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Editors
Yashpal Singh Malik
ICAR-Indian Veterinary Research Institute
(ICAR-IVRI)
Izatnagar, Uttar Pradesh, India

Raj Kumar Singh
ICAR-Indian Veterinary Research Institute
(ICAR-IVRI)
Izatnagar, Uttar Pradesh, India

Mahendra Pal Yadav
ICAR-Indian Veterinary Research Institute
(ICAR-IVRI)
Izatnagar, Uttar Pradesh, India

Sardar Vallabhbhai Patel University of
Agriculture and Technology
Meerut, India

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The book is dedicated to all the eminent virologists who own not only inventiveness and creativity but also astonishing compassion. Their resourceful contribution helped in accomplishing this mammoth compilation involving important aspects on animal viral pathogens/diseases.

Preface

A number of new infectious diseases in animals, humans, and plants have emerged in the last few decades in various parts of the globe. In other instances, diseases which were confined to certain regions of the world have now spread to new areas or new hosts. Amongst the various emerging infectious/contagious diseases, viral diseases/infections, including zoonoses, are on top of the list. In the absence of the safe and inexpensive antiviral drugs, the control and management of viral diseases is a challenging job requiring expertise in vaccine development, vaccinology, besides capacity building for detection and differentiation of the pathogen; development of rapid, sensitive, and cost-effective pen-side diagnostic tests/kits and regional and peripheral diagnostic laboratories; clinical and serosurveillance of the disease in the susceptible and in-contact animal populations; planning and implementation of appropriate strategies for control, prevention, and immunoprophylaxis; and border control for transboundary diseases, quarantine facilities, vector control, and restriction on the movement of animals from the affected areas, besides other relevant general health control measures, such as disposal of carcasses, zoo-sanitary measures, and management practices.

The book covers important viruses/viral diseases in animals of various chapters authored by eminent scientists and research scholars. The chapters deal both with single- and double stranded DNA viruses, including African swine fever virus, adenovirus, papillomavirus and polyomavirus, poxviruses (buffalo pox, camel pox, swine pox, fowl pox), porcine circoviruses, chicken anaemia virus, canine parvoviruses, bovine and equine herpesviruses, Marek's disease virus, and laryngotracheitis virus. The book provides an overview of evolving RNA viruses covering single- and double-stranded and segmented genome viruses including porcine coronaviruses, avian reovirus, avian pneumoviruses (APV) I (NDV), APV 2–15, bovine viral diarrhoea virus, avian infectious bronchitis virus, and infectious bursal disease virus to name a few.

Each chapter comprises of an introduction, emphasizing on the history, importance of the virus/disease, and recent scientific advancements in the area; virus structure/genome organization, types/variants, etc.; epidemiology of disease and risk factors; transmission, immunopathobiology, and diagnostics – conventional and modern; and prevention and control – vaccines, antivirals, and other measures.

The book also includes basic virology chapters on epidemiological perspective on managing viral epidemics in animals; virus-mediated cancers in animals;

antivirals, past, present, and future; and bioinformatics applications in animal virus research for updating the knowledge of the readers.

We believe that owing to the broader animal virology coverage with high-quality contributions, the present book will represent an excellent source of information for the readers. The chapters published could be useful for veterinary professionals, clinicians, public health experts, researchers, students/scholars, animal producers, faculty, and students having interest in virology, viral diseases, viral immunity and pathogenesis, epidemiology of viral diseases, viral zoonoses and management of viral diseases and epidemics, pharmaceutical industry, and biomedicine experts and pave the way towards designing and adapting effective and safer therapeutics from clinics to the laboratory for countering important animal viral diseases.

We, the editors, would like to express our gratitude to all the contributors for their support and hard work to make this book compilation a reality. We also extend special thanks to all the peer reviewers whose able expertise and rigorous reviewing of the manuscripts submitted in this book helped the authors to further improve their manuscript to reach publication stages. The guest editors are also grateful to the Springer Nature Publisher for accepting this book proposal. We extend our special thanks to Dr. Bhavik Sawhney, Associate Editor, Biomedicine, Springer Nature, for providing all the editorial help and high cooperation while processing the manuscripts for its successful publishing.

About World Society for Virology

World Society for Virology (WSV) is a nonprofit organization, 501c3-ID No. 001303257, that was established in 2017 with the main mission to strengthen virology research to viral diseases of humans, animals, and plants.

The main objectives of WSV includes but not limited to:

1. Gather the virologists worldwide in the main society that does not require a fee for its membership (a great obstacle for many virologists in many countries) and provide help to all whenever possible.
2. Build up a network of scientific collaborations among virologists worldwide.
3. Build international bridges for virology laboratories worldwide.
4. Help virologists worldwide to advance their careers and obtain awards.
5. Provide educational resources free of charge and freely available to all members.
6. Help and facilitate getting scholarships and vacancies for virologists worldwide.
7. Build up databases of virologists based on their field of specialization for remote assistance and guide in case of the existence of any disease outbreak.

For the details visit www.ws-virology.org

About the Indian Virological Society

1. The Indian Virological Society (IVS), a member of the International Union of Microbiological Society (IUMS), was established in December 1984 at Hisar, Haryana, with the objective to promote research & development in the field of virology. It provides a platform for those associated with the characterization and management of viruses affecting the animal, human, fish, insect, plant, and other living organisms. The society also organizes national and international conferences, trainings, seminars, workshops, invited lectures, academia-industry meet, etc. The society also recognizes the outstanding contributions of the individual members by electing them as Fellows of the society and conferring lifetime achievement awards, Springer Nature-IVS Best Publication Award and Young Scientist awards. IVS publishes an internationally reputed journal *VirusDisease* in collaboration with Springer Nature. The society also publishes a biannual Newsletter Virus Research News. The objective of the newsletter is to provide information on the latest developments in virology, news about IVS activities, and other connected events. At present, society has 500 active members.

Izatnagar, Uttar Pradesh, India

Yashpal Singh Malik
Raj Kumar Singh
Mahendra Pal Yadav

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Editors and Contributors

About the Editors

Yashpal Singh Malik is an "ICAR National Fellow" at the ICAR-Indian Veterinary Research Institute, Izatnagar, India. His work focuses on viral disease epidemiology, virus-host interactions, microbial biodiversity, and characterization and diagnosis of pathogens. He has completed training in molecular virology at the University of Minnesota, USA, and the Division of Virology, University of Ottawa, Ontario, Canada. He is a recipient of several prestigious national, state, and academy awards and honours, including the ICAR-Jawaharlal Nehru Award. Has authored 5 books and 25 book chapters and published 185 scientific research and review articles. He was the Editor-in-Chief of the *Journal of Immunology* and also edited special issues of *VirusDisease*, *The Open Virology Journal*, and *Current Drug Metabolism*.

Raj Kumar Singh is Director and Vice-Chancellor of the ICAR-Indian Veterinary Research Institute, Izatnagar. He is a respected Scientist, specializing in veterinary microbiology, biotechnology, molecular epidemiology, diagnostics, and vaccinology. He has served as Head, Division of Virology; station-in-charge at the IVRI, Mukteswar Campus, Uttarakhand; and later Director, NRC on Equines and VTCC, Hisar. He has filed 10 national patents, 2 of which have been granted, and has developed more than 8 live attenuated vaccines and 26 diagnostic tests and kits. He has authored 2 books and 23 book chapters and published over 234 scientific research papers, 52 reviews, 15 lead papers, and 24 guest editorials/compendium chapters. He has received several prestigious awards, including ICAR Rafi Ahmed Kidwai Award and Team Research Award, DBT Tata Innovation Fellowship Award, Agriculture Research Leadership Award, and FAO Fellowship for training at the University of California, Davis, USA. He is President of ISVIB and has distinguished fellowships and life memberships of prestigious professional societies.

Mahendra Pal Yadav is Former Director, ICAR-Indian Veterinary Research Institute, Izatnagar, and Vice-Chancellor, Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut. Dr Yadav is amongst the most respected scientists working in the field of virology. His major research contributions include the development of indigenous vaccines against equine influenza, infectious laryngotracheitis, and colisepticaemia in poultry, isolation and characterization of animal

viruses, and the development of several animal diseases diagnostic kits. He has also served as Director, NRC on Equines, Hisar. He is a recipient of several prestigious awards and fellowships including NAVS and NAAS. He served as President of the Indian Virological Society (1996–2003), IAUA (2006–2007), and NAVS (I) (2011–2013), as well as FAO National Consultant and Haryana Farmers' Commission Consultant, and since 1999, is the President of IAVMI.

Contributors

Adel M. Abdelaziz Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt
The Veterinary Diagnostic Laboratory, Ministry of Environment, Water, and Agriculture, Riyadh, Saudi Arabia

Ahmed S. Abdel-Moneim Microbiology Department, Virology Division, College of Medicine, Taif University, Al-Taif, Saudi Arabia
Virology Department, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef, Egypt

Elsayed M. Abdelwhab Institute of Molecular Virology and Cell Biology, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Greifswald-Insel Riems, Germany

R. K. Agarwal Division of Livestock Product Technology, ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Bareilly, Uttar Pradesh, India

Abdullah I. A. Al-Mubarak Department of Microbiology, College of Veterinary Medicine, King Faisal University, Al-Ahsa, Kingdom of Saudi Arabia

D. Ashokkumar Division of Virology, ICAR-Indian Veterinary Research Institute, Mukteswar, Uttarakhand, India

Lisanework E. Ayalew VIDO-InterVac and Veterinary Pathology Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada
Veterinary Pathology Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada

Sanjay Barua National Centre for Veterinary Type Cultures, ICAR-National Research Centre on Equines, Hisar, Haryana, India

Bidhan Chandra Bera National Centre for Veterinary Type Cultures, ICAR-National Research Centre on Equines, Hisar, Haryana, India

Mohammed Bule Department of Pharmacy, College of Medicine and Health Sciences, Ambo University, Ambo, Ethiopia

Vishal Chander Virology Laboratory, Centre for Animal Disease Research and Diagnosis (CADRAD), ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Uttar Pradesh, India

Madhan Mohan Chellappa Recombinant DNA Laboratory, Division of Veterinary Biotechnology, ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Uttar Pradesh, India

Ajai Lawrence D'Silva Recombinant DNA Laboratory, Division of Veterinary Biotechnology, ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Bareilly, Uttar Pradesh, India

Tareni Das Division of Pathology, ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Uttar Pradesh, India

Ashis Debnath Recombinant DNA Laboratory, Division of Veterinary Biotechnology, ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Uttar Pradesh, India

Sohini Dey Recombinant DNA Laboratory, Division of Veterinary Biotechnology, ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Uttar Pradesh, India

Kuldeep Dhama Avian Disease Section, Division of Pathology, ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Uttar Pradesh, India

Amit Gaba VIDO-InterVac, University of Saskatchewan, Saskatoon, SK, Canada

Ablesh Gautam Central Research Institute, Kasauli, Himachal Pradesh, India

V. Gowthaman Poultry Disease Diagnosis and Surveillance Laboratory, Veterinary College and Research Institute Campus, Tamil Nadu Veterinary and Animal Sciences University, Namakkal, India

Baldev Raj Gulati ICAR-National Research Centre on Equines, Hisar, Haryana, India

Praveen K. Gupta Division of Veterinary Biotechnology, ICAR-Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh, India

Maged Gomaa Hemida Department of Microbiology, College of Veterinary Medicine, King Faisal University, Al-Ahsa, Kingdom of Saudi Arabia
Department of Virology, Faculty of Veterinary Medicine, Kafrelsheikh University, Kafr El-Shaikh, Egypt

Abdulazim M. Ibrahim Department of Pathology, College of Veterinary Medicine, King Faisal University, Al-Ahsa, Kingdom of Saudi Arabia
Faculty of Veterinary Medicine, Department of Pathology, Suez Canal University, Ismailia, Egypt

S. Kalaiyarasu Indian Council of Agricultural Research- National Institute of High Security Animal Diseases, Bhopal, Madhya Pradesh, India

Deepthi Kappala Avian Immunology Laboratory, Immunology Section, ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Uttar Pradesh, India

Rajeev Kaul Department of Microbiology, University of Delhi, South Campus, New Delhi, India

Abdelmalik I. Khalafalla Veterinary Laboratories Division, Animal Wealth Sector, Abu Dhabi Agriculture and Food Safety Authority (ADAFSA), Abu Dhabi, UAE

Fazlullah Khan Department of Toxicology and Pharmacology, The Institute of Pharmaceutical Sciences, Tehran University of Medical Sciences, Tehran, Iran

Deepak Kumar Division of Veterinary Biotechnology, ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Uttar Pradesh, India

Sachin Kumar Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati, Assam, India

Asok Kumar Mariappan Division of Pathology, ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Bareilly, Uttar Pradesh, India

B. P. Mishra Division of Veterinary Biotechnology, ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Uttar Pradesh, India

Niranjan Mishra Indian Council of Agricultural Research- National Institute of High Security Animal Diseases, Bhopal, Madhya Pradesh, India

Palanivelu Munuswamy Division of Pathology, ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Bareilly, Uttar Pradesh, India

Sukdeb Nandi Centre for Animal Disease Research and Diagnosis (CADRAD), ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Uttar Pradesh, India

Kamal Niaz Department of Pharmacology and Toxicology, Faculty of Bio-Sciences, Cholistan University of Veterinary and Animal Sciences (CUVAS), Bahawalpur, Pakistan

Dinesh Chandra Pathak Recombinant DNA Laboratory, Division of Veterinary Biotechnology, ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Uttar Pradesh, India

Catherine Paul Department of Microbiology, University of Delhi, South Campus, New Delhi, India

Sachin S. Pawar National Institute of Abiotic Stress Management, Pune, Maharashtra, India

Rahul Recombinant DNA Laboratory, Division of Veterinary Biotechnology, ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Uttar Pradesh, India

P. Raja Department of Animal Biotechnology, Madras Veterinary College, TANUVAS, Chennai, India

M. A. Ramakrishnan Division of Virology, ICAR-Indian Veterinary Research Institute, Mukteswar, Uttarakhand, India

Saravanan Ramakrishnan Avian Immunology Laboratory, Immunology Section, ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Uttar Pradesh, India

Narayan Ramamurthy Recombinant DNA Laboratory, Division of Veterinary Biotechnology, ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Uttar Pradesh, India

G. Ravikumar Division of Veterinary Biotechnology, ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Uttar Pradesh, India

Maddula Ramakoti Reddy Division of Pathology, ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Bareilly, Uttar Pradesh, India

Thachamvally Riyesh National Centre for Veterinary Type Cultures, ICAR-National Research Centre on Equines, Hisar, Haryana, India

G. Saikumar Division of Pathology, ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Uttar Pradesh, India

Mithilesh Singh Immunology Section, ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Bareilly, Uttar Pradesh, India

Niraj K. Singh College of Animal Biotechnology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, India

Praveen Singh Division of Veterinary Biotechnology, ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Uttar Pradesh, India

Shambhu Dayal Singh Division of Pathology, ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Bareilly, Uttar Pradesh, India

Sonal Division of Veterinary Biotechnology, ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Uttar Pradesh, India

Suresh K. Tikoo VIDO-InterVac, University of Saskatchewan, Saskatoon, SK, Canada
Vaccinology & Immunotherapeutics Program, School of Public Health, University of Saskatchewan, Saskatoon, SK, Canada

Ashish Tiwari University of Kentucky, Louisville, KY, USA

Bhupendra Nath Tripathi ICAR-National Research Centre on Equines, Hisar, Haryana, India

Anuj Tyagi College of Fisheries, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, India

Nitin Virmani ICAR-National Research Centre on Equines, Hisar, Haryana, India

Part I

DNA Viruses

Animal Adenoviruses

1

Amit Gaba, Lisanework E. Ayalew, and Suresh K. Tikoo

Abstract

Since the first report on adenoviruses in the early 1950s, more than a hundred serotypes of this virus have been reported from reptiles, fish, mammalian, and avian species. These viruses exhibit different lineages based on the differences noticed in the genes located in the terminal regions. Adenoviruses are grouped into five genera including *Mastadenovirus, Aviadenovirus, Atadenovirus, Siadenovirus*, and *Ichtadenovirus.* Recently, a sixth genus, *Testadenovirus*, is proposed to include adenoviruses from turtles. Bats have been identified as prospective reservoir hosts of emerging and re-emerging diseases and playing an important role in the evolution of adenoviruses. This chapter details the information on epidemiology, clinical signs, pathology, diagnosis, prevention, and control aspects of various species-specific adenoviruses affecting bovine, ovine, porcine, canine, and equine that are reported from both healthy animals and those suffering from diarrhea and pneumoenteritis. There is no specific treatment or vaccine available for adenoviruses.

Keywords

Adenoviruses · Classification · Genome · Pathology · Diagnosis · Vaccines

A. Gaba
VIDO-InterVac, University of Saskatchewan, Saskatoon, SK, Canada

L. E. Ayalew
VIDO-InterVac and Veterinary Pathology Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada

Veterinary Pathology Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada

S. K. Tikoo (✉)
VIDO-InterVac, University of Saskatchewan, Saskatoon, SK, Canada

Vaccinology & Immunotherapeutics Program, School of Public Health, University of Saskatchewan, Saskatoon, SK, Canada
e-mail: Suresh.tik@usask.ca

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1.1 Prologue

Adenovirus was first isolated in 1953 from human adenoids (Enders et al. 1956). Since then, over 120 adenovirus serotypes have been isolated from mammals, birds, reptiles, and fish. Although adenoviruses infect a wide variety of animal species, most of these are involved with mild clinical infections. Unlike human adenovirus, animal adenoviruses are usually species specific. Although overall capsid structure and organization of adenovirus genome has remained largely unchanged, there are differences in the proteins encoded by selected regions of adenovirus genome. Interestingly, compared to genes located in the central region of genome, which are involved in virion capsid formation, DNA encapsidation, and DNA replication, the genes located in the terminal regions show distinct differences and may define distinct lineage (Davison et al. 2003).

