAG31066.1, BAG31111.1, BAG31120.1
Vpu	B	South Africa	Plasma	ABF00916.1
Vpu	B	Argentina	plasma	ABM67798.1, ABM67811.1, ABM67828.1, ABM67852.1, ABM67878.1, ABM67995.1, ABM67984.1, ABM67952.1, ABM67903.1, ABM67918.1
Vpu	B	Spain	Plasma	ACJ37115.1, ACJ37123.1, ACY70071.1, ACY70080.1, ACY70130.1, ACZ82305.1, ACZ82323.1, ADE34320.1, ADE34338.1, ADO32842.1, ADO32860.1, ADO32877.1, ADO32886.1

Vpu	B	Australia	Plasma	ADC55433.1, ADC55434.1, ADC55435.1, ADC55436.1
Vpu	B	China	Plasma	AGO06381.1, AGF43623.1, AGF43728.1, AGF43743.1, AGF43748.1, AGF43748.1, AEI88156.1, AEI88174.1
Vpu	B	Switzerland	Plasma	AGL45955.1, AGL45941.1, AGL45920.1, AGL45885.1, AGL45764.1, AGL45729.1, AFM44133.1, AFM44091.1, AFM43891.1, AFM43915.1, AFM43978.1
Vpu	B	Germany	Plasma	AFD62919.1, AFD62928.1, AFD62937.1, AFD62937.1, AFD62955.1
Vpu	B	Poland	Plasma	AEW28309.1
Vpu	B	Brazil	Plasma	AEW28046.1, AEW28054.1, AEW28062.1
Vpu	B	Sweden	Plasma	AEO22539.1, AEO22464.1, AEO22197.1, AEO22302.1, AEO22347.1
Vpu	B	Canada	Plasma	ADZ34992.1, ADZ34978.1, ADZ34936.1, ADZ34912.1, ADZ34857.1
Vpu	B	Peru	Plasma	ADZ33995.1, ADZ33974.1, ADZ33920.1, ADZ33834.1, ADZ33733.1, ADZ33618.1
Vpu	B	UK	Plasma	ADK75355.1, ADK75331.1, ADK75305.1, ADK75273.1, ADK75337.1
Vpu	B	Cyprus	Blood	AEB52479.1, AEB52496.1, AEB52526.1, AEB52603.1, AEB52795.1, AEB52846.1, AEB52879.1, AEB53025.1, AEB53016.1, AEB52440.1
Vpu	B	USA	Blood	AAA79530.1, AAA79550.1, AAA79643.1, AAA79621.1, AAA79657.1
Vpu	B	Netherlands	Blood	AAC54648.1
Vpu	B	USA	Blood	AAA79597.1, AAA79597.1, AAA79584.1, AAA79571.1, AAA79550.1, AAA79530.1, AAA79543.1, AAA79564.1, AAA79591.1, AAA79557.1, AAA81042.1, BAK41859.1, BAK41850.1, BAJ41166.1, BAJ41157.1, BAJ41148.1, BAJ41148.1, BAJ41139.1, BAJ41130.1, BAJ41121.1, BAJ41103.1
Vpu	B	Australia	Blood	AAD03246.1, AAD03230.1, AAV67938.1, AAV67931.1, AAV67924.1, AAD03196.1
Vpu	B	Russia	Blood	AAV90749.1, AAV30105.1, AAV30096.1, AAT76862.1

2.1.2 Capsid Protein (CA)

Among Gag proteins, the p24 polypeptide region or the capsid (CA) is the largest (~200–270 amino acids). The 20-amino-acid-long "major homology region" (MHR) of CA segment is highly conserved in the Gag protein for blood and brain HIV-1 B isolates. CA forms the conical core (Fig. 24.1) surround the viral RNA and delivers into the cell during infection. Unlike the majority of known CA proteins from diverse icosahedral viruses, which are characterized by eight β strands, CA in HIV-1 is helical, as predicted earlier from biochemical data [32]. The amino terminus itself is buried in the molecule. This implies a conformational change upon cleavage by PR, since the MA-CA junction must be stretched out to be readily accessible to viral protease [33].

The Cyclophilin A (CyPA) binding region of p24 is crucial for the incorporation of HIV particles [34, 35], it is conserved in both blood and brain derived HIV-1B isolates. A drug such as cyclosporine A disrupts the interaction between Gag and Cyclophilin A, and hence inhibits viral replication [36]. The 3D structure of the HIV-1 CA is being explored that may provide insight into its functions. A crystal structure of the hexameric building block fragment is available in PDB; entry 3h47 [37]. From the analyzed dataset, the brain derived HIV-1 p24 region showed only few variations than the blood derived sequences. This could be due to the lack of brain derived sequences. However, no variations were observed in the annotated MHR, CyPA, and Zinc binding regions of both brain and blood isolates.

2.1.3 Nucleocapsid (NC) Protein

The NC region of Gag is responsible for specifically recognizing the packaging signal of HIV [38]. The nucleocapsid (NC) protein (p7) is a small basic protein, typically about 60–90 amino acid residues long, which forms a stable complex with the viral RNA and protect it. The signal for packaging is coded in four stem loop structures located near the 5′ end of the viral RNA. This mediates the incorporation of a heterologous RNA into HIV-1 virions [39]. NC has two characteristic zinc finger motifs made up of conserved cysteines and histidines. The retroviral C-H motif has the structure CX2CX4 HX4C, (CCHC motif, in short) where "X" is not conserved either among retroviruses or between the two motifs of a single NC [33]. Two characteristic zinc finger motifs were observed near the clusters of arginine, asparagine, and glutamine residues of blood and brain derived HIV-1 B Gag sequences. Deletions or major alterations in the motif may result in the absence of RNA in virions or alter specificity during RNA packaging. NC also contains sequences that are stretches of basic residues, which act as "assembly domains" [27] and are required for the assembly or budding of virions. In HIV-1, the zinc finger motifs and series of basic residues are important for in vivo packaging [40]. The NC structure of HIV-1 is been worked out by De Guzman et al. (PDB entry 1alt) [41].

2.1.4 p6 Protein

Polypeptide (p6) of HIV-1 comprises 52 amino acids, cleaved from the Gag protein downstream from NC. The p6 polypeptide region mediates interactions between p55 Gag and the accessory protein Vpr and involved in the incorporation of Vpr into assembling virions [42]. The conserved segment is located near the N-terminal domain of p6 that are rich in proline residues and involved in Vpr binding. The C-terminal region of p6 contains a late domain that is important for the efficient release of budding virions from an infected cell [43, 44]. It is largely unstructured due to proline and currently no crystal structure is available in the PDB [25]. The Vpr binding sites of the blood derived sequences showed slight variation than the brain derived HIV-1 sequence. These variations could not be confirmed presently due to the lack of brain derived sequences.

2.1.5 Other Gag Proteins and Peptides

In addition to the proteins discussed above, many retroviral gag genes encode polypeptide segments that lie between MA and CA, between CA and NC, and/or downstream from NC. In most cases, the functions of these segments are poorly understood.

- p10
  p10 is a proline- and glycine-rich protein. It is preceded by a 22-amino-acid segment often referred as p2 that is processed into two smaller peptides, p2a and p2b [45]. p2b contains the sequence PPPY that is found approximately 150 amino acid residues from the amino terminus and conserved in large number of retroviral Gag proteins. Mutation in this motif results in blockage of budding and a defect in assembly.
- Spacer peptide (SP)
  SP is a short segment of 14 amino acids that separates the carboxyl terminus of CA and the amino terminus of NC. The spacer itself may undergo additional cleavage during processing of the Gag protein. During morphogenesis of HIV-1, the SP-NC site is the first site to be cleaved, whereas the CA-SP sites are cleaved later. Deletion at this site may result in loss of infectivity and anomalies during budding [46, 47].

2.2 *Envelope Proteins*

Like other animal viruses that carry a lipid envelope, the surface of retroviral virions is highly decorated with “nailhead like glycoproteins” (envelope or Env proteins). The spikes formed by these proteins contain carbohydrates, which makes them difficult to recognize by antibodies. Retroviruses contain a couple of Env proteins, the

surface (SU, representing gp120) and transmembrane (TM, representing gp41). The carbohydrates depleted structures are showed in Fig. 24.1 PDB entries SU (1g9m) and TM (2ezo). The synthesis of SU and TM and their incorporation into the virion are quite different from those of Gag and Pol.

Like cellular proteins, the nascent Env polypeptide is synthesized in the endoplasmic reticulum then it migrates through the Golgi complex, undergoes glycosylation at the consensus asparagine residue N-X-S/T with the addition of 25–30 complex *N*-linked carbohydrate side chains that are added. It is important for pathogenesis [48]. In the analyzed dataset, the NDT residues of blood derived Env sequences are found to be variable in V1 glycosylation site compared to brain isolates. Env is cleaved in the Golgi by a cellular protease, either furin or a related enzyme, to yield the mature SU and TM that are found in virions. The Env protein is the primary determinant of the cell type. Since all strains of HIV and SIV recognize the same CD4 receptor, in the analyzed dataset, no differences were found in the CD4 regions of blood and brain derived Env sequences of HIV-1 (Fig. 24.2). Nevertheless, major differences in amino acid sequence exist among diverse isolates of these viruses. These differences are largely due to several variable regions, denoted v1–v4, which form loops that are extend outward from the central domain of SU [33]. From the dataset, the V1 region is variable in blood derived Env HIV-1 sequences. No much variation was observed in the brain and blood derivatives of V2, and V3 containing CD4 regions. Except the start and stop regions, V4 is hypervariable in blood derivatives, but no variation in CD4 and glycosylation pattern of V4, as expected. However, no variation was observed in the V5 segment containing immuno-dominant region and fusion peptide of both brain and blood derivatives. These sequence variation may reflect mutations to escape from the immune system as well as in vivo variation in biological properties, such as tropism for macrophages or other cell types or ability to form syncytia [33].

2.3 Viral Enzymes

HIV carries three enzymes, reverse transcriptase (RT), integrase (IN), and protease (PR). RT also contains an additional enzymatic activity, RNase H that is mapped to a contiguous portion of the polypeptide. All these proteins are produced from Gag-Pol fusion protein [49]. During viral maturation, the virally encoded protease cleaves the Pol polypeptide from Gag. Further digestion separates the protease (p10), RT (p50), RNase H (p15), and integrase (p31) activities. These cleavages are not efficient. This is evident from the RT which is linked to RNase H as a single polypeptide (p65). Since RT, IN, and PR are essential for viral replication and have characteristics that distinguish them from related cellular enzymes, they all have become targets for drug intervention in AIDS.

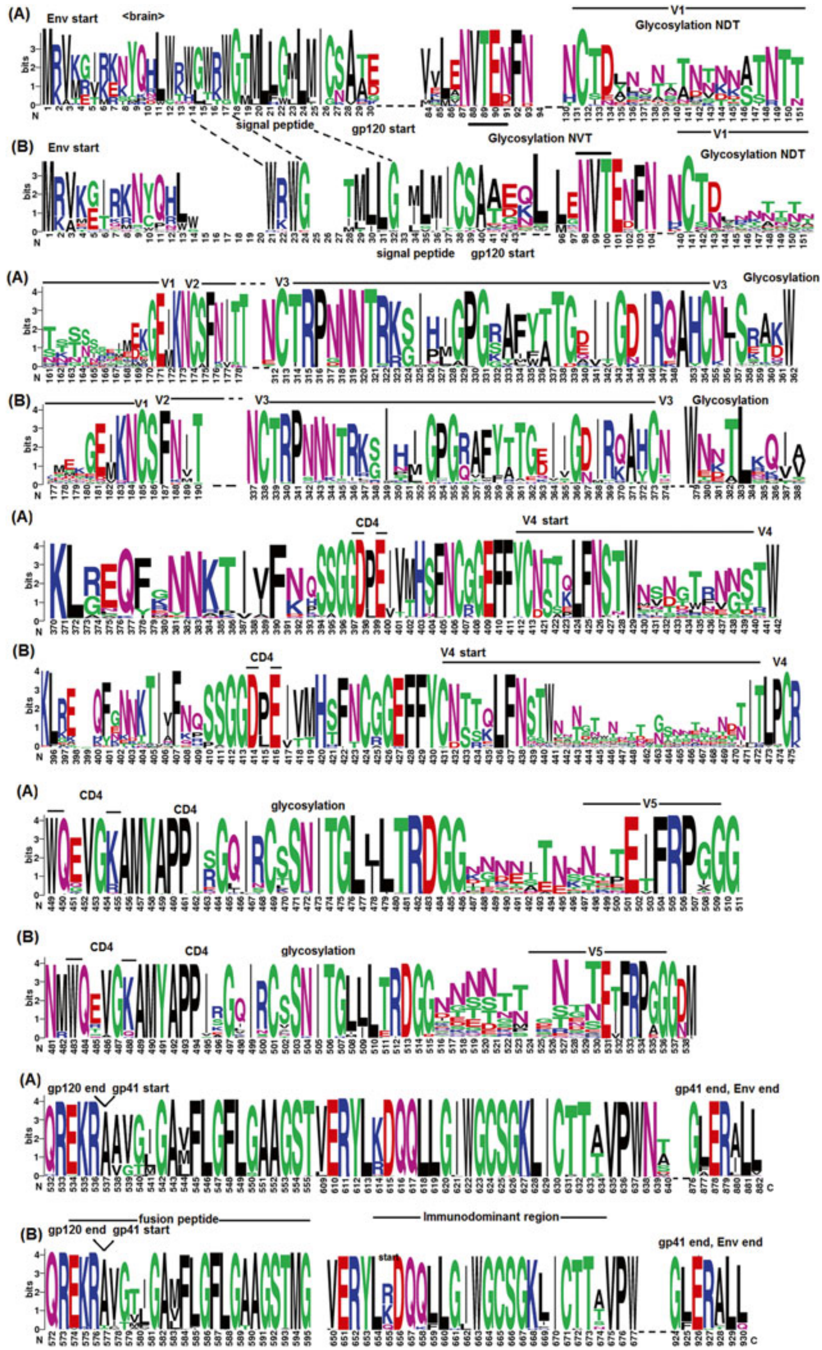

Fig. 24.2 Env proteins derived from brain (**a**) and blood (**b**) show sequence conservations (*tall characters*), semi-conserved substitutions (*stacked with similar colored characters*) and variations (*stacked with different colors*). The annotation is based on the benchmark HXB2 sequence (accession K03455) provided in HIV Sequence Compendium 2013 [89]

2.3.1 Reverse Transcriptase (RT)

The larger (p66) polypeptide of the HIV-1 contains both pol and RNase H domains but not IN. The smaller (p51) subunit is missing the complete RNase H domain. Biochemical studies show that p51 does not have an active role in catalysis [50], but it does have a role in the overall structure of the enzyme, consistent with the 3D structure of HIV-1 RT (PDB entry 1hys), which shows important differences in folding of the two subunits. The sequences of RT is conserved and used extensively to construct phylogenetic trees for retroviruses [51]. However, the subunit structure of RTs differs among viruses of different genera. The main function of RT is to build a DNA copy of the viral RNA genome, which is required to build new viruses. It builds a DNA strand from the viral RNA, then destroys it and builds a second DNA strand. Many of the drugs currently used to fight against HIV infection targets reverse transcriptase.

2.3.2 Integrase (IN)

Integrase (IN) takes the DNA copy of the viral genome and inserts it into the infected cellular genome and become dormant in cells for decades [25]. The best studied integrases are those of HIV-1, MLV, and ASLV. This enzyme was first identified as an endonuclease in ASLV virions [52]. Genetic analysis revealed the importance of IN domain for integrating viral DNA [53]. Purified IN has the ability to recognize the ends of the newly synthesized linear double-stranded viral DNA, to remove two nucleotides from the 3′ end of each strand, and to join this DNA end to a target DNA at random sites. Among the retroviral genera, the amino acid sequence of IN is less conserved than RT, except the conserved D-D-35-E motif that forms the active site and overall 3D structure (PDB entry 1ex4). Anti-HIV drugs that block Integrase have been developed.

2.3.3 Protease (PR)

Viral encoded proteases are typically involved in processing of the translated products and also in the maturation of the viral particle. The proteins in HIV are synthesized as long polyproteins, which are further cleaved into proper functional units by HIV PR. The 3D structure of HIV-1 PRs inferred from crystallography PDB entry 1hpv [54] shows that retroviral proteases are homodimers, the catalytic site is at the interface of each subunit. Hence, dimerization is crucial for enzymatic activity as well as for the virion formation. It is also involved in regulation of proteolysis of Gag and Pol proteins. Premature activation of PR in the infected cell leads to premature cleavage of Gag, thus aborting the assembly process [55]. PRs recognize stretches of amino acids, preferably hydrophobic sequences about 7–8 residues in length. The specificity of different PRs has been studied based on Gag and Pol cleavage sites and by experimental verification of synthetic peptides [56, 57].

From such studies, it is generalized that the cleavage between tyrosine and proline is common and efficient, whereas it is rare after isoleucine or valine [33]. Protease inhibitors are widely used as anti-HIV drugs, often in combination with other drugs that block reverse transcriptase and integrase.

2.4 Regulatory Proteins

2.4.1 Regulator of Virion (Rev)

Rev is a 13-kD sequence-specific RNA binding protein [58]. It induces the transition from the early to the late phase of HIV gene expression [59] Rev is encoded by two exons and accumulates within the nuclei and nucleoli of the infected cells. Rev binds to a 240-base region of complex RNA secondary structure, called the Rev response element (RRE) that lies within the second intron of HIV [60]. The binding of Rev to the RRE facilitates the regulation of splicing and transport of viral RNA. The portion of the protein that is bound to the RNA (PDB entry 1etf) is showed in Fig. 24.1. The whole protein is several times larger contain at least three functional domains [61]. An arginine-rich RNA binding mediates interactions with the RRE, an oligomerization domain [62] and an effector domain that act as nuclear export signal (NES) [63].

In the Rev sequences towards the end of exon 1, the hydrophobic amino acid phenylalanine (F) sandwiched between Lysine (K) and Leucine (L) is conserved in brain-derived sequences, whereas it is not conserved in blood-derived sequences. However, nuclear localization sequences (NLS) and effector domain is conserved. Slight variations were observed at the C-terminal of blood derived Rev sequences Fig. 24.3.

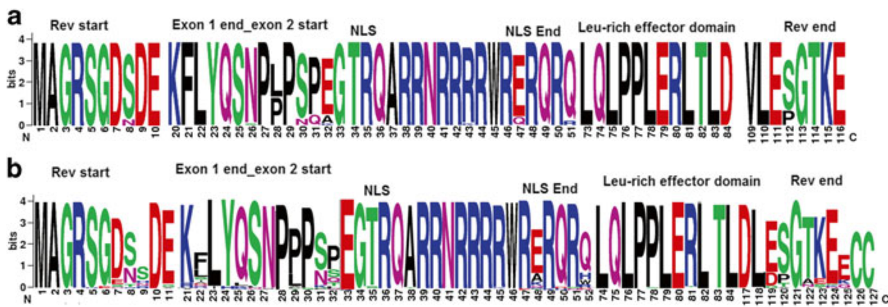

Fig. 24.3 Rev proteins derived from brain (**a**) and blood (**b**) show sequence conservations (*tall characters*), semi-conserved substitutions (*stacked with similar colored characters*) and variations (*stacked with different colors*). The annotation is based on the benchmark HXB2 sequence (accession K03455) provided in HIV Sequence Compendium 2013 [89]

2.4.2 Trans-Activator of Transcription (Tat)

Tat is essential for HIV-1 replication [64]. It is expressed by early fully spliced mRNAs or late incompletely spliced HIV mRNAs, 72 and 101 amino acids, respectively. Both forms function as transcription activators and are found within the nuclei and nucleoli of the infected cells. Tat is an RNA binding protein (PDB entries 1biv and 1jfw), unlike conventional transcription factors that interact with DNA [65]. Tat binds to a short-stem loop structure, known as the transactivation response element (TAR), which is located at the 5′ terminus of HIV RNAs. The binding of Tat to TAR activates transcription from the HIV LTR at least 1,000-fold. Tat has been shown to activate the expression of a number of cellular genes including tumor necrosis factor beta (TNFb) [66] and transforming growth factor beta (TGFb) [55] and to downregulate the expression of other cellular genes including bcl-2 and the chemokine, MIP-1 alpha [67–69].

In the Tat sequence data, exon 1 and 2 are annotated along with the NLS and disulfide bonding patterns. The amino acids followed by NLS of blood derived sequences showed more variation than the brain derived HIV-1B. Moreover, the ends of exon 1 and Tat of blood isolates are hypervariable (Fig. 24.4).

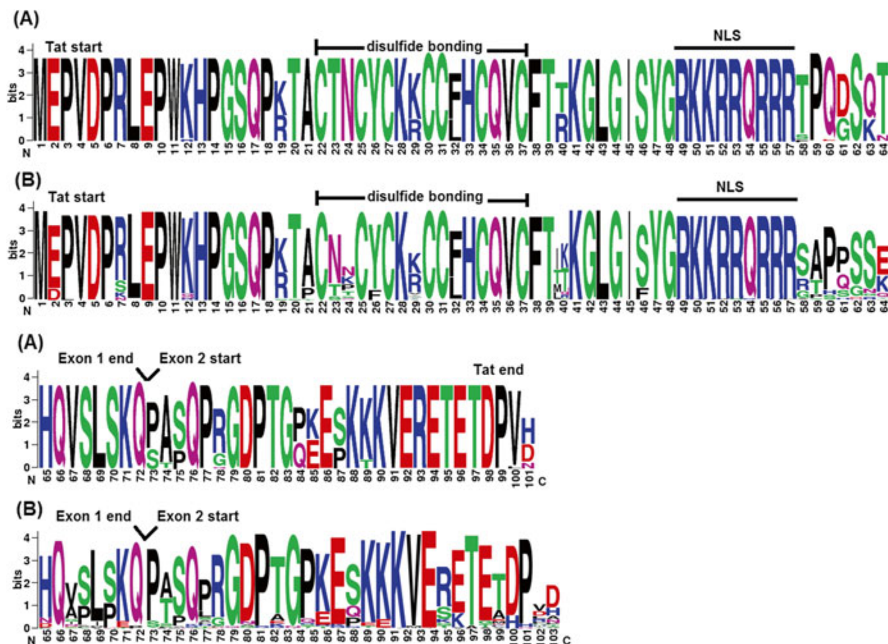

Fig. 24.4 Tat proteins derived from brain (**a**) and blood (**b**) show sequence conservations (*tall characters*), semi-conserved substitutions (*stacked with similar colored characters*) and variations (*stacked with different colors*). The annotation is based on the benchmark HXB2 sequence (accession K03455) provided in HIV Sequence Compendium 2013 [89]

2.5 *Accessory Proteins (Other Virus-Encoded Proteins in Virions)*

Products of most retroviral accessory genes are not incorporated into virions (for a detailed review, refer [70–72]).

2.5.1 Vpr (Viral Protein r)

Vpr (viral protein r) is essential for efficient replication of HIV-1 and is required for targeting the newly made viral DNA to the nucleus. This targeting function appears to be critical for the establishment of HIV infection in some non-dividing cells, which is a characteristic feature of lentiviruses [33]. Vpr also guides the viral genome into the nucleus after infection. The 3D structure is available in PDB (entry 1esx).

Although other accessory proteins are not incorporated into virions in substantial amounts, three HIV-1 proteins such as Vif, Vpu, and Nef besides Vpr appear to affect the structure, morphogenesis, or biological function of the mature viral particle.

From the analyzed blood isolates the oligomerization domains had slight variation but the C-terminal end had more variation. This could not be confirmed presently due to lack of brain derived sequences. However, H(S/N) RIG motifs seems to be conserved throughout the sequences derived from blood and brain.

2.5.2 Virion Infectivity Factor (Vif)

Vif is a 190–240-amino-acid protein that is relatively well conserved among HIV-1 strains. HIV-1 Vif has evolved to attack the cell's defense proteins, human antiretroviral DNA-editing enzymes especially APOBEC3G (A3G) and APOBEC3F (A3F) [73] for poly-ubiquitylation and proteasomal degradation [74] via the ubiquitin-proteasome pathway. A mutation of highly conserved cysteines or the deletion of a conserved SLQ(Y/F)LA motif in Vif results in mutants that fail to induce A3G degradation and produce non-infectious HIV-1. The potent activity of A3G directed research towards identification of small molecules that interrupt the Vif-induced degradative process [75]. These inhibitors might be useful in blocking Vif-mediated A3G destruction [76]. Only a small portion of Vif bound to proteins from the infected cell is shown in Fig. 24.1 (PDB entry 3dcg).

2.5.3 Viral Protein u (Vpu)

Vpu is a small integral membrane protein promotes the release of the budding virion at the plasma membrane [77]. Vpu also downregulates the levels of the CD4 receptor by accelerating its destruction. Vpu action is not specific for HIV-1, since it

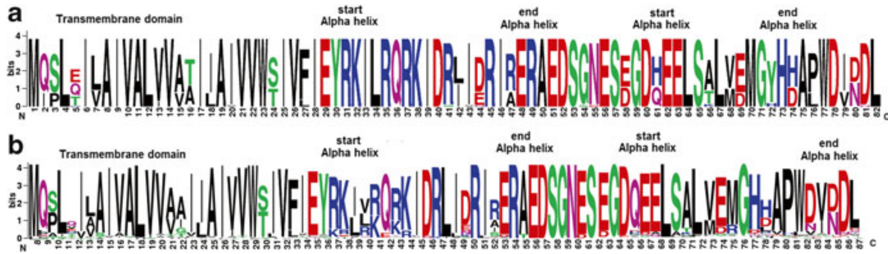

Fig. 24.5 Vpu proteins derived from brain (**a**) and blood (**b**) show sequence conservations (*tall characters*), semi-conserved substitutions (*stacked with similar colored characters*) and variations (*stacked with different colors*). The annotation is based on the benchmark HXB2 sequence (accession K03455) provided in HIV Sequence Compendium 2013 [89]

enhances release of other lentiviruses as well as MLV. The structure details are available in PDB entries 1pi7 and 1vpu. In the analyzed sequences the start of the first alpha helix in blood derived had conserved substitutions and in the second alpha helix of C-terminal region of blood isolates showed more variations than the brain isolates (Fig. 24.5).

2.5.4 Negative Regulatory Factor (Nef)

Nef is also membrane-associated. Nef is important in the progression of HIV infection to AIDS. Nef has complex effects on signal transduction pathways in the cell like Vpu, leads to loss of the CD4 receptor directly from the cell surface [78]. Virions released from cells in the presence or absence of Nef is indistinguishable in number. Virions produce Nef are capable of more viral DNA synthesis, suggesting that Nef directly or indirectly activates reverse transcriptase. Nef forces the infected cell to stop making several proteins that are important in cell defense. It is observed that some of this protein is found to have been cleaved by PR [79]. PDB entries 1avv and 1qa5. In the analyzed dataset, myristoylation site, acidic clusters, poly-P helix, phosphorylation site, premature and normal ends of Nef are annotated, which are shown in Fig. 24.6. Slight variations were observed in the myristoylation site of blood isolates and also in the acidic cluster of brain isolates.

2.6 Other Cellular Proteins in Virions

Only a few host proteins have been implicated as important virion constituents, including both cytoplasmic and membrane proteins.

Among cytoplasmic proteins perhaps the most interesting is Cyclophilin A. They are highly conserved proteins that have propyl-isomerase activity and serve as chaperonins to aid correct protein folding. These proteins are the targets for the

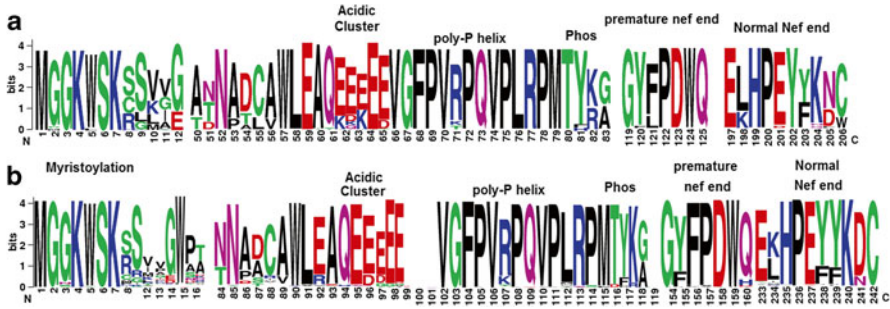

Fig. 24.6 The annotation of Nef proteins derived from brain (**a**) and blood (**b**) show sequence conservations (*tall characters*), semi-conserved substitutions (*stacked with similar colored characters*) and variations (*stacked with different colors*). The annotation is based on the benchmark HXB2 sequence (accession K03455) provided in HIV Sequence Compendium 2013 [89]

immunosuppressive drug Cyclosporin, and hence its name. Cyclophilins A and B were identified for human proteins capable of tightly binding to HIV-1 Gag protein [80]. Wild-type HIV-1 virions contain substantial amounts of Cyclophilin A [34, 35]. Gag-Cyclophilin interaction is specific for HIV-1. HIV-2 does not contain this protein, and hence, its replication is not affected by Cyclosporin treatment.

Another host protein that appears to be a physical constituent of virions is ubiquitin. This small, highly conserved protein is ubiquitous in all eukaryotes and has multiple functions in the cell. Its covalent attachment to lysine residues of a host protein marks that protein for degradation by the proteasome pathway [81]. Ubiquitin also becomes covalently conjugated to histones and to certain cell surface receptors. The possible functional role of ubiquitin in the retroviral life cycle has not been explored.

Of the other proteins that have been noted in preparations of retroviral particles, actin has been commonly seen in small amounts in preparations of retroviruses and other enveloped viruses. Due to its abundance in the cell, it is frequently dismissed as an artifact. However, in HIV, Gag binds to F-actin [82]. In addition, actin and other cytoskeletal proteins have been documented as genuine virion constituents [83].

Incorporation of cellular proteins into virions is not required for viral replication yet still has important practical consequences [33]. For instance HIV-1 includes large amounts of the major histocompatibility complex (MHC) class I proteins on its surface during budding. These surface glycoproteins have key roles in immune recognition and are present in virions similar to Env protein [84]. The presence of these proteins on virions is demonstrated by immunoprecipitation of Gag protein by treatment of intact virions with anti-class I antibody.

3 Conclusions

HIV-1-B protein sequences of different geographical locations from blood and brain isolates cluster into corresponding clades (Fig. 24.7). Average amino acid composition of each protein reveals variations in blood and brain isolates (Figs. 24.8 and 24.9).

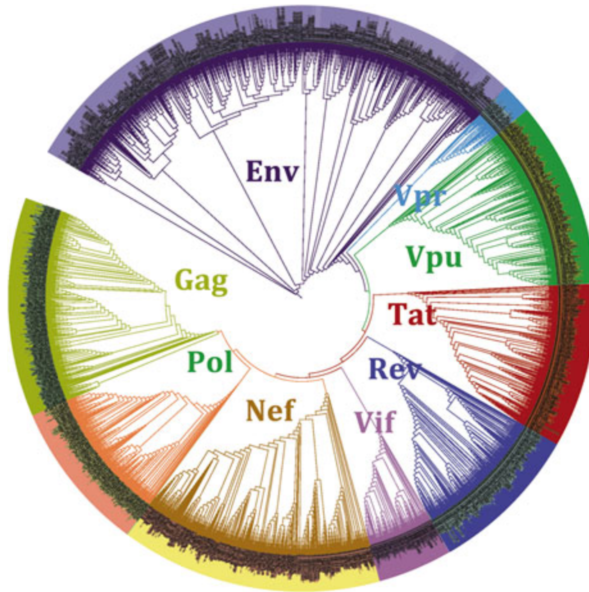

Fig. 24.7 Unrooted tree based on HIV-1B proteins from brain and blood using interactive tree of life (http://itol.embl.de) [90]

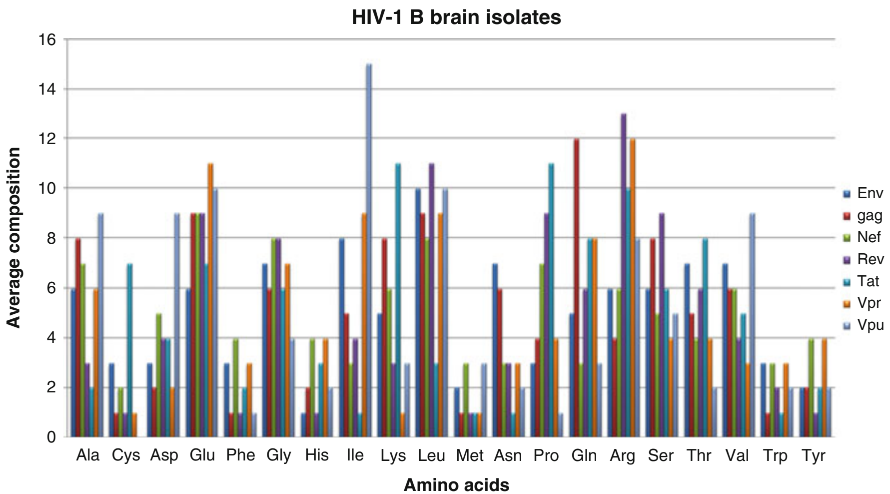

Fig. 24.8 Amino acid composition of HIV-1B brain isolates computed using MEGA 6.0 [91]

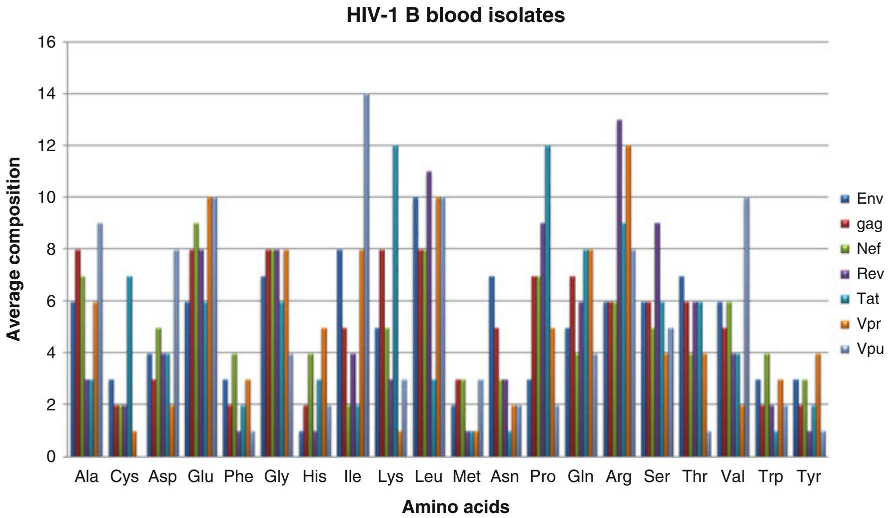

Fig. 24.9 Amino acid composition of HIV-1B blood isolates computed using MEGA 6.0 [91]

For instance, Glutamine in Gag of brain isolates is 12 %, whereas in blood isolates it is only 7 %. There were no cysteines found in Vpu of both blood and brain isolates. Glutamate in Vpr of brain is 1 % higher than that of blood isolates. In blood isolate Glutamate in both Vpr and Vpu are of equal proportions. Isoleucine in Vpu of brain is 1 % higher than that of blood isolate. Lysine and Proline in Tat is 1 % higher in blood than that of brain isolates. In Tat, equal amount of cysteine (7 %) was found in both blood and brain isolates of HIV-1 B clade. There is evidence to suggest that genetic differences in the Tat gene of the HIV clades may also alter the pathogenicity of the virus. For example, the cysteine in position 31 of clade B virus (Fig. 24.4) is mutated to a serine in clade C virus. This mutation results in decreased chemotactic properties of clade C virus and decreased neurotoxicity [85, 86]. However, there are indications that the Tat sequences of brain isolates (especially HAD patients) are poor transactivators of the HIV-LTR, which permits the virus to remain latent by escaping from the immune system [87]. These findings suggest that some HIV variants may be more capable of entering the CNS, but are less pathogenic in the brain environment, whereas other HIV variants might be more efficient in both brain infiltration and in setting up the HAD self-inflammatory macrophage environment [88]. The molecular determinants of these differences are unknown.

Due to lack of brain derived Gag proteins such as MA, CA, NC, and p6, the sequence variations could not be confirmed. In Env proteins no differences were observed in the CD4 regions of blood and brain derived Env sequences of HIV-1. The hydrophobic amino acid phenylalanine (F) sandwiched between Lysine (K) and Leucine (L) is conserved towards the end of exon 1 in brain-derived Rev sequences whereas it is not in blood-derived sequences. This could be due to the hydrophobic environment prevailing in the brain compartment and not in blood. The amino acids

followed by NLS of blood derived Tat sequences showed more variation than the brain derived Tat sequences. Moreover, the ends of exon 1 and Tat of blood isolates are hypervariable. In the Vpu of blood isolates, the second alpha helix at the C-terminal region showed more variations than the brain isolates. Analysis of Nef from blood isolates showed slight variations in the myristoylation site, whereas the brain isolates had slight variations in the acidic cluster of Nef. From the analyzed blood isolates of Vpr, the oligomerization domains had slight variation but the C-terminal end had more variation. At present, the sequence conservation, conserved and semi-conserved substitutions as well as the variation could not be confirmed for Gag, Pol, Vif, and Vpr proteins of brain isolates due to the lack of brain-derived sequences in public databases. In order to study such variations in the evolution of HIV-1 in the brain, autopsy specimens are required and it is impractical because most studies require large sample size. Alternatively, publicly available HIV-1 sequences could be used to develop prediction methods to detect molecular determinants of HIV and other associations like HAD.

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Chapter 25
Mutational Immune Escape in HIV-1 Infection

Aniqa Shahid and Zabrina L. Brumme

Core Message Within an infected individual, human immunodeficiency virus 1 (HIV-1) develops specific mutations that allow it to escape immune detection by its host. As such, the human immune response represents a major selective force driving the evolution and diversification of HIV-1 at the individual and population levels. Achieving a deeper understanding of the pathways, mechanisms, and implications of HIV-1 mutational immune escape, and how we can harness this information to design novel interventions, will bring us closer to our ultimate goal of ending the HIV-1 pandemic.

1 Introduction and Overview

The human immunodeficiency virus 1 (HIV-1) group M "pandemic" strain originated from a single zoonotic chimpanzee-to-human transmission event approximately 100 years ago [1, 2]. Since then, HIV-1 group M has diversified into 9 subtypes and more than 60 circulating recombinant forms that differ by up to 30 % in their envelope amino acid sequence (Fig. 25.1) [3–6]. This extraordinary global diversity has arisen as a result of evolutionary selection pressures imposed on HIV-1 by the estimated 75 million individuals infected since the epidemic's genesis [7]. Among the strongest of these evolutionary pressures is the human immune response itself.