1.1.1 Classification

Adenoviruses are members of *Adenoviridae* family. Based on the genome organization, phylogenetic relations including presence of conserved and unique genes, the adenoviruses are grouped into five genera including *Mastadenovirus, Aviadenovirus, Atadenovirus, Siadenovirus*, and *Ichtadenovirus* (Davison et al. 2003) (Fig. 1.1). Recently, a sixth genus, *Testadenovirus*, is proposed to include adenoviruses from

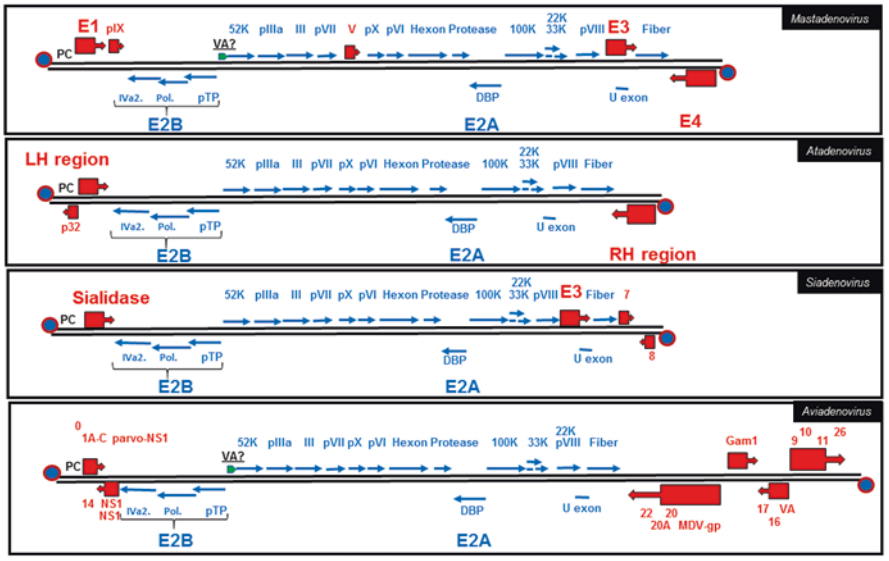

Fig. 1.1 Schematic diagram of adenovirus genomes of different genera. (Adapted from Davison et al. 2003). The common genes are depicted in blue text (⟶); unique genes are depicted in red text (➡) PC (cis-acting DNA packaging signal(s)); Terminal protein (TP) (●). E (early). The arrow heads show the direction of transcription

turtles (Order Testudines) (Doszpoly et al. 2013). Members of all genera share 16 genes [DNA polymerase (pol), terminal protein (TP), DNA binding protein (DBP), 52 K, IVa2, pIIIa, III, pVII, pX, pVI, hexon, protease, 100 K, 33 K, pVIII, and fiber].

Members of *Mastadenovirus* infect mammals and contain genus-specific proteins (protein IX, protein V, and few proteins encoded by E1, E3 and E4 regions). Members of *Atadenovirus* infect different hosts (ruminants, birds, reptiles, and marsupial) and (a) contain genome with high AT content and (b) encode genus-specific proteins p32 and LH3 but (c) do not contain genes encoding protein IX and protein V (Gorman et al. 2005).

Members of *Aviadenovirus* infect avian host (chicken, goose, turkey, and falcon) and (a) contain genomes of 43–45 kb (Kaján et al. 2012) with short inverted terminal repeats (ITRs) and (b) two fiber proteins per vertex of icosahedral virion capsid but (c) does not contain genes encoding protein IX, protein V, and E3 region proteins (Grgić et al. 2011). Members of *Siadenovirus* infect various hosts (reptiles, frogs, and turkeys) and (a) contain short genomes with short ITRs and (b) a viral gene encoding sialidase and (c) do not contain E1, E3 and E4 regions and genes encoding proteins IX and V (Davison et al. 2003). Members of *Ichtadenovirus* infect fish (white sturgeon) and contain the longest genome identified in all adenoviruses. Interestingly, unlike members of the other genera, the homologue of fiber gene appears to be located at the left end of the genome (Kovacs et al. 2003).

1.1.2 Virion Structure

Adenovirus is a non-enveloped icosahedral particle of 65–90 nm diameter in size (Fig. 1.2a), which contain a double-stranded linear genome of 26–48 kb (Kovacs et al. 2003). The ends of the genome contain inverted terminal repeats ranging from 36 bps to over 200 bps. In addition, a terminal protein (TP) is covalently attached to

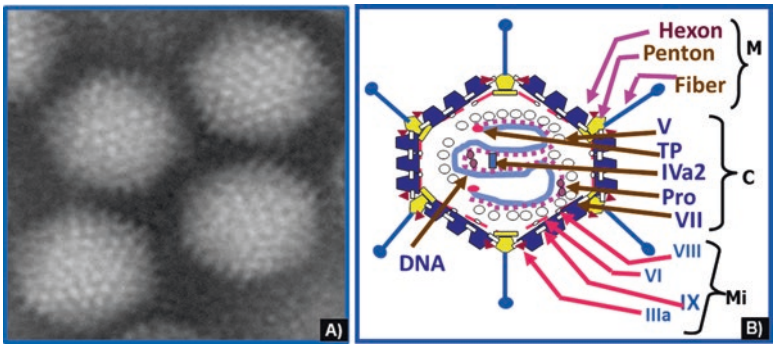

Fig. 1.2 (**a**) Electron micrograph of bovine adenovirus-3, a member of *Mastadenovirus*. (**b**) Schematic diagram of cross section of adenovirus virion. (Adapted from Russel et al. 2009.) *M* major capsid proteins, *Mi* minor capsid proteins, *C* core proteins, *Pro* protease, *TP* terminal protein

5′ ends of the genome, which is required for viral DNA replication (protein-primed DNA replication). Adenovirus genomes can encode between about 23 and 46 proteins (structural, core, and nonstructural). The icosahedral virion capsid contains major structural proteins (hexon, penton, fiber), minor structural proteins (IIIa, VI, VIII, IX), and core proteins (V, VII, Mu, TP, IVa2, cysteine protease) (Fig. 1.2b).

1.1.3 Adenovirus Life Cycle

Like other viruses, the adenovirus initiates attachment to host cell by interaction of fiber protein with cellular receptor. Following virus attachment, interaction of penton to cell surface receptors (e.g., integrins) activates rearrangement of actin cytoskeleton and initiates receptor-mediated virus endocytosis using clathrin-coated pits, which requires GTPase dynamin and adaptor protein 2 (Meier and Greber 2004). Stimulation of endosome acidification due to proton pump action leads to partial uncoating of viral capsid proteins. The exposure of lytic portion of protein VI by adenovirus protease cleavage induces disruption of endosomal membrane and results in microtubule motor protein dynein-mediated transport of partially disassembled virus capsid to microtubule organization center (MTOC) near the nucleus (Bremner et al. 2009). Next, the interaction of adenovirus hexon with cytoplasmic nucleoporin Nup214 located in fibrils on nuclear pore complex (NPC) associates disassembled virus capsid with NPC (Cassany et al. 2015). Interaction of protein IX with kinesin-1 bound to Nup358 induces further disruption of viral capsid (Strunze et al. 2011). Finally, viral DNA complex is transported to nucleus using cellular transport factors including transportin, importins, and histone H1.

Adenovirus genome in the nucleus is transcribed by host RNA polymerase II (Reviewed in Russel et al. 2009). The transcription of adenovirus genome is regulated temporally and can be divided into early (E) region before initiation of DNA replication, delayed early/intermediate (I) region during initiation of DNA replication and late (L) region after initiation of DNA replication. The proteins encoded by early (E) regions E1, E3, and E4 are nonstructural proteins and are involved in initiating viral gene transcription and cell cycle regulation (E1), evasion of host defense (E3) and viral gene transcription regulation and nuclear export (E4). The proteins encoded by early region E2 are structural and nonstructural proteins, which are involved in DNA replication. The proteins encoded by delayed early region/intermediate region are structural proteins (IX and IVa2) and are involved in virion stability, DNA packaging and activation of major late promoter.

The ITRs of adenovirus genome contain origin (ORI) of DNA replication sequences. Interestingly, a protein acts as a primer for the initiation of adenovirus DNA replication (reviewed in de Jong et al. 2003). The initiation of DNA replication occurs by covalent binding of hydroxyl group of serine (amino acid 580) of newly synthesized terminal protein (TP) to dCMP nucleotide residue on nascent DNA strand using Ser-dCMP phosphodiester bond. After initiation of adenovirus genome replication, chain elongation occurs by strand displacement mechanism in

the presence of viral (DBP, DNA polymerase) and cellular protein (nuclear factor II). The displaced strand can duplicate by formation of panhandle structure.

After adenovirus DNA replication, the L region is transcribed as one major transcript using major late promoter (MLP). This major transcript is processed in several overlapping transcripts using alternate splicing and usage of poly(A) signals addition sites. The L region encodes structural proteins (hexon, penton, fiber, IIIa, VI, VIII, IX, V, VII, Mu, TP, IVa2), protease and nonstructural proteins (100K, 32K,22K,52K). In addition, some adenovirus genomes carry virus-associated RNA genes transcribed by RNA polymerase III.

The transport of newly synthesized proteins to nucleus leads to the formation of empty capsids. Next, adenovirus DNA is packaged into empty capsid using cis-acting DNA sequences at the left end of the genome, viral proteins, and cellular proteins. Some reports suggest that capsid formation and DNA packaging occur simultaneously (Condezo and San Martín 2017). Final step in the production of infectious adenovirus virion involves the proteolytic cleavage of structural proteins pIIIa, pTP, pVI, pVII, pμ, and pVIII (reviewed in Russel 2009) by adenovirus cysteine protease. Although adenovirus E2 encoded 11.6K protein has been proposed to be involved in the lysis of infected cells (Tollefson et al. 1996), the virus usually remains in the infected cells till released by cell lysis.

1.2 Animal Adenoviruses

1.2.1 Bat Adenovirus

Bats are recognized as potential reservoir hosts of emerging and re-emerging diseases of humans and animals. They are also suggested to play an important role in the evolution of adenoviruses. The first bat adenovirus (BtAdV) strain FBV1\ BtAdV-1was isolated in 2008 from a fruit bat Ryukyu flying fox (*Pteropus dasymallus*) in Japan. The second BtAdV-designated as BtAdV-2 strain PPV1 was isolated from *Pteropus pipistrellus* in 2009. Subsequently, BtAdVs have been isolated from *Myotis chinensis* (BtAdV-3 strain TJM), *Rousettus leschenaultii* (BtAdV-4), *Eidolon helvum* (BtAdV-5), *Rhinolophus sinicus* (BtAdV WIV9–11), *Corynorhinus rafinesquii* (BtAdV 250-A), *Miniopterus schreibersii* (WIV12–13*), R. leschenaultii* (WIV17–18*)*, and *Eidolon helvum* (EhAdV 06–106) (summarized in Ogawa et al. 2017).

Based on analysis of complete genomic sequences, the BtAdV TJM and PPV1 strains, two species, namely, *Bat mastadenovirus-A* and *Bat mastadenovirus-B*, were established. Recently, analysis of additional novel BtAdVs genomes has led to the proposal of six more species: BtAdV C (WIV9–11), BtAdV D (250A), BtAdV E (WIV12), BtAdV F (WIV13), BtAdV G (WIV17–18), and BtAdV H (EhAdV 06–106). These species have been included into three groups, namely, Group 1 (*Bat mastadenovirus* A, B, and D), Group 2 (*Bat mastadenovirus* C), and Group 3 (*Bat mastadenovirus* E, F, G, and H) (summarized in Ogawa et al. 2017).

Since some BtAdVs are genetically closely related to *Canine mastadenovirus-A*, it was suggested that canine adenoviruses might have emerged from interspecies jumping of bat adenoviruses. However, analysis of novel BtAdVs WIV9–11 indicated that not all BtAdVs are closely related to *Canine mastadenovirus-A*. The epidemiology, pathogenesis, and molecular biology of BtAdVs are currently not well known.

1.2.2 Bovine Adenovirus

The bovine adenovirus (BAdV) was first isolated in 1959 from fecal samples of cow in the United States (Klein et al. 1959). Subsequently, BAdVs have been isolated from both healthy calves and those suffering from diarrhea and pneumoenteritis. Although shedding of BAdV by apparently healthy cattle and seroconversion with no apparent disease is widely reported, BAdV infection is also associated with several disease syndromes including respiratory disease in calves, digestive tract disease including enterocolitis, keratoconjunctivitis, and weak calf syndrome (Vaatstra et al. 2016). At present, based on viral neutralization tests, 10 serotypes of BAdV have been recognized, which are grouped into two genera: *Mastadenovirus* serotypes 1, 2, 3, 9, and 10 and *Atadenovirus* serotypes 4, 5, 6, 7, and 8 (ICTV 2016).

1.2.2.1 Epidemiology and Clinical Signs

Bovine adenovirus is a ubiquitous virus with a worldwide distribution in cattle population (Ursu et al. 2004). Various serological studies have reported the presence of antibodies to one or more BAdV serotypes in 25–87% of cattle sera tested. Virus transmission can occur directly by animal to animal contact or indirectly by contact with infectious virus excreted through saliva, feces, or nasal excretions via the conjunctival, oral, or nasopharyngeal route. BAdVs have also been implicated in cases of enzootic pneumonia in calves. A number of BAdV serotypes have also been associated with infectious keratoconjunctivitis. Repeated isolation of BAdV from bovine fetuses suggests that transplacental infection can occur (Bartha and Mate 1983).

BAdV's serotypes 3, 4, and 5 have been implicated in diseases of both upper and lower respiratory tract. Although isolation of BAdV-3 from outbreaks of acute respiratory disease showing symptoms of ocular and nasal discharge, pyrexia, pneumonia, and diarrhea in cattle has been frequently reported, the experimental infection of calves with BAdV-3 results in either no disease or disease with mild clinical signs (Akca et al. 2004). Repeated isolation of a number of BAdV serotypes including BAdV-4 and BAdV-8 from natural cases of pneumoenteritis with high mortality in calves has been reported. Calves 2 weeks to 4 months old are more susceptible to pneumoenteritis. Disease progresses from signs of upper respiratory tract infection followed by excessive salivation and diarrhea. Although calves usually recover with loss of condition, disease can be fatal in some of the calves that develop severe respiratory signs. Disease can be reproduced experimentally by using BAdV-4, but

symptoms are usually mild. In contrast, BAdV-8 could not produce the clinical signs in experimentally infected calves (Mohanty 1971).

BAdVs have been associated with abortion and weak calf syndrome. The affected calves are weak and listless at birth with pyrexia and polyarthritis. Occasionally, calves also have diarrhea. BAdV serotypes 5 and 7 have been isolated from the diseased calves. Moreover, experimental inoculation of calves with BAdV-5 resulted in a self-limiting, mild form of the disease with symptoms of pyrexia and diarrhea (Bartha and Mate 1983).

BAdV-10 has been associated with cases of fatal hemorrhagic colitis. The disease occurs in young calves and is characterized by signs of severe depression, recumbency, and severe hemorrhagic diarrhea resulting in death (Vaatstra et al. 2016).

1.2.2.2 Pathology

Adenovirus first infects lymphoid tissues of the oropharynx or epithelial cells of the respiratory tract. In case of alimentary tract infection, virus spreads to the intestinal epithelium, while, in case of respiratory tract infection, it spreads through the bloodstream to infect lungs and produce pneumonia. In cases of keratoconjunctivitis, virus infects conjunctival epithelium.

1.2.2.3 Diagnosis

Diagnosis of BAdV infections is difficult as clinical signs produced are indistinguishable from that produced by other bovine viruses. Moreover, since BAdV can also be isolated from healthy animal, virus isolation alone cannot lead to a definitive diagnosis. A definitive diagnosis thus requires virus isolation, identification of serotype, and seroconversion (Kahrs 1981). Diagnosis of BAdV can thus be done by combination of virus isolation, electron microscopy, PCR, and other tests like agar-gel immunodiffusion, ELISA, hemagglutination inhibition, complement fixation, and immunohistochemistry.

1.2.2.4 Prevention and Control

No specific treatment is available. Affected cattle should be treated according to symptoms. Antibiotics should be given to prevent secondary bacterial infection. The BAdV infection can be controlled by following good management practices. No vaccine is available in North America.

1.2.3 Canine Adenovirus

Canine adenovirus (CAdV) belongs to the genus *Mastadenovirus*. Based on virulence, genetic, and antigenic characteristics, CAdVs are classified into two groups: CAdV-1 and CAdV-2. Both viruses are grouped into the same species of *Canine mastadenovirus-A* (ICTV 2016). CAdV-1 was first recognized as the cause of infectious canine hepatitis (ICH) in 1947, which is characterized by acute necrohemorrhagic hepatitis formerly known as epizootic encephalitis of foxes. CAdV-2 was

first detected in 1962 and causes mild upper respiratory disease called infectious tracheobronchitis (ITB). CAdV-2 was also isolated in dogs that died from pneumonia and enteritis. Although CAdV-1 and CAdV-2 differ in their molecular characteristics, they show two-way cross protection.

1.2.3.1 Epidemiology and Clinical Signs

CAdVs are distributed worldwide in domestic and wild mammals in the family of Canidae, Ursidae, and Mustelidae. Red foxes, grey foxes, coyotes, wolves, and dogs are highly susceptible to infection. Serological surveys detected high prevalence of CAdV-specific antibodies in domestic dogs. CAdV-1, cause of ICH, is prevalent in wild canids as a subclinical infection and sporadic transmission can occur to unvaccinated susceptible domestic dogs. Virus transmission can occur directly by animal to animal contact or indirectly by contact with infectious virus excreted through urine; saliva; conjunctival, oral, and nasopharyngeal route; or feces (Willis 2000). Ectoparasites can act as mechanical vectors.

ICH occurs in young dogs less than 1 year of age. ICH has three forms: per-acute, acute, and mild. In per-acute disease, dogs die in 24–48 h without apparent clinical signs. The most common form of the disease is the acute form which causes high morbidity rate (10–30%). The incubation period of CAdV-1 is 4–7 days after ingestion of a material contaminated with virus or 6–9 days after direct contact with an infected animal. Acute form of the disease is characterized by acute or chronic hepatitis and interstitial nephritis. Common clinical signs are fever (>40°c), anorexia, blood in feces, vomiting, and diarrhea. Abdominal pain, conjunctivitis, photophobia, and bronchopneumonia are also common manifestations of CAdV-1 infection. Bilateral corneal opacity (blue eye) and uveitis develop in 25% of convalescent dogs but eventually disappears. In rare cases, infected dogs may develop encephalitis and show neurological signs. Virus persists in the kidney of recovered dogs, and virus is excreted in the urine for 6–9 months post infection. Mild form of ICH occurs in vaccinated animals that only developed partial immunity.

CAdV-2 is associated with canine respiratory disease complex or kennel cough syndrome. It causes mild respiratory disease with clinical signs that include tonsillitis, pharyngitis, tracheitis, and bronchitis (reviewed in Decaro et al. 2007).

1.2.3.2 Pathology

After infection of susceptible hosts, virus initially replicates in the tonsils and spreads to regional lymph nodes and other organs through the circulatory system. CAdV-1 has a tropism for vascular endothelial cells and hepatocytes, whereas CAdV-2 preferentially infects epithelial cells in the respiratory tract. During complications with secondary bacterial infections, CAdV-2 can also infect bronchial and alveolar epithelial cells. The disease is more severe in dogs less than 1 year of age, but unvaccinated dogs of all ages are susceptible. Virus replication takes place in the nucleus and forms characteristic large basophilic intranuclear inclusion bodies in hepatocytes. The inclusion bodies can also be observed in endothelial and epithelial cells of other virus-infected organs, mostly spleen and kidney (reviewed in Decaro et al. 2007). Chromatin condensation and margination occurs in infected

cells. Viruses are released by lysis of infected epithelial and endothelial cells causing tissue necrosis and disseminated intravascular coagulation.

Infected dogs with CAdV-1 show marked leucopenia, protein urea, and increased levels of liver enzymes. Due to impaired synthesis of clotting factors in the liver, clotting time of the blood is markedly reduced. Hence, bleeding in the oral cavity, ecchymotic hemorrhage in serosal surfaces, and lymph nodes are observed. Because of endothelial cell damage, multifocal vasculitis and hemorrhage and disseminated intravascular coagulation can occur. In addition, edema of the gall bladder can result in severe abdominal pain. Bilateral corneal opacity, uveitis, and interstitial nephritis occur as a result of deposition of circulating virus-antibody complexes.

1.2.3.3 Diagnosis

Diagnosis of CAdV infections is performed by virus isolation, electron microscopy, PCR, and serological test that include complement fixation test, hemagglutination inhibition test, and enzyme-linked immunosorbent assay (ELISA). The disease can also be diagnosed by histopathology and immunohistochemistry of infected tissue samples. CAdV-1 and CAdV-2 have 75% genetic sequence identity and can be differentiated by restriction enzyme analysis and DNA hybridization.

1.2.3.4 Prevention and Control

Maternal antibody is an important component of immunity that protects neonates from ICH and ITB. Modified live and killed vaccines are commercially available. CAdV-1-modified live vaccines are very effective; however, they produce corneal opacity and interstitial nephritis. Since CAdV-2 does not cause ocular or renal damage and antibodies induced against the virus cross-neutralize CAdV-1, current vaccines against ICH and ITB are mostly based on modified live CAdV-2. Lifelong immunity is conferred by live modified vaccine. Because of interference by maternal antibodies, 3 doses of vaccine in 4–5 weeks interval are recommended for puppies less than 16 weeks of age. Although CAdV-associated diseases are largely controlled by vaccination, in the recent past ICH outbreaks have been reported in different countries including Italy, Switzerland, and the United States.

1.2.4 Cervine Adenovirus

Cervine adenovirus (*Odocoileus adenovirus-1*) was first identified in 1993 as the cause of an epizootic of severe adenovirus hemorrhagic disease (AHD) that resulted in high mortality in mule deer in California, USA (Woods et al. 1996). Subsequently, *Odocoileus adenovirus-1* (OdAdV-1) has been isolated from white-tailed deer and black-tailed deer in the United States and a moose in Canada. OdAdV-1 has been tentatively placed in genus *Atadenovirus* (Boyce et al. 2000; Shilton et al. 2002).

1.2.4.1 Epidemiology and Clinical Signs

OdAdV-1 has been isolated from deer from various parts of North America. Virus transmission is directly by animal to animal contact or indirectly by contact with

infectious virus excreted through saliva, feces, or urine. Transmission through airborne routes, contaminated water, and contaminated equipment may also occur.

The AHD occurs in two forms: acute systemic form and chronic localized form. Animals suffering from acute form of disease show signs of weakness, difficulty in breathing, foaming or drooling from the mouth, and diarrhea that is often bloody. Progression of disease is rapid, and an infected animal can die within 3–5 days. Animals suffering from chronic infection show signs of extensive deep ulceration and necrosis in the mouth and throat and abscesses in oral cavity. Animals with localized lesions have difficulty in eating, which leads to weight loss and death. Rate of infection and mortality is higher in fawns compared to adults (Woods et al. 1996).

1.2.4.2 Pathology

The disease is characterized by pulmonary edema and erosions and hemorrhagic lesions and abscesses in the upper alimentary tract. Systemic vasculitis with endothelial intranuclear inclusions can be observed on histopathological examination (Boyce et al. 2000).

1.2.4.3 Diagnosis

OdAdV-1 infection can be diagnosed by virus isolation, detection of virions by electron microscopy, detection of virus antigen in tissues by immunofluorescence, and by virus- specific PCR assay.

1.2.4.4 Prevention and Control

No specific treatment or vaccine is available. Transmission of virus could be prevented by following standard biosecurity practices. Carcasses of animals should be disposed properly. Individuals handling animals should take adequate precautions to prevent spread of disease.

1.2.5 Equine Adenovirus

Equine adenovirus (EAdV) is widely distributed in horses and causes in apparent or subclinical infection in conventional foals. Two different serotypes designated as EAdV-1 and EAdV-2 have been isolated from horses. EAdV-1 is mainly isolated from the respiratory system of sick foals (Studdert and Blackney 1982). Pneumonia associated with EAdV infection has been reported in apparently immunocompetent foals, but virus has also been isolated from the respiratory tract of healthy foals. EAdV-2 is associated with clinical gastrointestinal tract infections of foals and subclinical gastroenteritis infection of horses.

1.2.5.1 Epidemiology and Clinical Signs

EAdV was first reported in the United States in 1969. Later, the virus was isolated and characterized from the pneumonic lung of an Arabian foal in California. EAdV-1 has been isolated from clinically healthy foals and foals with respiratory disease,

whereas EAdV-2 has been isolated from lymph node and feces of foals with respiratory disease and diarrhea (Studdert and Blackney 1982). Serological surveys have detected EAdV-specific antibodies in healthy horses. The mode of virus transmission is poorly characterized. However, foals potentially get infected from mares at birth via oral or nasopharyngeal route. Suckling Arabian foals with an autosomal recessive genetic disorder of severe combined immunodeficiency with a total lack of B and T cells are the most susceptible (Thompson et al. 1976). In these foals, EAdV-1 causes serious and often fatal respiratory infections. Adenoviral pneumonia in suckling Arabian foals is progressive and intractable. It is characterized by clear bilateral nasal discharge which later turns into yellow and slimy. Partial occlusion of the nostril makes suckling difficult and the foals gradually lose weight. A dry cough with extreme respiratory distress can be observed. The foals become dull and depressed. Secondary bacterial complications are common (Thompson et al. 1976). There have only been few reports of adenovirus pneumonia in non-Arabian foals. Experimentally infected thoroughbred yearlings showed watery nasal discharge starting between 4 and 12 days post infection with no significant changes in normal blood values, heart rate, and respiratory rate.

1.2.5.2 Pathology

Experimental infection of horses with EAdV causes pneumonia in horses regardless of breed. However, more severe lesions are observed in Arabian foals with combined immunodeficiency syndrome with extensive pneumonia of both lungs. Consolidated and firm lung especially the anterior ventral areas can be observed. The affected areas of the lung become depressed as compared to the unaffected parts of the lungs. The spleen can be very small and lymphoid follicles may not be present. On histopathology, distinctive lesions in the respiratory tract with focal areas of necrosis and large intranuclear inclusion bodies in the bronchial and bronchiolar epithelial cells are found. Purulent exudates with large number of leukocytes and hyperplastic bronchiolar epithelium can be observed (Webb et al. 1981).

1.2.5.3 Diagnosis

Diagnosis of EAdV infection is based on serological assays like complement fixation, agar gel diffusion, hemagglutination inhibition, and serum neutralization assays. Virus neutralization test is used as a gold standard to distinguish between EAdV-1 and EAdV-2 infection in horses. Isolation of virus and detection of virions by electron microscopy can also be performed. The use of PCR for the detection of EAdVs has also been reported. However, PCR and virus isolation do not necessarily suggest occurrence of clinical disease. Pulmonary histopathology is useful for postmortem diagnosis.

1.2.5.4 Prevention and Control

Although prevalence of equine adenoviral infection is widespread, infections are mild and self-limiting. Therefore, prevention and control measures are not economically feasible.

1.2.6 Murine Adenovirus

Murine adenovirus-1 (MAdV-1) (FL-1 strain) was first isolated in 1960 by Hartley and Rowe as a contaminant of Friend leukemia (FL) virus. Later, MAdV-2 (K87 strain) was isolated from the feces of clinically normal mice. In 2009, a novel murine adenovirus designated as MAdV-3 was isolated from a striped field mouse (*Apodemus agrarius*). MAdV-1, MAdV-2, and MAdV-3 are renamed as murine mastadenovirus A, B, and C, respectively, by the International Committee on Taxonomy of Viruses [ICTV] (ICTV 2016).

1.2.6.1 Epidemiology and Clinical Signs

Serological survey in the United Kingdom demonstrated that MAdV infections are common in wild house mouse populations with higher prevalence of MAdV-1 as compared to MAdV-2. Although mouse is the principal host of MAdV, rats can be infected. Naturally occurring disease due to MAdV infection has not been reported in immunocompetent adult mice. However, acute and persistent infections of MAdV-1with different disease conditions and associated clinical signs have been reported in experimentally infected immunocompetent or immunocompromised mice with MAdV-1 (Spindler et al. 2001). MAdV-2 is entrotropic and localizes in the intestine. The virus is shed for up to 3 weeks in the feces of immunocompetent mice and up to 6 months in athymic mice. Nevertheless, it is not known if it causes any disease conditions. MAdV-3 is known to be cardiotropic, but the virus is not well characterized.

MAdV-1 is transmitted by direct contact with virus-infected feces, urine, or nasal secretions. Clinical signs have never been observed in natural infections. Depending on age, immune status, strain of mouse, and virus dose, MAdV-1 can cause a fatal disease during experimental infections. Intraperitoneal infection of C57BL/6 mice with MAdV-1 produces encephalomyelitis as a result of endothelial cell activation and vasculitis. Although BALB/c mice are resistant to MAdV-1 infection, fatal generalized non-neurologic disease with focal hemorrhagic enteritis can be observed in BALB/c mice with severe combined immunodeficiency syndrome (SCID). Experimentally infected susceptible suckling mice show ruffled coat, lethargy, decreased food consumption, runting, and hunched posture usually resulting in death of mice in 3–10 days post infection. Neurological signs ranging from ataxia to flaccid paralysis can be observed in intraperitoneally infected adult immunocompetent CD-1 and NIHS mice with MAdV-1. Virus persists in adult immunocompetent mice with prolonged virus shedding in the urine for up to 2 years. Since it is not possible to study human adenoviruses pathogenesis in its natural host, MAdV-1 has been used as a model to understand the adenovirus virus host interactions and adenovirus pathogenesis in a natural host (mice) (Weinberg et al. 2007).

1.2.6.2 Pathology

MAdV-1 distributes widely in different tissues and organs post experimental infection. The major sites of MAdV-1 replication are the vascular endothelium and lymphoid tissues. Depending on the strain of mice, mononuclear phagocytes can be

systemically infected. Infected SCID BALB/c mice develop hemorrhagic enteritis after 17–19 day post infection. B cell or Bruton's tyrosine kinase (Btk) negative mice are highly susceptible to MAdV-1-induced disease. On postmortem examination of neonates infected with MAdV-1, necrosis of several tissues including the kidney, spleen, liver, pancreas, intestines, liver, and adrenal glands is observed. In histopathology, degenerative vascular changes, with infiltration of inflammatory cells, are observed in SJL/J mouse strains. Characteristic adenovirus intranuclear inclusion bodies are commonly seen in endothelial cells of the brain and epithelial cells of other infected tissues. Foci of necrosis with hemorrhage can also be observed in affected tissue. Cytotoxic CD8 + T cells are involved in immunopathology induced by MAdV-1. Survival of experimentally infected mice is dependent on both B and T lymphocytes. Antibodies play a great role in preventing the occurrence of generalized disease (Molloy et al. 2017).

1.2.6.3 Diagnosis

Complement fixation test, virus neutralization assay, indirect immunofluorescence (IF) test, and virus isolation and detection of virions by electron microscopy are used for diagnosis. Since MAdV-2 antiserum cross reacts with MAdV-1, but not vice versa, MAdV-2 antigens are commonly used for serological tests.

1.2.6.4 Prevention and Control

Most mouse colonies are currently free of MAdV infection. Biosecurity measures can be applied to prevent intrusion of wild mice to mouse colonies.

1.2.7 Ovine Adenovirus

Ovine adenovirus (OAdV) has worldwide prevalence as it has been isolated from different countries (Lehmkuhl et al. 1993). OAdVs are frequently isolated from lambs and are mainly associated with mild or inapparent infection of the respiratory and intestinal tract. Similar to BAdV, OAdVs are also grouped into two genera: *Mastadenovirus* and *Atadenovirus*. OAdV 1–5 and goat adenovirus-2 (GAdV-2) belong to *Mastadenovirus*, while OAdV-7 and goat adenovirus-1 are members of *Atadenovirus* (ICTV 2016).

1.2.7.1 Epidemiology and Clinical Signs

High prevalence of OAdV-specific antibodies has been detected in healthy sheep. Virus transmission can occur directly by animal to animal contact or indirectly by contact with infectious virus excreted through feces or nasal excretions. OAdVs have been isolated from apparently normal sheep as well as from sheep showing signs of respiratory tract infection and from sheep with intestinal tract infection.

OAdVs have been associated with pneumoenteritis in lambs. Disease starts with mild fever and diarrhea followed by signs of respiratory tract infection. Infected lambs show signs of sneezing, coughing, and nasal discharge. Frequently, there is

secondary bacterial infection which is characterized by signs of high fever and forced respiration (Belak et al. 1976).

Experimental infection of lambs with OAdV-4 or OAdV-5 failed to produce any clinical disease, but virus could be recovered from nasal and rectal swabs. However, experimental inoculation of 3 weeks old colostrum deprived lambs with OAdV-1 by intranasal and intratracheal route resulted in the production of clinical signs of respiratory and intestinal tract infection. Similarly, intranasal and intratracheal experimental infection of lambs with OAdV-6 showed signs of upper and lower respiratory tract infection.

1.2.7.2 Prevention and Control

No specific treatment or vaccine is available. Transmission of virus could be prevented by following standard biosecurity practices.

1.2.8 Porcine Adenovirus

Porcine adenovirus (PAdV) was first isolated in the 1960s from the rectal swab of a pig suffering from diarrhea (Haig et al. 1964). A few years later, another isolation of PAdV was reported from the brain of a pig suffering from encephalitis (Kasza 1966). In following years, PAdV isolation has been reported from samples from healthy pigs as well as from pigs suffering from respiratory disease, enteritis, encephalitis, nephritis, and reproductive disorders. Porcine adenovirus (PAdV) belongs to the genus *Mastadenovirus*. Based on serum neutralization tests, five serotypes of PAdV have been identified. These five serotypes are further grouped into three species: PAdV-A (serotypes 1, 2, and 3), PAdV-B (serotype 4), and PAdV-C (serotype 5). Recently, isolation of novel PAdVs, PAdV-WI and PAdV-SVN1, has led to the proposal of a new *Mastadenovirus* species.