It is now understood that a severe genetic bottleneck occurs at the time of HIV-1 infection [8–10] such that, depending on the transmission route, a single [8, 9] or a

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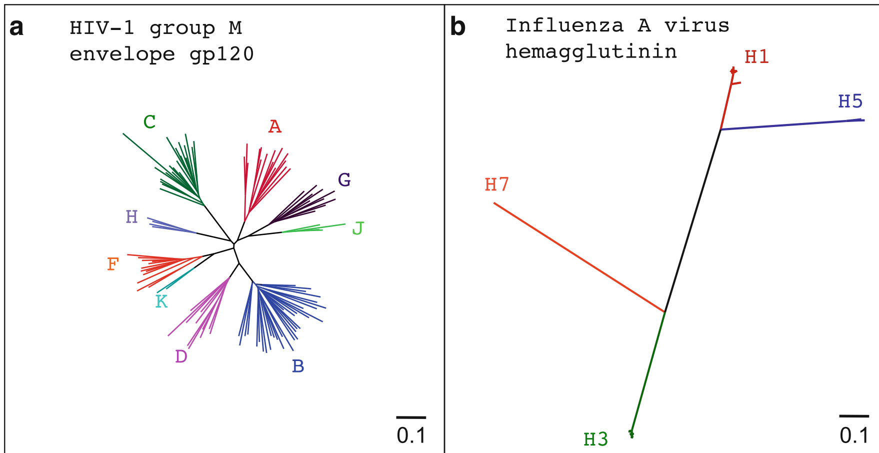

Fig. 25.1 Global genetic diversity of HIV-1 group M envelope and influenza A virus hemagglutinin, 2012. Unrooted maximum likelihood phylogenetic trees depicting global genetic diversity of $N = 112$ HIV-1 group M envelope gp120 (*panel A*) and $N = 138$ influenza A virus hemagglutinin (*panel B*) sequences sampled in the year 2012, drawn on the same genetic distance scale. HIV-1 group M sequences were obtained from the Los Alamos HIV Database 2012 compendium ([273] and http://www.hiv.lanl.gov/); influenza A virus sequences were obtained from the NCBI Influenza virus resource and flu database (http://www.ncbi.nlm.nih.gov/genomes/FLU/aboutdatabase.html). *Colored letters* denote HIV-1 group M subtypes A–K and influenza A virus hemagglutinin subtypes H1, H3, H5, H7. *Intra*-subtype diversity of HIV-1 group M vastly exceeds that of influenza A virus sequences circulating within a given year (though influenza A virus exceeds HIV-1 group M in terms of total *inter*-subtype diversity)

limited number [11, 12] of closely related founder viruses establish productive infection in the recipient. As a result of HIV-1's high replication rate [13], the high error rate of the virally encoded reverse transcriptase enzyme [14, 15], frequent recombination [16], and the mutation-inducing effects of host RNA editing enzymes such as APOBEC 3G [17], this transmitted/founder virus rapidly gives rise to a swarm of related viral variants within the infected individual [18]. This genetic pool becomes the evolutionary substrate upon which antibodies [19], T-lymphocytes [20], and possibly innate responses [21] exert immune pressures, driving the selection of escape mutations in the viral genome [22–26]. Mutational immune escape is a dynamic process that continues over the life of the infected person, shaping HIV-1 diversity within individuals [27–30] and host populations globally [31, 32]. Understanding the pathways, mechanisms, and biological implications of immune-mediated HIV-1 evolution is therefore of great importance to HIV-1 biomedical and clinical research, in particular to HIV-1 vaccine design.

Towards this end, this chapter provides an overview of past and recent advances in our understanding of mutational immune escape in HIV-1. Reflecting the authors' expertise, HIV-1 escape from cellular immune responses mediated by $CD8^+$ cytotoxic T-lymphocytes (CTL) comprises a major focus, but mutational escape from humoral (antibody), innate, and vaccine-induced immune responses is also covered. The relevance of immune escape to HIV-1 vaccine research, and its potential implications on the pandemic's future are also discussed.

2 HIV-1 Mutational Escape from Immune Recognition

2.1 Recognition and HIV-1 Immune Control by CD8⁺ Cytotoxic T-Lymphocytes (CTL)

CTL eliminate HIV-1-infected cells via the recognition of short, virus-derived peptide epitopes that are produced and processed within the infected cell and loaded onto Human Leukocyte Antigen (HLA) class I molecules for presentation at the cell surface (Fig. 25.2a). Located within the major histocompatibility complex (MHC) region on the short arm of chromosome 6, the HLA class I genes (comprising the

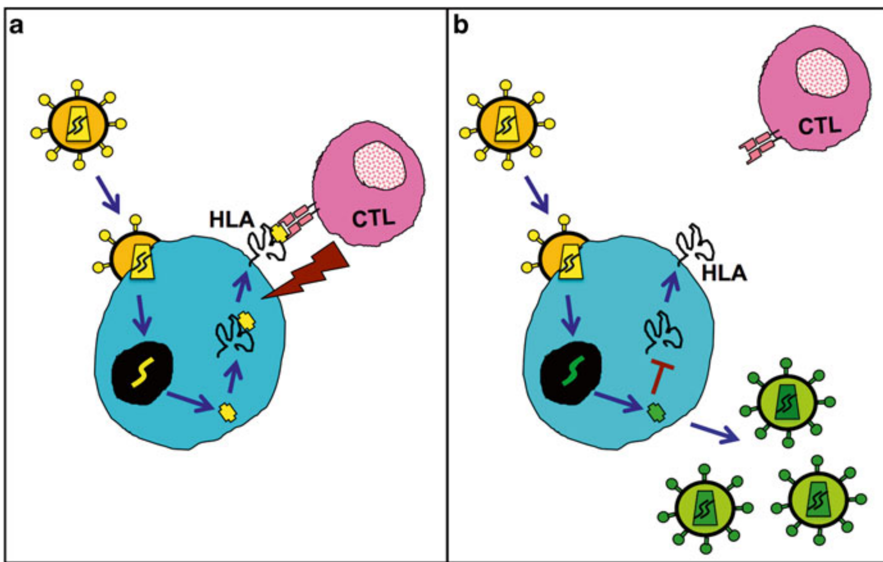

Fig. 25.2 Mutational escape in HIV-1 allows infected cells to evade detection and elimination by HLA class I-restricted CTL. *Panel A*: A simplified diagram depicting HIV-1 infection of a target cell, and the subsequent intracellular production of short virus-derived peptide epitopes that are processed and loaded onto Human Leukocyte Antigen (HLA) class I molecules for presentation at the cell surface. Recognition of the viral epitope-HLA complex by the T-cell receptor (TCR) complex of an HLA-restricted CD8⁺ cytotoxic T-Lymphocyte (CTL) results in elimination of the infected cell. For more details, see Sect. 2.1. *Panel B*: Under immune pressure, mutations are selected in HIV-1 that allow infected cells to evade detection by CTL. In this simplified diagram, a mutation occurs during the reverse transcription of RNA to proviral DNA, that, when translated into protein, abrogates the ability of the original encoded virus-derived epitope to bind HLA. As such, the viral epitope is not presented at the cell surface, allowing the infected cell to evade detection by the original HLA-restricted CTL. This results in the release of escape mutant HIV-1. In addition to the mechanism depicted here, CTL escape mutations may also interfere with proper processing of HIV-derived peptide epitopes prior to loading onto HLA ("antigen processing escape") and/or may abrogate CTL recognition of the mutant peptide-HLA complex ("TCR escape"), as described in Sect. 2.2

HLA-A, B, and C loci) are among the most polymorphic in the human genome [33]. Peptide-HLA binding is defined by HLA allele-specific amino acid motifs within the peptide, most commonly involving positions 2 and/or C-terminus [34], allowing CTL to recognize a broad range of pathogen-derived epitopes in an HLA-restricted manner.

HLA-restricted CTL play a major role in immune control of HIV-1 in vivo. It was long observed that HIV-1-specific CTL first appear around the time of the dramatic acute-phase viremia decline [35, 36] that occurs approximately 3–4 weeks following infection [37]; it is now known that the earliest CTL emerge *prior* to peak viremia and play an active role in its control to set-point levels [24]. Similarly, experimental depletion of $CD8^+$ T-cells in rhesus macaques resulted in an inability to control simian immunodeficiency virus (SIV) infection in early and chronic infection [38–40]. Strong epidemiological links between host carriage of specific HLA class I alleles and HIV-1 disease progression have also been demonstrated in natural history [41–45] and genome-wide association [46–49] studies. In particular, HLA-B*57 and B*27 are associated with lower viral loads and slower progression [44, 46, 50–52], whereas certain HLA-B*35 subtypes are associated with faster progression [41]. Independent effects of HLA-C expression level on HIV-1 control have also been demonstrated [53]. HLA class I alleles and their associated CTL responses may also protect against HIV-1 acquisition [54, 55] (though this remains controversial [56]). Similarly, evidence also suggests that vaccine-induced CTL could protect against SIV and HIV-1 acquisition and/or disease progression [57–59] (though readers new to the field should be aware that incomplete vaccine protection observed in the only "successful" HIV-1 vaccine trial to date was not likely attributable to CTL [60], and that three other trials evaluating a cellular HIV-1 vaccine delivered via a human adenovirus five vector ended in failure [61–63]). It is also worth noting that the efficacy of CTL-mediated control of HIV-1 differs based on the viral protein (and/or epitopes) targeted. In particular, recognition of key conserved epitopes in $p24^{Gag}$ [64–66] and to a lesser extent Pol may be most beneficial, whereas targeting of envelope may have negative clinical consequences [65]. However, the observation that HLA-restricted CTL exert potent immune pressures on HIV-1 in vivo is perhaps most clearly demonstrated by the virus' ability to escape this pressure via mutation. We now turn to the history of discovery in this area.

2.2 CTL Escape in HIV-1: Early Evidence and Mechanisms

Of the host immune responses targeting HIV-1 in vivo, those exerted by $CD8^+$ cytotoxic T-lymphocytes (CTL) are the best understood with respect to the specific mutational strategies employed by HIV-1 to evade them. Though CTL directly mediate the elimination of HIV-1-infected cells in vivo, HIV-1 mutational escape from CTL pressures is commonly conceptualized as "HLA-driven" or "HLA-associated" due to the requirement that the viral epitope be bound and presented by a specific HLA molecule for CTL recognition.

Selection of HIV-1 mutants capable of evading CTL recognition in vivo was first described in the early 1990s [20, 67, 68] when it was observed that "accumulation of such mutations in T-cell antigenic targets…provides a mechanism for immune escape" [20]. In the original 1991 study, researchers noted temporal shifts in the dominant HLA-B*08-restricted HIV-1 Gag epitopes targeted by patient-derived CTL, with some epitopes exhibiting permanent loss of recognition over time. These shifts in CTL epitope recognition coincided with the appearance of viral mutations within them. Whereas some mutants abolished in vitro CTL recognition, others retained some ability to be recognized by certain autologous CTL populations. The former observation led researchers to conclude that a major mechanism of in vivo CTL escape was the selection of a mutant epitope no longer capable of forming complexes with the relevant HLA, whereas the latter observation revealed that some escape mutants retained the ability to bind HLA, but stimulated a smaller and/or different pool of CTL following their selection [20].

CTL escape mutations can be broadly classified into three mechanistic categories (two of which were inferred in the original 1991 study [20]). The most intuitive is escape via mutation(s) that reduce or abrogate viral epitope binding to HLA, thereby impairing CTL recognition of infected cells (Fig. 25.2b). These mutations usually occur at HLA-specific epitope "anchor" residues—typically peptide positions two and/or C-terminus—and are commonly referred to as "anchor residue escape" mutations. A well-known example is the B*27-associated R264K mutation selected at position 2 of the B*27-restricted KK10 epitope in Gag (that spans codons 263–272) [69]. CTL escape can also act upon processes that occur prior to, or following, peptide-HLA binding. For example, some CTL escape mutant epitopes retain the capability to bind HLA, but reduce or abrogate recognition of the peptide-HLA complex by the T-cell receptor (TCR) expressed by some or all members of the original selecting CTL pool. Such "TCR escape mutations" usually occur at central epitope positions. The B*27-associated L268M mutation selected at position 6 of the KK10 epitope provides an example [20]. L268M-containing KK10 retains the ability to bind HLA-B*27, but abrogates its recognition by key B*27-restricted CTL clonotypes in the repertoire [70]. Other examples of TCR escape abound [70–75]. The original 1991 study was also the first to document de novo recognition of variant peptides by novel CTL populations following TCR escape [20], a now well-described phenomenon [76, 77] that underscores the dynamic and adaptable nature of the host cellular response to a rapidly evolving pathogen. The third category of CTL escape mutations inhibit epitope formation by interfering with their proper processing within the host cell. The first such "antigen processing escape mutation" to be mechanistically characterized was B*57:03-restricted Gag-A146P, occurring at the residue immediately upstream of the IW9 epitope (Gag codons 147–155) that acts via prevention of N-terminal aminopeptidase-mediated trimming of this epitope [78]. Though antigen processing mutations often occur at positions flanking the N and/or C-terminal epitope boundaries, they can also occur within the epitope [79]. For example, a mutation occurring at position 5 of a B*07-restricted epitope in a cryptic Gag reading frame acted via introduction of a proteasomal cleavage site at this position, yielding a profound reduction in epitope

formation [80]. Antigen processing CTL escape mutations may also occur at positions distal to the epitope [81].

Arguably the most foundational observation made in the original 1991 study was the HLA-restricted nature of CTL escape. Since “different HLA class I molecules select distinct HIV-derived epitopes to stimulate CTL responses” wrote the researchers, then “HLA type could have an effect on virus escape” [20]. This realization was key to the next major development in the field—namely, that the kinetics and nature of in vivo CTL escape was specific to, and thus broadly reproducible based on, the HLA class I alleles expressed by the host.

2.3 The Timing and Mutational Pathways of CTL Escape Are Reproducible Based on Host HLA

Despite HIV-1’s genetic plasticity, the timing and mutational pathways of CTL escape are broadly predictable based on host HLA—a phenomenon most strikingly illustrated by the near-identical patterns of CTL epitope targeting and escape in identical adult twins infected on the same date with the same virus via injection drug use [82]. Importantly though, CTL escape is also reproducible across unrelated hosts sharing the same HLA. For example, three-quarters of HIV-1 subtype B infected persons expressing the protective HLA-B*57 allele select a T-to-N mutation at Gag codon 242 (position 3 of the $p24^{Gag}$-TW10 epitope at Gag codons 240–249), usually within the first weeks to months following infection [83, 84]. Fifty percent of B*57-expressing persons will additionally select G248A at position 9 of this epitope [84–86]. Together, these two mutations confer complete escape from B*57-restricted, TW10-specific CTL [83]. In contrast, in B*27-expressing individuals, targeting of the immunodominant $p24^{Gag}$ KK10 epitope begins in early infection and is often sustained for years thereafter [42]. KK10 escape begins via selection of the L268M mutation at position 6 of the epitope a few months after infection [20], that abrogates its recognition by certain autologous B*27-restricted CTL [70]. Complete escape from KK10-expressing CTL does not generally occur until years later, via selection of R264K at epitope position 2 [69] that abrogates epitope binding to B*27 [87]. The lengthy timeline of selection of R264K is now known to be due to its substantial fitness cost, which necessitates the development of a distal compensatory mutation prior to its selection in vivo [88]. Though epitopes besides KK10 are targeted in B*27-expressing persons [89], KK10 escape remains one of the most clear-cut examples where in vivo HLA-mediated control of HIV-1 replication is largely mediated by sustained targeting of a single key epitope, and where escape leads directly to loss of HIV-1 control [27, 90].

That the first CTL escape mutations emerge rapidly following infection has long been known [22, 91, 92]. Recently, however, major advances in our understanding of the dynamics of HIV-1 infection and subsequent escape have been achieved via detailed studies of intra-host HIV-1 evolution using single-genome amplification (e.g., [9]) or next-generation sequencing (e.g., [93]). We now appreciate that HIV-1 transmission is characterized by a severe genetic bottleneck, where an estimated 80 % of

heterosexual transmissions are productively initiated by a single transmitted/founder virus [8–10], whereas infection in men who have sex with men or persons who inject drugs is generally established by a limited number of closely related donor/founder viruses [11, 12]. We also now appreciate that CTL-mediated killing of infected cells begins *prior* to acute-phase peak viremia, and that selection of the first CTL escape mutations occurs during this time [24, 93]. Indeed, the selection (and in some cases the fixation) of CTL escape variants has been observed as early as 21 days post-infection in humans [18, 24] and 17 days in macaque models of SIV infection [94].

The evolutionary pathways along which these early mutations arise have also recently been elucidated in detail. In the earliest days following infection, HIV-1 undergoes rapid population growth and exhibits star-like diversification, but immune selection (notably by CTL) dominates thereafter, leading to the survival of viral lineages harboring escape mutations [18]. As it turns out, the conceptually straightforward pathway whereby the first selected escape mutation gradually outcompetes the original transmitted form is likely to be true for only a minority of cases [24]. More commonly, the first escape variant tends to be rapidly followed by the emergence of numerous others, from which the "final" escape form is ultimately selected [24]. This is likely because the initially appearing pool of low frequency mutants often retain some ability to be targeted by existing (or de novo) CTL [95]. This drives the selection of more effective escape variants, often at HLA-anchor residues, that ultimately outcompete both transmitted founder and initial variants [10, 93]. For example, in a B*57:03-expressing individual, initial escape within the $p24^{Gag}$-TW10 epitope occurred approximately 5 months post-infection via a transient, minority G-to-E mutation at position 9 (G248E) that retained the ability to bind B*57:03 and reduced CTL recognition only modestly [95]. By approximately 1.5 years following infection, this mutation was outcompeted by variants expressing the "canonical" B*57-restricted G248A mutation at this position (along with T242N and V247I at epitope positions 3 and 8).

It is also now understood that CTL escape accounts for a major proportion of within-host HIV-1 evolution in the first year of infection [24, 84, 93]. To provide context, a detailed study of seven newly infected individuals revealed that, approximately 6 months following infection, between 9 and 18 positively selected substitutions were observed throughout the HIV-1 proteome [18], whereas another estimated that a minimum of 30 % of observed substitutions in Gag/Pol and 60 % in Nef were attributable to HLA-driven selection [84]. HLA-driven CTL escape continues to occur (albeit at a slower rate [96, 97]) over the infection course, with some escape mutations occurring on a time course of years [27, 98, 99].

2.4 Reversion, Compensation, and Fitness Costs of CTL Escape

When CTL escape mutations selected in a previous host are transmitted to an individual lacking the restricting HLA allele(s), many will revert to the original (usually subtype consensus) amino acid [83, 100–103]. Like escape, reversion is also

HLA-restricted, though in the opposite context (as it occurs in the *absence* of selection pressure by the original restricting HLA). The timing of reversion is also predictable to some extent. Whereas some escape mutations, for example the B*57-associated Gag T242N, revert consistently and rapidly following transmission [83, 93, 102], most revert more slowly [98, 104]. Yet others are so stable that they revert rarely or not at all [32, 105–107].

Certain CTL escape mutations occur at a cost to viral fitness [108]. Like the reversion of certain drug resistance mutations upon transmission to a therapy-naïve host [109], fitness costs of CTL escape mutations can be inferred by their tendency to revert following transmission to an HLA-mismatched host [83, 100, 108, 110]. Fitness costs of escape vary widely, depending on their location in the viral proteome. Broadly speaking, escape mutations within conserved viral regions tend to exhibit more pronounced fitness costs, whereas escape in more variable regions tends to be fitness-neutral [111]. An example of a highly fitness-costly mutation is the B*27-associated R264K substitution in the $p24^{Gag}$ KK10 epitope, which essentially abolishes in vitro viral replication when engineered alone into the reference strain NL4-3 ($HIV\text{-}1_{NL4-3}$), likely because this variant is unable to replicate efficiently in the presence of normal cellular levels of cyclophilin A [88]. Generally however, in vitro fitness costs of escape mutations observed in vivo tend to be subtler, often requiring multiple substitutions to reduce function. Alone, the B*57-driven Gag-T242N mutation reduces viral replicative capacity only modestly [112, 113], but dose-dependent replicative reductions are observed when it is present alongside other common B*57-driven mutations in $p24^{Gag}$ [114–116]. Fitness-costly escape mutations are numerous, widespread throughout HIV-1, and are restricted by a broad range of HLA alleles. Examples include A*74:01 [117] and Cw*03 [118]–driven mutations in $p24^{Gag}$, B*13-driven mutations in $p1^{Gag}$ [119], B*35-driven mutations in Nef [120], and Cw*05-driven mutations in integrase [121]. Furthermore, the consistent reversion of fitness-costly escape mutations explains why certain HIV-1 residues that are highly conserved at the population level remain so despite being under strong selection by one or more HLA alleles. For example, the Gag-T242N mutation is reproducibly selected in the vast majority of individuals expressing HLA-B*57 and/or B*58, but its consistent reversion [83] ensures that its prevalence remains $\approx$1 % among individuals lacking these alleles [83, 122].

Fitness costs associated with the primary escape event can be offset by the selection of compensatory mutations at secondary sites [123]. This was first demonstrated in the simian/human immunodeficiency virus (SHIV) model, where the fitness costs of a rare in vivo escape mutation in capsid were rescued by the selection of compensatory mutations 21 codons upstream and/or 24 codons downstream of the primary escape site [123]. Due to its routine late emergence following other clustered mutations within the $p24^{Gag}$ KK10 epitope, the B*27-restricted R264K mutation was long suspected to require compensation [69], however, it was not until 2007 that its compensatory mutation was identified to be S173A, nearly 100 residues upstream [88]. Indeed, the requirement that S173A be present prior to R264K selection in vivo provided an explanation for the latter's lengthy timeline of selection

and also resolved the seemingly paradoxical initial observation that R264K abolished HIV-1 replication when engineered alone in vitro (S173A rescues R264K replication to near wild-type levels [88]). Examples of compensatory mutations now abound. Whereas most occur in relatively close proximity to the primary escape site (e.g., S165N with A163G in B*5703-KF11 [98]; E260D with R264K in B*27-KK10 [124]; H219Q, I223V, and M228I with T242N in B*57-TW10 [112, 113], all in p24^{Gag}), others, such as S173A with R264K in B*27-KK10 [88], occur a substantial linear distance away, but may reside nearby in the folded protein structure. Compensatory mutations are also highly reproducible in context of their associated primary escape site. Indeed, the reproducibility of HLA-driven escape, reversion, and compensation is most clearly revealed by population-level studies [125–127], the subject of the following section.

2.5 *Identification of CTL Escape Mutations "at the Population-Level" by Statistical Association: Overview and Methods*

The predictable nature of HIV-1 adaptation to HLA has facilitated the systematic identification of HLA-associated viral polymorphisms "at the population level"—a term loosely used to describe the identification, via statistical association, of viral polymorphisms significantly over(or under)represented among persons expressing a given HLA allele, in cross-sectional datasets [31, 86, 126, 128]. An advantage of these approaches is that they are comprehensive and largely unbiased, allowing the identification of HLA-associated viral polymorphisms regardless of their proximity to known CTL epitopes. A disadvantage is their correlative nature, thus necessitating experimental validation to confirm HLA-associated polymorphisms as mutations directly conferring CTL escape, and to elucidate their mechanisms.

Population-level analyses identify two types of associations: adapted and non-adapted (Fig. 25.3). Adapted associations are viral polymorphisms that are significantly *overrepresented* in individuals harboring a particular HLA allele; these are likely to represent CTL escape mutations. Conversely, nonadapted forms are viral polymorphisms that are significantly *under represented* in individuals harboring a particular HLA allele; these represent the immunologically susceptible form for the HLA allele in question. In most cases, both nonadapted and adapted forms are identified at a given HIV-1 codon for a particular HLA allele. For example, at Gag codon 242, T and N represent nonadapted and adapted forms associated with HLA-B*57. Sometimes an HLA allele can select multiple escape pathways at a given viral site, yielding multiple adapted associations. For example Nef codon 94, position 5 of the B*08-restricted FL8 epitope, harbors four B*08-associated adapted forms: "E," "M," "N," and "Q" (whereas the subtype B consensus "K" represents the B*08-associated nonadapted form at this position) [126, 129]. In the majority of cases, nonadapted forms represent the HIV-1 subtype consensus whereas adapted forms represent variants, but exceptions occur. For example at Gag codon 147, "L" and "I"

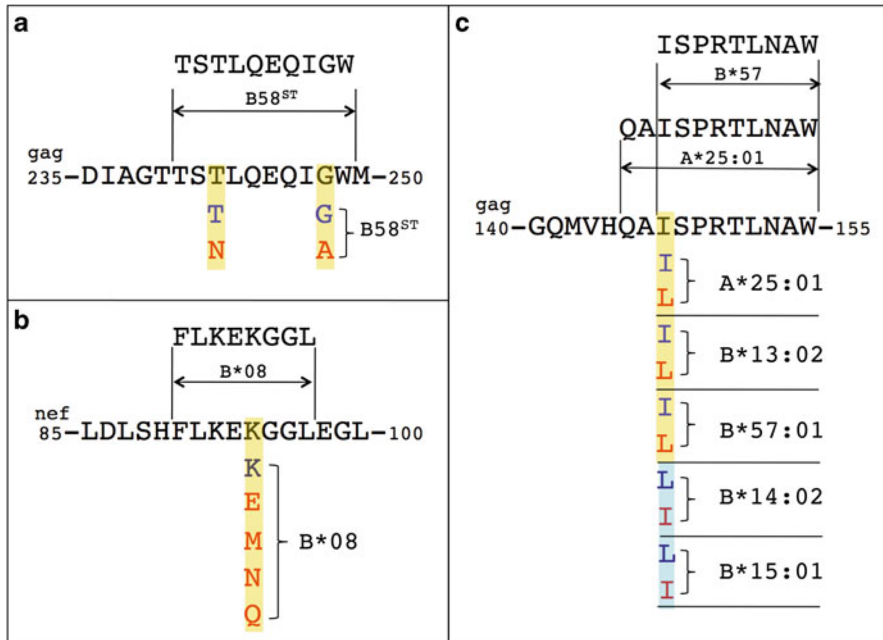

Fig. 25.3 Examples of HLA-associated polymorphisms in HIV-1 identified "at the population level." The predictable nature of HIV-1 adaptation to HLA has facilitated the systematic identification of HLA-associated viral polymorphisms "at the population level," via statistical association approaches. These associations can be depicted in "HIV-1 immune escape maps" which indicate their locations, specific amino acid residues, and HLA restrictions. A selection of HLA-associated HIV-1 sites, and their polymorphisms are shown here. The HIV-1 subtype B consensus amino acid is used as a reference. Known CD8^{+} epitopes in HIV-1 (available at http://www.hiv.lanl.gov/content/immunology), and their HLA-restrictions, are indicated above the consensus sequence. HLA-associated polymorphisms are listed below the consensus sequence: Nonadapted associations (HIV-1 residues under-represented among persons expressing the HLA) are in *blue*, whereas adapted associations (HIV-1 residues enriched among persons expressing the HLA) are in *red*. *Panel A*: At HIV-1 Gag codon 242, residue 3 of the TW10 epitope recognized by HLA alleles belonging to the B58 supertype (B58ST), T and N represent nonadapted and adapted forms associated with HLA B58 supertype alleles, respectively. At Gag codon 248, position 9 of this epitope, G and A represent nonadapted and adapted forms associated with the HLA B58 supertype alleles, respectively. *Panel B*: Sometimes HLA-driven escape can occur along multiple pathways at a given viral site, yielding multiple adapted associations. For example, at Nef codon 94, position 5 of the B*08-restricted FL8 epitope, the subtype B consensus "K" represents the B*08-associated nonadapted form at this position, whereas four B*08-associated adapted forms are observed: "E," "M," "N," and "Q." *Panel C*: Some HIV-1 codons harbor a large number of associations with different HLA alleles, some of which occur in opposing directions. Gag codon 147, which lies within numerous CTL epitopes, provides an example. Here, "L" and "I" represent the adapted and nonadapted forms associated with A*25:01, B*13:02, and B*57:01. In diametric opposition, "I" and "L" represent the adapted and nonadapted forms associated with B*14:02 and B*15:01. In the majority of cases, nonadapted forms tend to represent the HIV-1 subtype consensus, whereas adapted forms represent variants, but exceptions occur. The associations with B*14:02 and B*15:01 at this position also illustrate "exceptional" cases where the adapted form represents the subtype consensus and the nonadapted form represents a polymorphic variant (in most cases—including all other examples depicted in this figure, nonadapted forms represent the HIV-1 subtype consensus whereas adapted forms represent variants). For more detail, see Sect. 2.5

represent the nonadapted and adapted forms associated with B*14:02 and B*15:01 (but the subtype B consensus is “I”) [86]. HIV-1 codons harboring diametrically opposed HLA associations also exist, where a given viral polymorphism represents the nonadapted form for one HLA allele but the adapted form for another [129]. Gag codon 147 again provides an example: in opposition to the B*14:02 associations described above, “L” and “I” represent the adapted and nonadapted forms associated with A*25:01, B*13:02, and B*57:01, among others [86].

Published in 2002, the first study to identify HLA-associated polymorphisms “at the population level” identified nearly 100 polymorphisms in HIV-1 RT in a cohort of ≈400 patients, illustrating the extensive impact of CTL pressures on HIV-1 [31]. Since then, analytical methods have been refined. In particular it has been recognized that, since HIV-1 sequences are related to one another through descent from a common ancestor (some more closely than others, for example a transmission pair), they should not be analyzed using standard tests of association that assume independence of observations. The problem can be illustrated by the extreme example of a heterogeneous dataset comprising different host populations infected with different HIV-1 subtypes (e.g., Europeans infected with subtype B, and Africans infected with subtype C). Here, HIV-1 sequences belonging to each subtype will share lineage-specific polymorphisms reflecting their descent from the most recent common ancestor at the root of that lineage. Similarly, HLA allele frequencies will differ between the two host groups due to their descent from different ancestral populations. As such, standard tests of association would identify strong relationships between HIV-1 subtype C lineage-specific polymorphisms and HLA alleles enriched among Africans (and likewise HIV-1 subtype B lineage-specific polymorphisms and HLA alleles enriched among Europeans). However, such associations would be attributable to confounding due to viral lineage (“founder”) effects, rather than escape mutations directly selected by the associated HLA [130]. Though this is an extreme example, analyses comprising only a single HIV-1 subtype could similarly be confounded by subtle lineage effects.

To address this, “phylogenetically informed” methods that correct for the underlying evolutionary relationships (that is, the inferred phylogeny) linking HIV-1 sequences in the dataset have been developed, with the goal of distinguishing HIV-1 sites under HLA-driven selection in the present host from those likely to be explained by founder effects (i.e., neutral evolution in the tree) [125, 130, 131]. More recent strategies also correct for the confounding effects of linkage disequilibrium (LD) between HLA class I alleles [125] (thus identifying the HLA allele directly responsible for selecting the viral variant, rather than alleles in LD with it) and HIV-1 amino acid co-variation [125, 132, 133] (thus discriminating the specific viral variants directly selected by HLA, from those that indirectly arise as secondary or compensatory mutations). Strategies to address lineage effects and population stratification in both virus and host have also been implemented [134].

2.6 Insights from Population-Level Studies of CTL Escape

2.6.1 CTL Escape Pathways Are Highly HLA-Specific and a Major Driver of Viral Diversity

Population-level studies have yielded comprehensive maps of the locations, specific amino acids, relative frequencies, and statistical strengths of selection of HLA-associated polymorphisms in HIV-1 [125, 126, 129, 131, 135–138]. These "immune escape maps" are most detailed for HIV-1 subtype B [86, 126, 129, 138], followed by C [125, 128, 131, 139] and CRF01 (AE) [140], though other subtypes remain understudied in this context. Population-level studies have also confirmed escape (and reversion) as highly reproducible processes in context of host HLA. For example, the strongest HLA association in subtype B is the HLA-A*24:02-restricted Y135F escape mutation in Nef. In chronic infection, 81 % of A*24:02-expressing persons harbor this mutation, compared to only 12 % of persons who do not express an allele belonging to the A24 supertype, yielding a statistical association of very high magnitude (in this case, an odds ratio of approximately 30 and a *p*-value of 8×10^{-118}) [86]. By definition, such a strong statistical association can only be achieved if the mutation is near-universally selected in persons harboring the HLA, and reverts consistently in individuals lacking it [141]. Escape is also highly HLA-specific. When population-level analyses are undertaken at various HLA resolution levels (e.g., supertype, type, subtype), the majority (>60 %) of HLA-associated polymorphisms are identified as specific to a particular HLA subtype, whereas <10 % are identified as shared across HLA supertypes [86]. This high HLA-specificity remains true even for closely related HLA subtypes that present the same viral epitopes [128, 138]. For example, HLA-B*57:02, B*57:03, and B*58:01 all bind Gag-TW10, but they drive significantly different escape pathways within this epitope. In particular, escape at position 3 (via selection of T242N) is significantly stronger for B*57:02 compared to the others, escape at position 7 occurs via I247M in B*57:02 versus I247V in B*57:03, whereas escape at position 8 is essentially B*58:01-specific [128]. The specificity of HIV-1 adaptation to HLA may also shed mechanistic light on the long-standing observation that HLA alleles, sometimes differing by as little as one amino acid between them, can mediate differential rates of disease progression [142–144]. This observation also underscores the importance of identifying HLA-associated polymorphisms at the HLA subtype level.

Population-level studies also confirm immune escape as widespread throughout HIV-1. Over 2100 HLA-associated polymorphisms, occurring at ≈35 % of the virus' nonconserved codons have been identified across the HIV-1 subtype B proteome [86], though their distribution is somewhat nonuniform. Whereas HLA pressures greatly influence diversity of certain viral genes (e.g., the highly diverse *nef* gene and the relatively mutationally constrained $p24^{Gag}$ gene harbor HLA-associated polymorphisms at ≈70 % and ≈40 % of their nonconserved residues, respectively), the highly variable *vpu* gene exhibits evidence for HLA-mediated selection at only one-quarter of its nonconserved sites [86, 145]. Despite this, HLA is likely to represent the single most important host genetic factor influencing global HIV-1

diversity—an observation recently confirmed via genome-wide association. In a "genome-to-genome" analysis of >1,000 individuals for whom human genetic variation (assessed in terms of single nucleotide polymorphisms [SNPs]) and HIV-1 sequences were available, 48 HIV-1 amino acids associated with human SNPs were identified, all of which mapped to the HLA class I region [134]. Similarly, a study investigating immune-driven evolution of HIV-1 Gag and Nef during the North American subtype B epidemic observed that HIV-1 sites under HLA selection have diversified to the greatest extent over time, supporting a significant role of HLA in driving global HIV-1 diversification [146].

2.6.2 Population-Level Studies Illuminate Escape Mechanisms, Aid Novel Epitope Discovery, and Reveal Correlates of Protective Immunity

Though the mechanisms of immune escape cannot be determined via association studies alone, analysis of the distribution of HLA-associated polymorphisms within (or flanking) known or inferred epitopes can shed light on which mechanisms predominate. In particular, epitope-HLA anchor residues are significantly enriched for HLA-associated polymorphisms, identifying abrogation of peptide-HLA binding as a predominant in vivo escape mechanism [86, 147]. Moreover, bioinformatic predictions estimate that the "average" HLA-restricted anchor residue polymorphism confers a tenfold reduction in peptide binding affinity to HLA [86]. Inferred escape via TCR and/or antigen processing mechanisms also occurs, but less frequently than anchor residue escape. Population-level studies have also aided CTL epitope discovery, as the presence of HLA-associated viral polymorphisms generally indicates the presence of a CTL epitope nearby. Bioinformatic approaches can then be applied to predict its location and sequence for experimental validation—a type of "rational," polymorphism-guided approach to epitope discovery. Numerous CTL epitopes have been identified this way [121, 130, 148, 149], including HLA-restricted epitopes in nonstandard ("cryptic") [150] and/or antisense [151] HIV-1 reading frames.

Association studies of HLA-driven escape can also shed light on why certain HLA alleles are more effective at controlling HIV-1 than others [86]. This is because HLA-associated polymorphisms mark viral sites under strong and reproducible in vivo selection by a particular HLA allele. As such, analysis of the properties of these sites (i.e., their location, frequency, distribution, sequence conservation and their strength of association with the restricting HLA) can be used to identify features that discriminate protective from nonprotective HLA alleles. Analyses of this type have identified CTL response breadth as the most consistent correlate of immune protection: in general, protective HLA-A and -B alleles exert immune pressure on a larger overall number of HIV-1 sites compared to nonprotective alleles [86]. The strength and location of selection pressure is also important: protective HLA alleles also tended to strongly target highly mutationally constrained sites, notably in Gag and to a lesser extent Pol. Protective HLA alleles also exhibited a higher average number of escaping sites per epitope in Gag, supporting diversity of selection pressure (e.g., in terms of the clonal composition and/or diversity of

epitope-specific CTL repertoire [70, 152]) on key viral areas as an additional correlate of protection. It is important to emphasize that the unit of analysis in these investigations is the HLA allele, not the individual, and that conceptualizing HIV-1 codons as sites under HLA-mediated selection does not imply that CTL escape is protective at the individual level (on the contrary, escape is generally linked to negative clinical outcomes [27, 90, 128, 153]). Rather, these sites represent the total potential of individual HLA alleles to effectively target HIV-1. In support of the potential in vivo relevance of this novel analytical perspective, a recent population-level analysis in HIV-1 subtype C identified HLA-restricted viral polymorphisms as stronger predictors of HLA-plasma viral load correlations than CTL responses measured by traditional in vitro assays [128].

2.7 HLA Class II-Driven Immune Escape

Effective antiviral immunity generally requires $CD4^+$ T-lymphocyte help. HLA class II-restricted HIV-1-specific $CD4^+$ T-cell responses emerge rapidly following infection [154] (e.g., Gag-specific $CD4^+$ T-cell responses peak at a median of 28 days [155]), but the HIV-1-specific $CD4^+$ response rapidly becomes dysfunctional, in part because of the specific elimination of virus-specific $CD4^+$ cells [156, 157]. As such, the extent, durability and contribution of $CD4^+$ T-cells to HIV-1 control in vivo remains incompletely understood. It remains similarly unclear whether mutational escape from $CD4^+$ T-cell responses occurs to any great extent in vivo: whereas some early studies supported this possibility [158], others did not [159, 160]. Furthermore, attempts to identify HLA class II-restricted viral polymorphisms by statistical association have yielded no strong evidence of their existence [161], suggesting that mutational escape from HLA class II-restricted $CD4^+$ T-cells is far weaker, less specific and/or less reproducible compared to HLA class I-restricted escape from CTL pressures.

2.8 Escape from Neutralizing Antibodies

The HIV-1 envelope gene evolves rapidly within a host after infection and has diversified to an extraordinary extent at the population level [4]. Although CTL escape contributes to this process, the most significant factor driving HIV-1 envelope evolution is the autologous neutralizing antibody response. Beginning at approximately 3 months post-infection [162, 163] (though earlier in some [164]), HIV-1-infected individuals begin to develop antibodies capable of neutralizing their own virus (termed "autologous" or "strain-specific" neutralizing antibodies; NAbs) [19, 165] (non-neutralizing antibodies, directed against envelope and non-envelope targets, emerge earlier [166]). In contrast to acute-phase HIV-1-specific CTL responses, autologous NAbs do not contribute to virus containment to any appreciable extent,

likely because they drive the rapid selection and outgrowth of neutralization-resistant escape mutants [19, 162]. Initial NAb escape exposes novel envelope epitopes against which subsequent waves of autologous NAbs arise, driving further envelope evolution. That antibodies and virus coevolve in cycles of response and escape was first inferred via the ability of autologous sera to neutralize viral variants present in the infected individual 6 (or 12) months prior, but not those present at the time of serum sampling [23].

It is now understood that in approximately 80 % of infected individuals, this process results in the continued production of autologous NAbs that remain largely specific to the individual's evolving virus. However, in approximately 20 % of individuals, this process [30, 167, 168] leads to the emergence of antibodies that are capable of neutralizing a broad range of HIV-1 isolates across subtypes [169–171]. Though individuals producing such "broadly neutralizing antibodies" do not likely derive clinical benefit from them (presumably because their own virus has already escaped) [169, 170], the evolutionary mechanisms driving their development are of paramount interest as an effective preventive HIV-1 vaccine will likely require their elicitation (along with effective cellular responses) [172]. For this reason, HIV-1 antibody escape is being elucidated in the context of coevolution of HIV-1 founder viruses and their autologous NAbs, towards the goal of exploiting this natural process in HIV-1 vaccine design.