1.2.8.1 Epidemiology and Clinical Signs

PAdVs are distributed worldwide in swine population. Serologic studies have detected PAdV- specific antibodies in 50–90% of heathy pigs. PAdV-4 appears to be most widely distributed and is the most frequently isolated serotype. PAdVs are host specific and can only infect pigs. There has been no report of transmission of PAdV from pigs to other species of animals or humans. Since PAdV has been reported to be shed in feces, the virus transmission can occur by ingestion of contaminated material (fecal-oral route) or through inhalation (fecal-nasal route) (Benfield and Richard 2012). Although PAdV is considered a low-grade pathogen in pigs, it has been associated with enteritis, respiratory disease, encephalitis, and abortion.

PAdV-1, PAdV-3, and PAdV-4 have been isolated from pigs showing signs of gastrointestinal disease. One to four weeks of piglets are most susceptible. Infected animals show signs of depression, emaciation, and dehydration. The most consistent clinical sign observed is intermittent yellow watery diarrhea. Experimental inoculation of piglets with serotype 1, 2, 3, or 4 produces no symptoms or

occasionally mild diarrhea (Coussement et al. 1981; Shadduck et al. 1967; Sharpe and Jessett 1967). Death following experimental infection has never been reported.

PAdV-5 can be isolated from brain of pigs and from nasal secretions of pigs showing signs of respiratory disease. Interstitial pneumonia following experimental infection with PAdV-4 has also been reported. Isolation of PAdV-4 from the brain of pig suffering from encephalitis has been reported. Moreover, encephalitis can be reproduced by experimental intracerebral inoculation of less than 2 days old with PAdV-4 (Shadduck et al. 1967). Isolation of PAdV from aborted fetuses suggests that PAdV might contribute to reproductive failures mainly leading to abortion (Dee 1995).

1.2.8.2 Diagnosis

The PAdV infection can be diagnosed by virus isolation, detection of virus by electron microscopy, and serological tests like complement fixation, virus neutralization, and indirect immunofluorescence assay. An increase in anti-PAdV antibody titer and presence of clinical disease point to the role of PAdV in causing the disease (Benfield and Richard 2012).

1.2.8.3 Prevention and Control

There is no specific treatment or vaccine available. Transmission of virus could be prevented by following standard biosecurity practices.

1.2.9 Simian Adenovirus

The simian adenovirus (currently known as SAdV-21) was first isolated from feces of a chimpanzee suffering from respiratory disease (Rowe et al. 1956). At present, more than 50 serotypes of SAdV have been identified. Based on genomic properties, host origin, hemagglutinin properties, and a number of fiber genes, several identified simian adenovirus serotypes have been proposed to be grouped into seven species (*Simian mastadenovirus-A–G*) (Podgorski et al. 2016). The nonhuman primate adenoviruses which are genetically very similar to human adenoviruses are grouped under corresponding human adenovirus species or under officially accepted species for nonhuman primate adenoviruses designated as *Simian mastadenovirus-A*.

1.2.9.1 Epidemiology and Clinical Signs

Infections of nonhuman primates with simian adenoviruses are predominantly subclinical. Healthy chimpanzees, bonobos, gorillas, orangutans, and New World monkeys frequently shed significant amount of infectious adenovirus in their feces suggesting that persistent/latent infections could be established in nonhuman primates, without any clinical disease. So far, epidemiological studies are focused on isolation of adenoviruses from feces of asymptomatic captive nonhuman primates.

Though simian adenoviruses are associated with hepatitis, conjunctivitis, gastroenteritis, and respiratory disease problems in captive primates, their clinical

relevance in wild primates is unknown. Simian adenoviruses have been isolated from fecal samples of Western lowland gorillas (*Gorilla gorilla gorilla*) with prolonged diarrhea and self-limiting respiratory disease. Species D and E like adenoviruses have also been isolated from the stool of chimpanzees with acute upper respiratory signs in Western Tanzania. Interestingly, certain simian adenoviruses have the ability to cause neoplasia in hamsters.

Although most adenoviruses exhibit very narrow host specificity, there are reports of zoonotic transmission of simian adenoviruses from nonhuman primates to humans and between different primate species. Thus, virus host switching between different primate species might have contributed in the evolution of human and nonhuman primate adenoviruses. In a California research facility, an adenovirus outbreak characterized by fulminant pneumonia and hepatitis occurred in captive monkeys (*Callicebus cupreus*) with potential transmission to humans. The infection was assumed to be due to host switching from co-housed reservoir macaques. Serological surveys in Brazil and sub-Saharan Africa suggested infection of humans with New and Old World monkey adenoviruses. In addition, an adenovirus-associated acute respiratory disease outbreak occurred in a baboon colony in Texas, USA, which crossed over and infected staff personnel. Intra- and interspecies recombination between simian adenoviruses is also a common phenomenon.

1.2.9.2 Diagnosis

Simian adenoviruses can be diagnosed by virus isolation and detection of virus by electron microscopy, virus neutralization test, hemagglutination test, and PCR assay.

1.2.9.3 Treatment

There is no specific treatment or vaccine available.

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Papillomaviruses and Polyomaviruses

2

Abdelmalik I. Khalafalla

Abstract

In recent years, papillomaviruses and polyomaviruses have received consideration because of their association with malignancy development and their wide dissemination. Papillomaviruses are epithelia-tropic small circular DNA viruses belonging to the family *Papillomaviridae* that cause benign proliferative lesions in the skin (warts) and mucous membranes in different animal species and humans. Papillomavirus replication is firmly connected to the differentiation process of the host epithelial cells, and their transmission requires close cutaneous or mucosal contact that occurs via abrasions, or microlesions on the skin and mucosa. Papillomavirus infections may greatly influence animal health, and some diseases seen in farm animals are linked with great economic losses. The disease has a worldwide occurrence, seen generally in young animals as cutaneous and mucosal tumors. Papillomatosis in cattle is the most widely investigated animal papillomavirus infection. However, the disease is widely spread in horses, dogs, cats, sheep, goats, and camels. Most papillomavirus infections can be diagnosed clinically, but laboratory confirmation is sometimes needed. PCR followed by DNA sequencing represents the most sensitive method for papillomavirus identification and genetic characterization. Polyomaviruses are minute DNA viruses that belong to the family *Polyomaviridae*. These viruses infect different mammalian and avian hosts with a wide range of findings including asymptomatic infection, acute generalized disease, and tumor induction. They do not cause a significant animal disease but are widely distributed among mammalian and avian species.

A. I. Khalafalla (✉)
Veterinary Laboratories Division, Animal Wealth Sector, Abu Dhabi Agriculture and Food Safety Authority (ADAFSA), Abu Dhabi, UAE

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2.1 Prologue

In recent years, papillomaviruses and polyomaviruses have received consideration because of their association with malignancy development and their wide dissemination. Until recently, the terms *Papovaviridae* and *Papovaviruses* were used in the virus taxonomy and the scientific community to denote viruses of the current two families *Papillomaviridae* and *Polyomaviridae* mostly producing tumors in mammals and share numerous structural features, however, with dissimilar genomic configurations. The name PAPOVA was derived from three abbreviations: PA the first two letters of papilloma, PO for polyomavirus, and Va for "vacuolating" (simian vacuolating virus 40 [SV40], which is presently known to be part of the *Polyomavirus* genus). As the two virus families have different genome sizes, different genome configurations, and least nucleotide or amino acid sequence resemblances, these were officially recognized by the International Committee on the Taxonomy of Viruses [ICTV] as two different families; *Papillomaviridae* and *Polyomaviridae* (de Villiers et al. 2004).

Remarkably, two papilloma-polyomavirus hybrids (BPCV1 and BPCV2) harboring a novel prototype of viruses possessing both papillomaviral and polyomaviral characteristics have been recently detected in the marsupial species Western barred bandicoot (*Perameles bougainville*) and the southern brown bandicoot (*Isoodon obesulus*), animals suffering from various types of papillomatosis (Woolford et al. 2007; Bennett et al. 2008; Rector and Van Ranst 2013).

2.2 Papillomaviruses

Papillomavirus (PV) is an epithelia-tropic small circular DNA virus belonging to the family *Papillomaviridae* that cause benign proliferative lesions in the skin (warts) and mucous membranes in different animal species and humans as well as malignant tumors of the genital tract and the uterine cervix in people. It is believed that papillomaviruses are one of the oldest and the most extensive known virus families as they emerged simultaneously with tetrapods in the Carboniferous time of the Paleozoic period (330 million years ago) (Rector and Van Ranst 2013; Araldi et al. 2017). The molecular phylogeny analysis reveal that these viruses emerged in Africa and subsequently spread throughout the world (Bernard 1994).

Warts or papillomas have been known in animals predominantly in horses and dogs for decades, and genital warts were well known to physicians of the ancient world. However, it was not until 1933 when Dr. Richard Shope revealed the

discovery of the first papillomavirus that could cause papillomas in cottontail rabbits. This virus, later known as the Shope papillomavirus, was the first to etiologically link a DNA virus to malignant progression in mammals (Shope and Hurst 1933; Uberoi and Lambert 2017). After 50 years, Dr. Harald zur Hausen found that human papillomaviruses (HPV16 and HPV18) cause cervical cancer (Uberoi and Lambert 2017). In the current classification, there are 2 subfamilies in the *Papillomaviridae*; the first *is Papillomavirinae* with 52 genera, and the second *Papillomavirinae* with one genus (https://talk.ictvonline.org/taxonomy/).

2.2.1 Virion, Genome Structure, and Genotyping of Papillomavirus

The virus particles (virions) of papillomavirus consist of a round non-enveloped geometrically regular capsid with icosahedral symmetry. The isometric capsid has a diameter of 45–60 nm composed of 72 star-shaped pentameric capsomeres of the large capsid protein (L1) on the outer surface. A smaller capsid, L2, is located internal to the L1 shell, and the viral genomic DNA is bundled inside the L1/L2 capsid as a mini-chromosome (Zhou et al. 1993).

The viral genome varies from 5748 to 8607 bp. The genome comprises three functional regions, of which the early region encodes proteins involved in transcription, replication, and manipulation of the cellular milieu. The late region encodes the capsid proteins L1 and L2. The upstream regulatory region, located between the L1 and E6 open reading frames, contains the origin of replication as well as binding sites for viral and cellular transcription factors (Van Doorslaer et al. 2018). The genome of PV is a double-stranded circular DNA molecule ~8000 base pairs (bp) in length. In average there are 8 open reading frames (ORFs), which are separated into early and late gene expression classes.

Papillomavirus replication is firmly connected to the differentiation process of the host epithelial cells, and the expression of viral early genes takes place in the lower layers of the epithelium, while the rest of the multiplication process including replication of the viral genome, late gene expression, and virus assembly take place in more differentiated cells (Doorbar et al. 2012). The current classification of PVs is mainly based on nucleotide sequence identity of the open reading frame (ORF). A novel papillomavirus type is described as a cloned full-length papilloma viral genome, whose L1 nucleotide sequence is at least 10% not identical with that of any other PV type. New genera in the family *Papillomaviridae* are proposed when the nucleotide similarities are lower than 60%. ORF L1 differences between 2% and 10% ascertain a new subtype, and a variant is defined in case the difference is <2%. In spite of that, not just the percentage of nucleotide similarity should be considered to ascertain the PV's classification. The interpretation of the phylogenetic position, genome organization, biology, and pathogenicity ought to likewise be considered to define the PV's classification (Bernard et al. 2010; Daudt et al. 2018).

2.2.2 Clinical Picture of PV Infection

Skin warts are the widely recognized indication of disease with papillomavirus. There is skin surface elevations as of hyperplasia and also solid outgrowths of skin and connective tissue. Generally, PV lesions start as small, smooth, white-to-gray papules that grow into pedunculated outgrowth with multiple keratin projections. PV infections are classified morphologically into: (1) typical-exophytic masses, with "cauliflower" shape, having a wide or narrow insertion base; (2) pedunculated exophytic masses linked by a narrow base with a peduncle shape; (3) atypical or flat dense and flat exophytic masses completely linked with the tissue; (4) filamentous exophytic masses with very keratinized surface and a thin base, found in mammary glands; and (5) rice-form-small papillomas with rice-like shape (Monteiro et al. 2008; Araldi et al. 2017).

2.2.3 Epidemiology of Disease

Papillomavirus infections may greatly influence animal health, and a few diseases seen in farm animals are linked with great economic losses. It has a global occurrence, seen commonly in young animals as cutaneous and mucosal tumors. In addition, animal papillomavirus infections are interesting models for investigating molecular and cell biology. PVs are frequently recorded as causative agents of cutaneous benign hyperplasia and malignant epithelial lesions in many animal species, and over recent years, an increasing number of papillomaviruses that have been identified with bovine, canine, and feline papillomaviruses have been the most extensively studied PV infections.

Horses show development of cutaneous, genital, ocular, and oral papillomas and also squamous-cell carcinomas (Sykora and Brandt 2017). An equine papillomavirus (EcPV) was detected in cutaneous papillomatosis. The same viral type was identified in papillomatosis of the muzzle and the leg, but absent in penile papillomas, which points to the presence of two dissimilar types of EcPV (O'Banion et al. 1986).

Papillomavirus infection was also found in other animal spp. including monkeys (O'Banion et al. 1987; Kloster et al. 1988), different members of the cervidae family (Moreno-Lopez et al. 1981; Moar and Jarrett 1985), the captive reptiles (Eleni et al. 2017), the endangered western barred bandicoot (Perameles bougainville) (Woolford et al. 2007), rodents (Rogovskyy et al. 2012; Uberoi and Lambert 2017) and rabbits (Shope 1932), and California sea lion (Luff et al. 2018). Furthermore, PVs are also seen in birds and fish (Daudt et al. 2018). Papillomavirus infections are more frequent in young animals. Housing conditions, poor nutrition, absence of appropriate cleaning, and insufficient fittings are regarded as risk factors for the progress of papillomavirus infection in animals, as they can be the source of stress, and consequently immune depression, which impacts the occurrence and the sternness of the disease (Daudt et al. 2018).

2.2.4 Transmission

Transmission of PVs need a close cutaneous or mucosal contact. The need for close physical contact and the species-specific character of the virus as well as the steadiness of their double-stranded DNA genome makes it improbable that ongoing interspecies transmissions can represent the worldwide presence of a spectrum of papillomaviruses in many amniotes (Rector and Van Ranst 2013). PVs are entirely species- and tissue-specific albeit some established and abnormal cross-infections have been reported (Daudt et al. 2018).

It is hypothesized that transmission of papillomaviruses is typically horizontal and happens via contact, scratches, or microlesions on the skin and mucosa; however, vertical transmission and iatrogenic transmission have also been reported, and mechanical transmission by arthropods is possible (Campo and Bastianello 2004). BPV can spread from infected delivered females to susceptible calves via skin contact during suckling or from bulls to females amid breeding. Disruptions in the epidermis integrity caused by skin infections likely facilitate the virus entry to skin basal layers and thus predisposes to papilloma virus multiplication (Khalafalla et al. 2017).

2.2.5 Immunopathobiology

The assimilation of the PV genome with the host cells during virus infection brings about an anomalous regulation of cell cycle control. Infected cells undergo immune evasion resulting in inability of the virus to be recognized for a long time.

The immune system plays a critical role in modulating the severity of PV infection. According to Song et al. (2015), the immune system alterations prompted by human papillomavirus (HPV) infection incorporate tumor-associated macrophage differentiation, a compromised cellular immune response, an unusual imbalance between type 1 T-helper cells (Th1) and Th2 cells, regulatory T cell infiltration, and downregulated DC activation and maturation. Usually, the benign warts go through spontaneous, immune-mediated regression, probably prompted by T cells (especially CD4, but also CD8 subsets), though humoral immunity can avert the new infections (Nicholls and Stanley 2000). Clinical observations and experimental infections have indicated that dogs, horses, rabbits, and cows which heal from papillomas are immune to re-infection (Nicholls and Stanley 2000).

2.2.6 Diagnosis of Papillomavirus Infection

Most papillomavirus infections can be diagnosed clinically if the lesions are well characterized. However, laboratory techniques are indispensable for confirming the clinical diagnosis and determining the type of the causative PV. Different methods have been used to identify PVs; these include but not restricted to:

2.2.6.1 Polymerase Chain Reaction (PCR)-Based Methods

PCR is currently the most commonly used technique for PV identification first, due to its high sensitivity and the lack of the reliable serological tests and the difficulty of virus isolation in cell culture. Various gel-based PCR assays have been implemented to identify PVs using specific and/or degenerate primers (Stocco dos Santos et al. 1998; Forslund et al. 1999; Leto et al. 2011; Ogawa et al. 2004; Araldi et al. 2013, 2014a, b, 2015; Melo et al. 2014). Among the degenerate primer pairs mentioned in the literature, FAP59/64 (Forslund et al. 1999) is the most commonly employed in both BPV and HPV identification (Araldi et al. 2017). Furthermore, PCR followed by DNA sequencing represents the most sensitive method for PV identification and genetic characterization. Besides, restriction fragment length polymorphism of PCR products (PCR-RFLP) has been shown to allow identification of bovine PV (BPV) types (Carvalho et al. 2013).

2.2.6.2 Electron Microscopy (EM)

Electron microscopy (EM) has long been utilized for detection of many viruses. Although detection of viruses by EM requires generally substantial numbers of virions, it has the peculiar advantage of being simple, fast, and useful for those viruses possessing characteristic morphology such as PVs. Furthermore, some PVs were first described utilizing the EM, for instance, the first cases of dromedary camel papillomavirus infection reported by Munz et al. (1990) were exclusively diagnosed by the negative staining EM.