Initial studies of HIV-1 neutralization escape, the earliest of which date back to the late 1980s [173, 174], hinted at a variety of escape pathways including the accumulation of amino acid changes in envelope [23] (suggestive of escape through the selection of specific point mutations), changes in *N*-linked glycosylation patterns [162] and lengthening of certain hypervariable domains in gp120, notably V1/V2 [175, 176]. However, the identification of specific genetic events conferring escape from individual NAb responses has begun only recently (e.g., the first specific identification of an envelope escape mutation conferring neutralization escape at the single antibody level was not achieved until 2009 [177]). Unlike CTL epitopes whose (linear) sequences can be predicted from HLA anchor residue motifs without knowledge of the T-cell receptor sequence or structure, antibodies directly recognize three-dimensional epitopes whose sequences can span discontinuous sites on one or more members of the envelope trimer, rendering their locations difficult to predict based on HIV-1 sequence alone.

Recent studies have therefore taken the approach of longitudinally characterizing envelope evolution while simultaneously attempting to isolate individual neutralizing antibodies (and/or the B-cell clonal lineages producing them) in individual patients [30, 167, 168, 177, 178]. From these studies, a central role of immune-driven envelope evolution in driving autologous neutralization breadth is emerging. In one individual, initial autologous NAbs were directed against epitopes in the first and second hypervariable loops of gp120 (V1/V2), and escape was achieved via point mutations in this region including one in V2 that created a putative *N*-linked glycosylation site conferring escape from two distinct monoclonal antibodies isolated from this patient [177]. In a second individual, escape from the initial NAb pool occurred via convergent evolutionary pathways (one involving changes in the

V3–V5 gp120 outer domain and the other involving codependent changes in V1/V2 and gp41), whose lineage members subsequently oscillated in frequency over time [177]. NAb escape via distinct evolutionary pathways within a single host was confirmed in an individual in whom escape in a V3-proximal epitope occurred along three divergent viral lineages, each featuring a unique amino acid change [167]. A subsequent study of three acutely infected individuals whose initial response was directed against different conformational epitopes in envelope, where each escaped along distinct pathways [164], also supports the strain- and host-specific nature of initial epitope targeting and autologous neutralization escape. That escape occurs via distinct mechanisms (e.g., point mutations, glycan shifts, and cooperative conformational changes between two domains) both within and among hosts indicates that HIV-1 employs multiple mutational strategies to escape early autologous neutralizing antibodies [177].

Though autologous NAbs appear after HIV-1-specific CTL, NAb escape shares some similarities with CTL escape. Analogous to other HIV-1 regions, within-host envelope diversification is initially starlike—but, after the appearance of the first NAbs, multiple amino acids often transiently appear in regions under pressure, from which the final neutralization mutant(s) ultimately emerge [164, 168]. Of interest, the time course of selection (and subsequent fixation) of NAb escape mutations is on average slower than the corresponding process of CTL escape in early infection [164]. Moreover, fitness costs ranging from 0 to 24 % were observed for early envelope escape mutants, indicating that NAb escape can also be fitness-costly [164]. The extent to which neutralizing antibody epitopes—and their escape pathways—are shared across patients also remains a key question. The observation that, compared to transmitted/founder viruses, chronic subtype C viruses are significantly enriched for a glycan at envelope codon 332 (whose presence can help trigger the evolution of broadly neutralizing antibodies against this key conserved epitope region [30]), supports the idea of shared evolutionary pathways of neutralization escape. Finally and importantly, the discovery that broadly neutralizing antibodies evolve via complex and dynamic interplay between virus and host immune response has led to the hypothesis that this process could be recapitulated via vaccination with specific transmitted/founder envelopes and their sequential escape variants [168]. Though some experimental support already exists for such a strategy [179], further research will be required to move this exciting new idea forward.

2.9 Innate Immune Responses: KIR-Driven HIV-1 Polymorphisms?

Host-driven polymorphisms that do not map to known CTL or NAb escape sites are often identified in individual and population-level studies. Though many are likely attributable to incomplete epitope mapping, some could represent polymorphisms selected by immune responses other than CTL or NAb. In particular, evidence supports innate immune responses, notably Natural Killer (NK) cells, as mediators of HIV-1 immune control and potential drivers of immune escape.

NK cells express cell-surface receptors belonging to the highly polymorphic Killer cell Immunoglobulin-like Receptor (KIR) gene family, which comprise a variety of inhibitory and activating receptors that interact with HLA class I ligands on target cells [180]. Engagement of activating KIR (which generally exhibit short cytoplasmic tails, denoted by “S” in the gene name) delivers a stimulatory signal, whereas engagement of inhibitory KIR (which generally exhibit long cytoplasmic tails, denoted by “L” in the gene name) delivers a tolerance signal; when the former overcome the latter, NK effector functions are initiated [180]. Indeed, a major trigger for enhanced NK cell-mediated recognition of HIV-1-infected cells is the selective downregulation of their HLA-A and -B (though not C) ligands by the viral Nef protein [181], leading to a reduction in signaling through inhibitory KIR. Inhibitory KIR bind their HLA class I ligands in an allotype-specific manner. For example, KIR3DL1 receptors interact with HLA-B molecules belonging to the Bw4 allotype (determined by amino acids 77–83 of the HLA coding region), notably those harboring isoleucine at position 80 (Bw4-80I), and to a lesser extent those harboring threonine at this position (Bw4-80T) [182–184]. Some activating KIR also recognize HLA class I in an allotype-specific manner, though generally at lower avidity than their inhibitory counterparts [185]. An example is KIR2DS1, which binds HLA-C molecules belonging to the C2 allotype (determined by amino acids 77–80 of the HLA coding region) [186, 187]. Of note, despite high similarity between the extracellular domains of activating KIR to those of their inhibitory counterparts, many ligands for activating KIR remain unknown.

KIR, alone and in combination with their allotype-specific HLA ligands, may modulate HIV-1 susceptibility and pathogenesis [185]. HIV-1-infected individuals expressing the activating KIR3DS1 allele in combination with HLA-Bw4-80I exhibit lower viral loads [188], delayed clinical progression [189] and protection from opportunistic infections [188], though not in all studies [190, 191]. Higher frequencies of KIR3DS1 homozygosity [192, 193] and higher KIR3DS1/3DL1 transcript ratios [194] have been observed in HIV-1 exposed seronegative individuals, suggesting that activating KIR may also confer some level of protection against HIV-1 acquisition. Though protection via engagement of an activating receptor seems intuitive, the underlying mechanism remains unknown (KIR3DS1-expressing NK cells can inhibit Bw4-80I–expressing cells in vitro [195], but there remains no evidence that KIR3DS1 directly binds HLA-Bw4-80I [196]). Intriguingly, KIR3DL1 alleles possessing a high-expression, high-inhibitory phenotype (termed KIR3DL1*h/*y) may also be protective [197]. When present in combination with HLA-Bw4-80I alleles, notably HLA-B*57, KIR3DL1*h/*y alleles were associated with lower viral loads and conferred protection against HIV-1 disease progression [198]. KIR3DL1*h/*y-HLA-B*57 co-expression may also protect against HIV-1 acquisition [199] (though another study that did not discriminate KIR3DL1 alleles based on expression reported the opposite [193]). That highly inhibitory KIR receptor–ligand interactions can be protective seems somewhat counterintuitive, especially given that the opposing signals of activating KIR may also be protective. Nevertheless, the data support a role, albeit complex and incompletely elucidated, of KIR in HIV-1 control.

KIR-associated immune pressures may also drive the selection of viral polymorphisms that allow infected cells to evade NK-mediated killing. To shed light on how such mutations could arise in a reproducible manner, we must first briefly revisit KIR-ligand binding. Though not antigen-specific in the classical sense, KIR receptor–ligand interactions are nevertheless modulated in part by HLA polymorphism (within members of the relevant allotype [200]) and the sequence of the HLA-bound peptide [201–205]. In particular, C-terminal proximal epitope residues may play a role in KIR–HLA interaction [186, 203, 206]. The idea that naturally arising HIV-1 variants could affect KIR–HLA binding was supported by reduced in vitro binding of KIR3DL1 to its HLA B*57:03 ligand in the presence of the TW10 epitope harboring a G-to-E substitution at position 9 (though this was not claimed to be an in vivo NK-driven escape mutation, as failure to engage KIR3DL1 would render infected cells more, not less, susceptible to NK-mediated killing [95]). Rather, NK cell escape could theoretically be achieved via viral polymorphisms that reduce recognition by activating KIR, or enhance recognition by inhibitory KIR. Towards the identification of such mutations, statistical association approaches were applied to $N=91$ linked KIR/HIV-1 sequences, yielding 22 KIR-associated viral polymorphisms. The researchers identified two linked polymorphisms in Vpu (71M/71H) that were particularly overrepresented among KIR2DL2-expressing persons [21]. Consistent with the greater affinity of KIR2DL2 for HLA-C group 1 compared to group 2 ligands [207], these polymorphisms were even more enriched among KIR2DL2$^+$ individuals homozygous for HLA-C group 1 alleles [21]. Researchers further showed in vitro that the presence of these polymorphisms enhanced the ability of the inhibitory KIR2DL2 to bind HIV-1-infected cells, that KIR2DL2$^+$ NK cells failed to become activated in the presence of polymorphism-containing HIV-1, and that cells infected with polymorphism-containing HIV-1 were not inhibited by KIR2DL2$^+$ NK cells [21]. However, researchers were unable to elucidate the mechanism whereby these polymorphisms reduced the ability of KIR2DL2$^+$ NK cells to recognize variant virus-infected cells, nor were they able to identify whether specific peptide(s) played a role in this interaction.

Despite remaining questions, these findings suggest that immune pressure by an inhibitory KIR could select in vivo escape mutations conferring enhanced binding of the inhibitory receptor to HIV-1-infected cells, thereby allowing them to escape NK cell-mediated elimination. The recent identification of an HLA-C*01:02-restricted p24^{Gag} peptide variant that bound KIR2DL2, that conferred functional inhibition of KIR2DL2-expressing NK cells in vitro [208], provides theoretical support for this model. The idea that NK cells recognize antigen in a manner that is to some extent specific, leading to the reproducible selection of escape mutations in vivo, is intriguing. Further research is required to confirm and to elucidate the extent to which innate immune responses drive HIV-1 evolution.

3 Immune-Driven HIV-1 Evolution: Consequences and Implications

3.1 Fitness Consequences of Escape for Infection and Transmission

The protective effects of certain HLA class I alleles are attributable, at least in part, to their ability to mount strong CTL responses against mutationally constrained HIV-1 regions where escape can only occur at a functional and/or replicative cost to the virus (e.g., [209]). In these cases, the viral advantage gained via immune escape is offset in part by its associated replicative costs, thus conferring some residual biological "benefit" to the host in terms of lower viral loads. For example, HLA-B*81 is a protective allele in context of the South African HIV-1 subtype C epidemic. The B*81-driven Gag-T186S escape mutation (selected at position 7 of the immunodominant B*81-restricted TL9 epitope spanning Gag codons 180–188) is fitness-costly [210] and difficult to compensate [211]. Thus, although Gag-T186S confers escape from the B*81 TL9-mediated recognition of infected cells, the sustained replication defects conferred by this substitution may contribute to the long-term clinical benefits associated with HLA-B*81 expression. It is important to note however, that initial immune-driven viral fitness reductions are often of limited duration. For example, recombinant HIV-1_{NL4-3} encoding acute/early Gag-Protease sequences derived from individuals expressing protective HLA display replicative reductions in acute/early infection, but these defects are largely undetectable by chronic infection due to the selection of compensatory mutations [212].

The clinical "benefits" of immune-driven viral replicative costs can also be detected when viruses containing such mutations are transmitted to persons lacking the restricting HLA. Indeed, lower viral loads in individuals acquiring HIV-1 with key fitness-costly escape mutations in Gag [213, 214] (though not Nef [214]) have been demonstrated. That immune-driven mutations selected by past hosts inherently influence the pathogenicity of a given HIV-1 sequence is supported by the observation that a substantial fraction of set-point plasma viral load is "heritable" from one infection to the next [215]. That HIV-1 sequences are inherent determinants of pathogenesis is also supported by the observation that viral replication capacity correlates positively with viral load (and negatively with $CD4^+$ T-cell count) at various infection stages [139, 210, 212, 216, 217]. Extending these observations, one could hypothesize that the acquisition of attenuated HIV-1, followed by further within-host selection of fitness-costly escape mutations, would provide maximal clinical "benefit" to the host. Indeed, elite controllers, rare individuals who are able to spontaneously suppress plasma HIV-1 RNA to below limits of clinical detection without the need for antiretroviral therapy [218], provide a model for this phenomenon [219]. Elite controller-derived HIV-1 sequences generally exhibit reduced function compared to HIV-1 from noncontrollers at both early [220, 221] and chronic [116, 222–224] infection stages, supporting the acquisition of attenuated HIV-1 in at least some of these individuals. Two lines of evidence suggest that

these relative defects are also attributable to the within-host selection of fitness-costly mutations. First, elite controllers expressing protective HLA alleles exhibit even greater HIV-1 attenuation than those who lack them [222, 223], in a manner that is "dose dependent" on the number of mutations selected [223, 224]. Second, elite controllers harbor noncanonical escape mutations that confer greater fitness costs than conventional ones [225, 226], possibly as a result of enhanced immune recognition of common CTL escape variants in these persons [227].

The study of fitness consequences of escape in general—and in elite controllers in particular—is relevant to HIV-1 vaccine research. Specifically, immune-mediated containment of HIV-1 replication to levels that slow disease progression and possibly reduce transmission might be achievable through the design of vaccines that stimulate CTL responses focused against critically conserved viral regions where escape can only occur at substantial fitness costs [228, 229]. A related strategy would be to design immunogens featuring both "nonadapted" (susceptible) and "adapted" (escape variant) forms—provided the latter retain the ability to bind the relevant HLA molecules—with the goal of generating broad, potent, variant-reactive CTL responses that, upon infection, will drive HIV-1 evolution down unconventional pathways not unlike those selected in elite controllers [225, 226]. Strategies to comprehensively identify HLA-driven immune escape mutations, compensatory pathways and "vulnerable" sites across HIV-1 are thus paramount to achieving such goals.

3.2 Differential HIV-1 Adaptation to Global Populations

It is commonly said that HIV-1 adapts to its human hosts "at the population level" [31, 32]. This refers to the observation that, since HIV-1 genomes residing in an individual will exhibit adaptations to its host's immunogenetic profile, then HIV-1 sequences circulating in a given population will, by extension, exhibit adaptations that reflect the distinct immunogenetic profile of that host population [32, 136]. The existence of CTL escape mutations "unique" to particular host populations, because they are restricted by HLA alleles specific to these populations, provides one illustration of this phenomenon. For example, >50 % of HLA-associated polymorphisms identified in HIV-1 subtype B sequences in Mexico [136] and nearly two-thirds of those identified in Japan [230] are distinct from those observed in subtype B-infected cohorts from Canada/USA/Australia, because the former populations exhibit "unique" HLA alleles (e.g., B*39 in Mexico and B*67:01 in Japan) that are not found in the latter populations [136, 230]. That HIV-1 polymorphisms correlate with host ethnicity (a surrogate of HLA) also demonstrates population-specific viral adaptation [231].

Population-specific HIV-1 adaptation also manifests itself in terms of differential HIV-1 polymorphism frequencies among host groups. In particular, HLA-associated polymorphism frequencies among viral sequences circulating in a given population will generally reflect the frequencies of their restricting HLAs in that population [32].

This remains true even among individuals lacking the restricting HLA. This is because higher numbers of persons expressing the HLA will generally translate to higher numbers of polymorphisms selected and thus transmitted (though many factors, including the wide-ranging probabilities of polymorphism selection in context of their viral location and restricting HLA, the fact that multiple HLA alleles select the same—or opposing—mutations at a given viral location, the existence of "consensus" HLA-associations, and the timing of escape/reversion, will render this correlation less than perfect). The B*51-associated I135X mutation in Reverse Transcriptase (at the C-terminus of the B*51-TI8 epitope, RT codons 128–135) provides an example. In an analysis of nine cohorts spanning five continents, HLA-B*51 and RT-I135X prevalence exhibited a strong positive correlation [32], indicating that the more frequent an HLA allele is in a population, the more frequent its associated adaptations will be observed in circulating HIV-1 sequences.

Although a major portion of population-specific HIV-1 adaptation to host cellular immune responses is attributable to population-specific differences in HLA alleles and their frequencies, other host factors (e.g., variability in T-cell receptor genetics) also likely plays a role. A recent study comparing HLA-associated polymorphisms in HIV-1 subtype B cohorts in Japan versus Canada/USA/Australia identified numerous cases where the same HLA allele selected significantly different escape pathways across cohorts [230], implying factors beyond HLA in driving these differences. HLA-driven escape pathways also differ across HIV-1 subtypes, presumably as a result of genetic differences in the viral backbone. For example, Gag-T242N is commonly selected by B*57 in HIV-1 subtypes B [83, 126, 137, 138], C [125], and D [232] but rarely in subtype A1 [232]. Similarly, the fitness costs of escape can differ across subtypes. For example, Gag-M250I confers profound fitness costs in subtype B (where it represents a rare escape mutation selected by HLA B58 supertype alleles) but not subtype C (where it appears to be a minor non-HLA-associated polymorphism) [226]. Together, these observations highlight the relevance of HLA, along with other host and viral genetic determinants of HIV-1-specific CTL responses, in driving HIV-1 evolution. As such, cellular vaccine designs featuring immunogens that incorporate immune-relevant HIV-1 diversity may require us to distinguish escape pathways that are "universal" across host populations and/or HIV-1 subtypes, from those that are population and/or HIV-1 subtype-specific.

3.3 Is HIV-1 Becoming Increasingly "Resistant" to Host Immunity as the Epidemic Progresses?

As described in previous sections, many—though not all—immune escape mutations selected in the previous host will revert to consensus upon transmission to a host lacking the restricting HLA allele. As such, the persistence of certain escape mutations following transmission has led to concerns that these could gradually spread throughout the population (Fig. 25.4) [32, 105, 233–237]. Analogous to the

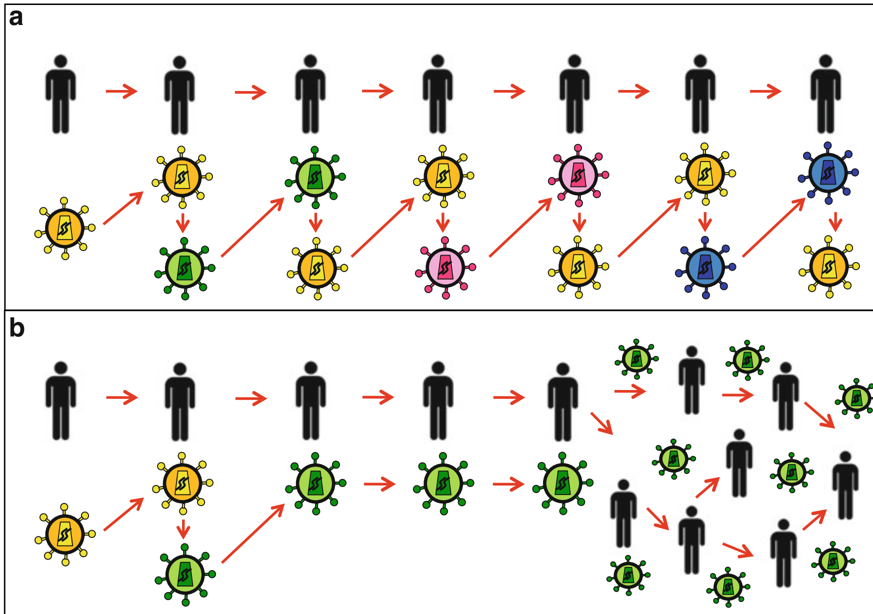

Fig. 25.4 The persistence of HIV-1 immune escape mutations upon transmission could lead to their gradual spread in the population over time. In this simplified diagram of HIV-1 transmission, viruses harboring the consensus amino acid at a particular codon are colored *yellow*, whereas *other colors* denote viruses harboring various HLA-associated escape mutations. *Arrows* depict HIV-1 transmission from host to host, and the subsequent selection and/or reversion of escape mutations within these hosts. *Panel A*: In this scenario, HIV-1 escape mutations selected in previous host(s) revert consistently upon transmission. When sampled at a given point in time, sites of viral escape would exhibit diversity at the population level (as the relevant polymorphism would be present in some hosts but not others); however, the population consensus sequence would remain stable (unchanged) over time. *Panel B*: In this scenario, an HIV-1 escape mutation stably persists upon transmission to hosts lacking the relevant HLA allele. Such mutations could gradually spread throughout the population, causing the subtype consensus to shift over time. For more detail, see Sect. 3.3

negative impact of transmitted drug resistance mutations on treatment efficacy [238], acquisition of "escape mutant" HIV-1 by persons expressing the relevant HLA could undermine the ability of their CTL to control infection. As such, the spread of HIV-1 strains harboring escape mutations throughout the population could gradually undermine host antiviral immune potential, and potentially diminish the protective effects of certain HLA alleles as the epidemic progresses [31, 32, 106]. Indeed, the S173A compensatory mutation has been shown to stabilize the B*27-associated R264K mutation in $p24^{Gag}$ upon transmission [106, 107] and the S165N compensatory mutation has been shown to stabilize B*57-associated mutations within the $p24^{Gag}$ KF11 epitope [98], supporting this concern. That certain (though not all) escape mutations are capable of spreading in HIV-1-infected populations has also been demonstrated via mathematical modeling [104].

The extent to which HLA-associated polymorphisms are spreading in HIV-1-infected populations remains incompletely known, in part due to the scarcity of historic data. Nevertheless, it has been suggested that CTL epitopes in European HIV-1 sequences are being “lost” through mutational escape from HLA-B mediated selective pressures [235]; similarly, higher viral polymorphism frequencies have been reported in modern compared to historic HIV-1 subtype B and F sequences in South America [236]. The high frequency of the B*51-associated HIV-1 Reverse Transcriptase (RT) I135X mutation in Japan, a population where B*51 prevalence approaches 20 %, is also suggestive of escape mutation accumulation [32] (though the possibility that the Japanese epidemic was founded by an HIV-1 sequence containing RT-I135X cannot be ruled out). A recent comparative study of historic (1979–1989) versus modern (2000+) HIV-1 subtype B cohorts in North America revealed modest, though statistically significant increases in the average background frequencies of HLA-associated polymorphisms, notably in Gag, over the study period which paralleled an approximate twofold increase in HIV-1 diversity during this time [146]. Although the extent of polymorphism spread appears relatively modest for the North American HIV-1 epidemic, corresponding rates of immune-driven polymorphism spread in regions with high HIV-1 prevalence, older epidemics, differential transmission dynamics and/or where host HLA diversity is relatively limited may be higher, and thus possess more immediate implications for host immunity in these populations.

The accumulation of CTL escape mutations in circulating HIV-1 sequences is paralleled by a similar phenomenon driven by humoral immunity. Two recent studies evaluating antibody neutralization resistance of historic versus modern HIV-1 envelope sequences suggest that HIV-1 is drifting towards a more neutralization-resistant phenotype over time [239, 240]. Furthermore, contemporary sera exhibited lower heterologous neutralizing activity than historic sera, consistent with a gradual undermining of humoral immunity as HIV-1 becomes increasingly neutralization resistant [240]. Taken together, evidence suggests that HIV-1 is becoming—albeit gradually—more “pre-adapted” to host immunity as escape mutations spread in circulation. Further studies are therefore warranted to explore the extent of HIV-1 adaptation to cellular and humoral immune pressures in different host populations as their respective epidemics increase in age and diversity, and the potential implications of this adaptation for natural and vaccine-induced immunity over time.

3.4 Implications of Immune Escape for Antiretroviral Therapy

Immune-driven HIV-1 polymorphisms can also influence HIV-1 susceptibility to antiretroviral drugs [241, 242]. Bevirimat, an HIV-1 $p24^{Gag}$ (capsid) maturation inhibitor whose development was halted in 2010 following poor efficacy in individuals harboring common Gag polymorphisms, provides an example. Bevirimat prevents capsid formation by inhibiting cleavage at the CA/SP1 site in Gag [243], but its activity is reduced in HIV-1 harboring naturally occurring substitutions

within the QVT motif of SP1 (Gag codons 369–371) and/or Gag substitutions V362I, S373P, and I376V [243–246]. Perhaps unsurprisingly, these "naturally occurring" substitutions are largely HLA-driven. Gag V362I, conferring high-level bevirimat resistance [245], and S373P, potentially associated with low-level resistance [244, 245] are selected by HLA-B*35 [126], whereas a variety of HLA alleles including C*03 select polymorphisms within the QVT motif [126]. Indeed, up to 50 % of subtype B sequences (and >90 % in other subtypes) harbor polymorphisms within the QVT motif [247], underscoring the relevance of immune-driven polymorphisms to drug development.

By definition, licensed antiretrovirals will have demonstrated potent activity against a range of HIV strains, so any impact of immune-driven polymorphisms on their activities will be subtler (or the relevant polymorphisms more rare) than the above example. Nevertheless, such effects have been documented for Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs). The first evidence arose via primary HIV-1 drug resistance surveys that identified a minority of HIV-1 RT sequences exhibiting intermediate-level reduced in vitro susceptibility to NNRTIs in the absence of major resistance mutations [248, 249]. These observations were not explained by shared viral ancestry, but rather by the presence of polymorphisms at sites not previously associated with resistance [249]. In particular, I135T/V/L and L283I in RT, present alone or in combination, conferred up to threefold reduced in vitro susceptibility to NNRTIs [250]. Notably, these polymorphisms are immune-driven: those at RT codon 135 are selected by a variety of HLA alleles including B*51 [32, 126, 149, 242] and B*52 [86, 149], whereas 283L is selected by B*15 [126]. Polymorphisms at RT codon 138 (E138G/A/K), selected by B*18 [86, 241] and B*46 [86], may also mediate up to sevenfold decreased susceptibility to the second-generation NNRTI inhibitor rilpivirine [241]. Naturally occurring polymorphisms modulating in vitro NNRTI susceptibility have also been identified in non-subtype B contexts [251].

Other immune-driven polymorphisms do not directly influence drug susceptibility, but rather facilitate the selection of major resistance mutations in vivo. The presence of I135T has been associated with the subsequent selection of the K103N resistance mutation during NNRTI therapy [252, 253], possibly because it enhances stability of the mutant enzyme active site [252]. Furthermore, in vitro passage of HIV-1 containing the I135V/T/R polymorphisms in the presence of NNRTI led to the selection of E138K, which together with I135V/T/R conferred significantly reduced in vitro susceptibility to both first and second-generation NNRTIs [242]. Similarly, the A*11-associated V106I polymorphism [86] confers significant in vitro resistance to some NNRTIs when present in combination with natural polymorphism V179D [254].

Despite the observations described above, it is important to note that no studies have conclusively demonstrated that specific HLA alleles (and/or the presence of specific immune-driven viral polymorphisms) enhance risk of treatment failure [253, 255], an observation attributable in part to the widespread use of HIV-1 drug resistance testing to guide treatment choices [256, 257]. Such risks nevertheless remain a concern, and argue for enhanced collaboration across the fields of viral

immune adaptation and drug resistance. A comprehensive understanding of HLA-associated polymorphisms across HIV-1 subtypes and host populations could facilitate the identification of immune escape mutations capable of modulating the efficacy of current and future antiretroviral agents.

3.5 Escape from Vaccine-Induced Antiviral Immunity

Our discussion of mutational HIV-1 escape has thus far focused on natural immune responses. However, vaccine-induced immune responses could also exert sufficient pressures to drive viral evolution [258–260]. Analysis of “breakthrough” HIV-1 sequences infecting participants of recent vaccine trials supports this idea. A recognized challenge in designing vaccines against genetically heterogeneous pathogens such as HIV-1 is the possibility that vaccine-induced immunity may protect against infection by strains most similar to the vaccine immunogen(s), but not against genetically divergent strains. That vaccine-induced immunity could induce a partial barrier through which antigenically divergent HIV-1 strains could penetrate has been termed the “acquisition sieve effect” [261–263]. A related—yet mechanistically distinct—possibility is that vaccine-induced immunity would fail to block HIV-1 infection regardless of strain, but would instead drive the outgrowth of escape variants at rates exceeding those observed in natural infection [259, 262], a phenomenon termed “postinfection sieve effect.” The latter is particularly relevant to vaccines designed to stimulate cellular responses, as these are unlikely to block HIV-1 transmission. Vaccine sieve effects can be identified by retrospectively comparing the HIV-1 sequences of vaccine vs. placebo trial participants who subsequently became infected, to determine differences between them (e.g., in terms of specific HIV-1 polymorphisms and/or differences in their average genetic distance from the vaccine strain) [261–263]. Notably, acquisition and post-infection sieve effects are difficult to distinguish from one another, as both may occur before HIV-1 RNA can be reliably detected in the bloodstream [37], and/or may manifest themselves via the presence of identical immune-associated polymorphisms.

HIV-1 vaccine sieve effects were first suggested by the presence of atypical V3 amino acid motifs in HIV-1 Env sequences from individuals vaccinated with recombinant HIV-1_{MN} gp120 [258]. Recent comparisons of founder HIV-1 strains from vaccine and placebo recipients of the RV144 “Thai” vaccine trial [60] identified differential amino acid frequencies at Env V2 codons 169 and 181 between the two groups [260], suggesting that the vaccine preferentially blocked viruses harboring specific substitutions at these positions [260], possibly via vaccine-induced V2-specific antibodies possessing antibody-dependent cellular cytotoxicity activity [264]. Rapid selection of CTL escape mutations by vaccine-induced cellular immune responses may also have occurred in the failed STEP vaccine trial [61, 265]. Inferred T-cell epitope sequences within Gag/Pol/Nef (the regions contained within the vaccine) from infected vaccine recipients exhibited greater genetic distances to the immunogen sequence compared to those of infected placebo recipients, presumably

as a result of extensive and rapid immune escape [259]. The lack of such differences for epitopes within other HIV-1 proteins also supported this conclusion [259]. HIV-1 sequences from vaccine recipients also exhibited substitutions at Gag codon 84 more frequently than placebo recipients, identifying this as a putative signature site of HIV-1 evolution in response to vaccine-induced CTL responses [259].

The implications of vaccine-induced immune responses on the transmission, selection and evolution of HIV-1 are potentially profound. Rapid vaccine-driven immune escape could yield clinical consequences for the infected individual [266], whereas the use of partially effective vaccines capable of blocking infection by certain HIV-1 strains raises concerns regarding potential shifts in HIV-1 strain and lineage distributions (and their clinical and pathogenic consequences) at the population level. The fact that vaccine-induced immune responses (notably CTL) may target slightly different epitopes than those generally targeted in natural infection [267] may further complicate this issue and highlights it as an area worthy of future investigation.

4 Concluding Remarks

Since its identification as a novel human retrovirus just over 30 years ago [268, 269], HIV-1 has claimed the lives of an estimated 40 million individuals, with approximately 35 million additional persons currently infected [7]. Although expansion of HIV-1 treatment can help to stem the pandemic's tide [270–272], HIV-1's substantial capacity for host adaptation and ever-increasing global diversification, driven in large part by selection pressures imposed by the host immune response, remain major challenges for the design of interventions, notably a vaccine [3, 4]. Achieving a deeper understanding of how immune selection pressures drive the evolution and diversification of HIV-1 both within and among hosts, and how these viral changes in turn affect our immune responses to the virus, will bring us closer to our ultimate goal of ending the HIV-1 pandemic.

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Chapter 26
The Biology of Quiescent CD4 T Cells, Their Role in HIV-1 Infection and Cocaine Drug Abuse

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Core Message This chapter discusses the biology of quiescent CD4 T cells, their resistance or block of HIV infection, and the factors including cocaine drug abuse that can alleviate this block. The permissiveness of resting T cells can be impacted by a variety of immune and non-immune factors. As resting T cells are the major component of the HIV latently infected population, elucidating the mechanisms of HIV infection in this cell type will have major implications on HIV treatment.

1 Quiescent T Cell Biology

Human T cells (both CD4 and CD8) are unique as they can remain at a non-dividing, low-metabolic state over prolonged periods of time. The majority of T lymphocytes circulating in blood are at the G_0 state of the cell cycle, a stage characterized by lack of DNA replication and RNA transcription [1–6]. Following exposure to an antigenic insult, T cells will be activated and expand rapidly to deal with the potential invader [1]. Even under these conditions, only a small subset of these resting T cells will respond to antigen and expand. Following clearance of infection, the majority of the expanded T cells will undergo apoptosis, with a small fraction of them transforming into memory cells. The latter allows for more effective and rapid responses, should the same antigen insult present itself. Interestingly, even memory T cells can survive for very long periods (months to years) of time under a non-dividing state.

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Table 26.1 The transcription factors involved in maintaining T cell quiescence

Quiescence factors	Function
Lung krupple like factor (*LKLF*) and krupple like factor 4 (*KLF4*)	• LKLF expression downregulated in activated CD4 and CD8 T cells [7] • Targets and decreases c-myc levels [8] • Targets important genes involved in cell cycle, cytoskeleton rearrangement, signaling, and cell metabolism [11] • Deregulation of proliferation [15]
Forkhead box class P1 (*FoxP1*)	• FoxP1 deficiency in murine thymocytes leads to increased spontaneous activation, higher IL2 production, proliferation and death after CD4 and CD8 stimulation [17] • Effect might stem from FoxP1 targeting IL-7Rα
Forkhead box class O (*FoxO*) 1,3,4	• Target D-type cyclins at transcriptional level [22] • During FoxO induced quiescence, p130 levels are increased and its phosphorylation is associated with quiescent cell specific forms [21]
Transducer of ERBB2, 1 (*Tob*)	• High mRNA levels in unstimulated CD4 T cells, then downregulated following T cell activation [3] • Blocked expression of effector cytokines at halt T cells at G1 → S transition point through downregulation of cyclin proteins [3]

Previously, T cell quiescence was considered a default state to which T cells will return in the absence of any stimulation. However, T cell quiescence is an actively maintained state, tightly regulated by a series of transcription factors [3, 5–13]. During quiescence, T cells maintain very low metabolic rates, minimal levels of RNA transcription—mostly limited to basic housekeeping genes—, small cell size, and very long periods of survival [5, 6]. This state is beneficial, as quiescence prevents cellular damage from metabolism [14] and most importantly prevents unregulated expansion of T cells that could lead to the development of lymphomas or cause major immunopathologies due to tissue damage [5, 6]. In this section, we review the transcription factors (summarized in Table 26.1) identified to date in establishing and maintaining this quiescent state.

1.1 Krupple-Like Factors

Krupple-Like factors (KLFs) and more specifically lung Kruppel-Like factor (LKLF) have been shown to regulate and maintain quiescence [7, 8, 11]. Early studies showed that LKLF was expressed on both mature CD4 and CD8 T cells, only to be downregulated following activation [7]. Through the development of chimeric murine models, where lymphocytes were derived from LKLF$^{-/-}$ embryonic stem cell lines, it was shown that T cell numbers were significantly decreased (approximately a 90 % reduction), with minimal CD4 and CD8 T cells [7]. Moreover, the loss of LKLF led to spontaneous T cell activation and apoptosis mediated by

Fas-FasL [7]. When the protein—KLKF—was overexpressed in Jurkat T cells, a transformed T cell line, the results supported the above observations [8]. The loss of proliferation mediated by LKLF was not due to a specific cell cycle stage block but rather a generalized decreased rate in cell division [8]. Finally, the authors demonstrated that LKLF targeted and decreased the levels of c-myc [8], thus providing a mechanism for the role of LKLF in T cell quiescence. A more comprehensive study focusing on the LKLF-regulated gene expression revealed that in addition to c-myc there were additional targets [11]. These targeted genes were largely involved in cell cycle, cytoskeleton rearrangement, signaling, and cell metabolism. More specifically, through upregulation of LKLF in Jurkat T cells, it was shown that CDw52, CXCR3, EDG-1, EMP3, lymphotoxin β, and paxillin genes were upregulated while APG-1, PTPLA, TSHR genes were downregulated [11].

In addition to LKLF, recent studies have shown that KLF4 is a negative regulator of CD8 T cell and B cell proliferation [12, 15]. The protein is regulated by ELF-4 [15]; knockdown of ELF-4 caused downregulation of KLF4 leading to deregulated proliferation, both homeostatic and antigen-induced, as well as increased spontaneous activation of CD8 T cells [15]. No studies on the role of the protein in CD4 T cells have been done yet.

1.2 FOX Proteins

Forkhead Box (FOX) proteins are a diverse family of transcription factors implicated in major cellular functions. FoxP1 may be involved in T cell quiescence [16, 17]. FoxP1 deficiency in murine thymocytes leads to increased spontaneous activation, higher IL-2 production, proliferation, and cell death after stimulation in both CD4 and CD8 single positive thymocytes [16]. The observed phenotype was acquired when thymocytes transitioned from double positive to single positive. This could be explained by the fact that FoxP1 seems to target the expression of IL-7Rα that is highly expressed in the single positive thymocyte populations. Subsequent studies showed that knockdown of FoxP1 led to increased levels of IL-7Rα, which resulted in increased responses of mature CD8 T cells to IL-7 in the absence of TcR stimulation [17]. Thus, these studies suggest that FoxP1 is essential for T cell homeostasis and actively regulate T cell quiescence.

In addition to FoxP1, forkhead Box class O (FOXO) factors have been shown to play a role in T cell quiescence, especially FOXO1, 3, and 4 [18, 19]. T cells activation by cytokines such as IL-2 leads to their inactivation and cell growth [10, 19–24]. These factors seem to mediate their effects by targeting D-type cyclins at the transcriptional level, a phenotype that can be reversed upon ectopic expression of cyclin D1 [22]. Moreover, during FOXO-induced quiescence, the levels of p130 are increased and its phosphorylation state is associated with quiescent cell specific forms [21]. Finally, FOXO3a has also been shown to provide protection to quiescent T cells against reactive oxygen species, thus preventing apoptosis [14].

1.3 Tob

Finally, Tob, another nuclear protein, has been recently shown to be involved in cellular quiescence. It belongs to a family of antiproliferative proteins known as APRO. These proteins, including Tob, are expressed in a wide array of different cells and regulate cell proliferation, development as well as immunity [13, 25]. Tob was identified as an antiproliferative factor following its overexpression in NIH3T3 fibroblasts [26]. The interaction of the protein with p185erbB2 resulted in inhibition of its antiproliferative effect [26]. Later studies showed that the protein was overexpressed in anergic CD4 T cells [3]. A closer examination showed that the levels of Tob mRNA were high in unstimulated CD4 T cells only to be downregulated following T cell activation [3]. Tob mRNA was found to be expressed in the spleen, bone marrow, thymus, and blood with the latter showing the highest levels. This is expected, as the majority of circulating T cells in the blood are at a resting state. In addition to blocking T cell proliferation, it was shown that Tob blocked the expression of effector cytokines such has IL-2, IFN-γ, IL-4, and IL-10 at the transcriptional level. T cell proliferation was halted at the G1 → S transition point through downregulation of cell cycle promoting protein cyclin A, cyclin E, and Cdk2 and upregulation of the $p27^{kip1}$ protein [3].

While the above factors have been identified as key proteins to maintain quiescence, they have not been implicated in mediating the block of HIV infection. One report tested if LKLF-induced quiescence inhibits HIV infection only to find that it had no effect [11]. In the sections below, we review the studies to date that have attempted to elucidate the block, potential factors identified, and means by which the block has been alleviated.

2 HIV Replication in Quiescent CD4+ T Cells

HIV replication is not cell cycle dependent as in other retroviruses that require cells to undergo mitosis; yet the virus is known to infect resting cells and establish viral latency. However, observations made during the early studies on the virus revealed that the activation state of CD4 T cells was crucial in the establishment of a productive infection [27–29]. This pattern of infection was challenging and encouraged a series of studies attempting to further elucidate this phenomenon.

2.1 Quiescent CD4 T Cells Are Refractory to HIV Infection

A series of studies by Zack and colleagues demonstrated that quiescent CD4 T cells are resistant to HIV infection. While the virus could enter the cells and efficiently initiate reverse transcription, it failed to complete synthesis of viral cDNA and thus to establish a productive infection [30]. In follow-up experiments [31], HIV infection in these cells was further characterized. More specifically, they showed that

reverse transcription not only failed to complete in quiescent T cells but also was quite slower. Moreover, infection was rescued after activation of quiescent T cells suggesting that the partial transcripts could serve as a template to restore infection. There was, though, one caveat; rescue was not proportional to the initial levels of infection suggesting that the partial transcripts were labile. These observations were further supported by studies from the Vitteta group [32–34]. Their approach involved separating $CD25^+$ from $CD25^-$ T cell populations, thus distinguishing the activated from the non-activated T cells, respectively, since CD25 is a marker of T cell activation. In their studies, they infected the two populations and showed that the $CD25^+$ T cells could be infected by the virus without the need of pre-stimulation. Interestingly, when they repeated the experiments with mixed peripheral blood mononuclear cells that include activated and resting T cells, dendritic cells, macrophages, monocytes, and NK cells, they noticed that the $CD25^-$ T cells were infected. They observed detectable levels of complete reverse transcribed viral cDNA. Based on their studies at the time, they concluded that productive infection of the $CD25^-$ T cells was only made possible in the presence of $CD25^+$ T cells. This would suggest that the productively infected $CD25^+$ T cells would efficiently transfer virus to their negative counterparts and potentially make them more permissive to infection.

2.2 *Quiescent CD4 T Cells Are Productively Infected by HIV and Are an Inducible Reservoir*

While the above groups demonstrated that there is a cell type resistant to HIV infection, some of their contemporaries suggested otherwise. Studies by Stevenson and colleagues showed that resting CD4 T cells can be infected by the virus but the viral cDNA fails to integrate until the these cells are activated [35]. Upon activation, the viral cDNA could then be integrated, establishing a productive infection. This suggested that reverse transcription was completed in resting T cells and that the viral cDNA was stable. This is in stark contrast to the studies by Zack and colleagues that showed reverse transcription did not complete and the viral reverse transcripts are very labile. These observations were further supported by Spina and colleagues [36] that demonstrated the presence of completely reverse transcribed viral cDNA for up to 10 days in culture but that reverse transcription occurring at a progressively lower rate.

2.3 *HIV Life Cycle Is Impacted at Different Stages in Quiescent T Cells Resulting in a Highly Inefficient Infection*

Based on the earlier studies it was unclear whether quiescent CD4 T cells were refractory to infection. There are several reasons for that. Cell purification technologies were quite limited at the time allowing for contaminating populations to skew data.

Table 26.2 The identified blocks to HIV-1 infection in quiescent T cells

Viral life cycle stage	Quiescent cell impact
Entry	Largely unaffected [44, 48]
Uncoating	Under investigation
Reverse transcription	Delayed, decreased compared to activated cells, inefficient, accumulation of labile viral cDNA [37–40, 44]
Integration	Occurs in quiescent cells, inefficient as demonstrated by high levels of 2-LTR circles, site selection may or may not be similar to activated cells; potential impact for latency [46, 47]
Viral synthesis and release	Nuclear retention of viral mRNA resulting in decrease viral release [57]

Moreover, real time PCR assays, while useful were not sufficiently sensitive. More importantly, as we outline in Sect. 1.3, the non-dividing CD4 T cell population is quite diverse, comprised of permissive and non-permissive cells, thus leading to divergent conclusions.

A series of recent studies by others and our group have shed more light on the interaction between HIV infection and quiescent CD4 T cells. These studies were mostly focused on characterizing the life cycle of HIV in these cells rather than examining restriction factors (reviewed in Sect. 1.4). These studies took advantage of more sensitive real-time and non-quantitative PCR protocols, improved cell-sorting technologies, and improved flow cytometric assays to assess the purity of the populations targeted (summarized in Table 26.2).

Through the use of a linker-mediated PCR assay, the Siliciano group was able to characterize the non-integrated reverse transcripts in HIV infected resting CD4 T cells [37]. They found that reverse transcription could be completed in this cell type, but with significant delays, 2–3 days later. This delay resulted, as shown by earlier studies, into a labile cDNA that had a half-life of approximately one day. Interestingly, follow-up studies showed that the viral cDNA made was integration competent [38]. However, the high rate of decay combined with the slower kinetics resulted in a block to infection [38].

The above studies demonstrated that the impact on HIV infection must occur at the early stages of infection. They also more closely supported the observations made by Zack and colleagues [30, 31]. The O'Doherty group and our group through a series of studies further characterized the life cycle of HIV in quiescent CD4 T cells and revealed some of the blocks that are taking place. In Swiggard and colleagues [39] it was first shown that in quiescent cells, the levels of reverse transcription were significantly decreased. However, any reverse transcripts made were stable for approximately 3 days. Moreover, the authors showed that the full-length viral cDNA accumulated over time in the infected cells. In a follow-up study [40], they took advantage of a very sensitive nested, Alu-based PCR assay to measure low levels of integration [41]. Through this assay, they showed that the quiescent T cells harbored integrated provirus, supporting the earlier studies by the Siliciano group of integration competent viral cDNA [40]. More interestingly, upon activation of these

cells they were able to induce expression [40]. Thus, quiescent T cells could be latently infected and potentially be a reservoir. Yet despite this observation, it was clear that quiescent T cells blocked the ability of HIV to efficiently replicate. Even after supplementing with nucleosides, a major building block in DNA synthesis limited in quiescent T cells, both the Zack and O'Doherty groups showed that despite improvement in the levels of reverse transcription, the deficiencies still persisted [42, 43].

We examined in detail HIV replication in quiescent T cells as we analyzed all stages of viral replication impacted in this cell type [44]. Following infection of CD4 T cells with HIV-1_{NL4-3}, we measured the levels of viral entry in both quiescent and activated CD4 T cells. The differences between the two groups were not significant. Reverse transcription was delayed in quiescent T cells by 18 h. In addition, it was decreased by 30-fold compared to the levels seen in activated T cells. Using the O'Doherty protocol, we measured viral integration as well. Due to the decreased reverse transcription, the levels of viral integration in quiescent cell were lower than that of activated ones. However, we saw further delays, as integration took place about 36 h later in quiescent cells. Nevertheless, the efficiency seemed to be similar to that of activated cells as the relative levels of integration were comparable. As these cells expressed viral cDNA, we assessed if there was any virus expression. Multiply spliced tat-rev was detected in quiescent cells but as expected at lower levels than that seen in integrated cells. Despite the expression of multiply spliced HIV RNA we did not detect any expression of virus from quiescent cells. The surprising observation in our studies was that we could not rescue the observed block in quiescent T cells [44]. More specifically, we stimulated quiescent T cell immediately after infection to see if we can rescue infection. Interestingly, these post-stimulated cells displayed similar patterns on infection with that of quiescent T cells. Thus, this block to infection was quite effective. Our studies have minor differences with that of the O'Doherty group [39, 40], which can be attributed to the different protocols of infection [45]. Nevertheless, both our studies underscore that quiescent T cells are quite but not completely resistant to infection.

The presence of integrated HIV warranted further investigation into the integration site selection in these cells. As quiescent T cells are distinct from activated ones, there could be some unique patterns that may impact the study of HIV latency and the viral reservoir. Two studies demonstrated that HIV was integrated in transcriptionally active sites of quiescent T cells [46, 47]. We showed that there were no differences between quiescent and activated T cells. More specifically, in both HIV was localized in gene dense regions, near CpG islands, and was associated with epigenetic patterns that correlate with transcriptional activation rather than repression [46]. Brady and colleagues demonstrated that there were modest differences between the two cell types as HIV in activated T cells integrated in gene dense regions and histone methylation patterns linked to transcriptionally active genes [47]. In addition to site selection, we looked at the state of the viral LTR through sequences analysis of 2-LTR circles and proviral DNA [46]. We noticed that in quiescent T cells there was a higher frequency of LTR mutants in both proviral and 2-LTR DNA. The mutations were not specific but rather a collection of additions to

LTR junctions of 2-LTR circles and large deletions ranging from 10 to 200 base pairs on either LTR end. Thus, integration efficiency is not as high as we initially thought due to these major defects. Moreover, the integrants found in quiescent T cells may be defective due to LTR attrition. While our LTR sequence survey was not quantitative, this would suggest that a significant fraction of quiescent T cells harboring provirus might not be capable of producing HIV upon stimulation.

Thus, despite differences among groups, it is well established that quiescent T cells, in light of some significant defects, can be latently infected by HIV. In the following section, we explore the potential restriction factors that have been suggested to cause these defects as well as means to render quiescent T cell more permissive to infection.

3 Maintenance of Restriction and Overcoming It

3.1 Restriction Factors

The majority of studies on HIV infection of quiescent T cells was focused on characterizing the changes in the viral life cycle. As this is a very unique phenomenon, identification of factors responsible for this block can have major implications for the development of new anti-HIV therapies. Initially it was believed that the block was due to limited raw materials needed by the virus to replicate, such as nucleotides. However, pretreatment of quiescent T cells with nucleosides failed to rescue infection [42, 43]. Thus, the focus has been shifted to the identification of factors (summarized in Table 26.3) the presence of which or lack thereof is responsible for the block.

Early events in the viral life cycle including entry, uncoating and reverse transcription are prime targets for the block. Studies have shown that HIV can efficiently enter quiescent CD4 T cells and it is not seen as a potential block [44, 48]. One study examined the efficiency at which HIV uncoats in quiescent T cells. Based on their findings, uncoating was impaired and was attributed to a yet to be identified

Table 26.3 A summary of HIV-1 restriction factors identified in quiescent T cells

Restriction Factors	Function
Sterile alpha motif (SAM) domain and HD domain-containing protein 1 (*SAMHD1*)	Regulates pool of available nucleosides through degradation, thus restricting RT in quiescent T cells [54, 55]
c-Jun N-terminal kinase (*JNK*) and peptidyl prolyl-isomerase enzyme (*Pin1*)	Lack of protein in quiescent T cells could impact integration step in viral life cycle [56]
Polypyrimidine tract-binding protein (*PTB*)	Low expression. Needed for cytoplasmic transport of viral mRNA from nucleus [57]
Copper metabolism domain containing 1 (*Murr1*)	Highly expressed. blocks Nf-κB activity [58]

protein factor [49]. In terms of reverse transcription, the Sterile Alpha Motif (SAM) domain and HD domain-containing protein 1 (SAMHD1) [50–53] has been recently implicated as a potential restriction factor in quiescent T cells [54, 55]. The protein regulates the pools of available nucleotides through their degradation, thus restricting reverse transcription in quiescent T cells where the pools are already limited.

While the efficiency of integration in quiescent T cells was shown to be largely unaffected, a recent study suggested that the lack of c-Jun N-terminal kinase (JNK) in quiescent T cells could impact this step in the viral life cycle. The protein phosphorylates viral integrase, which in turn interacts with the peptidyl prolyl-isomerase enzyme Pin1, causing a conformational change in integrase increasing the stability of the viral accessory protein and promoting efficient integration [56].

Viral gene expression s limited in quiescent T cells. Studies have indicated that resting T cells do not express high levels of polypyrimidine tract-binding protein (PTB) [57], which limits cytoplasmic transport of viral mRNA from the nucleus thus restricting expression of viral protein. Moreover, Murr1, a protein that blocks NFκB activity is highly expressed in resting CD4 T cells [58], which in turn can result in lower levels of virus expression.

Despite the plethora of restriction factors identified, not one in particular has been sequestered as the main restriction factor responsible for the block in quiescent T cells. These studies along with the work outlined in Sect. 1.2 point to the conclusion that HIV restriction in quiescent cells is more systemic, rather than limited to one particular aspect of the viral life cycle.

3.2 Alleviating Restriction

Early work by Korin and colleagues demonstrated that T cells need not be fully activated to be susceptible to infection. In her studies, she demonstrated that the non-dividing T cell population is quite diverse distinguished by the levels of cellular transcription. Using an elegant flow cytometry based assay, she was able to distinguish the resting T cells into two populations: (1) the $G_{0/1a}$ that was deemed as the truly quiescent T cells and (2) the G_{1b} that were resting CD4 T cells expressing high levels of RNA. The truly quiescent T cells are the subset that is resistant to infection, whereas the cells in G_{1b} are permissive. Based on this premise, we explored HIV permissiveness in differentially stimulated T cells [59]. More specifically, we stimulated using anti-CD3/anti-CD28 quiescent T cells for 1 or 2 days and then infected with HIV. One-day stimulation drove a subset of cells into the G_{1b} phase and it was sufficient to alleviate the observed block in quiescent cells. These studies were in agreement with earlier work that demonstrated that both the intensity and the duration of T cell stimulation could have a major impact on the establishment of a productive infection [60]. In addition to TCR crosslinking, increased permissiveness to infection can be achieved by exposure to cytokines. Through the exposure of resting cells to IL-2, IL-4, IL-7, or IL-15 and to some degree IL-6, resting T cells were transduced at a high rate with HIV based vectors as well as efficiently infected with

replication competent virus [61]. In addition to cytokines, engagement of toll-like receptors (TLR) as well as chemokine receptors can have a similar potentiating effect to infection. Studies have shown that engagement of resting T cells to TLR2 can lead to increased productive infection [62]. Moreover, crosslinking CCR7 to its ligands CCL19 and CCL21 leads to increased permissiveness to infection as well as establishment of latency [63, 64]. To further expand the list of ligands that can alleviate the block seen in quiescent T cells, the crosslinking of ICAM-3 can lead to increased infection [65, 66]. As this is a surface molecule involved in T cell adhesion, it also underscores the importance of cell-cell contact in productive transmission of HIV to resting cells. One study demonstrated infection of resting T cells in tonsillar tissues suggesting that in cell packed tissues such as lymphoid quiescent T cell restriction can be alleviated [67]. In all these studies, non-dividing CD4 T cells were permissive to infection.

3.3 Cocaine Exposure, Quiescent T Cells, and HIV Infection

Since the beginning of the AIDS epidemic, the use of illicit drugs has been widely examined in its role of human immunodeficiency virus (HIV) pathobiology. Not only are drug users at an increased risk of viral transmission by needle sharing or unsafe sexual practices, but also these drugs modulate immune function. One such drug, cocaine, has been shown to disturb normal immune functions by modulating distribution and effector functions of lymphocytes, neutrophils, NK cells, and helper T cells [68]. In addition, through the use of humanized mice, cocaine treated animals displayed higher levels of viral RNA levels and greater CD4 loss after infection with HIV [69]. Therefore, epidemiological and in vivo studies suggest cocaine use can influence HIV disease progression and further understanding of the stimulant's effect on immunopathology of virus is necessary.

One of our interests was to examine if cocaine falls under the category of non-immune factors that may minimally activate quiescent T cells to make them permissive to infection. There is a precedent for such effect; norepinephrine has been shown to enhance HIV replication [70]. In our studies, we treated quiescent T cells with 10^{-8} M cocaine for three days prior to infection [71]. Interestingly, a 3-day cocaine treatment led to increasing levels of cells present in the G1b stage of cell cycle, a stage when resting cells are permissive to infection. A more intriguing observation was that cocaine treatment did not impact classical T cell activation marker expression such as CD25, CD38, CD69, or HLA-DR. Together, these results suggested that cocaine may increase susceptibility of quiescent T cells to HIV infection through subtle changes of its quiescent state. Following infection, cocaine-treated cells harbored increased levels of full-length viral cDNA, accelerated kinetics of reverse transcription, tenfold higher levels of integrated DNA and higher viral production when compared with untreated quiescent T cells [71]. These data suggest that cocaine treatment circumvents the early block in efficient viral RT seen in quiescent cells and makes them permissive to infection. As cocaine is a

neurotropic factor, it was of interest to determine how this effect was mediated. Microarray data on quiescent cells revealed that the σ1 receptor (σ1R) and the dopamine 4 receptor (D4R) were expressed in these cells. Several studies have shown the σ1R as an integral signaling molecule in cocaine's potentiating effect on HIV infection. Using flow cytometry, we confirmed that both these receptors are expressed on quiescent T cells [71]. Through the use of agonists and antagonists for both receptors, we determined that the cocaine mediates its effects through both of these receptors with the D4R having a more pronounced effect [71]. We are currently carrying out studies to assess the mechanisms of this phenotype. Thus, our studies demonstrate that infection of resting cells can be impacted by other non-immune related factors that ultimately can have a major effect on the viral reservoir warranting a thorough investigation.

4 Conclusions

The relationship of quiescent T cell and HIV has been a much-debated area of HIV research. While significant progress has been made, there are still unanswered questions. It is clear that the major block to infection occurs very early, immediately following viral entry at the initiation of reverse transcription. While limited resources, such as restriction factors, can result in decreased levels of reverse transcription, downstream events prior to integration or even at integration play a major role. Therefore, further studies are needed to understand the block in quiescent T cells. To this date, it is unclear how the mechanisms of resistance in quiescent cells can translate into future therapies. Nevertheless, these studies will provide an improved understanding of the interactions between HIV and its various target cells that will ultimately lead to more effective interventions.

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Chapter 27
Role of Macrophages in the Immunopathogenesis of HIV-1 Infection

Jacqueline K. Flynn and Paul R. Gorry

Core Message Macrophages play a key role in the immunopathogenesis of HIV-1 infection. Expression of CD4 and both CCR5 and CXCR4 on their cellular surface renders them prime targets for HIV-1. They have a long-life span and reside in several tissues within the body, including immune privileged sites. Their ability to recruit immune cells to the site of infection, phagocytose infected cells and actively disseminate HIV-1 to multiple tissues creates complications for eradication of HIV-1 by both the immune system and current antiretroviral therapies.

1 Introduction

This chapter focuses on the role macrophages in HIV-1 disease progression. It examines their role in infection and dissemination of HIV-1, and characterizes macrophage involvement in acute HIV-1 infection and progression to chronic infection. It explores the role of macrophages in activating the immune system and reducing viral burden, but also in attracting cellular targets for HIV-1 and the transmission of

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HIV-1 between immune cells and distribution to multiple tissue sites. This chapter also discusses the effect of antiretroviral therapy on macrophages and the role restriction factors play in protecting macrophages from HIV-1 infection.

2 The Role of Macrophages in the Immune System

Macrophages are an integral part of the innate immune system. They are derived from the mononuclear phagocytic lineage, and released into circulation as bone-marrow-derived precursors, monocytes [1]. After monocytes migrate into the tissues through the endothelium of a blood vessel, via a process termed leukocyte extravasation, they differentiate into macrophages or dendritic cells. Therefore, monocytes play a key role in replenishing the macrophage population [1, 2]. Macrophages are found in most organs in the body and are named based on the tissue in which they reside. For example, alveolar macrophages reside in the lungs.

Macrophages are constantly surveying their environment for signals of tissue damage or invading organisms. They have the ability to phagocytose foreign antigens and present them to B and T cells acting as professional antigen presenting cells (APC), maintain healthy tissues by removing dead and dying cells, and also respond to danger signals detected by their surface receptors [1] (Fig. 27.1). Through their antigen presenting ability they can trigger antibody responses by presenting pathogen-derived peptides to $CD4^{+}$ T cells through the MHC class II pathway [1], and also activate $CD8^{+}$ T cells through the cross-presentation of phagocytosed extracellular antigens through MHC class I [3]. Thus, macrophages play an important role in both the innate and adaptive immune responses.

As well as the expression of a wide variety of receptors for phagocytosis on their cellular surface, macrophages also express the HIV-1 receptors CD4, CCR5 and CXCR4, making them a prime target for HIV-1 infection [2, 4]. The surface envelope glycoprotein of the HIV-1 particle, gp120, interacts with cellular CD4 and a co-receptor, CXCR4 or CCR5, to infect cells [reviewed in [5, 6]]. HIV-1 primarily infects $CD4^{+}$ T cells due to their high expression of CD4 on their cellular surface. However, macrophages, which have a comparatively lower expression of CD4 [4, 7, 8], also serve as key targets for HIV-1 [2, 9].

The ability of macrophages to infiltrate many organs and their dissemination over different tissues enhance their contribution to the spread of HIV-1 through the body [4, 10, 11]. The life span of a macrophage also varies greatly depending on their location and immunological roles, with some lasting days to months and others years [12, 13]. One consequence of the long life span of macrophages is that they can harbor HIV-1 for a long period, contributing to the viral reservoir and posing a major obstacle for eradication [4, 14, 15].

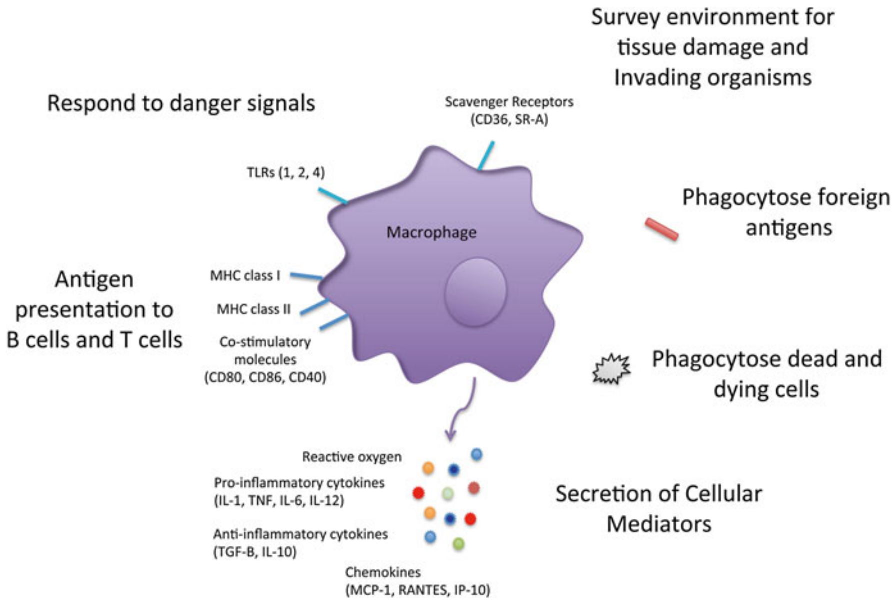

Fig. 27.1 The role of Macrophages in the immune response. Macrophages play an important role in both the innate and adaptive immune response. They can respond to danger signals detected by their pathogen recognition receptors, including toll-like receptors 1, 2, and 4 (TLR) [1]. Macrophages can also respond to foreign antigens through phagocytosis of foreign bodies and presentation to B and T cells. Macrophages are professional antigen presenting cells with the ability to present pathogen-derived peptides to both $CD4^+$ and $CD8^+$ T cells. Presentation to $CD4^+$ T cells occurs through MHC Class II and activation of $CD8^+$ T cells through the cross presentation of phagocytosed extracellular antigens through MHC Class I [3]. Macrophages commonly secrete cellular mediators, including cytokines and chemokines, often in response to foreign pathogens. Cytokines and chemokines are able to activate and attract surrounding cells, respectively. For example, secretion of anti-inflammatory cytokines, such as TGF-β and IL-10, can cause immune regulation and suppression, whereas secretion of pro-inflammatory cytokines, such as IL-12 and TNF-α, can promote a Th1 immune response, activation of dendritic cells, and promotion of host defense. Macrophages also play a role in tissue repair and phagocytosis of dead and dying cells [1]

3 Macrophage Classification in HIV-1 Infection

Macrophages can be classified into different subsets based on their immune function. M1 macrophages are known as classically activated macrophages, which mediate host defense from viruses, bacteria and protozoa [1, 14]. M1 macrophages are commonly associated with the secretion of proinflammatory cytokines that include interleukin (IL)-1β, IL-12, IL-15, IL-18 and tumor necrosis factor alpha (TNF-α) and also reactive oxygen [16, 17]. This response assists in modulating endocytic function and elimination of intracellular pathogens. M2 macrophages are known as alternatively activated macrophages that have an anti-inflammatory role and can regulate wound healing [1]. M1 macrophages can be activated by

interferon-gamma (IFN-γ) and usually by a microbial trigger, such as lipopolysaccharide (LPS), whereas M2 macrophages are activated by IL-4 and IL-13 [17, 18].

Deactivated macrophages (dM) also have a role in HIV-1 infection [reviewed in [18]]. The deactivated macrophages have strong anti-inflammatory functions and can downregulate MHC Class II on their cellular surface [19]. This can lead to immune suppression through the reduced MHC class II expression but also through the increased uptake of apoptotic cells creating an anti-inflammatory response. Deactivation of macrophages usually occurs through IL-10 secretion, but can also occur through transforming growth factor beta (TGF-β), macrophage colony-stimulating factor (M-CSF) and IFN-α/β secretion [18, 19]. Thus, deactivated macrophages are likely to play a regulatory role in HIV-1 infection.

The role of each macrophage subset during HIV-1 infection is not the main focus of this chapter. However, we briefly describe the prevalence of each subset during HIV-1 infection. In the acute stages of infection it has been proposed that M1 macrophages are the more dominant macrophages [18]. At this stage, viral proteins expressed early in the viral life cycle are present, particularly Nef, Tat and Vpr, which have been suggested to play a role in disease progression and in the formation of viral reservoirs in macrophages by activating transcription and interfering with apoptosis [20, 21]. High levels of Th1 (IFN-γ, IL-2, IL-12) and proinflammatory (TNF-α, IL-1β, IL-6) cytokines are present [18], and additionally chemokines, such as macrophage inflammatory protein (MIP)-1α/β and regulated on activation, normal T cell expressed and secreted protein (RANTES). These conditions favor the formation of viral reservoirs and the activation of M1 macrophages [21]. During acute HIV-1 infection, tissue injury in the lymph nodes and T cell apoptosis is common.

An increase in tissue injury and the presence of IL-4 and IL-13 cytokines during the later stages of HIV-1 infection are likely to contribute to a switch from the classically activated M1 macrophages to M2 macrophages [18]. M2 macrophages contribute to tissue repair, MHC Class II antigen presentation and T cell activation. During the onset of AIDS, opportunistic infections arise and T cell apoptosis increases [20]. This could result in increased clearance of apoptotic T cells by dM [22] and likely indicates an increase in IL-10 concentration [18]. A switch in Th1/Th2 immune responses and cytokine production, combined with an increase in IL-10 concentration, is likely to have detrimental affects on immune function and the progression to AIDS [23]. IL-10 in particular can have detrimental effects on viral infections, including its ability to suppress cytokine production and proliferation by $CD4^+$ and $CD8^+$ T cells, and alter the function of APC [24–27].

4 HIV-1 Infection of Macrophages

HIV-1 first attaches to macrophages through the interaction of the HIV-1 envelope glycoproteins on the viral surface with cellular CD4 on the macrophage (Fig. 27.2). The viral envelope includes the surface glycoprotein gp120 and the

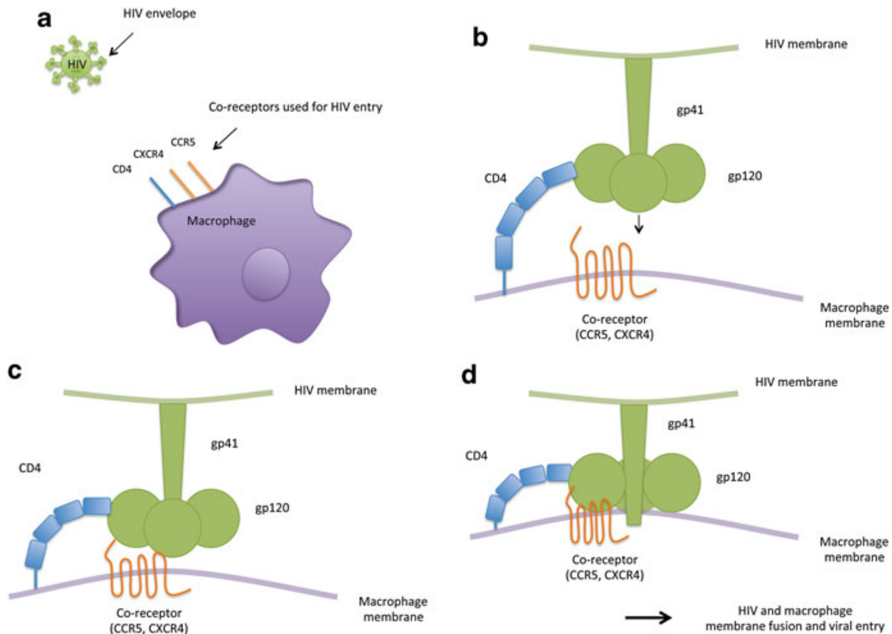

Fig. 27.2 HIV-1 entry into macrophages. (**a**) As well as expressing several cellular receptors for phagocytosis, macrophages express receptors for entry of HIV-1. These include CD4 and a co-receptor, such as CCR5 or CXCR4. (**b**) HIV-1 attaches to macrophages through the interaction of the HIV-1 envelope glycoprotein with cellular CD4 on the macrophage. HIV-1 gp120 binds to cellular CD4 anchoring the virus to the cell surface of the macrophage [28, 29]. (**c**) The binding of gp120 to CD4 triggers structural changes in gp120 that expose a binding site for the coreceptor [30]. gp120 binds to the coreceptor triggering conformational changes exposing the fusion peptide at the N-terminus of gp41 [31]. (**d**) The insertion of the fusion peptide into the macrophage membrane triggers the fusion of the viral and macrophage lipid bilayers and releases the HIV-1 particle core into the cytoplasm of the macrophage [29, 32]

transmembrane glycoprotein gp41. gp120 first binds to cellular CD4 anchoring the virus to the target cell surface [28, 29]. This triggers structural changes in gp120 that expose a binding site for the CCR5 or CXCR4 coreceptor [30]. gp120 binds to the coreceptor resulting in further conformational changes exposing the fusion peptide at the N-terminus of gp41 [31]. The insertion of the fusion peptide into the target cell membrane triggers the fusion of the viral and host cell lipid bilayers and release of the HIV-1 particle core into the cytoplasm of the target cell [29, 32]. Interestingly, macrophages are able to maintain unintergrated viral DNA for weeks after infection [33].

The cellular tropism of HIV-1 is influenced by CCR5 and CXCR4 coreceptor usage, in particular through the interactions of the envelope glycoproteins [5]. Macrophage-tropic viruses primarily use CCR5 [34, 35], whereas T cell-tropic viruses primarily use CXCR4 [36], and dual-tropic viruses use both coreceptors [37, 38]. However, the coreceptor specificity does not always define cellular tropism

[5, 39]. Non-macrophage-tropic CCR5-using (R5) viruses are able to replicate in primary CD4+ T cells and not in monocyte-derived macrophages (MDM) [40–43], and macrophage-tropic viruses can use CXCR4 for entry into MDM [40, 44]. Thus, cellular tropism of HIV-1 for macrophages is more complex than the coreceptor specificity of the virus [5]. Cellular tropism involves interactions between the envelope glycoproteins, gp120, and the N-terminus and extracellular loop 2 (ECL2) regions of each coreceptor [45]. Macrophage-tropic R5 viruses from blood have an increased dependence on the CCR5 ECL2 region [46] and decreased reliance on the N-terminus [47], whereas macrophage-tropic CXCR4-using viruses appear to have a increased reliance on the coreceptor N-terminus [47].

HIV-1 is most commonly transmitted sexually across the mucosal barrier. Macrophages, as well as CD4+ T cells and DCs, are regularly surveying the mucosal surface and are likely to be the first immune cells to come into contact with HIV-1 [48]. Macrophages are able to phagocytose HIV-1 and to process and present HIV-1 derived peptides via MHC class II to CD4+ T cells. Macrophages, similar to DC, also have the ability to cross present the virus through MHC class I to CD8+ T cells (commonly termed cytotoxic T lymphocytes, CTL). An effective CTL response can have a detrimental effect on HIV-1 replication [49, 50] and disease control in vivo [51]. HIV-1 infected macrophages are also killed by CTL [52]. However, HIV-1 has developed strategies to limit immune detection through downregulating MHC class I from the surface of both virally infected CD4+ T cells and macrophages [53–56].

Additionally, macrophages are able to support the establishment of HIV-1 infection by recruiting T cells to the site of infection through the secretion of chemokines and cytokines and thus enlarging the pool of target cells that HIV-1 can infect [57, 58] (Fig. 27.3). The interaction of HIV-1 surface components, particularly the gp120 envelope glycoprotein, with macrophages can lead to the production of chemokines and cytokines, for example, IFN-β, which can stimulate CC chemokine production (CCL2, CCL3 and CCL4) [58]. These chemokines are chemoattractants for monocytes/macrophages, DC and activated T cells, all of which can be infected by HIV-1 [57]. Additionally, macrophages are able to secrete IL-6, TNF-α, IL-1β, and IL-10 after HIV-1 infection and/or exposure to envelope glycoproteins and other viral proteins, including Nef and Tat [57–59]. These cytokines can also play a role in the regulation of CC chemokine expression. However, these soluble mediators have the ability to both regulate HIV-1 infection through controlling viral replication, and attract potential targets for HIV-1 through their chemoattractive role on immune cells [57]. Thus, the balance of their positive and negative effects on HIV-1 is likely to contribute to the pathogenesis of HIV-1 infection.

Fig. 27.3 (continued) cytokines (IFN-β, TNF-α, IL-1β, IL-10) and chemokines (CCL2, CCL3, CCL4), which can act as chemoattractants for T cells, dendritic cells, and macrophages [57–59]. (**b**) Macrophages can become infected with HIV-1 through the phagocytosis of dying infected cells, for example infected CD4+ T cells. (**c**) Macrophages are able to transmit HIV-1 through cell–cell contact. The cellular contact sites, termed virological synapses form between antigen presenting cells and T cells [32]. Macrophages form a virological synapse with T cells and are able to transmit virus to the uninfected cell [62, 63]. The T cells can extend a pseudopod/uropod extension to contact the infected macrophage and aid transmission of the virus [62, 63]

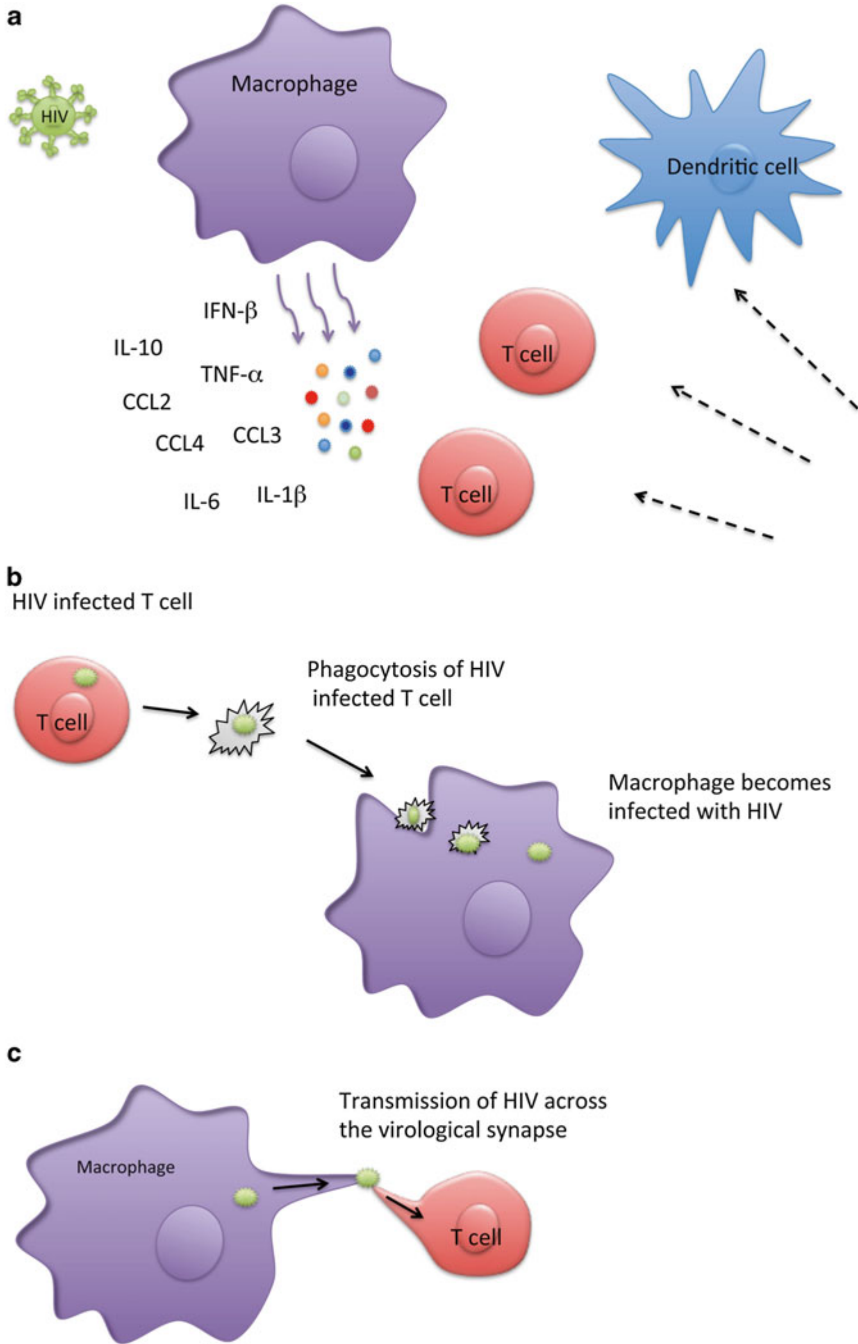

Fig. 27.3 The role of macrophages in the spread of HIV-1. (**a**) Macrophages are able to support the establishment of HIV-1 infection through the recruitment of T cells to the infection site. Interaction of the envelope glycoprotein gp120 with macrophages can lead to the secretion of

Furthermore, macrophage infection through the phagocytosis of dying HIV-1 infected CD4^{+} T cells can also contribute to the spread of HIV-1 [60] (Fig. 27.3). It is likely that apoptosis of immune cells, especially during acute HIV-1 infection in the mucosal lymphoid tissue, assists in a rapid recruitment of macrophages to uptake dying cells. This in turn provides a stable local viral reservoir for HIV-1 [60, 61].

Macrophages are able to transmit HIV-1 through cell–cell spread of the virus [62–64]. The cell contact sites, termed virological synapses (VS), resemble immunological synapses formed between APC and T cells [32] (Fig. 27.3). Imaging studies reveal that infected macrophages can form a VS between macrophages and T cells to transfer HIV-1 to the uninfected cell [62, 63]. In infected macrophages, viral particles assemble at the VS and in internal compartments that contain the tetraspanin markers CD81 and CD82 [62]. Upon coculture with uninfected T cells and formation of VS, the internal viral particles in the infected macrophages moved to the VS [62]. T cells also often extend a pseudopod/uropod-like extension that contacts the infected macrophage at the VS [62, 63]. The exact mechanism and the required molecular signals for internal viral particle movement and trafficking to the VS remain to be elucidated [32].