EM has been used for the identification many PV infections including human PV (HPV) infection (Alejo et al. 2018; Song et al. 2018), bovine papillomavirus (BPV) (Araldi et al. 2014a, b; Melo et al. 2015), feline PV infection (Egberink et al. 2013), canine PV infection (Lange et al. 2013), dromedary camel PV infection (Munz et al. 1990), and equine PV infection (Postey et al. 2007).

2.2.6.3 Immunohistochemistry (IHC)

Papillomavirus-induced skin lesions can be diagnosed by demonstration of papillomavirus proteins (antigens) in skin lesion biopsies. PV antigen has been identified using IHC techniques in different animal species (O'Banion et al. 1986; Araldi et al. 2015). Papillomavirus antigen could be detected in papillomas nonetheless in carcinomas (Junge et al. 1984; Olson 1987).

2.2.6.4 Histopathology

The histopathological findings are pathognomonic and useful for confirming the diagnosis of PV infection. Histologically, papillomas are characterized by hyperkeratosis, acanthosis of the spinal layer, and koilocytosis (da Silva et al. 2015; Lunardi et al. 2016; Monteiro et al. 2008). Additionally, cutaneous papillomas in cattle display epidermis proliferation and keratohyaline granules (Campo 2006; Grindatto et al. 2015; Lunardi et al. 2016). According to Lunardi et al. (2016), the presence of quite a few islands of degenerated epithelial cells bounded by a thick halo of hyperplastic epidermis can be appreciated.

2.2.7 Prevention and Control

Warts are largely not considered serious, as they usually disappear spontaneously. Warts can be expelled at the commencement stages. Prevention can be achieved by isolating sick animals from susceptible ones. However, in some cases PV infection do not respond to all kinds of treatment. For instance, a severe, naturally occurring, non-regressing canine oral papillomavirus (COPV) in which the lesion proved refractory to surgical and medical treatments, including autogenous vaccination and vaccination with capsid (L1) virus-like particles was reported (Nicholls et al. 1999).

Vaccination against PV in animals can be accomplished using live virus, formalin-inactivated virus, synthetic virus-like particles, and DNA vaccination. Nonetheless, there is no much advance in this regard owing to the mild nature of virus infection in animals. Another constraint is the fact that prophylactic immunity to a range of papillomaviruses is type-specific (Jarrett et al. 1990). However, progress in the development of a few vaccines for papillomavirus infections in rabbit, ox, and dog has been noticed (Nicholls and Stanley 2000).

Hitherto reports in cattle and rabbit experiment models suggest that vaccination with the amino terminus of the minor capsid protein (L2) containing residues 11–200 or 1–88 produced recombinantly in bacteria is protective (Campo 1994; Chandrachud et al. 1994; Gambhira et al. 2007). Jagu et al. (2011) found that inoculation with a multimeric L2 antigen derived from three bovine and three canine papillomavirus genotypes with divergent phenotypes purified from bacteria and formulated in adjuvant, like BPV1 L1 VLP, protected mice experimental challenged with BPV1 pseudovirus. Commercial wart vaccines for cattle are available in some countries such as the USA. The vaccine is given as 10 ml dose to calves or 15 ml to adults, injected subcutaneously at two sites along the neck with booster doses at 3–5 weeks.

Recently, an effective vaccine against BPV infection was produced using virus-like particles of BPV6 in silkworm pupae inducing specific IgG against the BPV6 antigen (Watanabe et al. 2017). Furthermore, it has been reported that vaccines inducing T-cell responses to E1, E2, E6, E7, E10, and L1 PV genes display higher protection (Christensen et al. 2017).

2.2.8 Papillomavirus Infection in Cattle

Papillomatosis in cattle is the most widely investigated animal PV infection and perhaps the second most studied PVs after human PVs. *Bos taurus* PV (formerly bovine PV) induces benign tumors of cutaneous or mucosal epithelia, called papillomas or warts. The disease generally regresses spontaneously without causing any serious clinical problems but may persist and progress to malignant neoplasms, and some disease conditions such as the bovine teat papillomatosis can prompt heavy economic losses in dairy cattle (Watanabe et al. 2017).

The persistence of papillomas predisposes animal to feeding and breathing troubles, lowers growth rate, results in weight loss, and also inclines to other bacterial

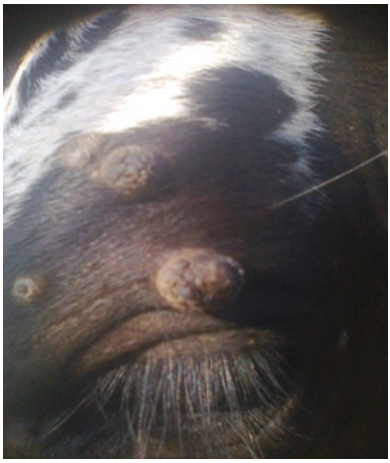

Fig. 2.1 Wart-like growth caused by BPV on the face of a bull

infections (Watanabe et al. 2017; Munday 2014; Campo 2002). Classified in several genera, currently, 24 BPV types from cattle have been identified and published at the *Papillomavirus* Episteme site (pave.niaid.nih.gov).

2.2.8.1 Clinical Presentation

Among the different bovine papillomatosis, fibropapilloma is the most widely recognized kind of benign tumors commonly found on the head (Fig. 2.1), legs, neck, penis, or teats. Additionally, epithelial papillomas of the upper gastrointestinal (GI) tract, mostly caused by BPV 4, can appear in every site from the mouth to the rumen (Borzacchiello and Roperto 2008). BPV types 2, 13, and 14 are associated with urinary bladder tumors in cattle (Roperto et al. 2014, 2016a, b; Cota et al. 2015).

The disease also disposes cattle to secondary bacterial infections, mostly affecting mammary glands and breast leading to mastitis, pain, and drop in milk yield and causing devaluation of leather value (Araldi et al. 2017).

2.2.8.2 Laboratory Diagnosis

Several assays are employed to recognize the PV infection in cattle including histopathology, PCR, restriction fragment length polymorphism-PCR (PCR-RFLP) immunohistochemistry (IHC), Southern blotting, and electron microscopy. PCR assays targeting L1 gene followed by sequencing is worthwhile for genotyping the BPV (Dagalp et al. 2017).

2.2.9 Papillomavirus Infection in Camels

The cutaneous papillomatosis related to PV in dromedaries was confirmed for the first time in 1990 (Munz et al. 1990), where dromedary camels in central Somalia in the age group of 6 months to 2 years were mainly affected. Subsequently, papillomatosis cases in young dromedaries have become common in Sudan, United Arab

Emirates, Kenya, and Saudi Arabia (Khalafalla et al. 2018). Notably, PV association with a 2-kilogram wart-like growth in a dromedary camel in India was reported (Sadana et al. 1980). As well, a corneal papilloma growth was seen in an old dromedary male camel possessing a history of chronic severe keratoconjunctivitis (Kilic et al. 2010). The majorities of the reported cases of papillomatosis in camel are commonly found in young animals and occur in the late rainy season, matching with episodes of camel contagious ecthyma and camel pox (Khalafalla et al. 1998; Munz et al. 1990). Papillomatosis is also seen in camelids of Southern America (llamas and alpacas; Schulman et al. 2003).

Currently, the genomes of two *Camelus dromedarius* PV types (type 1, CdPV1, and type 2, CdPV2) detected in field samples of the disease in Sudan have been completely characterized and both are genetically grouped within the genus *Deltapapillomavirus* (Ure et al. 2011). Sequencing of further eight DNA samples collected from Saudi Arabia disclosed the occurrence of both *Camelus dromedarius* papillomavirus types 1 and 2, previously documented (Khalafalla et al. 2017).

2.2.9.1 Clinical Presentation

The majorities of the described camel papillomatosis cases were seen in young animals and occur in the late rainy season. This may point that older animals have acquired immunity from a previous infection. The wart-like lesions that range between 0.2 and 2.5 cm in size are found mainly on the head, particularly the lips, eyelids, nares, and mandibles (Munz et al. 1990; Khalafalla et al. 1998, 2018; Ure et al. 2011). Often, wart-like lesion can be seen on the chest and forearm areas (Fig. 2.2), and the size in some cases can reach 5 cm due to coalition of more than one wart (Khalafalla et al. 2018).

2.2.9.2 Laboratory Diagnosis

Electron microscopy and histopathology were previously used to confirm the infection by PVs in camels. Additionally, PCR accompanied by L1 gene sequencing and immunohistochemistry have been utilized in the recent reports of the disease.

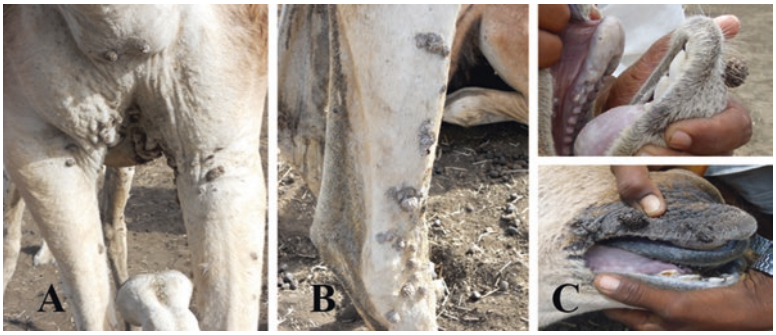

Fig. 2.2 Papillomatosis in dromedary camels in eastern Sudan. Cauliflower-like single and coalesced nodular warts on the chest (panel A), legs (panel B), and lips (panel C)

2.2.10 Papillomavirus Infection in Dogs and Cats

Papillomaviruses (PVs) affect both canines and the felines. In dogs, PV causes oral papillomatosis, cutaneous papillomas, and canine viral pigmented plaques. On the other hand, PVs have rarely been connected with the development of oral and cutaneous squamous cell carcinomas. Feline papillomavirus 2 is an emerging, widely prevalent virus in domestic cats, linked to a subset of malignant skin lesions (Gil da Costa et al. 2017). Increasing evidences are surfacing now claiming association of PVs with cutaneous squamous cell carcinomas and basal cell carcinomas in cats (Munday et al. 2017).

2.2.10.1 Clinical Presentation

Papillomavirus dermatoses in dogs and cats is associated with isolated sessile or pedunculated papilloma on the skin, while the canine genital papillomatosis is characterized by whitish, cauliflower-like hyperkeratotic papillomatosis on the tip of the penis or vaginal mucosa (Nagata 2013). On the other hand, dogs suffering from oral papillomatosis show signs that include bad breath linked to bleeding from the mouth, increased or decreased appetite, and excessive excretion of saliva.

2.2.10.2 Laboratory Diagnosis

Histopathology, immunohistochemistry, and/or PCR are useful for identifying PV infection in dogs and cats.

2.2.11 Papillomavirus Infection in Sheep and Goats

Gibbs et al. (1975) reported the first PV associating warts observed on the limbs of three sheep and the muzzle of one sheep. Later, PV in tumors of sheep and goats was reported from various countries (reviewed by Anon 2018). Three types of goat papillomas are described: mammary, cutaneous, and those other than mammary and genital (Theilen et al. 1985). According to Theilen et al. (1985), the development of mammary gland warts in caprine seems to depend on many factors, namely, nonpigmented skin, age of adult, excessive exposure to sunlight, and contact with a yet undefined infective agent. Recently, a new putative papillomavirus was detected in a case of teat papillomatosis in a Damascus goat in Turkey (Dogan et al. 2018).

2.3 Polyomavirus

Polyomavirus (PyV) is a minute DNA virus of the family *Polyomaviridae*, primarily affecting mammals and birds with some oncogenic (causing tumors) members. Polyomaviruses are double-stranded DNA, non-enveloped, spherical, and small size viruses (45 nm). The viral capsid symmetry is icosahedral where the VP1 protein constitutes the outer surface of virus (Henriksen et al. 2016).

It was reported first in 1953 in laboratory mice (caused tumors at multiple sites in neonatal mice) (Gross 1953). Consequently, the virus has been discovered in green monkeys, baboons, cage birds, and bovines. In addition to humans, they have been discovered in horses, monkeys, chimpanzees, rabbits, raccoons, mice, hamsters, bats, elephants, badgers, giant panda, sea lions, and a wide variety of birds (Qi et al. 2017). Recently, a novel pulmonary polyomavirus was detected in alpacas (Vicugna pacos) (Dela Cruz et al. 2017). Polyomaviruses lead to asymptomatic infection as well as acute generalized disease (Qi et al. 2017).

On the other hand, BK and JC viruses, the first human polyomaviruses, were reported in 1971. BKV was isolated from urine samples of a renal transplant patient while JCV from the brain tissue of a patient with Hodgkin's lymphoma (Jiang et al. 2009).

Advent of molecular cloning methods and the development of viral metagenomics platforms have encouraged the discovery of new animal viruses. For example, a unique polyomavirus species, called camel polyomavirus Abu Dhabi, was identified in UAE using next generation sequencing method, and the species was found clearly distant to prevailing polyomaviruses (Li et al. 2017).

Now, as per the ICTV *Polyomaviridae* study group recommendation, for defining a new polyomavirus species, we have to follow: (1) the whole genome sequence is available in public databases and published; (2) the genome shows an organization typical for polyomaviruses; (3) sufficient data on the natural host is available; (4) the demonstrated genetic distance to members of the most closely related species is >15% for the large T antigen (LTAg) coding sequence (Calvignac-Spencer et al. 2016; Qi et al. 2017). The family *Polyomaviridae* is currently comprised of 5 genera: genus *Alphapolyomavirus* (38 Species), genus *Betapolyomavirus* (32 Species), genus *Deltapolyomavirus* (4 Species), genus *Gammapolyomavirus* (9 Species), and genus unassigned (5 Species) (https://talk.ictvonline.org/taxonomy/, accessed May 12, 2018).

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Bovine Herpesvirus

3

Niraj K. Singh, Sachin S. Pawar, Anuj Tyagi, and Praveen K. Gupta

Abstract

Bovine herpesvirus 1 (BoHV-1) is an economically significant pathogen of livestock industry worldwide. The virus belongs to genus *Varicellovirus* under family *Herpesviridae*. The double-stranded DNA genome of BoHV-1 consists of approximately 135.3 kbp. Cattle is the primary host of BoHV-1 but other Artiodactyla like goats, sheep, water buffaloes and camelids are also susceptible to BoHV-1 infection. The virus has different stages of infection. The virus replicates at the entry portal or within the mucosa of nasal tract or genitalia, and from there the virus may enter into the nerve axons. Afterwards, through intra-axonal transportation, the virus goes to the neuron bodies of the regional ganglia and may remain in latency stage. During the stress conditions, latent form of BoHV-1 may revert back to activated form to cause clinical infections. With the beginning of virus replication, the host immune response to virus infection is activated. Seven days post-infection, predominant antibody-mediated humoral (AMI) and cell-mediated immune (CMI) responses arise. While AMI plays a critical part in prevention of viral spread and infection, CMI has a role in recovery from infection. BoHV-1 diagnosis is usually carried out using either serologic tests or by molecular diagnostic methods. The serologic tests involved either

N. K. Singh (✉)
College of Animal Biotechnology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, India

S. S. Pawar
National Institute of Abiotic Stress Management, Pune, Maharashtra, India

A. Tyagi
College of Fisheries, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, India

P. K. Gupta
Division of Veterinary Biotechnology, ICAR-Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh, India

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detection of virus/viral components or antibodies against virus. The molecular diagnostic methods involved detection of viral genome. The vaccines for BoHV-1 are the modified or attenuated vaccines (live vaccines) or inactivated (killed vaccines). In recent years, deletion mutant-based marker vaccines by removing one or more proteins have also been reported.

Keywords
Bovine herpesvirus · Latency · Antibody-mediated humoral immune response · Cell-mediated immune response · Serologic diagnostic tests · Molecular diagnostic tests · Live vaccines · Inactivated vaccines · Marker vaccines

3.1 Preamble

In agriculture sector worldwide, bovine herpesvirus 1 (BoHV-1) pathogen has significant impact on livestock economy. In several parts of world, including India, BoHV-1 is endemic and causes infectious bovine rhinotracheitis (IBR) and infectious pustular vulvovaginitis (IPV) in bovines. Common clinical symptoms of viral infection are tracheitis, rhinitis, conjunctivitis and fever. In addition, pustular lesions in the vulva and vagina of cow as well as in penile epithelium and prepuce of bull are also observed in venereal forms of the disease resulting in impaired reproduction. In already pregnant animals, BoHV-1 infection may result in abortion. As the virus may be excreted in semen of infected bull, proper screening of bull is very much necessary to avert the transfer of virus during the artificial insemination process.

Bläschenausschlag or coital vesicular exanthema (CVE) caused by bovine herpesvirus 1 (BoHV-1) infection was first observed in Germany in the nineteenth century. Till the 1950s, CVE was routinely referred to as infectious pustular balanoposthitis (IPB) and infectious pustular vulvovaginitis (IPV) in bulls and heifers (or cows), respectively (Graham 2013). The virus as aetiological agent of disease was reported by Reisinger and Reimann in 1928 (Biswas et al. 2013).