5 Involvement of Macrophages in Acute HIV-1 Infection

The acute stages of HIV-1 infection are often characterized by febrile illness and clinical signs associated with dissemination of the virus to the lymphoid tissue, the central nervous system (CNS) and other sites [65]. High titers of the virus in the plasma and lymphoid tissue reflect a high level of viral replication. The appearance of HIV-1-specific CTL during acute infection coincides with a decrease in the magnitude of HIV-1 replication, and in the absence of antiretroviral therapy (ART) a steady-state of HIV-1 replication usually occurs, the duration of which differs greatly among people [65–67].

Macrophages play a key role in the spread of HIV-1 to the lymphoid tissue and other organs through several mechanisms. Macrophages are likely to be an early target for HIV-1 infection through their proximity to the site of infection, commonly the mucosal tissue [48, 68]. Viral dynamics have also indicated R5 viruses are dominant early in infection and as macrophages express high levels of CCR5 [69], they can potentially be targeted early for HIV-1 infection. However, it must be noted that not all R5 viruses are macrophage-tropic [41, 43]. Studies on the transmitted/founder R5 viruses of HIV-1 infection found these viruses to be more T cell-tropic, requiring higher levels of CD4 for infection in in vitro studies [70, 71]. Thus it is not clear whether initial macrophage infection occurs directly from HIV-1 infection or indirectly through another mechanism, such as phagocytosis of infected T cells [60].

Macrophages also play a key role in the spread of HIV-1 through the secretion of cytokines and chemokines which assist in the recruitment of uninfected T cells to

the site of infection [57]. This accelerates HIV-1 infection, not only through the addition of new immune targets but also through the ability of macrophages to become infected indirectly through the phagocytosis of dying infected T cells [60]. Additionally, infected macrophages can spread HIV-1 through cell–cell contact with both uninfected macrophages and T cells [63].

One secondary lymphoid organ in particular in which HIV-1 is disseminated during acute infection is the gut-associated lymphoid tissue (GALT). The majority of $CD4^+$ T cells in this tissue are memory $CD4^+$ T cells and thus possess the coreceptor CCR5 [72]. During HIV-1 disease pathogenesis in the GALT, many $CD4^+$ T cells are depleted by HIV-1 and the integrity of the intestinal barrier is also decreased [73, 74]. This results in the translocation of microbial products into the blood stream, increasing immune activation and the spread and progression of HIV-1 [73, 75]. Despite intestinal macrophages being more resistant to HIV-1 infection compared to mucosal macrophages [76], they are likely to assist in the spread of HIV-1 through the phagocytosis of dying infected T cells and their role as APCs. At this stage of infection, virus is shed into the blood stream indicated by the rise in viral load detected in the plasma. Once in the blood stream, HIV-1 is able to infect perivascular macrophages, which migrate to other organs, including the lungs and brain [77]. Additionally, perivascular macrophages can survive up to three months playing an important part in the dissemination of HIV-1.

During acute infection, macrophages play a vital role in the induction of both the adaptive and humoral immune response, reducing viral burden on immune cells and lowering viremia [78]. However, macrophages also play a role in transmission of HIV-1 between immune cells and to multiple sites in the body. As macrophages are found in most tissues throughout the body, this creates multiple microenvironments in which HIV-1 is able to establish latent infection and form a long-lived viral reservoir [79]. This also poses a significant problem not only for viral eradication via immune cells, but also for effective ART. ART has a varying ability to penetrate different tissue microenvironments, causing a range in the antiviral activity and effectiveness of therapy against HIV-1 [14, 80].

6 Involvement of Macrophages in Chronic HIV-1 Infection and Progression to AIDS

During chronic infection, there is a balance between the immune regulation of HIV-1 and the impact of HIV-1 on the immune system. It is an environment in which $CD4^+$ T cells die from CTL responses and excessive immune activation, but also from HIV-1-induced cytotoxic effects and HIV-1 replication. Antibodies to HIV-1 are also fully developed. During chronic infection, $CD4^+$ T cells are replenished by the immune system and HIV-1 is still active. HIV-1 replicates at low levels, or a steady-state, during which most people are able to maintain a healthy $CD4^+$ T cell count (>500 cells/mm^3) [81, 82]. The duration of chronic infection varies greatly, but can last approximately 8 years, with the rate of disease progression relating to plasma HIV-1 RNA levels.

As the number of $CD4^+$ T cells decreases below 200 cells/mm^3, chronic infection progresses to the advanced stages of disease, commonly termed acquired immunodeficiency syndrome (AIDS). Opportunistic infections are common during AIDS due to the poor state of the immune system and life expectancy is decreased.

Over the course of HIV-1 infection, the coreceptor usage of the virus can change from CCR5 to CXCR4, and does so in approximately 50 % of individuals infected with HIV-1 subtype B [83]. HIV-1 can be divided into four groups (M, N, O and P), and subtype B is within group M (termed main or major). HIV-1 subtype B is predominant in Europe, North and South America, Japan and Australia. A switch in coreceptor usage, more commonly seen in HIV-1 subtype B, is also associated with worsened disease prognosis. Several hypotheses exist as to why a coreceptor switch may occur. These include a transmission mutation hypothesis whereby R5 viruses are favored in transmission. However, CXCR4-using viruses with higher fitness can emerge from R5 viruses via intermediate mutants with a lower fitness [83, 84]. Another hypothesis is that environmental conditions of the host change during disease progression and favor the emergence of CXCR4-using viruses [83]. These changes could include immune pressure or a decrease in available target cells for replication. For example, memory T cells express higher levels of CCR5, whereas naïve T cells express higher CXCR4 [84].

How a coreceptor switch affects the infectivity of macrophages remains to be elucidated. Macrophages express both CCR5 and CXCR4 on their surface [7]. Thus, although they are predominately infected by R5 viruses, they can also be infected by CXCR4-using viruses [5, 41, 47]. Some studies have demonstrated that during progression to advanced stages of infection, R5 viruses may have an enhancement in macrophage tropism [85, 86]. Due to the location of macrophages residing in many tissues in the body, including the brain, and the potential of macrophages to become a long-lived HIV-1 reservoir, this could contribute to HIV-1 disease progression.

7 Involvement of Macrophages in HIV-Associated Neurological Disorders

HIV-1 is able to cross the blood–brain barrier (BBB) and can cause neurological degenerative diseases termed HIV Associated Neurological Disorders (HAND). These range from a milder disease form which affects up to 50 % of HIV-1-infected individuals to more severe forms of neurological disease, including HIV-associated dementia (HAD) [87]. Incidences of HIV-associated dementia have declined with the introduction of ART, although it still can affect up to one third of infected adults and half of infected children [87, 88]. HAND is associated with mental and physical impairment, including memory loss, and can lead to seizures, hallucinations, and coma.

HAND is associated with a large infiltration of mononuclear cells into the brain parenchyma and the formation of multinucleated giant cells (MGC). The MGC are formed by the fusion of HIV-1-uninfected and -infected macrophages and are a large virus producer in the brain [89]. Viral load is not well correlated with disease or disease progression; however, the presence of large mononuclear infiltrates into perivascular areas, correlates with HIV-associated dementia [90].

Monocytes and macrophages have been described as mediating HIV-1 neuroinvasion [10, 91]. HIV-1 can enter the CNS early in infection and continues to do so throughout all infection stages [61, 92]. Invading monocytes and perivascular macrophages are able to transmit HIV-1 to microglial cells and astrocytes causing chronic inflammation [93, 94]. Infection by HIV-1 in the CNS is likely to alter the permeability of the BBB and cause neurotoxicity. The proteins of HIV-1 also have neurotoxic activities, which can contribute to disease progression.

The trans-activating protein (Tat) of HIV-1 plays a role in the induction of macrophage infiltration into the brain parenchyma and has strong chemoattractant properties [95]. Tat is also able to induce E-selectin, vascular cell adhesion molecule 1 (VCAM-1), and intercellular adhesion molecule 1 (ICAM-1) on endothelial cells, which increases the adhesion of monocytes to vessel walls and can induce matrix metalloproteinase-g (MMP-g) to facilitate migration through the BBB [95, 96]. Other HIV-1 proteins, such as gp120 can induce TNF-α and IL-6 cytokine production, which act as neurotoxic cytokines and promote pathogenesis of HIV-1 infection [97]. gp41, the envelope transmembrane protein, has also been implicated in the progression of HIV-associated dementia through the increase in MMP-2 expression, which facilitates monocyte migration into the brain [98]. HIV-1-infected macrophages are also able to increase expression of E-selectin and VCAM-1 adhesion molecules and the expression of chemokines, which can assist the migration of monocytes across the BBB [88].

8 Contribution of Macrophages to the HIV-1 Reservoir

Macrophages, as well as latently infected resting $CD4^+$ T cells, dendritic cells, and bone marrow hematopoietic stem cells all contribute to the HIV-1 reservoir [99–101]. A viral reservoir is a cell or anatomical site where replication-competent HIV-1 accumulates and persists stably [4]. Viral reservoirs can also serve to replenish the population of infected cells.

Macrophages contribute to the HIV-1 reservoir pool by secreting pro-inflammatory cytokines and chemokines, which attract T cells, in turn increasing the number of infected cells and viral reservoirs for HIV-1. HIV-1 itself can promote the formation of viral reservoirs through its proteins Nef, Tat and Vpr, which are able to activate the long terminal repeat (LTR) of HIV-1 resulting in sustained viral growth and anti-apoptotic pathways favoring both viral persistence and the formation of viral reservoirs [102]. HIV-1 proteins can also modulate the TNF receptor

signaling pathway, which can assist in the formation of viral reservoirs in macrophages [103].

The long life span of macrophages and their relative resistance to HIV-1 induced apoptosis suggests their potential as an important reservoir for HIV-1 [14, 61, 102]. Macrophages release high levels of HIV-1 over an extended period, and have been characterized as chronically and persistently infected cells [104, 105]. In contrast, T cells have a rapid exponential increase in virus replication followed by widespread cell death [4]. Macrophages are also able to avoid some of the cytopathic affects of HIV-1 by assembling virions within multivesicular bodies within the cytoplasm [106, 107]. This could also assist in evading immune detection due to reduced expression of viral proteins on the surface of macrophages.

Macrophages have been infected with HIV-1 across multiple tissue sites, including the liver, lungs and spleen [108–110]; however, it is not clear whether infection of macrophages at these tissue sites persists during combination ART [111]. Further research using techniques that allow for the detection of any infiltrating T cells by quantifying T cell receptor mRNA in sorted cell populations would assist in examining persistent infection of macrophages during ART [112, 113]. Macrophages play a significant role in contributing to the viral reservoir in the presence and absence of ART and create a large challenge for the eradication of HIV-1-infected macrophages for both the immune system and for current antiretroviral therapies.

9 Macrophages and Antiretroviral Therapy

Antiretroviral therapy uses a combination of at least three drugs to suppress HIV-1 at various stages in its life cycle. ART is able to suppress viral loads in patient's plasma to below clinically detectable levels [114]. However, ART does not eradicate HIV-1 and the virus can rapidly rebound if treatment is ceased [79, 99, 100, 114]. A rebound in viremia is a consequence of the virus forming latent viral reservoirs. Initiation of ART during the first 6 months of infection can reduce the HIV-1 reservoir size in $CD4^+$ T cells [115, 116]. This knowledge has significant implications for HIV-1 disease progression; however, there are current challenges for the distribution and penetration of ART.

Viral sanctuaries are able to promote the persistence of HIV-1 even during ART. Viral sanctuaries are infection sites, which are difficult to reach by ART, and usually are also immune privileged niches [79, 117]. One of these sites is the CNS, in particular the brain. The brain contains macrophages and astrocytes, both of which are able to be infected by HIV-1, and thus may contribute to viral persistence [101, 118, 119]. The brain is one site that causes a major challenge for HIV-1 therapies and for a cure for HIV-1.

Macrophages have been described as a cellular target for HIV-1 which are involved in HIV-1 rebound after cessation of ART [61]. Furthermore, macrophages are also known to store a large amount of unintegrated viral DNA and thus can contribute to viral rebound [120]. The ability of ART to target macrophages is not

universal, as ART has a range of effectiveness dependent upon the type of tissue and cellular microenvironment. Thus, ART may have a low bioactivity, or a range of bioactivities, for different macrophages found in different tissues and immune privileged sites [14, 61].

Further confounding the effect of ART on HIV-1-infected macrophages is the discovery of virus-containing compartments (VCC) in macrophages, which can harbor HIV-1 [121–123]. These intracellular compartments can act as sites of HIV-1 assembly in which budding, immature, and mature forms of HIV-1 have been found [121–123]. During time-course experiments VCC in infected macrophages migrate to virological synapses with T cells [62, 124], and HIV-1 has been found at the intracellular surface of these synapses [62]. Thus VCC, as well as potentially acting as holding compartments for the virus, can also facilitate HIV-1 infection of neighboring cells. The VCC potentially offers a level of protection from ART to HIV-1 and also from cellular restriction factors [125].

10 Restriction of HIV-1 in Macrophages

Human cells contain restriction factors, which are able to suppress various stages of the viral life cycle through different mechanisms. Restriction factors affecting the life cycle of HIV-1 include the apolipoprotein B messenger RNA (mRNA) editing enzyme catalytic polypeptide-like 3 family, more commonly known as APOBEC3. The wider researched proteins include APOBEC3G, bone marrow stromal cell antigen 2 (BST2) known as tetherin, and tripartite-motif containing 5α commonly abbreviated to TRIM5α [126].

These three restriction factors are able to inhibit HIV-1 via different mechanisms. APOBEC3G has polynucleotide cytidine deaminase activity, which results in the postsynthetic editing of cytidine residues to uridines, and thus alters the nucleotide sequence. APOBEC3G is packaged into the assembling HIV-1 particle and is transferred to target cells through viral infection [127]. In addition to causing mutations, APOBEC3G can also affect the level of cDNA that accumulates during subsequent HIV-1 infection, and can impede the translocation of reverse transcriptase along the viral RNA template [128, 129]. The second restriction factor, tetherin, is able to cause HIV-1 particles to remain at the surface of the infected cell, tethered to the plasma membrane, where they accumulate in endosomes following internalization [130]. Tetherin is not restricted to the cell surface and has also been found in VCC in macrophages [131]. The third restriction factor, TRIM5α, is a cytoplasmic protein, which acts on the release of retroviral capsids and their contents into the cytoplasm of cells. It assists in the failure to synthesize viral cDNA [132]. TRIM5α also binds to viral capsids [133] and it has been suggested that it causes capsid fragmentation [126].

However, HIV-1 is an effective evader of human cell restriction factors, frequently through the use of its accessory proteins, Vif, Nef, and Vpu. The HIV-1 Vif protein can counteract the cytidine deaminase APOBEC3G [134] and Vpu

inactivates tetherin [131, 135]. Nef and Vpu are able to regulate expression of host proteins during viral replication, in particular CD4 and MHC Class I [61] and Vpx, the SIV counterpart of Vpr, is able to counteract the actions of the dideoxynucleotide hydrolase SamHD1 [136, 137]. SamHD1 can block reverse transcription of HIV-1 by depleting deoxynucleoside triphosphates within cells [138, 139].

Macrophages express high levels of tetherin and SamHD1, especially with respect to CD4^{+} T cells which express no to low amounts [136, 140]. Higher expression of regulatory factors could possibly render macrophages less susceptible to infection. However, it must also be considered that HIV-1 infection of target cells can be partly restored by HIV-1 accessory proteins [demonstrated in vitro [136, 140]].

Cytokines, as well as restriction factors, play a role in inhibiting HIV-1 replication. Type 1 interferons (IFN) α and β have been well researched and are induced in response to viral infections. They trigger the transcription of a wide variety of IFN-stimulated genes (ISGs) through the signal transducer and activator transcription (Jak-STAT) pathway [141] and are able to induce an antiviral state in the cell. Furthermore, IFN-α can upregulate HIV-1 restriction factors, including APOBEC3G and the cellular membrane protein tetherin in macrophages [142].

IL-27 also plays a role in inhibiting HIV-1 replication in macrophages in vitro [128, 129]. IL-27 is able to induce antiviral genes in macrophages similar to IFN-α [128], and although IL-27 has not been shown to inhibit HIV-1 infection, macrophages induced with IL-27 show a reduction in the production of proviral cDNA of late HIV-1 gene products [129]. This study suggests that IL-27 may interfere with HIV-1 replication between viral entry and reverse transcription.

Further studies by Dai et al. [129] showed spectrin b non-erythrocyte 1 (SPTBN1) to be downregulated in IL-27-treated macrophages. Treating macrophages with IL-27 itself did not affect expression of macrophage differentiation markers nor its function. However, it was able to assist in resistance to HIV-1 infection by down-regulating SPTBN1 [129]. Knocking down SPTBN1 prevented infection of macrophages, whereas overexpression of SPTBN1 in IL-27-induced macrophages did not prevent infection. SPTBN1 was associated with Gag proteins in macrophages, and the authors suggested the interaction might be important for reverse transcription of the HIV-1 genome. However, this needs to be clearly elucidated and the role of IL-27 defined in in vivo models.

11 Conclusions

Macrophages play a key role in contributing to the HIV-1 reservoir and are an important vehicle for dissemination of HIV-1 throughout the body. The ability of macrophages to express both CCR5 and CXCR4 makes them a prime target for HIV-1 infection. Their location at many different tissue sites throughout the body and immune privileged sites, such as the brain, creates complications for effective eradication through current ART and immune surveillance.

Macrophages play a key role in viral dissemination and contribute to both the regulation and progression of HIV-1. They are able to assist in disease control through the phagocytosis of HIV-1 and HIV-1-infected cells, presenting HIV-1 antigens to CD4+ and CD8+ T cells, and initiating both innate and adaptive immune responses. Macrophages, however, are also able to progress HIV-1 infection through several mechanisms, including the secretion of chemokines and cytokines, which attract T cells to the site of infection and provide new cellular targets for HIV-1. Macrophages can also spread HIV-1 through their ability to phagocytose HIV-1-infected cells and through cell–cell transfer. Additionally, macrophages have a reduced sensitivity to the cytopathic effects of HIV-1 and are relatively stable sites of HIV-1 replication. HIV-1 can also reduce immune detection by locating to multivesicular bodies within the cytoplasm of macrophages.

Eradication of HIV-1-infected macrophages poses a challenge for both the immune system and for current ART regimens. Additional knowledge of the tissue microenvironments where macrophages reside and a better understanding the role of the cytokine and chemokine milieu in HIV-1 infection may lead to novel and effective therapeutics targeting HIV-1 in viral reservoirs.

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Chapter 28
Brain Imaging in People with HIV

Paul M. Thompson, Jeffry R. Alger, and Neda Jahanshad

1 Introduction

In the early days of the human immunodeficiency virus (HIV) epidemic, viral effects on the brain and all other systems of the human body were often devastating. Complications included opportunistic infections ranging from HIV encephalitis, cytomegalovirus (CMV) encephalitis, mycobacterial infections, and even neoplastic diseases such as primary central nervous system (CNS) lymphoma. In

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this context, brain imaging was commonly used to monitor the adverse effects of HIV on the brain, often offering insight into a whole host of vascular and neuronal changes.

More recently, combined antiretroviral therapy (cART) has become widely available. This has greatly increased the life expectancy and quality of life for many people living with HIV. Many people are now living into old age with HIV as a chronic disease, which treatment has largely stabilized; however, there are still significant effects of the virus on the brain [1].

Severe conditions such as HIV-associated dementia are not typical, but there is significant concern that the HIV virus may promote neuronal atrophy, inflammation, and other cellular changes. HIV may worsen the effects of cerebrovascular disorders common in the aging population. These changes may make the brain less resistant to other forms of neuropathology, such as the amyloid and tau accumulation that lead to Alzheimer's disease. As HIV infection becomes more of a stable disorder with a normal life expectancy, there is growing interest in knowing if age-related brain changes are accelerated in people living with HIV. If so, it would be vital to also know if certain brain regions are especially vulnerable, and whether any of these brain changes can be resisted.

In this chapter, we briefly survey the ways neuroimaging is used today, to understand brain changes in people with HIV. Rather than review all the papers that used imaging to study HIV, we focus on lessons learned and some key themes discovered from various worldwide studies of HIV and the brain. Our review is aimed at clinicians and neuroscientists interested in HIV, or medical students. We do not assume any specialized knowledge of brain scanning or radiology, or neurology.

2 Mapping Brain Changes with MRI (Magnetic Resonance Imaging)

MRI is the mainstay of brain imaging research, at least for clinical studies of HIV. MRI is relatively widely available and safe, and offers unmatched spatial resolution for understanding and mapping brain structure and anatomy. Computed tomography (CT) has also been used for many brain imaging studies of HIV [2] and some forms of CT offer excellent anatomical detail and tissue contrast in the brain. Even so, compared to CT, MRI has the advantage that no ionizing radiation is used at all. As such, it is safe for a person to be scanned repeatedly, opening the doors for advanced monitoring of brain changes over time, due to HIV infection.

MRI is based on the principle of nuclear magnetic resonance, which measures properties of molecules in living tissue, by sending radiofrequency pulses into it, and monitoring signals that come back out. An MRI scanner generates a high magnetic field using a large superconducting magnet, and the patient lies inside the bore of the scanner. Radiofrequency pulses are applied to the brain using coils that surround the patient's head. These stimuli do not interact with the key physiological properties of the brain, or its biochemistry; instead the magnetic field polarizes the

nuclear spins of the protons, specifically in hydrogen atoms, and detects the radio frequencies emitted, as the spins relax back to their original conformation. The density and the relaxation rate of these protons vary by tissue type (depending mainly on its water and lipid content). By acquiring images across the living brain, we can map different tissue types, leading to a high-resolution view of the living tissue as it matures and degenerates with disease. The main limitation of MRI is that it is sensitive to motion, so a patient should not move while in the scanner. It also cannot be used for people with pacemakers or other metallic implants, as it uses high magnetic fields to create images of the brain.

By applying calibrated sequences of pulses to the brain, the hydrogen nuclei in water, fat—and other molecules within the brain—are stimulated to emit a signal that depends on the chemical composition of the molecule, its environment, and its 3D position in the brain. Samples from the entire brain can be "de-coded" all at once, using a Fourier transform method to "tag" and de-scramble signals from different brain regions. The detection of all these signals, using a radio frequency antenna and software to map the brain in 3D, leads to a map of the gray and white matter in the brain, and fluid filled areas such as the ventricles.

If there is evidence of brain lesions, strokes, vascular disease or neoplastic tissue, then different varieties of MRI may also be used to better detect white matter disease or specific vascular changes. Contrast agents may also be injected intravenously, in combination with a patient's MRI scan, to better identify vascular or neoplastic changes.

The HIV virus penetrates the human brain within about 2 days of infection [3]. Untreated, the virus may multiply or lie dormant for long periods. The primary methods of degeneration induced by HIV are thought to be a combination of glial proliferation and inflammation, direct neurotoxicity, and eventual neuronal loss [4].

Cellular studies show that the viral load is not as high in neurons as in other cell types. It is often conjectured that the virus is transported across the blood-brain barrier by infected monocytes or macrophages; each has privileged access to the brain. Even when viral load is largely suppressed by antiretroviral treatments, there may be some evidence of ongoing viral damage to the brain. Not all medications are fully effective at crossing the blood brain barrier. In fact, a major direction of ongoing work is to study how well different treatments get into brain tissue. Imaging can help understand these effects by documenting any slowing of atrophy associated with treatment.

2.1 Pattern of Brain Atrophy in HIV/AIDS

Early radiologic studies of HIV noted the prevalence of white matter disease, subcortical atrophy and cortical damage, and sulcal widening [4, 5] suggesting that HIV might induce degenerative brain changes comparable to those seen in age-associated dementias.

In the cART era, some patients whose disease is stabilized by treatment show symptoms of cognitive impairment [6], perhaps associated with ongoing brain

Fig. 28.1 MRI scanning of a group of patients with Alzheimer's disease, and matched elderly controls, has been used to make a composite "movie" of the brain regions affected by the disease. Here, the *white* and *red colors* indicate brain regions where cortical gray matter is lost in Alzheimer's disease. The changes are not the same in every patient, but there is enough similarity for a typical trajectory to be identified. This pattern matches the trajectory of neurofibrillary tangle pathology, which accumulates in a similar sequence. Figure adapted from Thompson et al., J Neurosci 2003

atrophy. MRI has been used to understand whether the structural brain changes associated with HIV are diffuse—essentially random or uniform—or localized, with concentrated effects on specific systems.

The analogy with Alzheimer's disease is instructive. In contrast with HIV, Fig. 28.1 shows the typical progression of cortical gray matter loss in Alzheimer's disease (AD). Not all patients with AD have the same pattern of tissue loss, and their ages of onset and disease trajectories may differ.

Even so, there is a well-documented spread of brain tissue loss in Alzheimer's disease from medial temporal structures such as the hippocampus—which is crucial for learning and memory—into the association cortices of the parietal and frontal lobes [7]. There is also early limbic involvement in some patients. This may be associated with apathy, major depression, or other affective symptoms.

Several things are notable about the "time-lapse sequence" of Alzheimer's disease. First, the pattern of anatomical disease progression matches the typical pattern of accumulation of tau pathology [8]—one of the two major hallmarks of Alzheimer's disease—the other being beta-amyloid protein deposits, or plaques.

Second, the anatomical changes roughly parallel the behavioral and cognitive changes as disease progresses. Initially the main symptoms involve memory but may later diversify to involve emotional affect, language and executive function, self-control, and eventually all aspects of self-care. Despite decades of research mapping how AD affects the brain, the best biomarkers for picking up the effects of AD may change throughout the course of the disease. Also the best imaging methods to detect the earliest changes are somewhat disputed [9–11].

Even more debate surrounds the pathology of HIV infection; it is a highly heterogeneous disease that is also influenced by the levels of treatment, illicit drug use, and comorbidities; effects may even depend on the viral strain. In cognitively impaired elderly, HIV shows a somewhat different pattern of effects on the brain [12, 13]. It might be assumed that an infectious disease, such as HIV, should show no obvious anatomical pattern of effects, other than widespread or disseminated

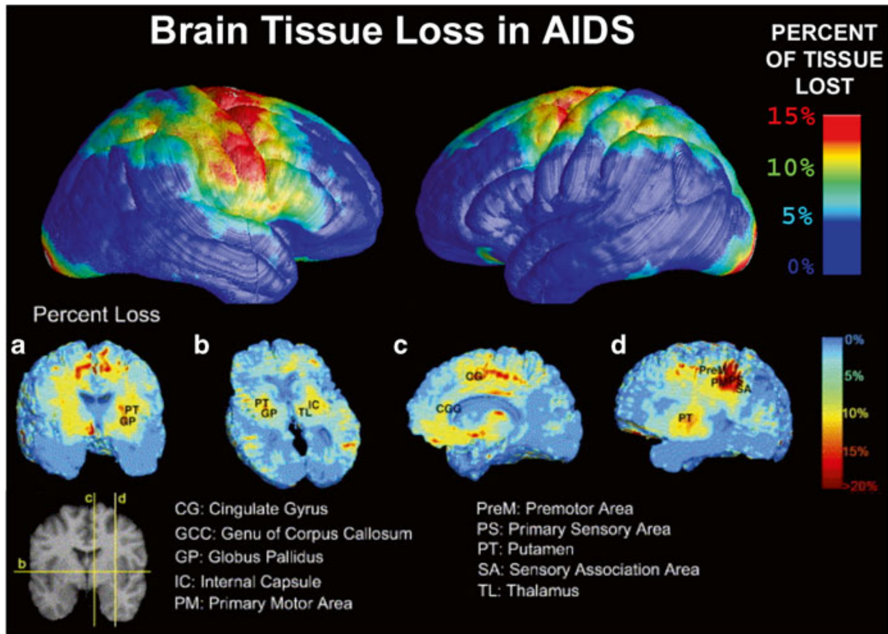

Fig. 28.2 Here the average pattern of cortical gray matter thinning in motor and other brain areas is shown (*top row; red colors*), in a group of people with HIV/AIDS compared to matched controls. Using an image analysis method called *tensor-based morphometry*, white matter deficits can also be mapped (*bottom row*). Brain atrophy in the HIV+ group is notable in the basal ganglia and premotor areas, consistent with alterations in fine motor skills seen in some patients. Figures adapted from Thompson et al., PNAS 2005 (*top*), and Chiang et al., Neuroimage 2007 (*bottom*)

atrophy. Or it could be argued that the pattern might depend on the distribution of the virus, which may differ from one patient to another. If that hypothesis were true, one might expect to see greatest brain changes in areas of highest viral load—namely, the caudate and basal ganglia that surround the CSF-filled ventricles, as these are highly enriched in the virus. As we describe below, early efforts to map the profile of cortical and subcortical atrophy in people with HIV largely confirmed these expectations, but with some exceptions.

Figure 28.2 (*top row*) shows a statistical comparison of cortical gray matter thickness between a group of adults with HIV and matched controls [14]. In these maps, the areas where patients show consistently thinner cortical gray matter are highlighted in red; areas in blue show no sign of difference, or at least not differences large enough to be detected with MRI in samples of this size. Clearly, atrophy is greater in some primary and supplementary motor areas, perhaps consistent with some of the deficits in fine motor control and concentration experienced by some patients.

To survey brain tissue atrophy, specialized MRI analysis methods may also be used. On a standard MRI scan, white matter fiber tracts are not clearly differentiated (although diffusion MRI overcomes these limitations; see below). But with standard anatomical scans, there is an approach to gauge atrophy relative to healthy controls, based on aligning, or “warping” brain scans from each individual to match a group

average template. In the process, brain regions may be identified in each person whose volumes are lower than average, after taking into account that person's age and sex, and other relevant modulating factors. Figure 28.2 (*bottom row*) shows extensive atrophy of the basal ganglia, and its white matter projections, consistent with predictions that brain regions closest to the ventricles might show greatest deficits [15]. Other studies focusing on subcortical structures—particularly the basal ganglia—confirm these findings [16].

Many MRI studies focus the imaging field of view on certain regions of the brain to speed up the scan times, but many do not include a full survey of the cerebellum, a key brain system for motor function and diverse aspects of cognition [17]. We and others found greater tissue loss within the cerebellum of people with HIV than their seronegative peers, with greatest effects in the *vermis*, a midline region of the cerebellum [18].

Although it might be tempting to relate atrophy in distinct brain systems to specific forms of cognitive dysfunction in specific patients, this has been more difficult. Studies of brain-cognition relationships have been successful in Alzheimer's disease and other dementias (such as FTD), but associations with cognition are harder to detect robustly in groups of patients who are less impaired overall. In fact, while studies of specific systems or cognitive dysfunctions are limited, the most robust cognitive correlates of the brain differences have been *global measures* of performance, such as the normalized neuropsychological summary Z-score (NPZ). The NPZ score pro-rates cognitive performance on a balanced range of tasks, relative to age-matched norms. This may be due to limited power in small studies of highly heterogeneous populations.

Similarly, comorbidities in people living with HIV are fairly prevalent. The disruptive effects of cerebrovascular disease (CVD) and its risk factors in the brains of HIV patients have led to inconsistent findings in neuroimaging studies. Some studies report added complications of metabolic risk factors [19], while others report minimal or no associations [20, 21].

Despite inconsistent findings with specific cognitive and CVD-associated factors, consistency has been established across multiple imaging studies of HIV+ cohorts. One of the best predictors of brain atrophy is the nadir CD4+ count [22]. Nadir refers to the lowest recorded value for plasma CD4+ T-cells, a sign of the extent of HIV disease before treatment. This association appears robust across many cohorts. The nadir CD4+ count seems to predict the level of cortical gray matter atrophy [14], expansion of the ventricular space [23, 24], overall brain tissue loss [25], and white matter microstructure [26, 27].

3 MR Spectroscopy

A variant of MRI—MR spectroscopy—has been able to identify some of the biochemical origins of brain changes in people with HIV [28, 29]. During a standard anatomical MRI scan, the main signal comes from the stimulated emission of

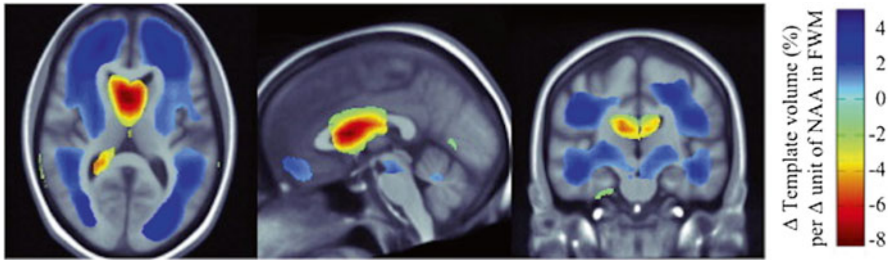

Fig. 28.3 In a group of HIV+ people, the level of *N*-acetyl-aspartate (NAA)—a marker of neuronal integrity—was measured using MR spectroscopy, in the frontal white matter. In standard MRI scans from the same participants, we also measured the level of volumetric atrophy relative to a standard brain template. *Blue colors* highlight regions with greater atrophy in people with lower frontal NAA; *red colors*—largely in the lateral ventricles—identify CSF expansions that are found in people with lower frontal NAA. Figure adapted from Hua et al., Neuroimage Clinical 2013

signals from hydrogen nuclei in water and lipids. These make up the largest fraction of the hydrogen nuclei in the brain. These signals can distinguish gray and white matter and the major nuclei of the brain, but do not tell us much about their biochemical content. When the hydrogen signal produced by water is suppressed using a specialized scan, a variety of metabolically relevant compounds can be measured, such as *N*-acetyl-aspartate (which provides a measure of neuronal integrity because this molecule is present in neurons but not in glia), choline and creatine, myoinositol, and the neurotransmitters glutamate and gamma-amino-butyric acid. In general, MRS studies show robust changes in several markers of neuronal integrity including NAA, as seen in Fig. 28.3 [30]. Levels of choline and creatine in the basal ganglia are elevated acutely after infection but stabilize after treatment [31].

4 Diffusion MRI

A further variant of MRI, called *diffusion-weighted MRI*, can map the neural fiber pathways in the brain—even whole networks of structural connections. This method, along with resting state functional MRI, has become an important neuroscience research tool [32]. It can reveal changes in the connections or wiring between different brain regions, and overall brain network properties, rather than observing each brain region in isolation.

Briefly, the MRI signal is attenuated because water is diffusing in the tissue being imaged. In white matter the diffusion is more pronounced in the direction parallel to the fiber bundles relative to the perpendicular direction, so we can often identify the direction of fiber bundle orientation throughout the white matter. This diffusion is measured by applying a magnetic field gradient at multiple angles around the brain and observing the attenuation of the MRI signal due to diffusion. This allows for a 3D reconstruction of the diffusion pathways. Mathematical

equations can model the degree of attenuation of the MRI signal in any direction, based on the rate and directional orientations of maximal and minimal diffusion. The scanner is programmed to detect diffusion in a range of directions (up, down, sideways, and multiple directions in between). Water diffusion can be tracked across the brain, and fibers reconstructed that traverse the brain and interconnect the major cortical and subcortical regions. The whole field of fiber tracking in brain diffusion MRI is rapidly evolving. Novel methods are constantly being proposed and tested to map finer scale circuits in a reasonable amount of time.

Anatomical connectivity of brain regions can be mapped using the principle shown in Fig. 28.4. By identifying a range of regions on the cortical surface, the fibers running between all pairs of these regions can be identified using tractography. And a measure of the density or integrity of these connections can be stored in a 2D matrix (*see figure*). By comparing groups of HIV+ people with

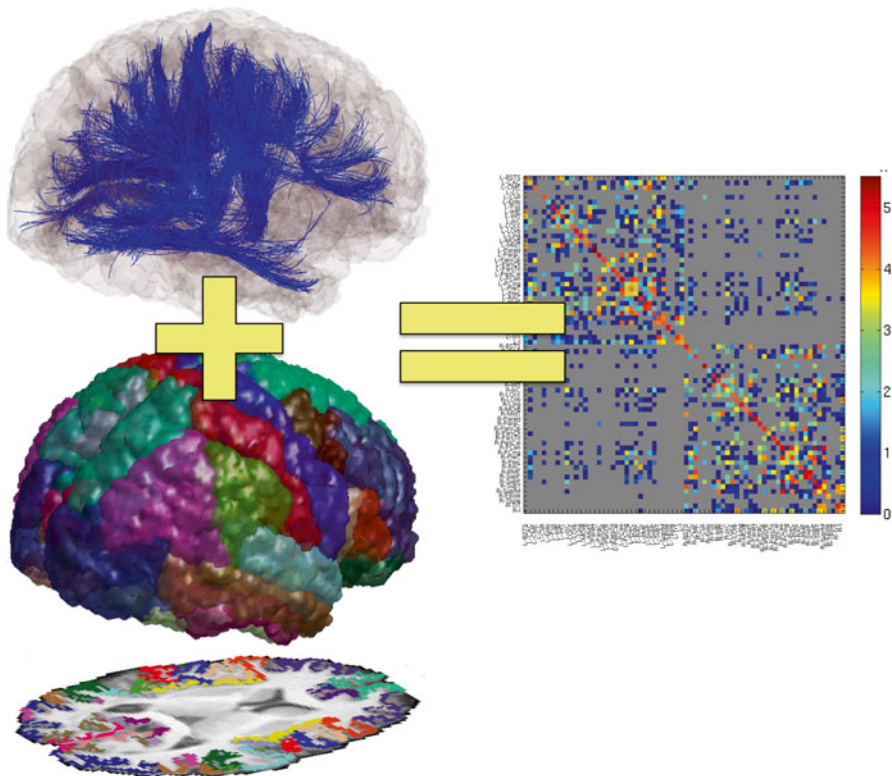

Fig. 28.4 Patterns of anatomical connectivity can be mapped by combining diffusion-weighted magnetic resonance imaging (dw-MRI) with high-resolution anatomical T1-weighted MRI. Tractography methods can be used on dw-MRI scans to map fiber bundles throughout the brain (*top left*) while detailed cortical labeling (*bottom left*) can define biologically and functionally significant cortical regions in each individual. By quantifying the tracts that connect one region to another, the strength of connections between two cortical landmarks can be estimated and represented by a matrix, where each element represents the physical connection between the regions defined on the axes

HIV− controls [33], we were able to show that some of the connections of motor areas were less dense in people with HIV, perhaps due to the cortical atrophy described earlier. In addition, greater deficits were seen in HIV+ people with prolonged infection who also carried the APOE4 risk gene, a risk factor for late-onset Alzheimer's disease (see Fig. 28.5). Whether or not these interactions with risk factors are robust or generally found will take large samples to verify.