In 1953, a new clinical complaint affecting cattle with morbidity and mortality rates of 7.6% and 3%, respectively, was reported in California (Los Angeles area). Short explosive cough with nasal discharge, high rate of salivation, inflamed nares mucous membrane and high fever (38.9–42.2 °C) were observed as characteristic clinical signs of the disease. During post-mortem examination, bronchitis, severe haemorrhagic tracheitis and typical lesion were also prominent. Nowadays, this report is accepted as the first peer-reviewed report of infectious bovine rhinotracheitis (IBR) in bovines with viral aetiology (Schroeder and Moys 1954). In subsequent year, a disease was reported in a Colorado feedlot. This particular disease was named as infectious necrotic rhinotracheitis with obvious inflammation of nasal pad and passage, and the 'red nose' term was used to describe its symptoms (Miller 1955). The term 'infectious bovine rhinotracheitis (IBR)' was used by the US Livestock Sanitary Association to describe this disease in 1955. The causative viral agent was first time isolated and characterized by Madin and co-workers (Madin

et al. 1956) and Tousimis and colleagues (Tousimis et al. 1958), respectively. As per Armstrong and colleagues (Armstrong et al. 1961), this virus belonged to herpesvirus group.

3.2 Epidemiological Studies

Various epidemiological studies have been conducted to evaluate the effect of BoHV-1 on bovine reproductive performance. In Turkey, the fertility losses due to natural subclinical infections were evaluated in non-vaccinated heifers and dairy cows. In case of seronegative and seropositive cows, conception rates were determined to be 38.98% and 33.33%, respectively. On the other hand, conception rates were 56.57% and 84.61% in seronegative and seropositive heifers, respectively (Ata et al. 2006). The BoHV-1 seroprevalence has been reported to be 36% in China (Yan et al. 2008), 43% in England (Woodbine et al. 2009), 63–86% in Egypt, 14–60% in Africa (Mahmoud et al. 2013) and 36–48% in South and Central America (Straub 1990). Successful IBR eradication programmes have been executed in several European nations, and as a result Sweden, Austria, Finland, Denmark and Switzerland have been officially declared IBR-free countries (Ackermann and Engels 2006). Presence of BoHV-1 has been reported in several parts of India. In a study, 38.01% of investigated bovine were found to be seropositive for BoHV-1 antibodies. Disease prevalence in the exotic and crossbred cattle was significantly higher than the indigenous breeds (Chatterjee et al. 2016). The seroprevalence of BoHV-1 in different states is as follows: 37.56% in Andhra Pradesh, 96.55% in Andaman and Nicobar, 69.05% in Arunachal Pradesh, 13.64% in Assam, 76.74% in Bihar, 42.50% in Goa, 10% in Gujarat, 9.095% in Haryana, 12.82% in Himachal Pradesh, 95.35% in Jammu and Kashmir, 64.22% in Karnataka, 46.67% in Madhya Pradesh, 77.90% in Maharashtra, 51.11% in Manipur, 13.64% in Mizoram, 100% in Orissa, 23.66% in Punjab, 60.16% in Rajasthan, 20.16% in Tamil Nadu and 82% in Uttar Pradesh (Suresh et al. 1999).

3.3 Virus Classification

BoHV-1 is a member of genus *Varicellovirus* of subfamily *Alphaherpesvirinae* and family *Herpesviridae*. Based on genomic analysis and antigenic properties, the BoHV-1 has been further subdivided into BoHV-1.1 (1), BoHV-1.2a (2a) and BoHV-1.2b (2b) subtypes. Virus belonging to subtype 1 is mainly prevalent in South America, North America and Europe, whereas subtype 2 is present in Brazil and Europe. On the other hand, BoHV-1 virus prevalent in Australia belongs to subtype 2b. Subtype 1 virus causes infectious bovine rhinotracheitis (IBR), and it is normally present in aborted foetuses and in the respiratory tract. Subtype 2a is commonly related with genital tract and respiratory clinical conditions like balanoposthitis (IPB), infectious pustular vulvovaginitis (IPV), IBR and abortions. In comparison to subtype 1, virus belonging to subtype 2b has low pathogenicity, and this mainly causes IPV/IPB and respiratory diseases (Jones and Chowdhury 2007).

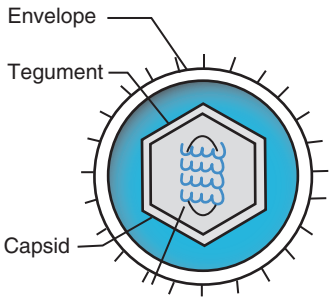

Fig. 3.1 Structure of BoHV-1. (Chatterjee et al. 2016)

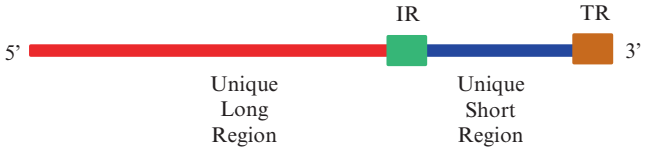

Fig. 3.2 Genome of BoHV-1

The icosadeltahedron-shaped capsid of BoHV-1 is approximately 100 nm in diameter and it consists of 162 capsomeres (Roizman and Pellett 2001). Inside the capsid, core consists of a fibrous spool-like structure rounded by linear DNA double-stranded genome of virus. On the outer side of the capsid, a globular material coating known as tegument is present which is further encircled by lipoprotein envelope having small glycoprotein spikes (Biswas et al. 2013) (Fig. 3.1).

The virus genome size is approximately 135.3 kilobase pairs (kb) having unique short (US) and unique long (UL) regions. The US region is flanked by inverted internal repeat (IR) and terminal repeat (TR) sequences. Thus, BoHV-1 genome is organized as 5'-UL-IR-US-TR-3' (Fig. 3.2). Virus genome has 73 open reading frames (ORFs) enclosing ten glycoprotein encoding genes. Among ten glycoprotein genes, six are located in the UL region and four are situated in the US region. The glycoproteins located in the UL region are gL (UL1), gM (UL10), gH (UL22), gB (UL27), gC (UL44) and gK (UL53), whereas those in the US region are gG (UL4), gD (UL6), gI (US7) and gE (US8). Based on various studies on ability of mutant virus with single gene deletion to grow and multiply in cell culture, genes of BoHV-1 have been grouped into nonessential and essential ones. During cell culture, deletion of nonessential genes shows insignificant or no effect on in vitro multiplication of virus. On the other hand, deletion of essential genes has been shown to interfere with the replicative abilities of mutant BoHV-1. The *gE* gene is one of the well-known nonessential genes in BoHV-1 (Muylkens et al. 2007).

3.4 Host Range

Though cattle is the primary host of BoHV-1, other Artiodactyla like goats, sheep, water buffaloes and camelids are also susceptible to BoHV-1 infection. Asian elephants without any clinical symptoms have also been reported to carry antibodies against BoHV-1. BoHV-1 has been also detected in wildebeest, pronghorn antelope, healthy mink and ferrets as well as in soft-shelled tick (*Ornithodoros coriaceus*). BoHV-1 can also be carried by face flies (*Musca autumnalis*), but the flies cannot transmit virus to the cattle. No natural BoHV infection has been reported in guinea pigs, rats, mice or chick embryos till date. However, experimental infection in immunocompromised mice has been achieved by intra-peritoneal injection of the virus. Similar types of intra-conjunctival injection-induced experimental infections have also been reported. Till date, there is no report of BoHV-1 infection in human beings (Biswas et al. 2013).

3.5 Life Cycle of BoHV-1 Virus

After infection, virus replicates at the entry portal or within the mucosa of nasal tract or genitalia, and from there the virus may enter into the nerve axons. Subsequently, virus is transported intra-axonally and reaches into the neuron bodies of the regional ganglia and may remain in latency stage (Nandi et al. 2009). Virus transmission in the body occurs through nerves, blood and cell-to-cell interaction of infected tissues. Initially virus causes transient viraemia followed by infection of secondary sites such as the udder, digestive tract, ovaries and foetus. From the initial site of infection, the virus moves through the nerve axonal tract to the peripheral nerves, i.e. sacral nerve ganglion and trigeminal nerve ganglion in case of genital and respiratory infections, respectively (Biswas et al. 2013). The entry of BoHV-1 into host cells is a multi-step process, which starts with attachment of viral glycoproteins with specific receptors on the cell followed by penetration. Only the cells producing specific types of proteins required for BoHV entry and subsequent multiplication are prone to virus infection. BoHV-1 infects macrophages and monocytes (Forman et al. 1982; Nyaga and McKercher 1979), CD4 T cells (Lovato et al. 2003) as well as epithelial cells of prepuce or vaginal mucous membranes, respiratory tract and conjunctivae (Tikoo et al. 1995a). The complementary binding partners on host cell (surface heparan sulphate) and virus (glycoproteins) are required for virus entry to cells (Tyler and Nathanson 2001). The initiation of virus entry to host cell starts with loose binding of viral glycoprotein gC to cell surface heparan sulphate proteoglycans, which further leads to static binding of viral gD to cellular putative second receptor. For entry of the virus into the cell, binding of viral gD is an essential prerequisite (Karger et al. 1995). Further, the final entry of the virus into host cell is facilitated by the fusion of viral envelope with the cell membrane (Liang et al. 1996). After the host cell entry, BoHV is carried to the nucleus by microtubule to facilitate its replication by using the host cell protein synthesis machinery. After the completion of capsid formation and viral DNA

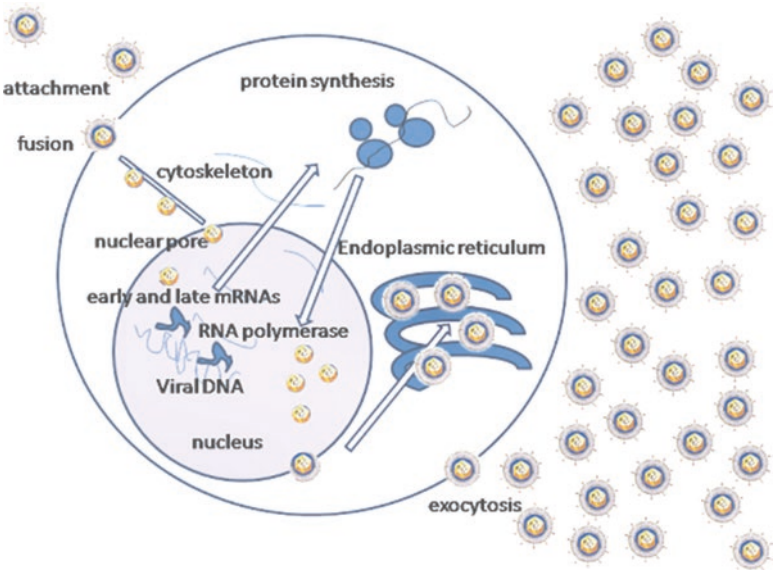

Fig. 3.3 Replication of BoHV-1. (Chatterjee et al. 2016)

packaging processes, BoHV leaves the nucleus by the process of budding. During the budding event, an envelope resulting from the internal portion of nuclear membrane covers the BoHV capsid (Knipe et al. 2001) (Fig. 3.3).

3.6 Latency

Latent infections of BoHV-1 are established at immuno-privileged locations succeeding acute infection (OIE 2010). In host animal, BoHV-1 generally remains in latent stage of infection and there is absence of persistent infection. The virus latency takes place at immune-privileged spots in peripheral nervous system proceeding into productive viral infection (OIE 2010). The virus in latency stage has also been reported in peripheral blood lymphocytes and tonsillar lymphoid cells (Mweene et al. 1996). During the latent phase of the virus, proteins related to latency, with a role in protecting the infected cells from programmed cell death, are only produced (Schang et al. 1996). Studies have also suggested that animal with latent infection may be seronegative for anti-BoHV-1 antibodies (Hage et al. 1998). However, young calves having latent infections may show seropositive results due to occurrence of maternal antibodies (SCAHW 2000). Vaccination by using live attenuated virus may also proceed to latent infection (Kit et al. 1985). During the stress conditions, latent form of BoHV-1 may revert back to activated form to cause clinical infections (Thiry et al. 1987).

3.7 Immunology

Studies have also been conducted to understand the immune response of host to viral infection as well as strategies deployed by the virus against the host immune response. Understanding of these mechanisms is very much crucial to decipher host-pathogen interactions during BoHV-1 infection.

3.8 Immune Response in BoHV-1 Infection

Immune response of the host is activated with the beginning of virus replication (Babiuk et al. 1996). Seven days post-infection, predominant antibody-mediated humoral (AMI) and cell-mediated immune (CMI) responses arise (OIE 2000). While AMI plays a critical part in prevention of viral spread and infection, CMI plays a crucial role in the recovery of host from the infection (Babiuk et al. 1996).

3.9 Antibody-Mediated Immune Response

The AMI response includes production of neutralizing antibodies, which play a role in antiviral response through antibody-dependent cellular cytotoxicity (ADCC) (Tikoo et al. 1995a), and complement-mediated response (Rouse et al. 1977). Antibody production against gE, gD, gC and gB glycoproteins (Tikoo et al. 1995a) has been suggested to protect the host from viraemia and related critical disease (Mechor et al. 1987).

3.10 Cell-Mediated Immune Response

The CMI responses to BoHV-1 infection are controlled by macrophages, interferon-γ and interleukin-2 production, natural killer (NK) cell activity, viral gD- and gC-specific CD4 T-cell proliferation and activation of cytotoxic T lymphocyte (Tikoo et al. 1995b). In mice model experimentation, interferon-α, interferon-β and interferon-γ have been reported to contribute in protection against infection as well as in prevention of viral spread (Abril et al. 2004). In contrast to T helper 1-type response in most of intracellular pathogens (Mena et al. 2002), both T helper 1-type and T helper 2-type responses play a role in BoHV-1 infection (Babiuk et al. 1996).

3.11 Immune Evasion

BoHV-1 has immunomodulatory property facilitated by viral protein, which mimics crucial molecules of the host immune system (Raftery et al. 2000). Immunomodulatory proteins of BoHV can bind to C3 complement in a species-specific manner to modulate host immune response (Engels and Ackermann 1996).

Therefore, BoHV-1 can evade host immune system by modulating macrophages and monocytes through antigen processing and presentation (Forman et al. 1982). Moreover, entry of BoHV-1 in latent phase is also a mechanism of immune evasion, as no viral proteins are produced throughout the latency period. Moreover, during the latent phase, the virus remains at immuno-privileged sites due to which class I major histocompatibility antigens are not expressed (Tyler and Nathanson 2001).

3.12 Immunosuppression

Broad immunosuppression has been reported in clinical BoHV-1 infection cases in cattle, which further progresses to secondary infections due to bacterial and viral pathogens (Winkler et al. 1999), resulting to bovine respiratory disease (BRD). Alteration of lymphocyte, polymorphonuclear neutrophil and macrophage functions is the predominant cause of immunosuppression (Tikoo et al. 1995a). In addition, immunosuppression may also occur due to reduced IL-2 receptor expression, decreased circulating T lymphocyte counts and poor stimulation of peripheral blood mononuclear cells (PBMCs) by mitogen (Winkler et al. 1999). Viral infection to macrophages and monocytes causes altered phagocytosis, deprived T-cell stimulation and weakened ADCC function (Forman et al. 1982). Partial immunosuppression is facilitated by glycoprotein G (gG) of BoHV-1, which behaves like broad-spectrum chemokine-binding protein. The activity of chemokine is inhibited by glycoprotein gG binding (Bryant et al. 2003).

3.13 BoHV-1-Related Diseases

BoHV-1 or IBR virus causes conjunctival infection, respiratory infection and abortion. The clinical manifestation is dependent on the subtypes of virus. While the BoHV-1.1 and BoHV-1.2 are responsible for respiratory and genital infections, respectively, the neurological disease is caused by the BoHV-1.3. During respiratory infection, symptoms like high fever, excessive salivation and mucopurulent nasal discharge are observed. The infection is characterized by lesions like laryngotracheitis, pharyngitis and rhinotracheitis (Yates 1982). Normally, secondary bacterial infection also occurs which is a worse respiratory symptom in clinical BoHV-1 infection. Lesions like pustules and ulceration in the vulva and vagina mucosa are common in genital infection. Generally, abortion may also occur at the end of the fifth month of pregnancy. The virus is assumed to spread from nasal mucosa to the brain through the trigeminal nerve ganglion causing encephalitis in calves. BoHV-1 remains in latent stage in the trigeminal sacral nerve ganglion for long periods. In calves, due to replication of virus in the digestive tract, frequent incidences of enteritis have also been reported.

3.14 Clinical Course

In BoHV-1, most of the infections have mild or subclinical manifestations (van Oirschot et al. 1993). During clinical infection, symptoms include fever, anorexia, salivation, depression and serous nasal discharge after 2–4 days of onset of disease which further proceed to mucopurulent ocular and nasal discharges. Balanoposthitis or pustular vulvovaginitis may also develop due to genital infection caused by natural mating practice. Normally, uncomplicated genital or respiratory disease caused by BoHV-1 lasts for 5–10 days, but secondary viral or bacterial infections may lead to a complex disease like 'crowding fever' or 'shipping' in young animals. In case of airborne route of BoHV-1 infection, the virus multiplies in the mucous membrane of the tonsil and upper respiratory tract. Then, the virus moves to conjunctivae and further spreads to the trigeminal nerve ganglia using neuro-axonal transport. In case of genital infection by natural mating practice, the virus multiplies in the mucous membranes of prepuce or vagina and enters latent stage in the sacral nerve ganglia. The virus can remain in neuron ganglia throughout the life cycle of the host. The latent infection can be converted to clinical infection due to stressful situations such as parturition and transport. Corticosteroid use is also responsible for conversion of latent stage to clinical infection (Muylkens et al. 2007).