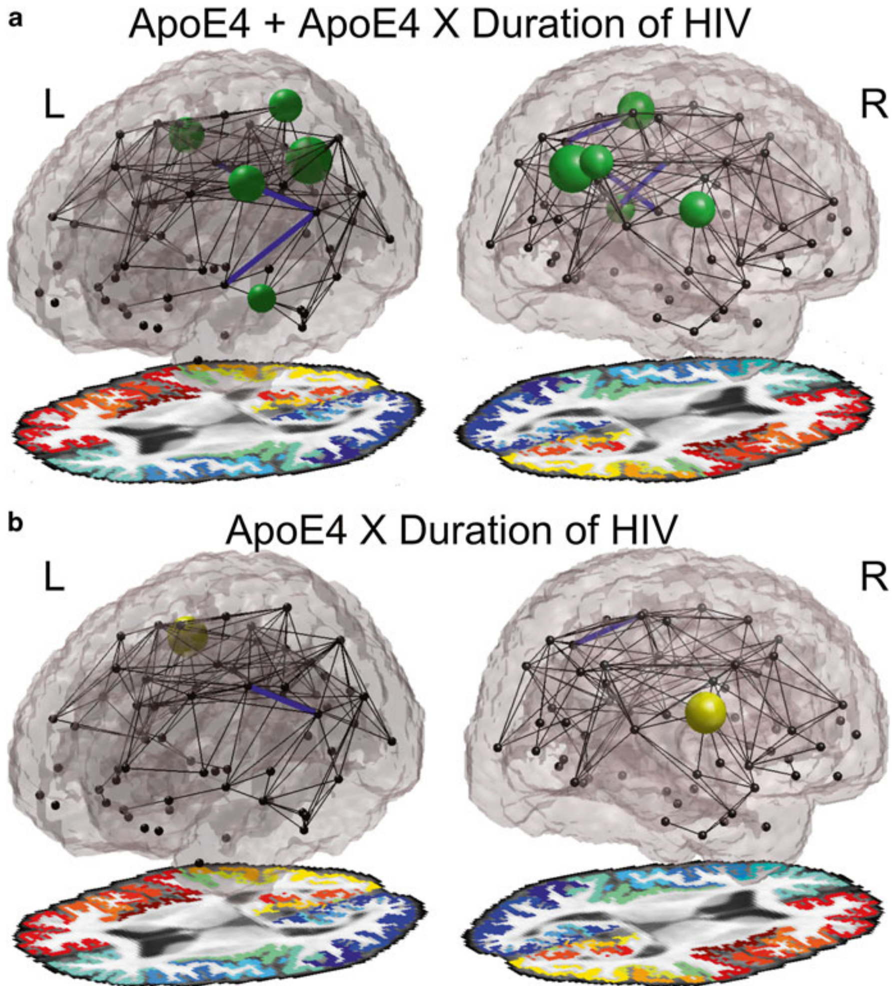

Fig. 28.5 Brain Connectivity in HIV/AIDS. Here we show nodes and regions in the brain's structural network, where the density of connections is different, on average, in people with HIV versus controls. Carriers of the Alzheimer's disease risk gene, APOE4, also have lower connectivity in some brain regions, and carrying this gene and the HIV virus may lead to greater reduction in connectivity. (**a**) Highlights the connections where the APOE4 genotype shows a significant effect on connectivity, and (**b**) shows the effect of the interaction of APOE4 with duration alone. Larger spheres indicate greater effect-sizes. These maps are computed from diffusion-weighted MRI, which can be collected at the same time as standard anatomical MRI, on the same scanner. Figure adapted from Jahanshad et al., Brain Connectivity 2012

4.1 MRI to Characterize Brain Development in Children with HIV Exposure or Infection

cART has improved and lengthened lives of many adults who have contracted HIV, but is also conversely responsible for the growing number of children who contracted HIV from their HIV+ mother, and are living past infancy. Many of these children were exposed to the virus or treatment prenatally. Understanding how treatments and moderators (such as nutrition; [34, 35]) may help these children develop normal cognitive abilities is critical. To date, there have been few imaging studies of brain development in HIV-infected children [36]. Recently, large collaborative studies such as Pediatric HIV/AIDS Cohort Study (PHACS) [37] and the Pediatric Randomized Early vs. Deferred Initiation in Cambodia and Thailand (PREDICT) study [38] are underway to better categorize the developmental trajectories and determine necessary interventions that may promote healthy brain growth. The PREDICT study aims to determine whether the trajectory of childhood brain development is affected by deferred versus immediate access to antiretroviral treatment. It also assesses how prenatal HIV exposure may impact the developing brain [39]. As exquisitely sensitive methods are refined to detect and measure growth rates in individual children [40, 41] scanned annually with MRI, treatment effects are likely to be mapped and discovered in this kind of study. Using standard structural MRI, diffusion and MRS techniques to image these developing children should provide insight into brain network alterations and disrupted brain physiology and biochemistry. Future studies will also determine how generalizable these developmental changes are, across multiple populations of HIV-affected youth.

5 ENIGMA-HIV and Diversity of Cohorts

MRI and its variants offer much insight into how HIV affects the living human brain—how it spreads, the systems it affects, the connections the virus impairs, and the covariates that amplify these effects. However, many of these factors are subtle, hard to detect, or may only be relevant for particular groups of patients—those of a certain age, treatment plan, drug history, or even with specific genetic clades of the virus [42].

In many areas of neurology, it is relatively easy to map the profile of disease effects on the brain, and show that patients' neuroanatomy is different from healthy controls. It is more challenging to discover factors that reduce or accelerate these effects, and predict what is going to happen in the future. This would have an immediate clinical impact and may lead to a more personalized treatment approach. For example, some researchers have tried to predict future brain decline in patients, based on MRI, and blood markers, such as cytokines, inflammatory and immune system markers. Clearly, if an intervention or treatment could slow the rate of brain atrophy, MRI would play a vital role in proving it.

Having performed many neuroimaging studies of disease for decades, several groups came to the conclusion that large sample sizes are required to detect consistent disease effects on the brain, with enough power to identify modulators. The Enhancing Neuro Imaging Genetics through Meta Analysis (ENIGMA) Consortium, for example, amassed 29,000 brain scans and genome-wide DNA data from the same subjects, and found a number of common variants in our DNA that predict the size and volumes of several key brain structures [43, 44]. As most treatment effects are subtle—perhaps slowing atrophy by no more than 1–5 %—international consortia are forming to assess factors that affect HIV disease progression in the brain. Such efforts include the HIV Neuroimaging Consortium (HIVNC) [45], CHARTER [46], PHACS [37] and many others, as well as consortia that meta-analyze effects across hundreds of studies (ENIGMA; [47]). Along with genetic risk factors such as *APOE* and others yet to be discovered [48], additional factors promoting brain atrophy in people with HIV could include: adverse environment, drug use, time from infection to treatment, comorbid vascular disease, advanced age, duration of illness, and many others.

A significant factor of interest in HIV research is the confounding effects of drugs of abuse—such as alcohol and methamphetamine. Many such drugs have complex and lasting effects on brain chemistry and structure, visible on brain MRI in users [49] as well as children exposed to the drugs prenatally [50]. Figure 28.6 shows effects of methamphetamine on the brain; although gray matter deficits are shown here, other research groups report white matter hypertrophy in methamphetamine users [51]—perhaps denoting inflammation that may resolve later.

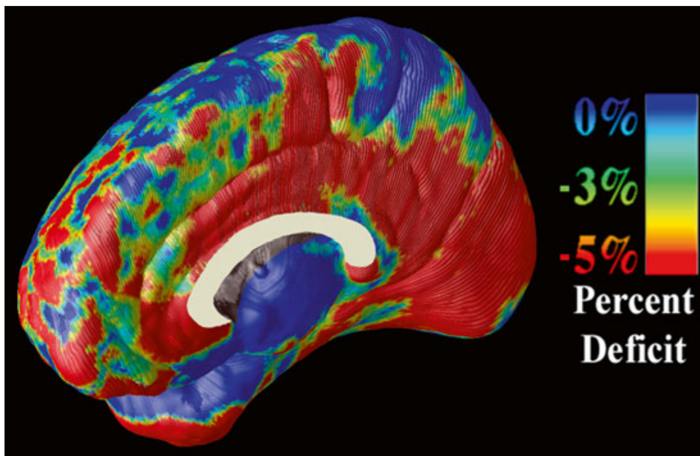

Fig. 28.6 Here we show brain regions in the limbic system (*red colors*) where cortical gray matter is reduced in people who chronically use methamphetamine, relative to matched controls who do not. The Alzheimer-like pattern is striking but the neurotoxic effects of drugs operate through different mechanisms and may involve white matter inflammation as well. The relationship between drug- and virus-related brain changes is of interest in HIV+ drug users. Figure adapted from Thompson et al., J Neuroscience 2004

Due to the heterogeneity in HIV infected populations, and the diffuse, time-dependent nature of CNS involvement, the power to detect subtle brain changes is limited by available sample sizes. Some studies report additive effects of methamphetamine abuse and HIV infection [52, 53]; common CVD risk factors may worsen HIV effects on the brain. Whether or not any of these modulators can be shown to affect the disease trajectory in the brain will probably rely on large and diverse samples assessed with neuroimaging as well as standard blood biomarkers and clinical evaluations. Large consortia such as ENIGMA may provide new insights into the infection by pooling existing data. Meta-analytical approaches may then detect important genetic and environmental moderators that may be too subtle to identify in any single study.

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Chapter 29
Seasonal and Pandemic Influenza Surveillance and Disease Severity

Tamara V. Feldblyum and David M. Segal

Core Message This chapter addresses the disease burden on the US population caused by the annual influenza epidemics or pandemic and the methods of influenza surveillance used to monitor and prevent the spread of the disease. The meaningful use of electronic health records for influenza research and surveillance are discussed with a focus on variations of influenza disease severity between seasons and between individual patients. Surveillance of severe disease cases can contribute to a more effective public health preparedness and response.

1 Introduction: Influenza Surveillance and Disease Burden

The recent influenza pandemic in 2009 caused by influenza A/H1N1 reassortant with high human-to-human transmissibility, demonstrated the unpredictable nature of emerging viruses and importance of continuous surveillance. During the 2009–2010 influenza season, the 2009 H1N1 virus infected approximately 61 million persons and caused an estimated 274,000 hospitalizations and 12,500 deaths in the USA [1]. This novel virus caused severe morbidity and mortality in pregnant women [2–4] and younger adults with 87 % of deaths occurring in persons younger than 65 years of age [5]. In addition to the human toll, annual influenza epidemics and pandemics carry substantial economic consequences in health-care utilization costs, intervention costs, and reduced productivity. The cost of annual influenza epidemics in the USA is estimated to range between $52 and $199 billion [6].

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Individual risk factors for severe outcomes of influenza infection vary between seasons and are associated with circulating influenza virus types and subtypes, as well as with individual demographic characteristics, such as age, ethnicity, and clinical conditions, such as asthma, diabetes, cardiovascular, lung, and neurological diseases [8–12]. Due to variations in influenza virus activity, the capacity to respond to seasonal epidemics and pandemics depends on the availability of accurate and timely information and swift and early identification of pandemic and epidemic strains.

The US national influenza surveillance systems include syndromic, clinical, and virologic monitoring. Information on influenza-like illness (ILI), influenza hospitalizations, influenza and pneumonia associated mortality, influenza-associated pediatric mortality, and laboratory testing of a subset of specimens from patients with ILI to characterize the circulating viruses are reported. These surveillance systems are resource-intensive [13, 14] and require sustained funding for epidemiologic and virologic information gathering at the national and local levels [15]. Enhanced and timely syndromic surveillance methods that use electronic health records (EHR) could improve the assessment of influenza medical and economic disease burden and associated risk factors leading to identification of at risk population groups, targeted and appropriate public health interventions, and estimates of economic burden associated with the disease [14, 16–18]. EHRs capturing information using the International Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9-CM) codes lend themselves to efficient quantitative analyses and have been used in numerous epidemiologic studies and influenza surveillance [14, 17, 19–21].

With the growing focus of the US health care system on the meaningful use of electronic medical records, one of the practical applications is expanding biosurveillance and preparedness capabilities, such as surveillance of influenza severity and associated risk factors during seasonal epidemics and pandemics [18, 22]. Traditional influenza surveillance data are based on laboratory testing of a limited number of samples, case reporting by participating health care providers, hospital-based primary data, and deaths reported by statistics offices [24]. Data extracted from electronic medical records can enrich reporting of risk factors for disease severity or clinical diagnoses, even in the absence of laboratory testing, and augment the traditional surveillance. In addition, monitoring patients EHRs may enable detection of disease outbreaks for which no laboratory diagnostics were requested including emerging pathogens and biothreat events [25].

The timely reporting of information on circulating influenza viruses and the disease burden associated with seasonal and pandemic influenza is essential for optimal public health response, identification of vulnerable populations, and for prevention and patient management strategies. Large electronic datasets of hospital discharge records, such as the Nationwide Inpatient Sample (NIS), could provide information on risk factors for disease enhancing influenza surveillance methods [7, 21]. The use of much larger more representative national population repositories from existing electronic medical records can potentially augment or replace small hospital case series studies often employed for assessment of influenza severity.

2 Influenza Surveillance

Every year, emerging and reemerging influenza viruses lead to tens of millions of respiratory infections and up to 500,000 fatalities worldwide. Unpredictability of antigenic drift or antigenic shift leading to emergence of viral strains with limited or no immunity in human population results in variable disease spread and severity. A novel high pathogenicity virus adapted to human-to-human transmission could cause a global pandemic with millions of deaths [25]. Timely detection and reporting of disease in specific populations through an effective biosurveillance system is the most promising strategy for mitigating the impact from disease outbreaks caused by naturally occurring epidemics or bioterrorism events [26]. Influenza virus surveillance informs selection of the annual vaccine strains and guides antiviral therapy. Monitoring influenza outbreaks is of particular interest because they represent a proxy for research of potential biothreat surveillance systems. Early clinical symptoms of many biologic warfare agents such as aerosolized *B. anthracis*, tularemia, and smallpox resemble influenza like illness [17, 27].

Surveillance of infectious diseases can be conducted using passive or active approaches. Active methods based on laboratory testing and case reporting are usually resource intensive and require ongoing reporting by participating physicians, hospitals, and laboratories [14]. Only a subset of specimens can be tested [28] and cases are often underreported. Passive syndromic surveillance methods may be less accurate but they are also less expansive and enable assessment of the disease spread and severity in the population. Implementing syndromic surveillance based on signs and symptoms, diagnosis, and large volumes of other health related data for disease of interest can greatly improve the quality and timeliness of passive surveillance [29]. Information acquired integrating both methods can generate a more complete picture of an outbreak or an epidemic [14].

2.1 Active Influenza Surveillance

In the USA, the national influenza surveillance is lead by the CDC as a collaborative effort of state and local health departments and laboratories, health-care providers, hospitals, and clinical laboratories. The data on circulating influenza viruses and the disease activity including incidence, morbidity, and mortality is collected year round, compiled, and published weekly with a 1–2-week reporting delay [25]. Influenza virologic surveillance throughout the USA is conducted by approximately 140 laboratories comprising the WHO and National Respiratory and Enteric Virus Surveillance System (NREVSS) laboratory networks. They collect information on the proportion of influenza A and B positive respiratory specimens and determine influenza A subtypes. A subset of the influenza positive samples, especially if the subtypes cannot be determined by standard diagnostic tests, are sent to CDC for further characterization by gene sequencing to monitor emergence of novel viruses

and antiviral resistance [23]. The second component of the surveillance system is the Illness Surveillance Network (ILINet) comprised of approximately 3,000 health-care providers voluntarily reporting all outpatient visits and the number of visits due to influenza-like illness (ILI) stratified by age group. The percentage of weekly ILI visits weighted to reflect the population size of reporting states are compared to the national baseline of ILI visits outside of influenza season to monitor ILI activity levels in each state [23]. Vital statistics offices in 122 participating US cities report the total number of deaths and the number of deaths caused by pneumonia or influenza (P&I) stratified by age groups. Statistical methods are used to calculate the weekly level of P&I mortality above the seasonal baseline or epidemic threshold. In 2004, pediatric influenza-associated mortality for children 0–18 years of age became a nationally notifiable condition. Influenza Hospitalization Network comprised of hospitals in over 80 counties in 14 states collects information from hospital records and reports on laboratory-confirmed influenza hospitalizations for children and adults. The information on geographic spread of influenza activity is augmented by State and Territorial health department epidemiologists' reports [23].

In addition to the CDC surveillance systems, the armed forces operate the Global Emerging Infections Surveillance and Response System (GEIS) to protect military personnel and their families [30]. Respiratory Infections surveillance is one of the GEIS programs contributing to the global influenza surveillance network. The program leverages established laboratory and research facilities in host countries and collaborations with global partners. Its activities are coordinated and information regarding circulating influenza viruses, disease burden, and epidemiology is shared with CDC, WHO, and host countries. The data is also used in research and for development of vaccines and diagnostics [31]. International influenza surveillance is accomplished through the WHO Global Influenza Surveillance Network collaborating centers including the CDC. Global influenza surveillance information is shared through the WHO FluNet tool and it provides advance signals of influenza activity and trends, informs selection of annual vaccine strains, and enables member countries to better prepare for upcoming influenza season [32].

2.2 Alternative Surveillance Methods

In addition to the active surveillance efforts, alternative methods such as syndromic surveillance, electronic patient records from emergency room or ambulatory doctor visits, and hospital discharge records have been used for surveillance of influenza and other infectious diseases with growing frequency [19, 20, 27, 33]. Syndromic surveillance provides clues on disease patterns collected from multiple information sources such as emergency department visits, ambulatory health-care visits, calls to health information hotlines, Internet health information seeking, and over-the-counter medication purchases. Indication of potential disease outbreak from syndromic surveillance is usually available before laboratory test results are reported [14]. ESSENCE, the Electronic Surveillance System for the Early Notification of

Community-based Epidemics is an example of syndromic surveillance system implemented by the Department of Defense (DoD) to automatically download data from the electronic health records of military personnel and their families. The system captures information coded in accordance with the International Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9-CM) standards from over 300,000 weekly outpatient visits to US military treatment facilities [17]. It monitors disease outbreaks based on health care utilization patterns and uses ICD-9-CM codes to group diagnosis into one of the eight disease syndromes. Another national electronic surveillance system, BioSense, launched in 2003 and operated by the CDC collects and analyzes ICD-9-CM coded data from outpatient visits to health-care facilities and emergency departments, hospitalized patients, laboratory tests, and information on over-the-counter medications sold in pharmacies [34]. Although different studies reported variable utility of syndromic disease surveillance systems for local disease outbreaks, the majority of them indicated that it was useful for monitoring respiratory disease activity and the annual influenza seasons [19, 35, 36]. Sensitivity of ICD-9-CM based detectors of acute respiratory disease and influenza epidemics varied from 44 to 79 % for acute respiratory disease to 100 % for influenza outbreak [37] and specificity ranged between 96 and 97 % [20, 27]. Sensitivity was found to be moderate and likely not sufficient to detect a small disease outbreak, e.g., in the event of a local bioterrorism incidence. However, ICD-9-CM coded data can be useful for influenza surveillance when accuracy, completeness, and timeliness are carefully considered [29] before using such data for decision making.

For a comprehensive influenza surveillance system, it is critical to include hospitals that would collect epidemiological and virological information on severe cases. This data enables characterization of severe ILI, identification of at risk population groups, tracking of genetic changes in the circulating viruses, and serve as a monitoring tool for emerging pandemics [38]. Hospital based case series studies yield valuable information on risk factors for sever influenza during an ongoing or past influenza seasons. Although these studies can inform vaccination and therapy decisions, majority of them have a limited sample size, are recourse intensive, and the results are not generalizable on the national level. The lack of this data became especially apparent during the 2009 H1N1 pandemic when the disease incidence rate was very high resulting in declaration of phase 6 pandemic while the disease severity on a national level was not ascertained [28]. Hospital-based electronic surveillance is a cost-effective approach to identify influenza season-specific populations at high risk for ILI complications and fatal outcomes. Detailed clinical information on each individual case is coded in patients' records and can be used to augment active surveillance in public health response planning and implementation [16].

Advances in information technologies enabled new global and national surveillance methods and real time information sharing among multiple stakeholders. Monitoring indicators other than the traditional information captured by health-care providers can be a cost effective approach to augment respiratory disease surveillance. Rise in purchases of over-the-counter cold medications, school absenteeism, Internet health information searches, and utilization of health advice

phone lines were shown to correlate with increased influenza activity [14]. The rise in health information seeking preceded doctors' visits by about 1 week and was also correlated with media coverage of the health concern [39].

An approach to influenza surveillance monitoring ILI health-seeking Internet queries was launched by Google and CDC during the 2007–2008 influenza season. The system analyzed logs of Web searches related to ILI information and reported data with only 1 day lag instead of the usual delay of 1–2 weeks. The accuracy of the ILI estimates was 85–96 % as compared to the actual disease incidence reported by CDC influenza surveillance [25]. A Health Map Web based data collection system was employed during the 2009 H1N1 pandemic to monitor the Internet, compile, and report influenza activity in geographically diverse locations through an interactive map. Data was collected from news media, blogs, and other nontraditional sources as well as from the WHO, CDC, and the public health agency of Canada. The median lag between reported and confirmed cases ranged from 9 to 18 days with considerable variations between the countries influenced by public health infrastructure, political system restricting information, and media coverage. The nontraditional information sources may enable earlier detection of outbreaks and epidemics, expand population coverage, improve sensitivity of emerging diseases detection, and place the epidemic or pandemic in the context of the affected population [40].

2.3 Utilization of Electronic Health Records in Influenza Research and Surveillance

While electronic surveillance based on nonclinical data such as over-the-counter medication sales, school absenteeism, and health information seeking may provide preliminary signs of potential infection spread, prompt release of electronic health records (EHR) containing diagnosis and clinical outcomes can lead to a more informative and timely disease surveillance [20]. Increasing utilization of patient electronic records could play an important role in attaining public health objectives and complimenting other information sources.

Information from electronic medical records captured through surveillance platforms or stored in local or centralized databases has been used in numerous studies for monitoring disease incidence, prevalence, severity, risk factors, and medical care decisions. Analyses of electronic medical records were employed to augment the traditional approaches [17, 18] during respiratory seasons in the USA. Standard surveillance was not sufficient during the recent influenza 2009 H1N1 pandemic when several states, including New York [41], Wisconsin [42], and California [43] implemented additional information gathering methods based on electronic medical records to gain a more complete understanding of the ongoing pandemic severity. EHR-based surveillance systems such as Electronic Medical Record Support for Public Health (ESP) implemented in Ohio and Massachusetts and BioSense were successfully used for analyzing ICD-9 diagnosis codes, reporting notifiable disease

cases, surveillance of ILI, identification of influenza or upper respiratory infection risk factors among hospitalized patients, and for monitoring diabetes prevalence, risk factors, and disease severity [13, 19]. The results of influenza risk factor analyses based on ICD-9 coded data overall agreed with earlier observations based on primary data collected through the Emerging Infections Program during 2005–2008 influenza seasons [44] and in Manitoba, Canada during 2009 H1N1 pandemic [45] as well as with laboratory confirmed influenza hospitalizations reported to the CDC during the 2009 pandemic [17, 46, 47] demonstrated that optimally selected ICD-9 code groups can be used in an automated surveillance system drawing information from electronic medical records for accurate monitoring of influenza activity. In this study of the US Air Force personnel and their dependents outpatient visits the syndromic surveillance results correlated with the results of sentinel ILI surveillance conducted by the CDC. Placzek and Madoff (2011) used administrative hospital discharge records to estimate the hospitalization rates and characterize patients hospitalized with ILI during the seasonal flu epidemics and the 2009 H1N1 pandemic in Massachusetts. They evaluated two sets ("maximum" and "minimum") of ICD-9 diagnosis codes for their relevance and accuracy in identifying influenza-associated hospitalizations and disease severity and concluded the proposed minimum ICD-9 criteria more accurately reflected the actual influenza cases. ICD-9 coded diagnosis alone or in conjunction with other electronic health data were used in monitoring of ILI severity and risk factors [18, 48, 49], and for modeling early detection of local respiratory disease outbreak [24]. This approach was adopted for other disease surveillance, such as SARS [50], diabetes incidence and management [13], and pertussis [51]. The study results suggest that timely ILI surveillance is feasible using ICD-9-CM coded electronic medical records and emphasized the importance of the appropriate ICD-9-CM code selection for case definition for accurate assessment of disease activity and severity [18, 20].

Current influenza surveillance systems are resource intensive and provide limited information on patients at-risk for severe influenza. To date, no study has been conducted using a large sample of electronic health records (EHR) to examine the risk factors for influenza in hospitalized patients across the USA. Larger data sets of EHRs will enable the creation of statistically significant age-specific models of influenza severity and predict more representative influenza risk factors and vulnerable groups. A recent study utilized the Nationwide Inpatient Sample (NIS) which is a repository of eight million electronic hospital discharge records from 1,000 participating hospitals in over 43 states representing approximately 20 % of all US hospitalizations [7]. This data source is maintained by the Healthcare Cost and Utilization Project (HCUP) sponsored by the Agency for Healthcare Research and Quality (AHRQ). Results from the retrospective unmatched case–control study of NIS patients hospitalized with influenza during the 2009 H1N1 pandemic and severe A/H3N2 2007–2008 epidemic seasons confirmed the utility of using an existing electronic resource to identify comorbidities and demographic risk factors for severity of clinical outcomes associated with pandemic and epidemic influenza viruses [21]. The use of primary diagnosis ICD-9-CM codes 487.xx–488.xx to correctly identify influenza hospitalizations from NIS was verified by comparing

Table 29.1 Number of influenza cases reported in NIS and CDC surveillance systems during the 2007–2008 season and 2009 pandemic

Surveillance system	2007–2008	2009
WHO/NREVSS	41,809	177,814
FluSurv-NET	3,933	8,278
NIS	17,767	30,613

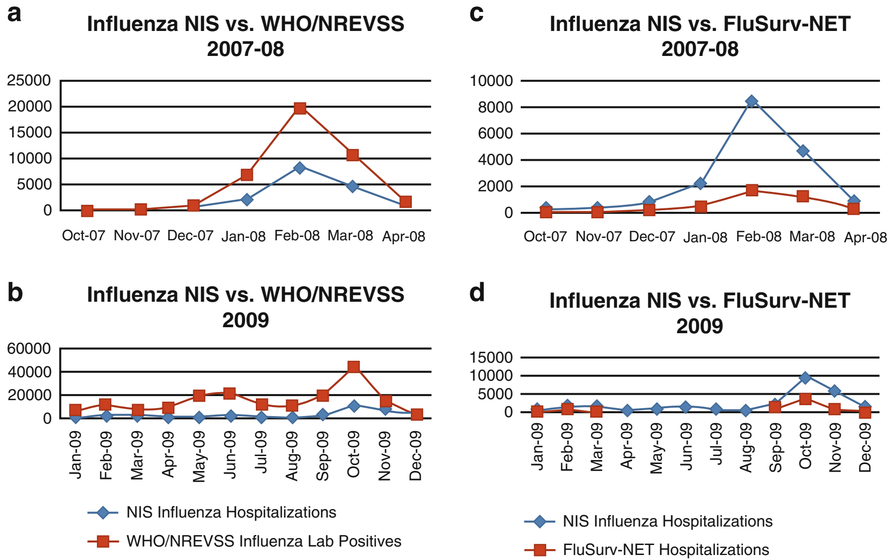

Fig. 29.1 Number of monthly influenza hospitalizations in NIS compared with WHO/NREVSS reported laboratory confirmed influenza infections during October 2007–April 2008 (Panel **a**) and January to December 2009 (Panel **b**) and FluSurv-NET during October 2007 to April 2008 (Panel **c**) and January to December 2009 (Panel **d**)

the temporal trends of monthly hospitalization counts identified in NIS records (Table 29.1) with influenza cases reported by the National Respiratory and Enteric Virus Surveillance System WHO/NREVSS and Hospitalization Surveillance Network (FluSurv-NET) during the 2007–2008 and 2009 influenza seasons (Fig. 29.1).

Findings from these studies demonstrate that large datasets of electronic medical records are an essential component of influenza epidemic surveillance. Integration of ICD-9 diagnosis codes into more complex disease detection algorithms can further improve the sensitivity and specificity of surveillance systems based on electronic medical records [52]. However, this approach is limited if electronic records are fragmented between different providers using different disease algorithms whereas the ICD-9 codes even though potentially less specific are standardized among all users and may be more applicable to nationwide surveillance [13]. Further

standardization of data coding and selection criteria, and interoperability among private and government surveillance efforts has the potential to enhance the electronic data quality and timeliness [14, 18]. This methodology can be especially advantageous for public health applications as it uses routinely collected data and requires modest investments for maintenance and operation [50].

3 Influenza Virus

Influenza virus is a zoonotic pathogen causing annual epidemics and pandemics resulting in human toll and economic losses all over the world. Influenza-associated morbidity and mortality are especially high among persons with chronic health conditions and usually among the very old or the very young [53]. Although the virus was identified and isolated only 80 years ago, influenza disease outbreaks can be traced back to Middle Ages and identified by signs and symptoms, sudden start of the epidemic, and excess mortality in historical sources dating to 1650 [54]. Shope demonstrated in 1930s that the infectious agent causing flu in humans could adapt to other species and cause similar disease in swine. The influenza virus adaptability to the host immune system enables sustained human-to-human transmission and the emergence of novel viral strains [55]. It also poses a challenge to the public health efforts to predict and control the annual influenza epidemics and pandemics.

3.1 Pathophysiology

Influenza viruses belong to the *Orthomyxoviridae* family and are divided into three genera or types, Influenza virus A, B, and C [56]. Influenza A viruses are further classified into subtypes defined by one of the 18 hemagglutinin and one of the ten neuraminidase subtypes present in the virus [57]. Influenza B viruses are not classified into distinct subtypes but are divided into two genetic lineages, Yamagata and Victoria [58].

It is an enveloped single stranded RNA virus with a genome fragmented into eight segments encoding 11 proteins. The surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) play the most important role in viral infection and transmission. HA attaches the virus to the target cell's sialic acids receptors facilitating the viral RNA entry into the cell. The NA enzymatic activity cleaves the sialic acid releasing the newly produced viral particles [53, 59].

The annual epidemics are caused by influenza A and influenza B, but only influenza A can adapt to multiple hosts and emerge as a novel virus causing pandemics. Antigenic drift due to mutations in HA and NA genes allows the virus to evade preexisting antibodies in the human immune system conferring the pathogenicity and virulence. Antigenic shift occurs when influenza viruses containing diverse HA and NA subtypes coinfect the same host, triggering a reassortment event and

producing progeny with genomic segments from both parental viral subtypes [53]. Wild birds are the natural reservoir for influenza viruses. Sixteen hemagglutinin and nine neuraminidase influenza A subtypes were isolated from aquatic birds and only the most recent HA17 was isolated from fruit bats [60]. According to the mixing vessel theory, pigs are considered the main mammalian host where the adaptation of an avian influenza viruses to human host and reassortment events occur [61]. Pigs' cell-receptors match both human and avian influenza, rendering them susceptible to infection with viruses from both hosts [53]. Influenza A viruses have been also isolated from other animals, including a horse, dog, cat, tiger, and leopard [59]. Influenza Type B and C is rarely found in hosts other than humans, although influenza B has been found in seals and influenza C has been reported in swine and dogs [53].

3.2 Epidemiology

Despite the investments in influenza research, surveillance, and prevention efforts, influenza virus remains a cause of respiratory infection in the USA and in the world. Annual influenza-associated deaths in the USA range between 3,349 and 48,614 [62] and, on the average, 200,000 are hospitalized due to severe disease [63]. The variations in mortality can be attributed to difference in the circulating viral types and subtypes. The average mortality rates are 2.7 times higher during the seasons when influenza A(H3N2) subtype is predominant as compared to seasons when influenza B or other influenza A subtypes are the predominantly circulating viruses. During a typical influenza season, severe illness and death occur most frequently among individuals 65 years and older (89.4 %) or children younger than 2 years of age [62]. Persons of any age with underlying health conditions are also at a greater risk for severe outcomes associated with influenza infections [64].

Influenza viruses are transmissible among humans via the respiratory rout. During seasonal epidemics and pandemics, each case transmits the virus at 2–3 day interval to 1.1–1.8 and 1.5–5.5 individuals respectively [65]. The human-to-human transmission occurs in one of the three ways: direct contact with infected persons, touching object contaminated with the virus and then transferring it from hands to mucus surfaces of the nose or eyes, and inhaling virus-containing droplets produced by infected person when coughing or sneezing [66, 67]. The efficiency of influenza transmission aerosolized in droplets depends on the size of the droplet, viral concentration, and humidity. Yang and Marr (2011) demonstrated that the concentration of infectious influenza virus in cough droplets is inversely related to the relative humidity (RH) in indoor settings, while the droplet size is directly related to relative humidity. In a dryer environment, the smaller droplets tend to stay in the air longer, infecting larger number of sensitive hosts. In a humid environment, the virus in large droplets settles on objects (fomites) and can survive for several days. Viable influenza viruses in mucus were detected on paper money bills after 48 h and in some cases up to 17 days [68].

In temperate climates, influenza epidemics occur in a seasonal pattern during the colder months of the year, while in the tropical climates, influenza circulates all year round with patterns associated with rainy seasons. Multiple reasons for this periodicity such as sunlight, temperature, humidity, human mobility, and contact rates, and functions of the immune system have been explored without arriving at a definitive conclusion [69, 70]. Yang and Marr (2011) suggested that the winter seasonality can be partially explained by higher concentration of droplet-suspended influenza viruses in heated buildings due to lower humidity. Other environmental factors, such as colder temperature and reduced ultraviolet radiation, are also independently associated with virus survival and seasonality. Temperature and humidity also effect the human immune system, diminishing the blood flow and leukocyte supply in low temperatures while increasing viral shedding [69]. Lowen and Palese (2011) confirmed that cold and dry conditions facilitated viral transmission through aerosolized droplets, while warm or humid environment (30 °C, 80 % RH) prevented the viral spread. They proposed that seasonal pattern of influenza epidemics in temperate climate occurs due to viral transmission by aerosolized droplets, while year-round infections occur through fomites or direct contacts in tropical climate. The exception to this pattern was the 2009 spring outbreak of the swine-origin influenza A H1N1, which possibly could be explained by the increased frequency of transmission via direct contact due to the absence of human immunity to the novel antigenic strain. Variations in temperature and humidity did not affect viral spread by direct contact [66].

Influenza pandemics are caused by novel viruses for which the world population has no immunity [54, 72]. Each of the six pandemics in the last 120 years were caused by a different novel influenza A virus that has undergone antigenic shift, reassortment of gene segments encoding HA and/or NA, and successfully adapted to the human host [53]. However, of the multiple possible combinations between 17 HA genes and 10 NA genes, influenza viruses with only three combinations (H1N1, H2N2, and H3N2) have adapted to enable human-to-human transmission suggesting the presence of inherent limitations in viral ability to adapt [72, 73].

Of the documented pandemics, the most devastating occurred in 1918–1919 (the Spanish influenza), causing more than 500,000 deaths in the USA and over 50 million deaths in the world [74]. The avian origin influenza A H1N1 virus which caused the pandemic had a case-fatality rate of 2.5 %, with the majority of the deaths occurring among otherwise healthy young adults 20–40 years of age [130]. The high mortality appeared to be associated with pneumonia caused by bacterial coinfection [72]. World War I potentially contributed to the spread and severity of the pandemic. Crowded conditions, increased stress, and malnutrition could have weakened the immune system of the troops while increased travel of the armed forces and civilians facilitated the spread of the virus throughout the world [75].

The sequence data of the 1918 influenza a H1N1 virus suggest that the virus was not a reassortant but rather all eight viral segments were novel with no prior immunity in the human population. In contrast, the viruses that caused the 1957 (H2N2) and 1968 (H3N2) pandemics were direct descendants of the 1918 influenza and evolved from the existing strains through reassortment events with genes from

avian influenza viruses [76]. The H2N2 virus with two surface proteins new to humans caused the Asian pandemic, resulting in approximately 70,000 deaths in the USA and two million deaths worldwide. The 1968 H3N2 "Hong Kong'" virus was associated with 34,000 deaths in the USA and approximately 1,000,000 excess deaths globally. The disease caused by the pandemic H3N2 was relatively mild and the virus became seasonal and is circulating to date [53, 77].

Predictions that high pathogenicity avian influenza (HPAI) H5N1 would be the next pandemic strain were the subject of public health concern. The H5N1 continues to spread, causing disease in poultry and occasional human infections through direct contacts with infected poultry. Data pertaining to the H5N1 IAV strain adaptation to human host is limited, but it appears that human-to-human transmission has not occurred. Meanwhile, a fourth generation swine origin descendant of the 1918 virus caused a pandemic in 2009 [53, 72]. Three strains of viruses, derived from birds, pigs, and humans, gave rise to the pandemic virus by antigenic shift, reassortment, and recombination in pigs [78]. Human infections with the novel triple reassortment swine origin virus pdm2009H1N1 were first detected in Mexico and then in California in April of 2009, followed by the declaration of public health emergency in the USA [79]. Due to the fast spread of the virus worldwide, the WHO declared influenza pandemic in June 2009 [78].

Despite the high transmissibility, the disease severity was moderate which is not typical of most pandemic strains [79]. A distinguishing feature of the 2009 H1N1 virus, also observed in previous pandemics, was the off-season timing for the start of the pandemic and young age prevalence among influenza cases, hospitalizations, and deaths. In Mexico in the early stage of the pandemic, 87 % of deaths were reported for patients 5- to 59-years-old [80]. In the Northern Hemisphere, the majority of deaths, 65.5–91.7 %, occurred among adults 25–64 years and only 4.2–20.7 % of deaths were reported in adults older than 65 years [81] compared to a typical influenza season when estimated 90 % of deaths occur in this age group [82]. Among hospitalized patients 68.8 % of fatalities occurred among adults 19–64 years of age during the 2009 pandemic while 74.9 % fatalities occurred among patients 65 years and older during the preceding influenza season [21]. Underlying medical conditions contributed to disease severity in all age groups. Cross-reactive immunity was found more frequently among persons older than 60 years of age due to earlier exposure to influenza A/H1N1 strains derived from the 1918 pandemic virus [83, 84].

4 Influenza Disease

The impact of influenza epidemics or pandemics on the affected population has been associated with predominantly circulating viral types and subtypes and their relation to the preexisting immunity of the human host [62, 72]. Influenza infections may cause especially severe disease in populations already burdened with a high prevalence of chronic pulmonary conditions [85]. Galiano et al. (2012) suggested that the major determinant of influenza disease severity was host-related and

included immune response, individual genetic background, and likely environmental factors surrounding human host and the virus. They based their hypothesis on the fact that a complete sequence of the A/Fujian/411/2002-like H3N2 virus isolates from cases that died and those who survived did not reveal any genetic differences that could be associated with disease severity or increased mortality [64]. Because the mechanisms by which viruses evolve and adapt to human hosts remain undetermined and the seasonal influenza disease continues to cause substantial public health threat, identifying the most vulnerable population groups in a timely manner remains a critical component of public health response.

Interventions to prevent or mitigate the impact of epidemics and pandemics include vaccination, antiviral drug therapies, and non-pharmaceutical methods. Vaccination is considered the most effective prevention method because it creates herd immunity by protecting not only the vaccinated individual but also precluding the viral transmission to those who did not receive the vaccine. However, effective protection can be achieved only if the vaccine strains antigenically match the circulating viral strains [86]. Antiviral therapy is beneficial, especially when a new viral strain emerges for which there is no vaccine. Novel therapeutic technologies against influenza offer great promise such as the use of siRNA and ribozymes delivered by intranasal spray or retroviral carriage [79]. Non-pharmaceutical methods include social distancing to reduce crowding and personal interactions and travel restrictions [71].

4.1 Clinical Symptoms and Patient Management

Influenza symptoms range from mild upper respiratory ailment to severe complications resulting in patient hospitalizations and in extreme cases, death [87]. The symptoms of influenza-like-illness (ILI) include fever, chills, sore throat, or cough [46, 88]. Depending on the circulating viral strains, diarrhea or vomiting may also be associated with influenza infection, especially in children [47]. Influenza may be difficult to diagnose based on clinical symptoms alone because the clinical presentation may be similar to other respiratory viral and some bacterial infections [90]. Presence of influenza virus can be confirmed by laboratory testing. The disease severity can be characterized by outcome indicators such as hospitalizations, admissions to intensive care units, length of hospital stay (LOS), utilization of mechanical ventilators, and flu-associated mortality [8, 42, 90, 91].