3.15 Diagnosis of BoHV-1

BoHV-1 diagnosis is generally carried out using either serologic tests for detecting virus, viral components or antibodies to virus or by molecular diagnostic methods that are based on detecting presence of BoHV-1 genome. At present several diagnostic methodologies are available for BoHV-1 detection. Viral isolation and characterization provides definitive diagnosis, and it is considered as the gold standard for detection of BoHV-1 (Schultz et al. 1982; Weiblen et al. 1992). Electron microscopy for BoHV-1 detection has also been used (Brunner et al. 1988). Recently, methods for detection of viral nucleic acid such as dot-blot hybridization (Xia et al. 1995), polymerase chain reaction (van Engelenburg et al. 1993; Kataria et al. 1997), nested PCR (Rocha et al. 1998; Oliveira et al. 2011), real-time PCR (Wang et al. 2007; Diallo et al. 2011) and loop-mediated isothermal assay (Pawar et al. 2014) have also been employed for BoHV-1 detection. Reference laboratories in many countries also use PCR-based and antibody-specific enzyme immunoassays for virus detection (Murphy et al. 1999).

3.16 Vaccines for BoHV-1

The first report on isolation of BoHV-1 was published in 1956 by Madin et al., and in the same year first live attenuated vaccine was developed (Kendrick et al. 1956). Conventional BoHV-1 vaccines are the modified or attenuated vaccines (live vaccines) or inactivated vaccines (killed vaccines). In case of live vaccines, an

attenuated BoHV-1 strain with capability to replicate in the host is used. On the other hand, BoHV-1 strain in killed vaccines remains non-infectious due to its inability to replicate in the host (van Oirschot et al. 1996). Vaccines containing live virus can produce latent infection and may induce viral shedding of reactivated vaccine virus (Graham 2007). A recent study suggests inactivated forms of the vaccine provide similar efficacies as modified live vaccines, but they may be safer to give during gestation (Zimmerman et al. 2007).

New-generation vaccines include DNA vaccines, subunit vaccines and marker vaccines. Immunization of cattle and mice with a DNA vaccine encoding for BoHV-1 gD results in immune response (Cox et al. 1993; Lewis et al. 1997). Moreover, the immunization of mice with plasmid encoding for BoHV-1 gD in secretary form also results in both humoral and cell-mediated immune responses in the face of pre-existing passive antibodies. Mice studies demonstrated that DNA vaccine for BoHV-1 may overcome the important shortcoming of conventional BoHV-1 vaccine, i.e. antibody-mediated suppression of the immune response (Lewis et al. 1999; Deshpande et al. 2002). During studies in bovines, glycoprotein C (gC) of BoHV-1 encoded by a DNA vaccine was able to induce neutralizing antibody and lymphoproliferative responses with BoHV-1-responsive memory B cells in bovines. However, during the BoHV-1 challenge, this developed immunity was not enough to completely protect the calves (Gupta et al. 2001). In contrast to whole killed vaccines containing entire virion particle as antigen, the subunit vaccine contains only selected components of virion particle such as envelope glycoproteins (Brun et al. 1988). The subunit vaccines primarily stimulate Th2 response (Cox et al. 1993). For preparation of subunit vaccines against BoHV-1, immunogenic viral glycoproteins (gB, gC and gD) are either separated from virus-infected cell or synthesized as peptide. Immunization with these proteins results in development of high antibody titres in targeted animal, resulting in protection during experimental BoHV-1 challenge (Nandi et al. 2009). It has also been reported that serum antibody titre-based immunity and protection against experimental challenge due to individual glycoproteins are much higher than with the commercially available inactivated vaccines (Babiuk et al. 1987).

A marker vaccine is a deletion mutant on one or more microbial proteins that enable a clear distinction between vaccinated and infected animals by checking the antibody responses. These vaccines (marker) are employed in conjunction with a test (companion test) capable of detecting antibodies against the protein that was deleted in the marker vaccine strain backbone. The marker vaccine plays a crucial role in the implementation of any disease control programme with the ultimate aim of disease eradication. These programmes contain the following: (1) intensive vaccination using novel marker vaccines; (2) monitoring of infection incidence using companion diagnostic tests; (3) if necessary, ‘economic’ culling of seropositive animal; and (4) prohibition of vaccination (van Oirschot et al. 1996). Live and killed marker vaccines based on a deletion mutant of BoHV-1, lacking glycoprotein gE encoding gene, are available commercially (van Oirschot et al. 1996, 1997). Eradication schemes have been successful in several European countries using vaccination methods or culling of seropositive animals in regions with low seropositivity (Ackermann and Engels 2006).

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Conflict of Interest The authors declare no conflict of interest.

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Equine Herpesviruses

4

Baldev Raj Gulati, Nitin Virmani, and Bhupendra Nath Tripathi

Abstract

Equine herpesviruses (EHVs) are a group of 11 viruses of the family *Herpesviridae* affecting horses globally. Of these, two EHVs (EHV1 and EHV2) are the most devastating to the equine industry, causing acute upper respiratory tract disease in young horses, late-term abortion in pregnant mares, neonatal foal mortality and neurological disease termed equine herpesvirus myeloencephalopathy (EHM). The global incidence of abortion and rhinopneumonitis is on decline, while that of EHM is on the rise, due to widespread use of vaccines to control respiratory infections and abortions. Although the clinical form of EHM is less frequently observed, it can cause serious economic losses in breeding horses and has very negative impact on the equine industry. Following infection, EHV1 establishes a life-long latent infection within the host-specific tissues where viral genome persists with very limited transcription. The latency enables the virus to evade the radar of the host immune system for its perpetuation in nature. The viral factors that influence EHV disease severity and latency are poorly understood, and this has hampered vaccine development. EHVs are the most common infections among horses worldwide; however, their diagnosis, treatment and prevention are very challenging, partly because of the complexity of the virus-host interactions. This chapter provides an update on the recent developments in our understanding of the aetiology, pathogenesis, diagnosis and control of EHV-associated diseases, with an emphasis on understanding the mechanisms of EHM and latency.

Keywords

Equine herpesviruses · EHV · *Equidae* · Latency · Pathogenesis · Diagnosis · Treatment · Prevention

B. R. Gulati (✉) · N. Virmani · B. N. Tripathi
ICAR-National Research Centre on Equines, Hisar, Haryana, India

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4.1 Prologue

Equine herpesviruses (EHVs) are highly significant pathogens affecting all members of family *Equidae* globally. Five subtypes of herpesviruses (EHV1–EHV5) have been reported in horses, while donkeys are host to EHV6 to EHV8 (asinine herpesviruses, AHV1 to AHV3) and Thomson's gazelles, giraffe and zebras are hosts to the newest member of the equine herpesvirus EHV9 (gazelle herpesvirus) with encephalitis (Davison et al. 2009; Brosnahan and Osterrieder 2009; Schrenzel et al. 2008). EHV1 and EHV4 are the most important equine herpesviruses that infect 80–90% of horses globally by 2 years of age, resulting in respiratory infection, characterized by fever, anorexia and nasal and ocular discharge (Allen 2008). EHV1 causes upper respiratory tract infection in young horses at the time of weaning (Allen 2008), abortion in pregnant mares, neonatal foal mortality and neurological disorders. Abortion is economically the most crippling outcome of EHV1 infection with 95% of EHV1-associated abortions occurring in the last 4 months of pregnancy. Neurological disease associated with EHV1 is called equine herpesvirus myeloencephalopathy (EHM), which can cause serious economic loss in breeding horses (Henninger et al. 2007; Pronost et al. 2010).

EHV3 is responsible for equine coital exanthema (ECE), a venereal and highly contagious disease. EHV3 infection causes the formation of papules, vesicles and pustules on the external genitalia of mares and stallions. The affected stallions may have stiff gait and loss of the libido and refuse to mate mares (Vissani et al. 2018, Barrandeguy and Thiry 2012). EHV2 and EHV5 cause upper respiratory tract infection, inappetence and immunosuppression. They also cause keratoconjunctivitis, lymphadenopathy and general malaise (Fortier et al. 2010). EHV5 is routinely detected in blood and nasal secretions of healthy horses and generally does not cause disease in the horse. However, recent reports associate EHV5 with lung infection leading to equine multinodular pulmonary fibrosis (EMPF) (Williams et al. 2007).

This chapter discusses the aetiology, pathogenesis and epidemiology of EHV infections and gives an overview of prevention, control and treatment of EHV infections.

4.2 Etiology

Equine herpesviruses (EHVs) are a group of 11 viruses of the family *Herpesviridae* causing infections in equines, including nine equine herpesviruses (EHV1–EHV9) and two asinine herpesviruses (AHV4–AHV5) (Davison et al. 2009, Davison 2002; Fortier et al. 2010). EHVs reported so far belong to subfamilies *Alphaherpesvirinae* and *Gammaherpesvirinae* and none belong to subfamily *Betaherpesvirinae* (Table 4.1).

EHV1 and EHV4 have linear double-stranded DNA genomes that are approximately 150 (EHV1) and 145 (EHV4) kbp in size, respectively, with a unique long region (U_L) and a unique short region (U_S). The U_S is flanked by identical pair of terminal repeat (T_R) and internal repeat (I_R) regions. Sequence analysis showed

Table 4.1 Classification and diseases associated with equine herpesviruses

Species	Subfamily	Genus	Disease
EHV1	α	*Varicellovirus*	Rhinopneumonitis, abortion, myeloencephalopathy
EHV2	γ	*Percavirus*	Respiratory tract infection, keratoconjunctivitis, malaise
EHV3	α	*Varicellovirus*	Coital exanthema
EHV4	α	*Varicellovirus*	Rhinopneumonitis
EHV5	γ	*Percavirus*	Equine multinodular pulmonary fibrosis
EHV6	α	Unassigned	Coital exanthema
EHV7	γ	Unassigned	NA
EHV8	α	*Varicellovirus*	Rhinitis
EHV9	α	*Varicellovirus*	Gazelle and equine neurological disease

α, *Alphaherpesvirinae*; γ, *Gammaherpesvirinae*

55–84% DNA homology at nucleotide level and 55%–96% homology at amino acid level (Teleford et al. 1992, 1998). The genomes of both viruses encode for 76 homologous genes, with three duplicated genes in EHV4 and four duplicated genes in EHV1 within the repeat regions (Teleford et al. 1992). Two more genes were reported for EHV1 genome as regulatory genes IR2 (ORF77) (Kim et al. 2006) and IR3 (ORF78) (Holden et al. 1992). Thus, EHV1 genome has a total of 78 ORFs, including six regulatory genes 64, 65, 66, 67, 77 and 78 that are present as duplicate genes both in the internal and terminal repeats of the EHV1 genome (Shakya et al. 2017). The expression of EHV1 and EHV4 genes takes place in an orderly and tightly controlled cascade and is accordingly categorized into immediate-early (IE) or α-genes, early (E) or β-genes and late (L) or γ-genes. The regulatory genes playing an important role in coordinated gene expression in EHV1 include one immediate-early protein (IEP, ORF64), four early proteins (IR2, truncated ORF64; EICP0, ORF63; UL5; and IR4) and one late tegument protein (equine α-*trans*-inducing factor, ETIF) (Charvat et al. 2011).

EHV2 has a 184-kbp genome and 79 open reading frames (ORF) encoding 77 proteins, while EHV5 has a shorter, 179-kbp genome (Agius et al. 1992). Both are distinct viruses with only 60% shared identity at DNA and amino acid levels between all the conserved EHV2 and EHV5 sequences (Agius et al. 1994). The EHV2 genome has unique central region (149 kbp) flanked at both ends by long (17.5 kbp) direct terminal repeats. The unique central region has a pair of unrelated internal, short inverted repeats at separate locations. However, the 179-kbp genome of EHV5 lacks both internal and terminal sequence repeats (Allen and Murray 2004).

4.3 Clinical Syndromes

Respiratory Illness Respiratory disease caused by EHV is widespread among young horses, in the period between weaning and 2–3 years of age. Exposure to EHV occurs through inhalation of aerosolized virus-infective respiratory secretions. The virus multiplies in the epithelia of the nasal cavity, pharynx, trachea and

bronchi, causing primarily upper respiratory tract disease. The infection of the lower respiratory tract may also result from dissemination through airway surfaces or via blood vessels and cell-associated viraemia. The clinical signs include acute fever, inappetence, serous nasal discharge and cough.

Abortion Abortion can be initiated either by exogenous infection or by reactivation of latent virus. EHV1 infection leads to late-gestation abortion, stillbirth and weak neonatal foals. EHV1 replicates in endothelial cells and induces thrombosis and ischaemia in the microcotyledons of the placenta, causing abortion (Smith et al. 1992). Sometimes, a live foetus may be born if infection occurs in later stages of pregnancies. Such foals die but soon after birth due to respiratory distress, pneumonia and other respiratory complications.

Equine Herpesvirus Myeloencephalopathy Neurological disease can affect horses of all ages, including unweaned foals, and often horses exhibiting neurologic diseases can shed the virus in their nasal secretions and transmit the disease to in-contact animals (Henninger et al. 2007). Clinical signs of EHM usually occur 6–10 dpi following the onset of viraemia. It includes fever, ataxia, paresis/paralysis of hind limbs, bladder dysfunction, urinary incontinence and sensory deficit in the perineal area. In addition ventral oedema, scrotal or preputial oedema in male horses and limb oedema are also noticed.

The ORF30 spanning the nucleotide region 51522-55184 (3662 nt) in EHV1 genome encodes for a protein referred to as *Pol*, the putative DNA polymerase catalytic subunit, which possesses DNA synthesis activity. This gene is highly conserved throughout its length. Recently, a single nucleotide polymorphism (SNP) of guanine (G) for adenine (A) at 2254 nucleotide position of the ORF30 region resulting in an amino acid variation, from asparagine to aspartic acid (N/D752), has been proven to be associated with the neuropathogenic potential of the EHV1 strain (Nugent et al. 2006). This DNA polymerase enzyme of EHV1 has two sets of identical protein subunits, each of which contains two catalytic pockets (Liu et al. 2006), serving as site for polymerase activity and the site for 3′–5′ exonuclease activity. In EHV1 neuropathogenic strains, the point mutation results in a switch from no charge to a negative charge and induces a conformational change within the viral polymerase structure and thereby increases the replicative capacity of the virus and produces significantly higher viral loads (Nugent et al. 2006; Liu et al. 2006).

4.4 Equine Herpesvirus Latency

Herpesviruses establish life-long latent infection within the host-specific tissues (Hogk et al. 2013). During latency, expression of viral genes is highly restricted with expression of few or no viral proteins. EHVs belonging to *Alphaherpesvirinae* establish latency in neurological tissues, lymphoid tissues and peripheral blood leucocytes (PBL). On the other hand, EHVs belonging to *Gammaherpesvirinae* establish latency

in lymphoid tissue and PBL (Ma et al. 2013). In EHVs, only a few transcripts of early genes are transcribed and none of early and late genes are expressed during latency. This enables the virus to evade the radar of the host immune system. The virus from the latently infected animals can be reactivated to the productive infection by stress and administration of corticosteroids or other drugs (Barrandeguy et al. 2008).

4.5 Pathogenesis

Following inhalation of infectious aerosol or contact with infectious fomites, EHV1 and EHV4 infect and replicate in mucosal epithelium of the respiratory tract. Within 24 h, the virus is transported via infected leucocytes to lymph nodes associated with the respiratory tract and from there into the blood circulation in monocytes and T lymphocyte. Subsequently, the virus moves to different target organs and replicates in endothelial cells of target organs. EHV1 replicates in endothelial cells of the uterus, showing marked thrombosis and ischaemia, which is supposed to be the primary cause of abortion. The virus might cross the placenta and infects foetus leading to late-term abortion. In some cases, fetus may be born alive if EHV1 infection occurs at a later stage of pregnancy, but it does not survive more than 24 h (Paillot et al. 2008; Patel and Heldens 2005). Similarly in CNS, the virus induces myeloencephalitis by replicating in endothelial cells (and not in neural cells), leading to the development of nervous disorders due to equine herpesvirus myeloencephalopathy (EHM) (Azab and Osterrieder 2012).

There is a strong association between EHM and the G_{2254} mutation in ORF30. However, this nucleotide substitution is not the only determinant of neurological disease. A number of EHV1 strains with A_{2254} genotype have been isolated from EHM cases. Similarly, G_{2254} genotype EHV1 isolates from numerous horses with no evidence of neurological symptoms have been identified. One of the possible reasons for this observation could be the fact that besides $A_{2254} \rightarrow G_{2254}$ substitution, other non-synonymous nucleotide substitutions in ORF30 can also have effect on the production of neurological disease by either enhancing or attenuating the capability of viral replication rates in vivo.

Furthermore, DNA polymerase is only one out of six proteins involved in 'elongation complex' of DNA replication machinery. Substitutions occurring in the ORF of any one of these proteins could have a considerable impact on viral replication rates, which will in turn have an effect on neuropathogenicity. This is an area of research that needs further investigation. Comparative whole genome sequencing of neuropathogenic EHV1 strains from different geographical locations might decipher other markers related to neuropathogenicity.

During latency, the virus reaches to the site (lymph nodes, PBL, trigeminal ganglia, etc.) and viral genome translocates to nucleus of target cells, circularizes and maintains as episome without integrating into the host genome. Horses with latent EHV1 infection periodically experience viral reactivation and shed the virus in respiratory tract secretions. Viral reactivation occurs due to stress during transport, weaning, racing or intensive management practices or corticosteroid treatment (Slater et al. 1994).