On the average, the frequency of severe cases requiring hospitalization or resulting in death is higher during the seasons when A(H3N2) viruses are predominant [62, 92]. During the 2009 pandemic, an estimated 0.45 % of the pdmH1N1 influenza cases required hospitalization and could be characterized as severe; approximately 12,500 of the cases or 0.02 % died [93]. In a review of studies characterizing the disease severity in the beginning of the 2009 H1N1 pandemic, Falagas et al. (2010) found a wide range of hospitalization rates (0–93.8 %), ICU admission rates (0–36.4 %), and fatality rates (0–38.5 %) among influenza cases. The fatality rate was significantly higher (25–41.4 %) among patients admitted to the ICU. A

prospective study in Canadian population measured the outcomes of severe 2009 influenza A (H1N1) cases as mortality, length of stay (LOS) in an ICU, and duration of mechanical ventilation. In this study of 215 critically ill patients, 81 % required mechanical ventilation, the median ICU stay was 12 days, and 17.3 % died within 90 days [94].

Annual influenza vaccination is universally recommended in the USA as the most effective prevention method for children older than 6 months of age and for all adults [95]. Vaccinating in advance 70 % of the US population even with low-efficacy vaccine in combination with school closure could be a cost-effective approach to reducing the disease burden [71].

4.2 Influenza Risk Factors and Vulnerable Population Groups

Susceptibility to influenza and severity of the disease is affected by multiple factors including characteristics of the circulating virus strain, genetics of the host, prior infection history, comorbidities, age, and environmental factors [87, 96]. Higher proportion of younger adults aged 20–50 [97] were more frequently infected during the 2009 H1N1 pandemic than traditionally more vulnerable age group 65 years or older during the seasonal influenza epidemics while pediatric mortality and morbidity was of a greater concern during the 2003–2004 season [90, 98]. This unpredictability of the virus–host interactions and consequences to population's health underscores the need for continuous timely and informative influenza surveillance.

Multiple studies conducted during different influenza seasons demonstrated increased severity of influenza when chronic conditions such as asthma, diabetes, neurologic disorders, obesity, and cardiovascular disease are present in children and adults [42, 47, 89, 99]. Underlying health conditions, especially chronic lung and heart disease [12] were more prevalent among the cases admitted to ICU or those who died compared to other hospitalized patients diagnosed with influenza [100]. In an international study of more than 70,000 hospitalized patients with laboratory confirmed H1N1pdm influenza proportion of patients with underlying chronic conditions increased with disease severity and constituted 52.3 % of those admitted into ICU and 61.8 % of those who died [99]. During the 2009 pandemic, mortality was higher among individuals with underlying medical conditions regardless of their age [83]. The presence of any chronic disease was also associated with influenza severity among hospitalized cases in the USA during the 2009 pandemic and preceding seasonal epidemics [21, 101].

4.2.1 Clinical Influenza Risk Factors

Underlying health conditions including HIV, cancer, heart disease, lung and respiratory conditions, diabetes, neuromuscular and neurological disorders, obesity, and pregnancy were reported to be associated with increased risk for influenza infection

or disease severity. However, results were often controversial or not confirmed to be statistically significant.

Slightly more than half of a sample from the NIS hospitalization records (54.4 % in 2007–2008 and 53 % in 2009) reported at least one underlying health condition assessed (Fig. 29.2) [21]. For both the 2009 H1N1 pandemic and A/H3N2 2007–2008 epidemic seasons, the proportion of records with comorbidities among severe cases (64.7 % and 62.9 % respectively) and among those who died in the hospital (62 and 63.4 %) was similar and significantly higher than among the hospitalizations with moderate disease (45.4 and 46.2 % respectively). The hospitalized patients with any comorbidity had greater odds of severe seasonal and pandemic influenza (OR = 2.21 and 1.97 respectively) and inpatient death (OR = 1.96 and 2.02 respectively) [21].

During the 2009 H1N1 pandemic, a greater proportion of immunocompromised HIV-positive persons were hospitalized with influenza compared to HIV prevalence in general population but the H1N1pdm-associated disease severity and mortality were not substantially affected. In a US study of hospitalized patients with confirmed 2009 pandemic influenza A H1N1, there was no statistically significant difference between the proportion of immunosuppressed patients among those with pneumonia (10 %) compared to patients without pneumonia (14 %) [102]. In low prevalence settings the severity of seasonal influenza does not appear to change significantly in adults infected with HIV. However, in high HIV prevalence populations, influenza may pose a higher morbidity and mortality risk due to compromised immune functions and the presence of tuberculosis, hepatitis, and other comorbidities [103]. In South African population with high prevalence of HIV among patients with confirmed influenza A (H1N1) infection referred to ICU, 31.5 % were immunosuppressed due to either HIV or immunosuppressive therapy [85].

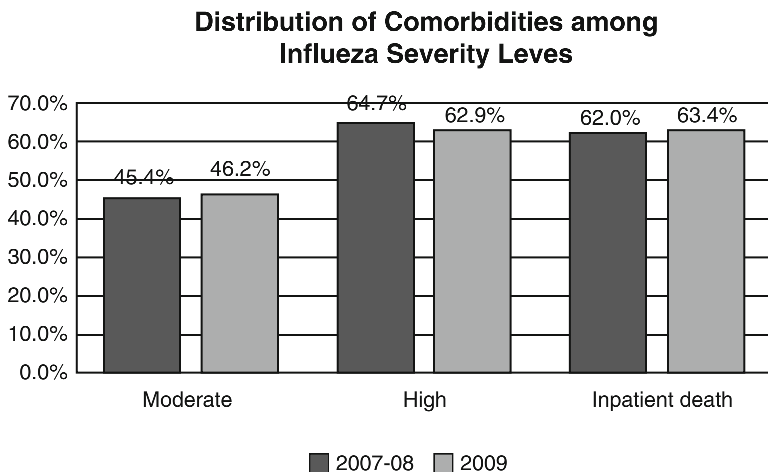

Fig. 29.2 Distribution of cases in the influenza severity groups among hospitalizations with at least one underlying medical condition during the 2007–2008 epidemic and 2009 pandemic

Cancer patients receiving chemotherapy or after hematopoietic cell transplant (HCT) have suppressed immune functions and are susceptible to infections including seasonal or pandemic influenza viruses. Influenza infection outcomes in HCT recipients vary depending on the influenza virus type and subtype [104]. Studies comparing seasonal and pandemic influenza disease in children and adults undergoing cancer therapy found significant differences in clinical symptoms at presentation and in clinical outcomes [105–107]. Although children infected with 2009 H1N1 were healthier at presentation and had fewer comorbidities they more frequently had pneumonia, stayed longer in the hospital, were more frequently admitted to ICU [106], and experienced higher mortality (10 % vs. 0 %) due to complications compared to children with seasonal influenza infections. Males were especially at high risk for developing pneumonia. Timely antiviral therapy mitigated the influenza disease severity in children and adult recipients of HCT [104, 107].

Chronic heart disease is a known risk factor for severe outcomes among persons with influenza-like illness. During the 2009 H1N1 pandemic, heart disease was the second most prevalent medical comorbidity present in approximately 25 % of reported deaths among adults and in almost 50 % of fatalities among persons 65 years or older [83]. Heart and lung disease were also frequent comorbidities with diabetes and kidney disease among the influenza case fatalities. In a dataset pooled from multiple countries in Europe, Asia, and America chronic heart disease was present in 7.1 % of all hospitalized patients with pH1N1 infection, 10.9 % of ICU admissions, and 12.1 % of deaths [99].

Lung diseases were the most frequently reported chronic conditions for the 2009 H1N1 influenza case fatalities with the chronic obstructive pulmonary disease (COPD) most prevalent in adults and asthma in children [83]. Regardless of asthma severity, its prevalence tends to grow with escalating influenza disease severity [99]. Influenza virus infection is known to exacerbate asthma and asthma is a known risk factor for influenza infection. It was the most frequently reported underlying medical condition in pediatric deaths associated with influenza A/2009 H1N [83]. The impact of asthma may also depend on the circulating influenza viruses. In a Canadian studies of pediatric population hospitalized with influenza, children with pandemic H1N1 influenza in 2009 were significantly more likely to have asthma (22 %) than those with seasonal influenza during the 2004–2009 seasons (6 %) although there were no difference in severity or clinical presentation of asthma between the pandemic and seasonal pediatric influenza cases [108]. Asthma was also more prevalent among the children admitted to ICU with pH1N1 and developing pH1N1-associated pneumonia compared with seasonal influenza in 2006–2009 [109]. Patients with chronic lung and airways diseases such as COPD are at a greater risk for severe morbidity and mortality associated with influenza infection. Evidence suggests that bacterial coinfections in COPD cases may further impact the disease severity. In a study of patients hospitalized with severe COPD in Italy, viral infection was detected in 23.4 % and viral-bacterial coinfection in 25 % of patients hospitalized with COPD exacerbation. Influenza was one of the most frequently identified infections adversely effecting lung function and extending hospital stay [110]. Although many national guidelines recommend influenza vaccination, there is only limited evidence

that vaccine is effective in COPD patients. However, some observational studies suggest that vaccine reduces both hospitalizations and mortality [111].

The association between diabetes Type1 and Type 2 and a greater risk for influenza associated complications may be explained by adverse impact of excessive blood glucose on immunity, as well as heart, kidney, and lung functions [112]. Influenza surveillance data in Wisconsin and New Mexico during the 2009 H1N1 pandemic indicated that diabetes was the second most frequent comorbidity following asthma and was present in 16–20 % of hospitalized influenza cases [42, 105]. Van Kerkhove et al. (2011) reported that diabetes was an underlying chronic condition in 9 % of influenza-associated hospitalizations and 13.6 % of cases admitted to the ICU in a sample representing 19 countries with diverse populations and healthcare systems. Diabetes was present in 8 % of influenza A/H1N1 associated fatalities in England [113], 14.4 % fatalities in a large international sample [99], and 29 % of fatalities in New Mexico [105]. The higher proportion of diabetes in New Mexico potentially could be due to a higher than 50 % obesity among hospitalized patients older than 18 years. Diabetes prevalence is on the rise in the USA, especially among the aging population, reaching almost 27 % prevalence among persons 65 years of age or older [114]. Influenza surveillance and timely characterization of clinical disease course are important for potential prevention and treatment of diabetic influenza cases [112].

Neurological and neuromuscular disorders (NNMD) are risk factors for influenza infections possibly due to difficulty clearing secretions from respiratory tract due to impaired or reduced muscle tone and lung function could lead to severe disease [9]. Persons with NNMD also may have an increased susceptibility to recurrent respiratory infection due to diminished ability to protect airways through cough [90] and a higher risk (OR, 5.6) of influenza-related neurologic complications such as seizures [115].

In a study of influenza-associated pediatric deaths during the 2003–2004 influenza season, 33 % of the children had neuromuscular or neurologic disorder [116]. Louie et al. (2006) further confirmed that neurologic diseases with the potential to compromise respiratory function were present in more than 25 % of severe influenza cases among children. NNMD were the most prevalent chronic diseases associated with respiratory failure in hospitalized children with laboratory-confirmed influenza diagnosis followed by chronic lung and chronic heart conditions [9]. A study of pediatric deaths reported to CDC during the 2009 H1N1 pandemic showed that 43 % of case fatalities had neurologic disorders. Majority of the children also had additional comorbidities such as heart disease [117]. Adult patients who developed pneumonia as a consequence of influenza 2009 H1N1 infection were more than twice as likely to have a neurological disease compared to patients who had no complications [102]. Neurological disorders found among patients hospitalized due to influenza included Down syndrome, cerebral palsy, developmental delay, history of stroke [102], seizures, spinal cord injuries [90], neuromuscular disorders, hydrocephalus, and epilepsy [117]. Pediatric deaths due to pandemic influenza five times exceeded the annual average number of deaths caused by seasonal influenza viruses during the five proceeding seasons. Neurologic

disorders were the most frequent comorbidities found in influenza-associated pediatric deaths [117] underscoring the importance of continues surveillance of disease severity and the need for timely characterization of risk factors during an ongoing influenza season.

During the 2009 H1N1 pandemic, obese individuals with body mass index (BMI) exceeding 30 kg/m^2 were at a higher risk for influenza infection; they were more likely to be hospitalized and were disproportionately represented among the patients in ICUs, those with longer duration of mechanical ventilation, longer hospital stay, and those who died compared with those who were not obese [99, 118, 119]. In a study of California adults the prevalence of obesity and extreme obesity among influenza cases was 1.5 and 2.8 times higher respectively than the US population average. The odds ratio (OR) for fatality among the extremely obese (BMI > 40) patients was 2.8–4.2 [120]. These findings corroborated the results of Kwong, Campitelli, and Rosella (2011) suggesting that obese individuals were at a greater risk for hospitalization than persons with normal weight during 12 pre-pandemic influenza seasons with OR = 1.45 and 2.1, for individuals with BMI 30-34.9 and ≥35 respectively.

The association between obesity and infection can be explained by impaired immune response or by strain of infection on respiratory system and reduced mechanical function of lungs and airways. Obese persons consume high percentage of oxygen to maintain normal respiratory function; they have increased airway resistance and may suffer from hypoventilation and chronic inflammation of the respiratory tract altering the immune function and the ability to respond to challenges to respiratory system [119, 120]. The role of obesity as an independent risk factor may be difficult to ascertain, especially in studies with a limited sample size, as it is often directly correlated with other underlying health conditions (e.g., diabetes and heart disease) known to increase risk for influenza infections and severe outcomes [118]. However, because more than 35 % of adults in the USA [121] and 500 million worldwide [122] are obese it may be a major contributor to excess morbidity and mortality associated with influenza and warrants further investigation.

Pregnancy has been reported as a risk factor for seasonal and pandemic influenza infections and severe disease outcomes using historical and current data. About 50 % of pregnant women infected with influenza developed pneumonia during the 1918 and 1957 pandemics [123]. Pregnancy was reported to be a risk factor for infection with influenza and severe disease outcome during the influenza A/2009 H1N1 pandemic as well. In a review of publications on 2009 H1N1 pandemic epidemiology in the Northern Hemisphere, Falagas et al. (2010) reported that 4.5–17.4 % of hospitalized cases were pregnant women and they comprised 11.5–18.2 % of ICU admissions. Compared to nonpregnant women diagnosed with influenza, they were seven times more likely to be hospitalized and twice more likely to have fatal outcomes [99]. In a UK study of a population with an estimated 6 % prevalence of pregnancy, 21 % of patients hospitalized with laboratory confirmed influenza 2009 H1N1 were pregnant and the majority of them were in the second or third trimester. The case fatality rate ranged between 1 and 6 % [2]. The rate of respiratory hospitalizations among pregnant women in Nova Scotia during non-pandemic

influenza seasons between 1990 and 2002 was almost 8 times higher for pregnant women than the year before they became pregnant [124]. Pregnant women with comorbidities such as asthma, anemia, and heart or renal disease were at the greatest risk for influenza-associated hospitalization.

The findings on influenza severity association with pregnancy were not consistent. In several countries as the level of disease severity increased the proportion of pregnant women diminished and the odds ratio for death among hospitalized pregnant women was <1 [99]. Interestingly, in a study of ILI hospitalized patients during the 2007–2010 influenza seasons, pregnancy was protective against pneumonia (OR 0.4), possibly due higher likelihood of hospitalizing pregnant women with severe respiratory infection [125]. This observation was supported by a UK study reporting that maternal outcomes were no more severe that for nonpregnant women of similar age hospitalized for influenza [2]. An increased susceptibility to influenza infection and severe disease among pregnant women could be partially explained by changes in immune response due to lower plasma levels of adiponectin regulating macrophage activity [119]. An additional explanation could be psychosocial changes that may occur during pregnancy such as perceived increased stress, anxiety, and negative mood which also were shown to alter the immune functions and increase the risk for respiratory tract infections [126].

4.2.2 Demographic Influenza Risk Factors

In addition to clinical comorbidities demographic characteristics and socioeconomic conditions also can increase the risk for influenza infections. Close human contacts in crowded housing during the influenza season, influenza vaccine uptake in a community, awareness of influenza transmission routs, and following the nonpharmaceutical prevention practices effect influenza virus spread and attack rates in population. The risk for influenza infection may also vary in individuals from different racial/ethnic backgrounds and age groups.

Historically, higher attack rates and more severe disease outcomes were observed among minorities since 1900s including during the 1918 influenza pandemic [45, 127]. In an analysis of influenza 2009 H1N1 cases pooled from 19 countries, Van Kerkhove et al. (2011) reported that indigenous populations and minority groups were disproportionately represented among hospitalized influenza cases and fatalities in Canada, Australia, and New Zealand, while in Mexico and Thailand minority groups did not carry excess disease burden. In a Canadian case–control study of laboratory-confirmed pH1N1 cases, 37 % were represented by the First Nation residents. The odds ratio was 6.52 for the First Nation individuals being admitted to the ICU compared to other ethnic groups even when controlling for socioeconomic status, age, residency settings, comorbidities, and time to treatment [45]. Similar results for influenza severity were observed in the USA where the risk for pH1N1 influenza hospitalization in New Mexico was 2.6 times higher among American Indians, 1.7 times higher for Blacks, and 1.8 times higher for Hispanics compared to non-Hispanic Whites [105]. Surveillance data from 12 states showed that the rate

of mortality attributed to pH1N1 was four times higher among the American Indians and Alaska Natives (AI/AN) and they had the highest rate (81.0 %) of underlying health conditions than all other ethnic groups [128]. Higher proportion of pediatric hospitalizations among minorities was observed during the pre-pandemic seasons as well, including the 2000–2001 season [129] and 2003–2004 when influenza A/ Fujiian was the prevalent circulating virus [116].

Although the reasons for disparities in influenza susceptibility and severity among the racial and ethnic populations are not fully identified several explanations have been proposed including socioeconomic status and resulting differences in living conditions, crowding, health behaviors, and access to medical care [96]. Cultural differences may affect utilization of available health care or vaccination uptake. Difference in genetic susceptibility and higher prevalence of chronic conditions associated with increased risk for influenza disease severity may also impact the attack rates and the disease outcome in ethnic minority communities [99].

Traditionally populations at the extremes of the age spectrum, young children and older adults are the most vulnerable groups during seasonal influenza epidemics while pandemics exhibit a characteristic shift towards younger adults in influenza-related deaths [65, 80, 130]. Persons younger than 65 years of age accounted for a greater proportion of deaths during all three pandemics in the twentieth century as well as during the 2009 H1N1 pandemic when young adults were at an increased risk for morbidity and mortality. Age was an independent risk factor for severe disease outcomes and death. In a study of hospitalized influenza cases in Washington State the odds of ICU admission or death were 4.4 and 5.9 times greater among adults 18–49 years and 50–64 years of age respectively compared with children younger than 18 years when controlling for other risk factors [11]. The lower influenza incidence rate and mortality among adults over 64 years observed during pandemics could be explained by antigen recycling mechanism, a partial protection due to earlier exposure to a similar virus [65]. However, if infected, this age group had the highest mortality rate among the hospitalized patients [99] potentially due to the presence of comorbidities, effect of medications, and bacterial coinfections. Explanations for severe disease among young adults included antibody-dependent enhanced infection and strong inflammatory response in the lungs leading to lung injury and ARDS [11]. Once infected with a novel influenza virus younger persons may retain long-lasting immunity better than older persons [130].

During the seasonal influenza epidemics older adults and young children are usually at a higher risk for severe disease and death. The proportion of influenza-attributable deaths during the 1994–2000 influenza seasons in Canada increased with age from 2 % in 65–69 age group to 5 % in persons 90 years and older. The case fatality rate for influenza hospitalized patients increased from 4 to 30 % for population 50–64 years to 90 years or older respectively and over 90 % of deaths occurred in persons older than 65 years of age [12]. During the 2003–2004 season when Influenza A Fujian strain was predominantly circulating virus increased morbidity and mortality was observed among children younger than 5 years of age [90, 98] while children hospitalized due to severe influenza during the 2009 H1N1 pandemic were significantly older with a larger proportion older than 5 years of age as

compared to pediatric admissions during the pre-pandemic influenza seasons [108]. Developing immune system and absence of immunity to circulating viruses in young children and weakened immune response to vaccination among the older adults renders both groups especially susceptible to seasonal influenza infection [11, 12, 99].

Although the health conditions described in this chapter contribute to influenza virus susceptibility and severity of the disease, their prevalence and impact may vary during different influenza seasons. During the 2009 influenza pandemic, only one third of the 70,000 hospitalized cases representing 19 countries had an identified chronic clinical comorbidity while approximately two thirds of hospitalized cases and 40 % of fatal cases did not have any identified preexisting disease. For the 2009 influenza pandemic, the overall difference in demographic and clinical factors between the disease severity groups and moderate disease controls suggests that age, sex, race, and all clinical conditions of interest showed overall statistically significant association with influenza severity. However, pregnancy was not associated with influenza severity for women of childbearing age [21]. The differences of risk factors and clinical outcomes in different countries further highlighted the need for country-specific and global surveillance as well as data sharing internationally [99].

5 Conclusion

Timely information on circulating influenza viruses and the disease burden associated with seasonal and pandemic influenza is essential for optimal public health response, identification of vulnerable populations, and for prevention and patient management strategies. Susceptibility to influenza and severity of the disease is affected by multiple factors including characteristics of the circulating virus strain, genetics of the host, prior infection history, comorbidities, age, and environmental factors. The unpredictability of the virus–host interactions and consequences to population's health underscores the need for continuous timely and informative influenza surveillance. Clinical surveillance is critical for identification of at risk population groups which also may change depending on the circulating virus as well as for monitoring the disease spread in the population and severity. Syndromic surveillance based on nonclinical indicators may contribute to a signal of epidemic spread and increase of cases. To better predict viral strains for effective vaccines and monitor novel emerging viral strains that could cause epidemics it is critical to continue and expand viral surveillance on an International level. While electronic surveillance based on nonclinical data such as over-the-counter medication sales, school absenteeism, and health information seeking may provide preliminary signs of potential infection spread, prompt release of electronic health records (EHR) containing diagnosis and clinical outcomes can lead to a more informative and timely disease surveillance. Increasing utilization of patient electronic records could play an important role in attaining public health objectives and complimenting other information sources.

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Chapter 30
The Role of Viral Protein Phosphorylation During Filovirus Infection

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Core Message Following the emergence of the *Filoviridae* family members Marburg virus and Ebola virus nearly five decades ago, there has been great interest in deciphering the molecular events that underlie the severe pathologies associated with infections by these viruses. Although there is significant insight into the pathological events that occur following infection gained from both investigations of human patients and from experimental animal infections, there is a relative paucity of information regarding the role of molecular interactions between the host and pathogen during disease progression or resolution. There is now an increasing appreciation that host-mediated phosphorylation of filovirus proteins serves a regulatory role for filovirus protein function/activity. Here, we discuss the role of these phosphorylation events in the filovirus life cycle.

1 Introduction

The mononegaviral family *Filoviridae* includes three genera, *Marburgvirus*, *Ebolavirus*, and *Cuevavirus* [1, 2]. The members of these genera possess non-segmented, single-stranded, negative-sense RNA genomes and produce enveloped, filamentous virions. Ebolaviruses and marburgviruses are etiological agents of severe hemorrhagic fevers in Africa that are associated with unusually

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high case-fatality rates in humans and nonhuman primates (NHPs) [3, 4]. These viruses are of global concern, considering the potential for their accidental introduction from endemic to nonnative regions or intentional manipulation for nefarious purposes [5, 6]. Concerns regarding virus spread from rural areas, where filoviruses tend to emerge, to urban areas, such as during recent disease outbreaks of Sudan virus in Uganda and Ebola virus in Guinea/Liberia, have increased fears of rapid spread of these highly lethal viruses [7, 8]. These concerns have been further exacerbated by importation of Marburg virus (MARV) by tourists returning from Uganda to the Netherlands and the USA [9, 10]. Currently licensed vaccines for filovirus infections are not available, and treatment is primarily based on supportive care.

Although many investigations have focused on the molecular and pathological events of the host following filovirus infection there is a relative paucity of information regarding the role of viral proteins and their respective function and/or activities in these processes. From this perspective, the role of posttranslational modification of proteins through phosphorylation is one of the most well-characterized modifications in terms of the regulation of protein function and/or activity. The role of protein phosphorylation in the regulation of cellular events in both prokaryotic and eukaryotic organisms has been well documented. Phosphorylation also plays an essential role in regard to the modulation of viral protein function in multiple members of the order *Mononegavirales* [11–13]. Given the importance of the role for modulation of viral protein function and activity through phosphorylation, this review focuses on the current state of knowledge regarding the role of filovirus protein phosphorylation in viral infection.

2 Etiologic Agents and Natural History

The three filovirus genera *Marburgvirus*, *Ebolavirus*, and *Cuevavirus* include a total of seven species and eight viruses (Table 30.1). The first documented human filovirus disease outbreak occurred in 1967 in West Germany and Yugoslavia following the importation of grivets/African green monkeys (*Chlorocebus aethiops*) infected with Marburg virus (MARV) from Uganda [14]. Although the case-fatality rate associated with this outbreak was 23 %, more recent disease outbreaks were characterized by much higher case-fatality rates (updated overall mean ≈ 80.3 %) [15]. Together with Ravn virus (RAVV), which thus far has only caused sporadic human infections, MARV is a member of the *Marburgvirus* genus. The disease these two viruses cause is referred to as Marburg virus disease (MVD).

The genus *Ebolavirus* has five members: Bundibugyo virus, Ebola virus, Reston virus, Sudan virus, and Taï Forest virus. Ebolaviruses were first identified in 1976, when a disease with similar clinical presentation to MVD was noted in two simultaneous viral hemorrhagic fever outbreaks in southern Sudan (now South Sudan)

Table 30.1 Current filovirus taxonomy as accepted by the International Committee on Taxonomy of Viruses (ICTV) and the ICTV *Filoviridae* Study Group [125, 126]

Genus	Species	Virus
Marburgvirus	*Marburg marburgvirus*	Marburg virus (MARV)
		Ravn virus (RAVV)
Ebolavirus	*Bundibugyo ebolavirus*	Bundibugyo virus (BDBV)
	Reston ebolavirus	Reston virus (RESTV)
	Sudan ebolavirus	Sudan virus (SUDV)
	Taï Forest ebolavirus	Taï Forest virus (TAFV)
	Zaire ebolavirus	Ebola virus (EBOV)
Cuevavirus	*Lloviu cuevavirus*	Lloviu virus (LLOV)

and northern Zaire (now Democratic Republic of the Congo). Surprisingly, two distinct viruses were found to be the causative agents of these outbreaks, and both were only distantly related to MARV. Today, these viruses are known as Sudan virus (SUDV) and Ebola virus (EBOV), respectively. SUDV and EBOV, as well as the later discovered Taï Forest virus (TAFV) and Bundibugyo virus (BDBV), are the etiological agents of Ebola virus disease (EVD) [16–19]. The number of EVD outbreaks has steadily increased from 2000 onwards and has been largely due to the emergence or reemergence of EBOV and SUDV [20]. However, it should also be appreciated that this increase may result from multiple factors, including ecological changes, increased contact between humans and intermediate and/or reservoir hosts, or improved disease surveillance.

EBOV appears to be the most lethal member of the *Ebolavirus* genus causing EVD with an updated mean case-fatality rate of 77 % [15]. In addition, EBOV is also suspected to be responsible for fatal infections of central chimpanzees (*Pan troglodytes troglodytes*) and western lowland gorillas (*Gorilla gorilla gorilla*) and may contribute to the rapid decline in the populations of these animals [21]. SUDV and BDBV, which have only been associated with human infections, have updated associated case-fatality rates of ≈53 % and 34 %, respectively [15]. In contrast, only one nonfatal human case of TAFV infection has been reported [15], and RESTV is considered to be nonpathogenic in humans [22, 23].

RESTV was first detected in 1989 in Reston, Virginia, USA, following the importation of crab-eating macaques (*Macaca fascicularis*) from the Philippines. RESTV was therefore the first ebolavirus to emerge outside of Africa [24, 25]. Several other epizootics due to RESTV infection occurred in the USA and Italy in captive macaques in subsequent years, and all originated in the Philippines. Interestingly, simian hemorrhagic fever virus (SHFV), an arterivirus, was always found during these outbreaks as well. In 2008, RESTV was coincidentally detected in domestic pig tissue samples during a highly fatal outbreak of atypical porcine

reproductive and respiratory syndrome in the Philippines [5, 26]. However, RESTV was only found in sample groups that also tested positive for the arterivirus porcine reproductive and respiratory syndrome virus (PRRSV). Adding further complexity to the epizootiology, PCR analysis also identified a third virus, porcine circovirus type 2, thus further precluding the identity of the causative agent behind the outbreak. The ecological connection between these viruses and their individual contribution to the observed disease outbreaks remains to be determined.

Recently, a phylogenetically distinct, filovirus was discovered in carcasses of Schreiber's long-fingered bats (*Miniopterus schreibersii*) found in Cueva del Lloviu, Asturias, Spain. This virus, Lloviu virus (LLOV), is the founding member of the genus *Cuevavirus*. The transcriptional features of LLOV have suggested marked differences from other filoviruses [6]. However, as infectious LLOV has not yet been isolated, the biological significance of these differences has not been established. At the moment, LLOV is not thought to be pathogenic for humans.

3 Clinical Signs of Filovirus Infection in Humans

The clinical presentation of human filovirus infections has been reviewed elsewhere in detail [20, 27, 28]. Here, we briefly discuss clinical signs that have been associated with MVD and EVD. Much of the information that is available regarding clinical disease features of MVD and EVD is based on single, well-defined exposures, including those associated with laboratory accidents [28]. The mean incubation times associated with MARV infections range from 5 to 9 days and from 3 to 12 days during EBOV infection with a median survival of 9 days from the onset of illness. Interestingly, Sadek et al. reported that during the 1995 EVD outbreak patients who survived to day 14 had >75 % chance of survival [29]. In the case of both EVD and MVD, clinical sign onset occurs rapidly with fairly nondescript manifestations that predominantly include fever, rash, and thrombocytopenia, often accompanied with gastrointestinal, respiratory, vascular, and neurological manifestations [30–34]. Many of these early signs can confound the early diagnosis of MVD and EVD with more common illnesses, such as malaria [28]. Clinically, EVD and MVD are indistinguishable based on either physical examination or clinical laboratory analysis. Although hemorrhagic manifestations associated with MVD and EVD can be impressive, they are found in less than half of the patients [20]. When present, hemorrhage is often found in multiple foci of the mucosa and commonly in the conjunctivae with associated ease of bruising and persistent bleeding from venipuncture sites [31, 33, 35–39]. Petechiae and mucosal hemorrhage arise during the peak of illness that is associated with altered fluid distribution, hypotension, and aberrant coagulopathy [40–43].

In terms of common laboratory parameters, thrombocytopenia is the most common feature associated with MVD and EVD. Thrombocytopenia develops early during the course of infection and continually declines in patients with severe disease [30–34]. Leukocytosis has also been described, with the presentation of leukopenia

at the time of clinical presentation of disease with subsequent neutrophilia [30–34]. Elevations in liver enzyme concentrations are common features of both diseases with elevated concentrations of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) during clinical disease presentation. In addition, coagulation defects, including disseminated intravascular coagulation (DIC), prolonged prothrombin time (PT), and partial thromboplastin time (PTT), are typical [9, 30, 31, 33, 34, 44, 45]. Viremia appears to be largely associated with the onset of illness in infected patients and appears to remain elevated in patients that die. Higher viral titers are associated with fatal disease as compared to those in patients with resolution of infection [46–49]. Progression to severe hypotension and shock is generally associated with progression to death [33, 35, 50–52]. Nonfatal disease or asymptomatic infection has been associated with a transient strong pro-inflammatory response early in the course of disease with elevations in circulating interleukin β (IL-β), IL-6, and tumor necrosis factor α (TNFα) concentrations, and may also be associated with specific IgM and IgG responses [20]. However, the underlying cause and effect of these responses in protection from disease and/or death remains to be elucidated.

There is relatively limited information regarding the pathology or pathogenesis associated with MVD and EVD in human patients. The majority of human infections appear to be associated with direct contact with infected patients or cadavers. Filoviruses appear to enter the host predominantly through mucosal surfaces, skin abrasions, or parenteral introduction [51–54]. Interestingly, the route of viral entry appears to be related to both disease course and outcome as the mean incubation period for contact exposures is 9.5 days as compared to 6.3 days for introduction through puncture/injection [55]. Examination of tissue samples from human patients with fatal disease revealed that monocytes, macrophages, and dendritic cells likely play central roles in the dissemination of virus [56–58]. These investigations have also demonstrated that viral replication is supported in these cells, as well as in endothelial cells, fibroblasts, hepatocytes, adrenal cortical cells, and epithelial cells. Although it has been postulated that structural damage within infected endothelial cells likely contributes to hemorrhagic diathesis, there has been no supporting histological evidence for this hypothesis [20].

4 Host Immune Responses to Filovirus Infection

There has been limited information regarding the host immune response in MVD or EVD. Investigations of EVD in human patients have suggested that infection results in the expression of several pro-inflammatory mediators, including IL-2, IL-6, IL-8, IL-10, multiple IFNs, interferon-inducible protein 10 (IP10), monocyte chemoattractant protein 1 (MCP1), RANTES, TNFα, and reactive oxygen and nitrogen [40, 46, 47, 59]. As a result of these limited investigations, the immune response to filovirus infection has been studied predominantly in vitro or in experimental animals. To this end, genome-wide expression studies have contributed markedly to

our current state of knowledge regarding host responses to filovirus infections [60–62]. Perhaps unsurprisingly, host gene expression cluster analysis in human hepatocytes demonstrated that the responses of EBOV- and MARV-infected cells shared greater similarity to one another than to the response of cells infected with RESTV [62]. One of the key findings was that filovirus infection resulted in the global suppression of host antiviral responses, including Toll-like receptor (TLR)-, IFN regulatory factor 3- (IRF3), and protein kinase R (PKR)-mediated pathways [62]. Further, RESTV infection resulted in the activation of more than 20 % of the IFN-stimulated genes (ISGs), whereas EBOV and MARV infection resulted in inhibition of these responses. Importantly, analysis of signal transducers and activators of transcription (STAT) phosphorylation demonstrated that MARV and EBOV differentially modulated the activation state of these molecules. This analysis suggested that these two filoviruses inhibit IFN signaling and activation through differential mechanisms. The potential molecular mechanisms through which the filoviruses subvert these responses are detailed in Sect. 5.

Rubins et al. characterized the temporal gene expression profiles of peripheral blood mononuclear cells from EBOV-infected crab-eating macaques compared to baseline [41]. Few changes occurred in the early stages (1–2 days) following infection; however, broad changes to host gene expression profiles were observed on days 4 and 6 post-inoculation. These changes included significant upregulation of pro-inflammatory cytokines (including IL-1β, IL-6, IL-8, and TNF-α) and chemokines (e.g., macrophage inflammatory protein 1α and monocyte chemoattractant protein 1–4). In addition, multiple genes related to apoptosis regulation (e.g., Bcl-2 family members, multiple caspases, Fas-associated death domain protein, TNF superfamily member 10) were also upregulated in the later stages of infection. EBOV infection also resulted in the upregulation of IFN-regulated genes starting early during the course of infection (day 2) and remained upregulated through to the endpoint of the study (day 6). More recently, Wahl-Jensen and colleagues demonstrated that EBOV particle attachment and entry into human macrophages induce pro-inflammatory mediators (including IL-6, IL-8, and TNF-α) 1 h post-inoculation [61]. While such studies of global gene expression have been informative, many questions remain regarding the molecular pathogenesis of filovirus infection.

Analyses of host responses to filovirus infection have focused predominantly on humans or NHPs. Following the isolation of RESTV from pigs [5], subsequent investigations have demonstrated that pigs were susceptible to both RESTV and EBOV infection with preferential targeting of macrophages in the lungs [63]. Nfon et al. therefore recently investigated the modulation of host gene expression patterns in the lungs of EBOV-infected pigs [63]. In pigs inoculated with EBOV, upregulation of chemokine expression began at day 3 post-inoculation compared to mock-infected pigs. The most pronounced changes in gene expression were found at 5 and 7 days post-inoculation and included the upregulation of a broad set of cytokines (IL-5, IL-6, IL-8, IL-10, IL-22, IL-26, IL-27, resistin), chemokines (CCL2, CCL10, CCL19, CCL20, AMCF-II, CCL3L1, CCL4), cell adhesion protein (selectin), antimicrobial protein, palate, lung, and nasal epithelium clone protein, and pro-apoptotic molecules (multiple caspases, caspase recruitment domain-containing protein 6 (CARD),

apoptosis-associated tyrosine kinase (AATK), Fas, Fas-associated protein with death domain (FADD), TNF receptor-associated factor 3 (TRAF3), TNFα-induced protein 3-interacting protein 1 (TNIP1)). In addition, expression of multiple genes related to microbial sensing (pattern recognition receptors) or antiviral responses (ISGs) was upregulated in the lungs of infected animals. Although the localization of the cytokine response of pigs and humans or NHPs differs during the course of EBOV infection (localized responses in the lungs of pigs versus a predominantly systemic response in humans and NHPs), the cytokine profiles of pigs, humans, and NHPs were quite similar. This similarity is also evident when the results from the NHP study by Rubins et al. are compared to those from the pig study by Nfon et al. as both investigations reported on the induction of specific host responses that were common across both studies (i.e., IL-6, IL-8, several caspases). Given the increasing use of pigs for modeling human diseases, observations such as these could provide important information regarding EVD pathogenesis in humans.

5 Filovirus Genome and Particle Structure

Filovirus genomes are nonsegmented, single-stranded RNAs of negative polarity (≈19 kb) and contain seven genes (in the order 3′-NP-VP35-VP40-GP-VP30-VP24-L-5′). Each gene usually contains a single open reading frame, each of which encodes one protein. An exception is the ebolavirus GP gene, which encodes four proteins from three overlapping open reading frames via co-transcriptional editing and proteolytic processing [64–67]. The open reading frames of each gene are flanked by long non-translated sequences of somewhat unclear function [68]. Although the organization of the filovirus genome is conserved across the filovirus family members, the individual genome sequences are highly variable. The filovirus genes encode for four structural proteins that are involved in the formation of the nucleocapsid: a nucleoprotein (NP), an RNA-dependent RNA polymerase (L), a polymerase cofactor (VP35), and a minor nucleocapsid protein (VP30) [69]. In addition, these proteins also facilitate viral replication and transcription. The filovirus nucleocapsid is helical in structure, and together with the two matrix proteins VP40 and VP24 assembles in the form of long filamentous structures. Although the average lengths of particles formed by different filoviruses vary (≈800–≈1,000 nm), they do have similar widths (80 nm) [26].

As with other negative-strand RNA viruses, filovirus particles are formed following viral component assembly at the plasma membrane and subsequent release from infected cells by budding [70–74]. NP is the major determinant of the nucleocapsid and expression of NP results in the formation of intracellular inclusion bodies. Overall, the matrix protein VP40 is transported to the plasma membrane following expression by the retrograde late endosomal pathway. Following this, VP40 re-localizes cellular budding machinery to the site of viral assembly and budding (Ascenzi ippolito 2008 151). VP40 is also associated with viral inclusions that contain the assembled nucleocapsids. NP-RNA interactions are sufficient for the

formation of nucleocapsids with recruitment of VP30, VP35, and L. Small amounts of VP40 co-localize with the accumulating nucleocapsids in cellular inclusions. Following expression through the secretory pathway and proteolytic cleavage in the *trans*-Golgi network, GP localizes to the late endosome and accumulates in multivesicular bodies that are enriched with VP40 followed by transport to the site of viral particle assembly and binding. The nucleocapsid complexes are then transported to these sites leading to virus particle assembly and release [75].

In this section we focus on the immunomodulatory properties that have been described for filovirus proteins VP35, VP24, VP40, and $GP_{1,2}$.

5.1 VP35

In addition to core functions in the nucleocapsid complex, multiple immunomodulatory functions have been described for VP35 (Fig. 30.1). These functions include antagonism of type I IFN responses through disruption of the interaction between retinoic acid inducible gene-1 (RIG-1) and IRF3 and inhibition of double-stranded RNA-dependent protein kinase activation [76–78]. VP35 suppresses IFN-β responses in a similar manner as the influenza A virus NS1 protein [76]. These activities are localized to the C-terminal region of VP35, as specific mutations within a region of basic amino acids ablated both the binding of dsRNA and IFN inhibition [79, 80]. Suppression of IFN responses by VP35 is mediated by the inhibition of TANK-binding kinase 1 (TBK-1)/IKKε-mediated phosphorylation of IRF 3/7 by acting as an alternative IFN kinase substrate [81]. VP35 also modulates small ubiquitin-like modifier conjugation (SUMOylation) in the host through interaction with the SUMO E2 enzyme Ubc9 and the SUMO E3 ligase PIAS1, resulting in the promotion of SUMOylation of IRF-7 and repression of IFN transcription [82]. The inhibition of protein kinase R (PKR) activity by VP35 may result from the binding of dsRNA by VP35 [83]. The binding of dsRNA is important for inhibition of the host RNAi pathway by acting as an antagonist of RNA interference (RNAi) [84, 85]. The high degree of sequence conservation in regions of VP35 related to modulation of host responses across the filovirus family members suggests that these activities are likely conserved broadly across the filovirus family.

5.2 VP24

VP24 is a minor matrix protein involved in nucleocapsid formation and assembly and plays a role in the transition from viral transcription/replication to virion assembly [43, 86–90]. Following formation of NP-derived helical tubes, VP24 and VP35 interact with these helices to form nucleocapsid-like structures [91]. Further, VP24 is also a determinant of host cell tropism as demonstrated by the adoption of mutations during EBOV adaptation to mice and guinea pigs [81, 92–94]. EBOV VP24 can also modulate host IFN responses (IFN-α, IFN-β, and IFN-γ signaling) primarily

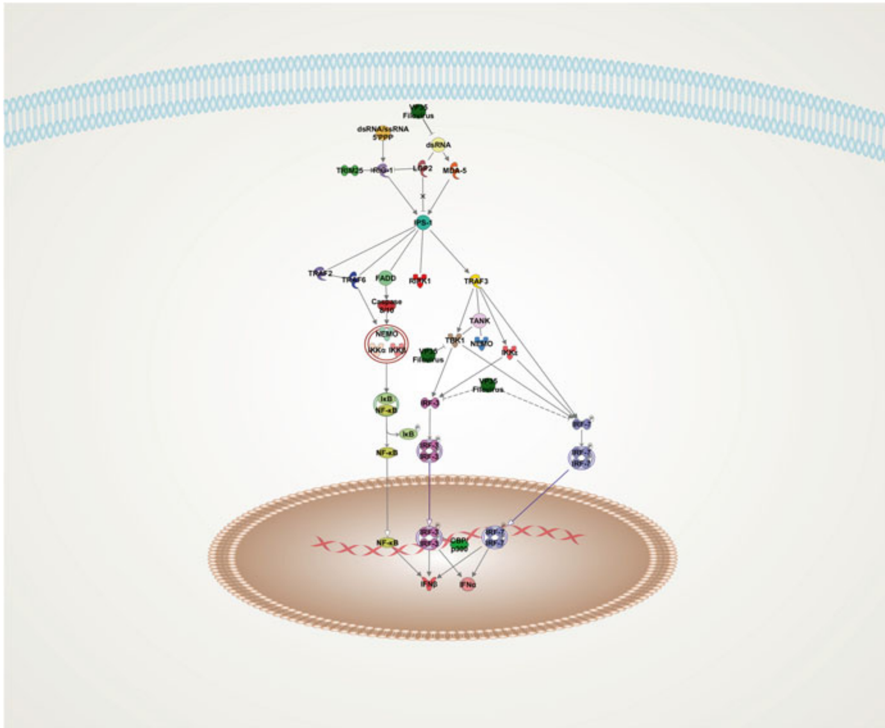

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Fig. 30.1 EBOV VP35 modulation of the RIG-1 signaling pathway and inhibition of host downstream IFN responses. The filovirus VP35 protein can antagonize type I IFN responses through interaction with multiple host proteins. VP35 can bind dsRNA directly through a region of basic amino acids in the C-terminus of the proteins, thus inhibiting the activation of IFN in response to dsRNA. These events may also be responsible for the inhibitory activity of VP35 on PKR. In addition, VP35 can inhibit TBK-1/IKKε-mediated phosphorylation of IRF 3/7 by acting as an alternative IFN kinase substrate. VP35 also modulates host SUMOylation through interaction with the SUMO E2 enzyme Ubc9 and the SUMO E3 ligase PIAS1, resulting in the promotion of SUMOylation of IRF-7 and repression of IFN transcription. Images were generated using the Ingenuity Pathway Analysis software suite

through the blockade of the karyopherin α1 (KPN α1)-STAT1 interaction and subsequent inhibition of IFN-induced nuclear accumulation of phosphorylated STAT1 [95] (Fig. 30.2). Although the role of VP24 in the viral life cycle is conserved across the filovirus family members, overlap in the host immunomodulatory properties of this protein between ebolaviruses and Marburg virus is limited [96]. Subversion of host IFN responses in MARV infection appears to occur through a differential inhibitory mechanism and is discussed in the next section. Try42 and Lys142 of EBOV VP24 are critical residues for modulation of IFN responses as mutation of either of these residues results in inhibition of the interaction between VP24 and KPN α1 [97]. Subsequent experiments suggest that EBOV VP24 may block IFN responses by binding KPN α5 and KPN α6, which also bind to STAT1 [98].

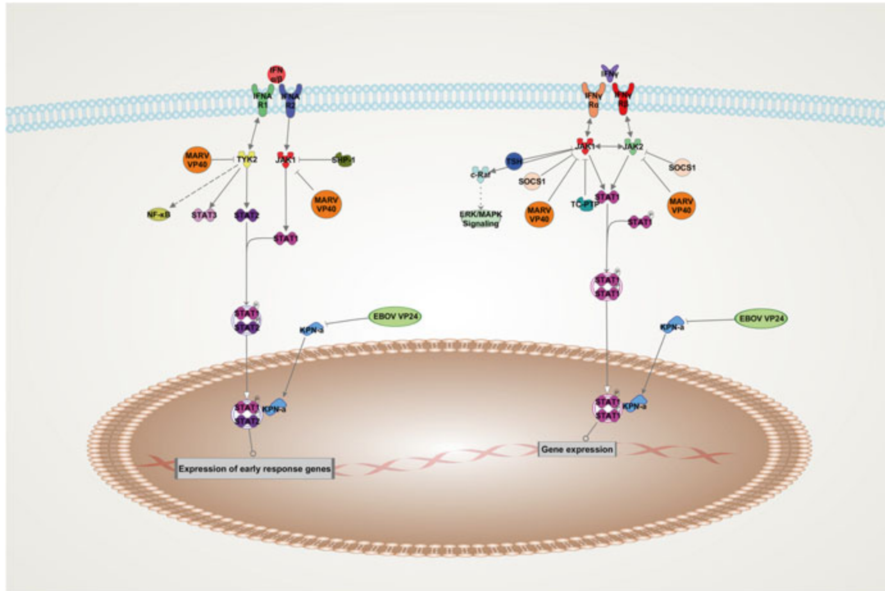

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Fig. 30.2 EBOV VP24- and MARV VP40-mediated repression of IFN signaling pathways. EBOV VP24 has been demonstrated to repress host IFN responses (IFN-α, IFN-β, and IFN-γ signaling) through inhibition of the interaction between KPN α1 and STAT1 resulting in the inhibition of IFN-induced nuclear accumulation of phosphorylated STAT1. Further, EBOV VP24 may also repress IFN responses by binding KPN α5 and KPN α6, which also bind to STAT1. MARV VP40 and EBOV VP24 can also inhibit STAT2 nuclear accumulation in response to IFN-α and repress host IFN responses. MARV VP40 can also block Jak1 and Tyk2 phosphorylation upstream of STAT1 and STAT2 in response to IFN-α/β through blockade of JAK1 auto-phosphorylation. Images were generated using the Ingenuity Pathway Analysis software suite

5.3 VP40

VP40 is a peripheral membrane protein involved in transportation of the filovirus ribonucleocapsids to the plasma membrane, incorporation into viral particles, and viral particle budding [90, 99]. VP40 can also modulate host IFN responses (Fig. 30.2). Valmas et al. demonstrated that MARV VP40 inhibited IFN-α/β and IFN-γ-mediated gene expression, host antiviral responses in an IFN-α/β-dependent fashion, and Janus kinase-1 (Jak1)-dependent IL-6 signaling [96]. Interestingly, EBOV VP24 and MARV VP40 both inhibit STAT2 nuclear accumulation in response to IFN-α, suggesting that MARV VP40 has a similar role to EBOV VP24 in interfering with IFN signaling [96]. As MARV VP40 blocked Jak1 and tyrosine protein kinase 2 (Tyk2) phosphorylation, upstream of STAT1 and STAT2 in response to IFN-α/β through blockade of JAK1 auto-phosphorylation, MARV VP40 may inhibit host antiviral responses through a mechanism distinct from that of EBOV VP24.

5.4 $GP_{1,2}$

The filovirus genome encodes a single surface protein, the type I transmembrane glycoprotein ($GP_{1,2}$). This protein mediates host cell attachment, fusion, and cell entry [72, 100, 101]. In addition, $GP_{1,2}$ induces cytopathic effects (e.g., cell rounding, detachment, increased vascular permeability) and downregulates the expression of various cell surface proteins at least in vitro [69, 102]. Mutation of an editing site in the GP genome resulted in a recombinant mutant EBOV with enhanced cytopathic effect and overexpression of $GP_{1,2}$ as compared to wild-type virus [103]. These results suggest that the overall expression of $GP_{1,2}$ is a primary determinant for the cytopathic effect of this protein. More recently, Groseth et al. investigated the effect of exchanging $GP_{1,2}$ from RESTV with that of EBOV on virulence [104]. Interestingly, although the incorporation of RESTV $GP_{1,2}$ into EBOV (EBOV-GPRESTV) resulted in decreased lethality and prolonged mean time to death in mice, the incorporation of EBOV $GP_{1,2}$ into RESTV (RESTV-GP_{EBOV}) did not result in lethal disease. Thus, although $GP_{1,2}$ contributes to virulence, the lack of disease in the recombinant RESTV-GP_{EBOV} suggests that other factors are required for full virulence. The cytotoxicity of $GP_{1,2}$ may be related to modulation of cellular responses within the host through extracellular signal-related kinase (ERK)/mitogen-activated protein kinase (MAPK) signaling. An investigation by Zampieri et al. showed that $GP_{1,2}$ expression decreased phosphorylation of ERK1/2 compared to a mutant form of $GP_{1,2}$ that lacked a mucin-like domain and had reduced cellular cytotoxicity [102]. These effects appeared to be largely related to a preferential modulation of the phosphorylation state of the ERK2 isoform.

5.5 Posttranslational Modification of Ebola Virus Proteins by Phosphorylation

The posttranslational modification of proteins (PTMs) provides an essential mechanism to diversify the activities or functions of proteins beyond that of the synthesized transcript [105]. These modifications provide a dynamic mechanism to rapidly alter cellular responses to changes in the surrounding environment. Further, PTMs are likely responsible for the regulation of virtually all cellular events including transcription and translation, signal transduction, and communication between the internal and external cellular environments [105]. Although PTMs may involve a broad array of enzymatic (including lipidation, acylation, glycosylation, and phosphorylation) and nonenzymatic modifications (biotinylation) we focus on Ebola protein PTMs through phosphorylation in this review.

Protein modification through reversible protein phosphorylation is the primary mechanism by which proteins regulate cellular responses. These events are controlled by the kinase class of enzymes and involve the transfer of γ-phosphate from ATP to a specific phospho-acceptor, primarily a serine, threonine, or tyrosine residue. Kinase-mediated phosphorylation events regulate a diverse range of cellular

responses including metabolic processes, transcription, immune responses, and apoptosis [106]. Kinases constitute approximately 2 % of the human genome and are among the largest and most well-studied families of genes. Manning et al. demonstrated that eukaryotic protein kinases could be divided into seven major groups based on similarities in their sequence identification features and similar function: AGC [protein kinase A (PKA), PKG and PKC families], Ca^{2+}/calmodulin-regulated kinase (CAMK), casein kinase 1 (CK1), GMGC [including cyclin-dependent kinases (CDK), mitogen-activated protein kinases (MAPK), glycogen synthase kinase (GSK) and CDK-like kinases], STE (related to yeast sterile kinases), tyrosine kinases, and tyrosine-kinase like (TKL) [107]. Kinase-mediated phosphorylation events are largely dominated by phosphorylation of serine residues (86 %), followed by threonine (12 %) and tyrosine (2 %) [106]. Importantly, although much of the information regarding kinases and their role in cellular processes has focused on host proteins, there is an increasing appreciation for the role that host kinase-mediated phosphorylation plays in viral protein modification and/or function.

5.6 Posttranslational Phosphorylation of EBOV VP30

Posttranslational phosphorylation of EBOV proteins by eukaryotic proteins plays a significant role in viral pathogenesis. In 2002, Modrof et al. reported on the role of phosphorylation of EBOV VP30 in the regulation of viral transcription and binding to NP inclusions [108]. Two serine clusters (Ser29–31 and Ser42–46) and a single threonine (Thr52) in the N-terminal domain of the protein were sites of phosphorylation. Mutation of these six serine residues to alanine resulted in loss of ability of EBOV VP30 to bind to NP inclusions, even distribution of VP30 throughout the cytoplasm, and had no significant effect on transcription activation. Treatment of VP30 with okadaic acid, an inhibitor of protein phosphatase (PP) 1, PP2A, and PPC resulted in dephosphorylation of VP30 and loss of transcription activity. Intermediate phosphorylation of VP30, achieved by mutation of only one of the serine clusters (i.e., VP30 Ser29–31A), resulted in activation of both viral transcription and assembly. Interestingly, the replacement of the six phosphoserine residues with aspartate resulted in impaired transcription activity. These results suggest that the negative charge imparted by the phosphorylation of these residues played an important role in the regulation of VP30 activity. Martinez et al. demonstrated that the mutation of the VP30 serine clusters resulted in the inability to rescue infectious recombinant virus as only wild-type EBOV genome was rescued [109]. VP30-AA (both serine clusters mutated to alanine residues), VP30-DD (Ser → Asp), VP30-AD, and VP30-DA supported transcription and were incorporated into virion-like particles (VLPs). While VP30-AA was able to support full viral transcription, the VP30-AD and VP30-DA mutants could only initiate transcription of the first gene of full-length viral RNA and could not support transcription reinitiation of subsequent genes. Although VP30-AA was able to activate transcription removal of the negative charge that would accompany phosphorylation, mutation of the two N-terminal serine clusters resulted in the loss of a subsequent function required for generation

of fully infectious virions. Taken together, these observations suggest that the dynamic phosphorylation and dephosphorylation of EBOV VP30 act to balance transcription and replication processes during infection [110].

The role of phosphorylation MARV VP30 has also been investigated. MARV VP30 contains seven serine residues in the C-terminal region of the protein similar to that seen for the ebolaviruses [111]. Replacement of serines 40–42 in MARV VP30 resulted in ablation of interactions between VP30 and NP-induced inclusion bodies whereas substitution of serine for alanine between amino acids 43–51 had relatively no effect on this interaction. This interaction was largely regulated by the negative charge of the phosphorylation at these residues as Ala → Asp in the VP30-Ala40–42 restored the interaction between VP30 and NP inclusions.

The availability of amino acid sequence information for the proteins from all of the ebolaviruses, as well as from specific isolates, has enabled comparative analysis of the viral proteins. This information could help guide investigations into the prospective roles of specific posttranslation modifications (PTMs) in the differential virulence of these viruses. For example, sequence alignments of the VP30 proteins from EBOV variant Mayinga and EBOV variant Kikwit demonstrate homology of the amino acid sequences with 100 % conservation of sequence identity and thus presumably conservation of the phosphorylation patterns reported for VP30 (Fig. 30.3).

```
VP30_MARVV    MQQ--PRGRSRTRNHQVTPTIYHETQLPSKPHYTNYHPRARSMSSTRSSAESSPTNHIPR    58
VP30_RAVV     MQQ--PRGRSRNRSHQVALSTYHENQLPSKPQYINHHPRARSMSSTRSSTEGSPTNHASR    58
VP30_RESTV    MEHSRERGRSSNMRHNSREPY-------------ENPSRSRSLSRDPNQVDRRQPRSASQ    47
VP30_SUDV     MERGRERGRSRNSRADQQNST-------------GPQFRTRSISRDKTTTDYRSSRSTSQ    47
VP30_EBOV     MEASYERGRPRAARQHSRDGH-------------DHHVRARSSSRENYRGEYRQSRSASQ    47
VP30_BDBV     MDSFHERGRSRTIRQSARDGP-------------SHQVRTRSSSRDSHRSEYHTPRSSSQ    47
VP30_TAFV     MEVVHERGRSRISRQNTRDGP-------------SHLVRARSSSRASYRSEYHTPRSASQ    47
              *:    ***                             *:** *      :    .   :

VP30_MARVV    ARPPSTF-----NLSKPPPPPKDMCRNMKIGLPCADPTCNRDHDLDNLTNRELLLLMARK    113
VP30_RAVV     ARPLSTF-----NLSKPPPPPKDMCRNMKIGLPCTDPACNRDHDLDNLTNRELLLLMARK    113
VP30_RESTV    IRVPNLFHRKKTDALIVPPAPKDICPTLKKGFLCDSKFCKKDHQLDSLNDHELLLLIARR    107
VP30_SUDV     VRVPTVFHKKGTGTLTVPPAPKDVCPTLRKGFLCDSNFCKKDHQLESLTDRELLLLIARK    107
VP30_EBOV     VRVPTVFHKKRVEPLTVPPAPKDICPTLKKGFLCDSSFCKKDHQLESLTDRELLLLIARK    107
VP30_BDBV     VRVPTVFHRKRTDSLTVPPAPKDICPTLRKGFLCDSNFCKKDHQLESLTDRELLLLIARK    107
VP30_TAFV     IRVPTVFHRKKTDLLTVPPAPKDVCPTLKKGFLCDSNFCKKDHQLESLTDRELLLLIARK    107
               *  . *            ** ***:* .:: *: * .  *::**:*:.*.::*****:**:

VP30_MARVV    MLPNTDKTFR--SPQDCGSPSLSKGLSKDKQEQTKDVLTLENLGHILSYLHRSEIGKLDE    171
VP30_RAVV     MLPNTDKAFK--SQQDCGSPSLSKGLSKDKQEQAKDVLTLENLGHILNYLHRSEIGKLDE    171
VP30_RESTV    TCGIIESNSQITSPKDM---RLANPTAEDFSQGNSPKLTLAVLLQIAEHWATRDLRQIED    164
VP30_SUDV     TCGSTDSSLNIAAPKDL---RLANPTADDFKQDGSPKLTLKLLVETAEFWANQNINEVDD    164
VP30_EBOV     TCGSVEQQLNITAPKDS---RLANPTADDFQQEEGPKITLLTLIKTAEHWARQDIRTIED    164
VP30_BDBV     TCGSLEQQLNITAPKDT---RLANPIADDFQQKDGPKITLLTLLETAEYWSKQDIKGIDD    164
VP30_TAFV     TCGSTEQQLSIVAPKDS---RLANPIAEDFQQKDGPKVTLSMLIETAEYWSKQDIKNIDD    164
                   :.    : :*     *::  :.* .:     :**  * .  ..    ::  :::

VP30_MARVV    TSLRAALSLTCAGIRKTNRSLINTMTELHMNHENLPQDQNGVIKQTYTGIHLDKGGQFEA    231
VP30_RAVV     TSLRAALSLTCAGIRKTNRSLINTMTELHINHENLPQDQNGVIKQTYTGIHLDKGGQFEA    231
VP30_RESTV    SKLRALLTLCAVLTRKFSKSQLGLLCETHLRHEGLGQDQADSVLEVYQRLHSDKGGNFEA    224
VP30_SUDV     AKLRALLTLSAVLVRKFSKSQLSQLCESHLRRENLGQDQAESVLEVYQRLHSDKGGAFEA    224
VP30_EBOV     SKLRALLTLCAVMTRKFSKSQLSLLCETHLRREGLGQDQAEPVLEVYQRLHSDKGGSFEA    224
VP30_BDBV     SRLRALLTLCAVMTRKFSKSQLSLLCESHLRREGLGQDQSESVLEVYQRLHSDKGGNFEA    224
VP30_TAFV     SRLRALLTLCAVMTRKFSKSQLSLLCESHLRREGLGQDQSESVLEVYQRLHSDKGGNFEA    224
              : *** *:* ..  ** .:* :  : * *:.:* * ***   : :.*  :* **** ***

VP30_MARVV    ALWQGWDKRSISLFVQAALYVMNNIPCESSISVQASYDHFILPQSQGKGQ----------    281
VP30_RAVV     ALWQGWDKKSISLFVQAALYVMNNIPCESSISVQASYDHFILPRNQGERQ----------    281
VP30_RESTV    ALWQQWDRQSLIMFISAFLNIALQIPCESSSVVVSGLAT-LYPAQDNSTPSEATNDTTWS    283
VP30_SUDV     ALWQQWDRQSLTMFISAFLHVALQLSCESSTVVISGLRL-LAPPSVNEGLPPAPGEYTWS    283
VP30_EBOV     ALWQQWDRQSLIMFITAFLNIALQLPCESSAVVVSGLRT-LVPQSDNEEASTNPGTCSWS    283
VP30_BDBV     ALWQQWDRQSLIMFITAFLNIALQLPCESSSVVISGLRL-LVPQSEDTETSTYTETRAWS    283
VP30_TAFV     ALWQQWDRQSLIMFITAFLNIALQLPCESSSVVISGLRM-LIPQSEATEVVTPSETCTWS    283
              **** **::*: :*: * * :  :: ****   * :.    : * .

VP30_MARVV    ------      281
VP30_RAVV     ------      281
VP30_RESTV    STVE--      287
VP30_SUDV     EDSTT-      288
VP30_EBOV     DEGTP-      288
VP30_BDBV     EEGGPH      289
VP30_TAFV     EGGSSH      289
```

Fig. 30.3 Multiple sequence alignment of ebolavirus and marburgvirus VP30. Sequence alignments were performed using ClustalW Omega. *Red lines* and *blue line* denote serine cluster(s) of functional importance for EBOV VP30 and MARV VP30, respectively

```
VP30_RAVV (Ravn)      MQQPRGRSRNRSHQVALSTYHENQLPSKPQYINHHPRARSMSSTRSSTEGSPTNHASRAR    60
VP30_MARV (Popp)      MQQPRGRSRTRNHQTASSIYHETQLPSKPHYTNHHPRARSMSSTRSSAESSPTNHIPRAR    60
VP30_MARV (Ozolin)    MQQPRGRSRTRNHQAIPSIYHETQLPSKPNYTNHHPRARSMSSTRSSTESSPTNHIPRAR    60
VP30_MARV (Musoke)    MQQPRGRSRTRNHQVTPTIYHETQLPSKPHYTNYHPRARSMSSTRSSAESSPTNHIPRAR    60
VP30_MARV (Angola)    MQQPRGRSRTRNHQATPSIYHETQLPSKPHYTNHHPRARSMSSTRSSAESSPTNHIPRAR    60
                      *********.*.**.  : ***.******.* *:*************:*.*****  ***

VP30_RAVV (RAVN)      PLSTFNLSKPPPPPKDMCRNMKIGLPCTDPACNRDHDLDNLTNRELLLLMARKMLPNTDK    120
VP30_MARV (Popp)      PPPTFNLSKPPPPPKDMCRNMKIGLPCTDPTCNRDHDLDNLTNRELLLLMARKMLPNTDK    120
VP30_MARV (Ozolin)    PPSTFNLSKPPPPPKDMCRNMKIGLPCTDLTCNRDHDLDNLTNRELLLLMARKMLPNTDK    120
VP30_MARV (Musoke)    PPSTFNLSKPPPPPKDMCRNMKIGLPCADPTCNRDHDLDNLTNRELLLLMARKMLPNTDK    120
VP30_MARV (Angola)    PPSTFNLSKPPPPPKDMCRNMKIGLPCTDPTCNRDHDLDNLTNRELLLLMARKMLPNTDK    120
                      *  ************************:* :*****************************

VP30_RAVV (RAVN)      AFKSQQDCGSPSLSKGLSKDKQEQAKDVLTLENLGHILNYLHRSEIGKLDETSLRAALSL    180
VP30_MARV (Popp)      TFRSLQDCGSPSLSKGLSKDKQEQTKDVLTLENLGHILNYLHRSDIGKLDETSLRAALSL    180
VP30_MARV (Ozolin)    VFKSPQDCGSPSLSKGLSKDKQEQTKDVLTLENLGHILNYLHRSEIGKLDETSLRAALSL    180
VP30_MARV (Musoke)    TFRSPQDCGSPSLSKGLSKDKQEQTKDVLTLENLGHILSYLHRSEIGKLDETSLRAALSL    180
VP30_MARV (Angola)    TFRSPQDCGSPSLSKGLSKDKQEQTKDVLTLENLGHILSYLHRSEIGKLDETSLRAALSL    180
                      .*:* *******************:*************.*****:***************

VP30_RAVV (RAVN)      TCAGIRKTNRSLINTMTELHINHENLPQDQNGVIKQTYTGIHLDKGGQFEAALWQGWDKK    240
VP30_MARV (Popp)      TCAGIRKTNRSLINTMTELHINHENLPQDQNGVIKQTYTGIHLDKGGQFEAALWQGWDKR    240
VP30_MARV (Ozolin)    TCAGIRKTNRSLINTMTELHINHENLPQDQNGVIKQTYTGIHLDKGGQFEAALWQGWDKR    240
VP30_MARV (Musoke)    TCAGIRKTNRSLINTMTELHMNHENLPQDQNGVIKQTYTGIHLDKGGQFEAALWQGWDKR    240
VP30_MARV (Angola)    TCAGIRKTNRSLINTMTELHINHENLPQDQNGVIKQTYTGIHLDKGGQFEAALWQGWDKR    240
                      ********************:**************************************:

VP30_RAVV (RAVN)      SISLFVQAALYVMNNIPCESSISVQASYDHFILPRNQGERQ  281
VP30_MARV (Popp)      SISLFVQAALYVMNNIPCESSTSVQASYDHFILPQSQSKGQ  281
VP30_MARV (Ozolin)    SISLFVQAALYVMNNIPCESSISVQASYDHFILPQSQGKGQ  281
VP30_MARV (Musoke)    SISLFVQAALYVMNNIPCESSISVQASYDHFILPQSQGKGQ  281
VP30_MARV (Angola)    SISLFVQAALYVMNNIPCESSISVQASYDHFILPQSQGKGQ  281
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Fig. 30.4 Multiple sequence alignment of MARV and RAVV VP30. Sequence alignments were performed using ClustalW Omega. The *blue line* denotes the serine cluster of functional importance for MARV VP30

In contrast, alignments of VP30 from the available MARV variants Musoke, Popp, and Angola demonstrate lower homology with many individual differences related to differences in Ser (S), Thr (T), or Tyr (Y) residues (Fig. 30.4). The potential relation between these substitutions and strain-dependent virulence remains to be investigated. Multiple sequence alignment (MSA) of the VP30 proteins from the most well-characterized variants of each member of the filovirus family demonstrates that the first serine cluster of Ebola virus is only partially conserved in Sudan virus, Reston virus, and Marburg virus (Fig. 30.3). In contrast, the serine cluster (Ser43–51) is nearly completely conserved across all of the available VP30 sequences from MARV and RAVV with the exception of a Ser → Gly substitution in position 50 in RAVV VP30 (Fig. 30.4).

5.7 *Phosphorylation of the Nucleocapsid Protein NP*

Although almost three decades have passed since the first description of the phosphorylation state of EBOV proteins within virions [112], information regarding the role of these phosphorylations in the regulation of EBOV protein function is generally lacking with the exception of VP30. However, a role for NP phosphorylation in the MARV viral replication cycle has been reported [113]. Phosphorylation

of MARV NP may regulate recruitment of NP into viral particles [114]. The phosphorylation of MARV NP is largely relegated to seven regions within the C-terminus and few of these phosphorylations have been studied in detail [115]. Of these seven regions, region II of the NP C-terminus contains six consecutive serine residues (Ser 450–455), multiple threonine residues, and several negatively charged amino acids [113]. Becker et al. demonstrated by phosphoamino acid analysis that the major phospho-acceptor sites within region II were serine sites with a minor signal for phospho-threonine. In a subsequent study, Ser446 and Ser453–455 were identified as the major phosphorylation sites for this region of the NP protein [113]. However, the authors demonstrated that the phosphorylation of NP region II had no influence on self-interactions (NP-NP) or interactions with VP30 or VP35.

Analysis of the role of these phosphorylation events during the early stages of infection was investigated using a minigenome system. Here, it was demonstrated that mutants that mimicked completely dephosphorylated (Ser → Ala) or hyperphosphorylated (Ser → Asp) NP were unable to fully support RNA synthesis. Interestingly, substitution of Ser → Ala in Ser450–452 and Ser → Asp in Ser453–455 (NP-AD) increased viral RNA synthesis as compared to wild-type NP and conserved viral transcription/replication activities [113]. NP-DA had impaired ability to support viral transcription and replication, suggesting that Ser453–455 phosphorylation is likely the most important region for regulation of NP function. MSA comparisons of EBOV variant Mayinga and MARV variant Musoke demonstrate low conservation of sequence (37 % similarity) and limited homology between Ser450–455 of MARV and EBOV (Fig. 30.5). There is limited conservation of sequence between EBOV and MARV in these regions with direct alignment of only two Ser residues and the alignment of Tyr in EBOV with a Ser from MARV.

5.8 *Phosphorylation of the Matrix Protein VP40*

Although previous investigations of EBOV VP40 had demonstrated that PTMs result in the assembly of homo-oligomers that influence plasma membrane transport and RNA binding [116, 117], investigation into the role of PTMs in the regulation of VP40 function is limited. Results of several studies indicate that VP40 is phosphorylated at Tyr residues at positions 7, 10, 13, and 19, major phosphorylation sites, and at certain serine sites. Importantly, resolution of MARV VP40 by SDS-PAGE results in two protein species with bands of ≈36 and 38 kDa in mass [118]. A similar phenomenon had been described previously for EBOV VP40 by Jasenosky et al.: mutation of a second start codon (Met14) resulted in the loss of this doublet [119]. However, mutation of a similar Met residue (Met11) in MARV VP40 had no effect on the doublet, suggesting the possibility that this second band might represent a modified VP40 [118]. The authors subsequently demonstrated that this heavier second band represented a phosphorylated form of VP40 and phosphoamino acid analysis suggested a strong signal for phospho-tyrosine and a weak signal for phosphoserine. Site-directed mutagenesis of Tyr residues to Phe demonstrated that the

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NP_EBOV (Mayinga)        MDSRPQKIWMAPSLTESDMDYHKILTAGLSVQQGIVRQRVIPVYQVNNLEEICQLIIQAF    60
NP_MARV (Musoke)         ------------------MDLHSLLELGTKPTAPHVRNKKVILFDTNHQVSICNQIIDAI    42
                                           ** *.:*  * .     **:: : :::.*.  .**: **:*:

NP_EBOV (Mayinga)        EAGVDFQESADSFLLMLCLHHAYQGDYKLFLESGAVKYLEGHGFRFEVKKRDGVKRLEEL    120
NP_MARV (Musoke)         NSGIDLGDLLEGGLLTLCVEHYYNSDKDKFNTSPVAKYLRDAGYEFDVIKNADATRFLDV    102
                         ::*:*: :  :. ** **:.* *:.* . *  * ..***.  *:.*:* *.  ..*: ::

NP_EBOV (Mayinga)        LPAVSSGKNIKRTLAAMPEEETTEANAGQFLSFASLFLPKLVVGEKACLEKVQRQIQVHA    180
NP_MARV (Musoke)         SPNEPHYSPLILALKTLESTESQRGRIGLFLSFCSLFLPKLVVGDRASIEKALRQVTVHQ    162
                          *     . :  :* :: . *: ... * ****.**********::*.:**. **: **

NP_EBOV (Mayinga)        EQGLIQYPTAWQSVGHMMVIFRLMRTNFLIKFLLIHQGMHMVAGHDANDAVISNSVAQAR    240
NP_MARV (Musoke)         EQGIVTYPNHWLTTGHMKVIFGILRSSFILKFVLIHQGVNLVTGHDAYDSIISNSVGQTR    222
                         ***:: **. * :.*** *** ::*:.*::**:*****:.:*:**** *::*****.*:*

NP_EBOV (Mayinga)        FSGLLIVKTVLDHILQKTERGVRLHPLARTAKVKNEVNSFKAALSSLAKHGEYAPFARLL    300
NP_MARV (Musoke)         FSGLLIVKTVLEFILQKTDSGVTLHPLVRTSKVKNEVASFKQALSNLARHGEYAPFARVL    282
                         ***********:.*****: ** ****.**:****** *** ***.**:*********:*

NP_EBOV (Mayinga)        NLSGVNNLEHGLFPQLSAIALGVATAHGSTLAGVNVGEQYQQLREAATEAEKQLQQYAES    360
NP_MARV (Musoke)         NLSGINNLEHGLYPQLSAIALGVATAHGSTLAGVNVGEQYQQLREAAHDAEVKLQRRHEH    342
                         ****:*******:********************************** :** :**:  *

NP_EBOV (Mayinga)        RELDHLGLDDQEKKILMNFHQKKNEISFQQTNAMVTLRKERLAKLTEAITAASLPKTSGH    420
NP_MARV (Musoke)         QEIQAIAEDDEERKILEQFHLQKTEITHSQTLAVLSQKREKLARLAAEIENNIVEDQ-GF    401
                         :*:: :. **:*:*** :** :*.**:..** *::: ::*:**:*:  *     : .  *.

NP_EBOV (Mayinga)        YDDDDD-----IPFPGPINDDDNPGHQDDDPTDSQDTTIPDVVVDPDDGSYGEYQSYSEN    475
NP_MARV (Musoke)         KQSQNRVSQSFLNDPTPVEVTVQ-----ARPMN-RPTALPPPVDDKIEHESTEDSSSSSS    455
                          :.::       :  * *::    :       * : : *::*  * *  : .  * .* *..

NP_EBOV (Mayinga)        GMNAPDDLVLFDLDEDDEDT-KPVPNRSTKGGQQKNSQKGQHIEGRQTQSRPIQNVPGPH    534
NP_MARV (Musoke)         FVDLNDPFALLNEDEDTLDDSVMIPG---------------------TTSREFQGIPEPP    494
                          ::  * :.*:: ***  *    :*                    * ** :* :* *

NP_EBOV (Mayinga)        RTIHHASAPLTDNDRRNEPSGSTSPRMLTPINEEADPLDDADDETSSLPPLESDD-----    589
NP_MARV (Musoke)         RQSQDLNNSQGK--QEDESTNRIKKQF---------------LRYQELPPVQEDDESEYT    537
                         *  :. .    .  :.:* :   . ::                    . ..***::.**

NP_EBOV (Mayinga)        ----EEQDRDGTSNRTPTVAPPAPVYRDHSEKKELPQDEQQDQDHTQEA-----------    634
NP_MARV (Musoke)         TDSQESIDQPGSDNEQGVDLPPPPLYAQEKRQDPIQHPAANPQDPFGSIGDVNGDILEPI    597
                             *. *: *:.*.  .  ** *:* :...:. : :   : **   .

NP_EBOV (Mayinga)        RNQDSD-NTQSEHSFEEMYRHILRSQGPFDAVLYYHMMKDEPVVFSTSDGKEYTYPDSLE    693
NP_MARV (Musoke)         RSPSSPSAPQEDTRMREAYELSP----DFTN--DEDNQQNWPQRVVTKKGRTFLYPNDLL    651
                         *. .*    *.:  :.* *.        *       .  :: *  . *..*: : **:.*

NP_EBOV (Mayinga)        EEYPPWLTEKEAMNEENRFVTLDGQQFYWPVMNHKNKFMAILQHHQ   739
NP_MARV (Musoke)         QTNPPESLITALVEEYQNPVSAKELQADWPDMSFDERRHVAMNL--   695
                         :  **     .  ::* :. *: .  *  ** *...::  . ::
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Fig. 30.5 Multiple sequence alignment of EBOV and MARV NP. Sequence alignments were performed using ClustalW Omega. The *blue line* denotes the serine cluster of functional importance for MARV NP aligned with the corresponding region in EBOV NP

phospho-tyrosine signal within the heavier second band of the doublet was related to phosphorylation of Tyr7, 10, 13, and 19. Mutation of these Tyr phosphorylation sites did not perturb membrane binding; however, phosphorylated VP40 preferentially bound to membranes. In addition, phosphorylated VP40 influenced the length of filamentous protrusions from cells expressing VP40. Further, nonphosphorylated VP40 retained the ability to form filamentous virion-like particles (with only a modest effect on the length of the particles) and was not required for the inhibition of IFN signaling. However, the role of VP40 phosphorylation was related to the formation of infectious VLPs (iVLPs), also known as transcription- and replication-competent virus-like particle systems (trVLPs) [120], as the infectivity of iVLPs was reduced by tenfold when mutant VP40 (Tyr → Phe at residues 7, 10, 13, and 19) was substituted for wild-type VP40. Additional analysis demonstrated that VP40 phosphorylation is an important regulator of the interaction between nucleocapsid-like structures and VP40-enriched membranes, including recruitment of the nucleocapsids to filamentous protrusions within the cell, viral particle assembly,

and infectivity. MSA of EBOV and MARV VP40 shows that MARV VP40 Tyr 10 and 19 align with corresponding Tyr and Ser residues, respectively, in EBOV VP40 and the functional significance of these residues to similar processes for EBOV remains to be investigated.

6 Discussion

Members of the *Filoviridae* family are the etiological agents of severe hemorrhagic fevers that are associated with unusually high fatality rates in humans and NHPs [20, 121]. As many previous reviews of filovirus infections have focused primarily on the pathology associated with disease, we have summarized the potential role of molecular interactions between the host and pathogen during filovirus infections. Importantly, many of these interactions involve the direct modulation of host responses by viral proteins, in particular those associated with innate immunity. Conversely, host-mediated phosphorylation of filovirus proteins plays a central role in the activation state of viral proteins. Thus, we believe that an increased understanding of these PTMs will provide critical information regarding the mechanisms of molecular pathogenesis for these viruses, as well as aid the development of novel therapeutic strategies for the treatment of filovirus infections. Novel technologies that provide insight into these processes, such as system kinome analysis [122–124], could therefore further increase our understanding of filovirus disease, identify novel biomarker targets associated with filovirus disease, and help evaluate the mechanism(s) of action of targeted therapeutics.

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