The viral transcription and translation is blocked during latency, except for transcription of latency-associated transcripts (LATs) from the region antisense to immediate-early (IE) genes (Allen and Murray 2004, Preston and Efstathiou 2007). These LATs have been thought to play a role in latency by promoting cell survival by inhibiting apoptosis or by down-regulating the expression of viral genes. The molecular mechanisms by which LATs produce such function are poorly understood. The miRNAs are also thought to play a pivotal role in establishment and maintenance of latency. Currently, the role of miRNAs in alphaherpesviruses is known for six viruses (HSV1, HSV2, herpes B virus, BoHV1, BoHV5, pseudorabies) (Jurak et al. 2014; Tang et al. 2014). Identification of miRNAs and deciphering their role in equine herpesvirus biology might unravel their role in latency and subsequent reactivation (Gulati et al. 2015).

4.6 Pathology

Allen et al. (1999) described EHV1 infection as a mucosally acquired, infectious viral disease of equines with a multi-organ, systemic pathogenesis causing a spectrum of disease conditions including abortions, respiratory affections, paresis and perinatal foal mortality. It involves epithelium, leucocytes and endothelium in three separate systems/organs/tissues, viz. the respiratory tract, immune system and pregnant uterus.

Foals infected experimentally through intranasal route develop distinct herpetic lesions in all parts of the respiratory tract. The lesions are characterized by the presence of intranuclear inclusions and necrosis of the respiratory epithelium and lymphoid germinal centres. Histopathological changes include inflammation and necrosis of nasal, pharyngeal and occasionally tracheal epithelium along with the presence of intranuclear inclusion bodies (Kydd et al. 1994; Whitwell and Blunden 1992; Allen and Bryans 1986; Edington et al. 1986; Jackson et al. 1977; Prickett 1970). Bryans et al. (1977) suggested that neonatal EHV1 infection causes pneumonitis with extensive depletion and/or degeneration of lymphocytes in the spleen and thymus, which predisposes animals for local secondary bacterial infections. In EHV1-associated perinatal mortality grossly, lungs are voluminous and firm with massive atelectasis, and microscopic lesions include extensive non-suppurative histiocytic-type alveolitis, with acute focal necrotizing bronchitis and presence of intranuclear eosinophilic inclusions (Hartley and Dixon 1979). The gross lesions in the respiratory tract include hyperaemia of nasopharynx and tracheal mucosa, purulent mucus present in the trachea, oedema of the nasopharyngeal mucosa and little change in the pharyngeal lymphoid follicles (Sutton et al. 1998).

Lesions in aborted foetus differ depending on the stage of gestation (Prickett 1970). The early abortions are characterized by a severely autolysed foetus, with presence of numerous intranuclear inclusions without a local inflammatory response. In contrast, the prominent macroscopic lesions in late abortions include jaundice and petechiation of visible mucous membranes. In addition, subcutaneous oedema, excessive pleural fluid, pulmonary oedema and splenic enlargement with prominent

lymphoid follicles and white foci of hepatic necrosis are observed. The characteristic microscopic lesions include bronchiolitis, pneumonitis, severe necrosis of the splenic white pulp and focal hepatic necrosis with inflammatory cellular response. The most consistent feature in aborted foetus is necrotizing bronchitis, interstitial pneumonia, focal hepatic necrosis and necrosis of germinal centres in all lymphoid tissues (Prickett 1970). In mares, the only gross lesion observed following foetal inoculation, and before abortion, was distention of the regional lymphatics, whereas after abortion, the most consistent lesion observed was an intense perivascular infiltration of lymphocytes and plasma cells in association with those vessels located just beneath the glandular layer of the endometrium (Prickett 1970). Edington et al. (1991) were the first to demonstrate endothelial cell infection and thrombosis in the uterus of pregnant mares. Smith et al. (1992) reported that endothelial infection when associated with severe thrombo-ischaemic necrosis could result in abortion without viral infection of the foetus. Histological lesions in the endometrium include congestion, widespread vascular changes including perivascular oedema, ischemia associated with avascular necrosis and perivascular infiltration of lymphocytes, neutrophils and monocytes (Carlton and McGavin 1995; Smith et al. 1992).

Neurological disease is associated with the thrombi formation in vessels in grey and white matter of the CNS and the leptomeninges. This is followed by focal vasculitis and necrosis of the brain stem, cerebrum and white matter of the spinal cord. Vasculitis lesions involved both arteries and veins, which led to ischemia of white matter in brain and left ventral white columns of the spinal cord (Jackson et al. 1977; Edington et al. 1986; Whitwell and Blunden 1992; McCartan et al. 1995; Walker et al. 1999).

4.7 Experimental Animal Models for EHV1

The earlier studies have documented that EHV1 induces abortions in rabbits, guinea pigs and hamsters, but infection is not characterized well (Dimock et al. 1942). The hamsters were used for purpose of passaged KyD strain (Stokes et al. 1989), passive protection (Wilks and Coggins 1977), monoclonal antibodies for viral glycoproteins (Stokes et al. 1989; Shimizu et al. 1989; Sinclair et al. 1989) and response of antiviral agents and also for the virus-induced immunosuppression studies (Rollinson and White 1983). The baby mice were earlier used for the adaptation of EHV1 by intracerebral inoculation, and intranuclear inclusions were observed in the neurological cells (Hatziolos and Reagan 1960). The 2-day-old unweaned mice have been used to distinguish EHV1 from EHV4 (Patel and Edington 1983).

Awan et al. (1990) developed a murine model using BALB/c mice infected intranasally under general anaesthesia which mimics many of the features of EHV1 in the natural host. Abortions could also be produced in this model (Awan et al., 1991, 1995). The clinical signs observed in mice model include body weight loss, hunched posture, depression, dyspnoea, ruffled fur, crouching in corners and neurological signs (Awan et al. 1990, 1991; Inazu et al. 1993, Singh et al. 2009). Awan et al. (1990) reported that mice lost weight from 12 hrs pi, and the preinoculation body

weights were not regained until at least 14 days pi. Baxi et al. (1996) could reisolate virus from lungs and nasal turbinates till 8 dpi with maximum titre on day 3. The lung histopathology in EHV1-infected mice was characterized by acute focal alveolitis and bronchiolitis, eosinophilic intranulear inclusion bodies in bronchiolar epithelial cells, focal necrosis of pneumocytes and perivascular and peribronchiolar infiltration of inflammatory cells (Awan et al. 1990; Field and Awan 1990; Walker et al. 1998).

The mouse model has been used for the molecular pathogenesis and virulence of EHV1 by the recombinant mutants. The EHV1 mutants devoid of glycoprotein B or M were apathogenic for mice but induced protection against challenge infection (Neubauer et al. 1997). The EHV1 gD deletion mutant induced a protective immune response (Csellner et al. 2000). Frampton et al. (2004) reported the role of gE and gI in neurovirulence with the help of KyA mutants in CBA mouse model. The role of the gp2 in the virulence of EHV1 has been revealed with help of recombinant mutants in the mouse model (vonEinem et al. 2004; Smith et al. 2005). vonEinem et al. (2007) reported that gG deletion mutant was successfully studied in mice and compared with the parent virus RacL11. Kasem et al. (2010) documented mouse encephalitis model for studying the role of ORF37 with the help of recombinant mutants.

4.8 Laboratory Diagnosis

Accurate and timely confirmatory diagnosis of EHV infection in horses is important as clinical signs of respiratory disease, abortion and neurological disease can be caused by several other equine viral and bacterial pathogens. The diagnosis of EHV infection is currently based on at least one of the following criteria: clinical symptoms, cerebrospinal fluid examination, serological testing, virus isolation, molecular detection methods and post-mortem examination. Differential diagnosis should also be made from other viral causes of encephalitis, rabies, protozoal myeloencephalitis and different plant/chemical intoxications (Pusterla et al. 2009; Pusterla and Hussey 2014). Definitive laboratory diagnosis of EHV infection relies on collection of right clinical samples at appropriate time and can be done either by direct detection of virus (virus isolation) or demonstration of viral antigen or viral nucleic acid or indirectly through serologic evidence of recent infection.

4.9 Collection of Clinical Samples

Samples should be collected from in-contact febrile horses, which might not be showing any other clinical signs at the time. The nasopharyngeal swabs must be collected in the febrile phase of respiratory infection (1–5 days post-infection). Virus can also be detected in the blood sample (in EDTA) and cerebrospinal fluid on appearance of neurological signs. The brain and spinal cord samples collected at autopsy are useful for confirmation of viral DNA by PCR. The placenta, lungs, liver, spleen and thymus of aborted foetus should be collected aseptically for virus

detection and also in 10% buffered formalin along with the spinal cord and brain from suspect cases of EHM for histopathology and immunohistochemical (IHC) examinations. Samples for virus isolation should be stored and transported at 4 °C (Balasuriya et al. 2015).

Virus Isolation Virus isolation has been the gold standard for diagnosing EHV1 and EHV4 infections. EHV1 isolation can be done from nasal swabs, bronchoalveolar lavage fluid, aborted foetal tissues (placenta, lungs, liver, thymus, spleen), PBMCs and brain tissues. The virus isolation is done in continuous cell lines such as RK13, equine dermis and BHK21, MDBK and Vero or in primary equine cells (lungs, kidney cells or endothelial cells). EHV1 produces characteristic cytopathic effect (CPE) in infected cell cultures within 5–7 days. The identity of virus isolates must be confirmed by PCR, indirect fluorescent antibody testing (IFAT) or neutralization assays using EHV1-specific antisera or monoclonal antibodies. In our laboratory, EHV1 isolations were made from 24 of 64 abortion cases investigated during 2006–2015 using RK13 cells. Nowadays, PCR has largely superseded virus isolation in most laboratories, as virus isolation is a cumbersome and time-consuming procedure.

Viral Nucleic Acid Detection PCR-based sensitive, specific and rapid assays have been developed to detect EHVs in clinical specimens. A variety of type-specific PCR primers and probes can distinguish between different EHVs (EHV1, EHV2, EHV3, EHV4 and EHV5) (Wagner et al. 1992; Borchers and Slater 1993; Kirisawa et al. 1993; Wang et al. 2007). A sensitive nested PCR assay targeting the glycoprotein B genes (gB) has been described for identification and discrimination of these of EHV1 and EHV4 (OIE 2017). A number of real-time PCR assays targeting various EHV1 genes (gB, gD and ORF30) have been described in the literature. Real-time PCR assays targeting gB and gD are used for measuring the viral load in samples by some laboratories (Pusterla et al. 2009).

Serological Assays Assays to demonstrate antibodies to EHV1 in the serum specimen can also help to demonstrate exposure to EHV1. Serological assays such as virus neutralization test (VNT), complement fixation test and ELISAs are commonly employed for demonstration of significant increase (fourfold or greater) in antibody titres in paired sera taken during the acute and convalescent stages of the disease (OIE 2017). However, because of pre-existing antibodies to either EHV1 or EHV4 as a result of prior infection or vaccination, serologic assays are less useful as a diagnostic tool. Sera from mares that abort or from those of neurologic cases may already contain peak levels of antibodies, and no increase in titres may be detectable in sera collected subsequently.

Commonly employed serological assays (CF, VNT or ELISA) which detect IgG response are not specific and cross-react with antibodies to both EHV1 and EHV4. Therefore, type-specific ELISAs targeting glycoprotein G (gG) have been developed, using epitopes in gG of both viruses which can discriminate between IgG immune responses to two viruses (Crabb et al. 1995; Yasunaga et al. 2000). We have

developed a monoclonal antibody-based type-specific ELISA that is alternate to more cumbersome virus neutralization for screening of large number of equine sera (Singh et al. 2001, 2004). Further, a recombinant glycoprotein G-based ELISA developed in our laboratory is being employed for type-specific EHV diagnosis. EHV1 seroprevalence in 17 states of India was recorded during 2006–2015, and overall 666 out of 12935 (5.15%) equines were found positive for EHV1 antibodies.

Recently, linear immunodominant epitopes in different glycoproteins of EHV1 and EHV4 have been identified, which do not cross-react between two viruses and have been used to develop ELISA that is able to identify horses that had been infected with EHV1 or EHV4 using paired serum samples (Andoh et al. 2013, Lang et al. 2013). We recently developed an ELISA using synthetic peptide corresponding to immunodominant epitopes on glycoprotein E of EHV1 and compared its performance with VNT using EHV1-positive (n=108) and EHV1-negative (n=34) serum samples. The relative sensitivity and specificity of the peptide ELISA vis-à-vis VNT were found to be 96.42% and 87.71%, respectively (unpublished data). Such ELISAs are being further validated with large number of samples for use in routine testing. Virmani et al. (2004) developed immunohistochemical method based on microwave-based irradiation for detection of EHV1 antigen in tissue sections.

Detection of EHM Detection of EHM is not straightforward. Serological and molecular methods mentioned above are unable to differentiate between neuropathogenic and non-neuropathogenic viruses. PCR assays based on ORF30 followed by sequence analysis can be used to differentiate neuropathogenic and non-neuropathogenic EHV1 isolates (Nugent et al. 2006; Allen 2007; Leutenegger et al. 2008; Pusterla and Hussey 2014). More sensitive allelic discrimination EHV1 real-time PCR (Allen 2007; Leutenegger et al. 2008, Smith et al. 2012) and a primer-probe energy transfer method (Malik et al. 2010) were later added to the list of diagnostics for detection of neuropathogenic EHVs. A single nucleotide polymorphism-based real-time PCR has been developed in our laboratory that is able to differentiate neuropathogenic and non-neuropathogenic EHV1 strains. Analysis of the A/G SNP by using this assay and sequence analysis revealed that 54 of 56 (96.43%) of EHV1 in aborted mares were of the non-neuropathogenic genotype (A2254), while two (3.57%) had the neuropathogenic marker (G2254). This has been the first report circulation of neuropathogenic EHV1 among Indian equine population (Anagha et al. 2016).

Detection of Latency Ante-mortem diagnosis of latent EHV infection is a major challenge clinically as well as by the currently available diagnostic methods. This is because the viral proteins are not expressed by latently infected cells, thus escaping immune detection, and numbers of latently infected cells are rare (1 per 10^4 or 10 (Allen 2007)). Latent infection in the experimentally infected ponies can be detected by reactivation of infection following corticosteroid treatment and by in vitro co-cultivation of lymphoid tissues, which provide the unequivocal evidence of latent infection (Ma et al. 2013; Gulati et al. 2015).

The latency can be detected by showing the expression of latency-associated transcripts (LATs) in total RNA derived from equine PBL or neural tissues (Baxi et al. 1995). RT-PCR (Chesters et al. 1997, Borchers et al. 1999, Allen et al. 2008) and real-time PCR (Pusterla et al. 2009) have been developed for demonstration of LAT in equines.

Latency can also be demonstrated by expression of late structural genes. During latency, EHV DNA is present in selected tissues but no transcription of late proteins takes place. The latency is confirmed when tissue samples are found PCR positive for the late structural glycoprotein B (gB) DNA in the absence of detectable gB mRNA in the samples (Pusterla et al. 2012). In our laboratory, we have developed RT-PCR and quantitative PCR for detection of latent infection in equines. Using these assays, 59 out of 113 (52.2%) of equines in northern India were found to be latently infected (unpublished data).

4.10 Control

Prevention of EHV infections can be done by vaccination. Available EHV1 vaccines are licensed for prevention of EHV respiratory disease and/or abortion. In addition, there are at least 12 multivalent EHV1 and EHV4 inactivated and modified live vaccines available in the market, which only provide protection against respiratory diseases due to EHV1 and EHV4. EHV1 is considered to be genetically and antigenically stable, and no impact of strain variation on vaccine efficiency has been demonstrated. For preventing respiratory infection, vaccination of foals is done around 3–5 months of age, with a second immunization within 4 to 6 weeks, followed by booster vaccination every 3 or 6 months, depending on type of vaccines. To avoid EHV1-induced abortion, it is recommended to vaccinate pregnant mares at fifth, seventh and ninth months of pregnancy.

Inactivated Vaccines Majority of commercially available vaccines have been inactivated whole virus or subunit vaccines (Table 4.2). Viruses are usually inactivated by formaldehyde or β-propriolactone. Many subunit vaccines and recombinant vaccines targeting gB, gC, gD or gH of EHV1 have been tested in experimental animals, and they produce VN antibody and afford protection (Paillot et al. 2008). ICAR-National Research Centre on Equines has developed an inactivated oil-adjuvanted vaccine using an indigenous isolate, which is very effective in controlling abortions mediated through EHV1. The vaccinated pregnant mares show good immune responses as estimated through serum neutralization assay.

DNA Vaccines Studies on antigenicity and efficacy of DNA vaccination against EHV1 infection have shown promising results in mice. A DNA vaccine encoding gD has elicited humoral and cell-mediated immune responses and reduced respiratory lesions, virus shedding and abortion induced on challenge infection (Walker et al. 2000). However, results on vaccine trials in ponies have not shown much protection against challenge in one trial. The glycoprotein gene-vaccinated

Table 4.2 Commercially available equine vaccines for control of diseases caused by EHV1 and EHV4

Vaccine	Manufacturer (market)	Vaccine components[a] and type	Protection claim[b]
Duvaxyn EHV1/4	Fort Dodge (Europe)	EHV 1 and EHV4, inactivated	Abortion and respiratory disease
Equiffa	Merial (Europe)	EHV1, EIV-1 and EIV-2, inactivated	Respiratory disease
Equi Guard	Boehringer Ingelheim (USA)	EHV1 and EHV4, inactivated	Respiratory disease
EquiVac EHV1/4	Fort Dodge (USA)	EHV1 and EHV4, inactivated	Respiratory disease
Fluvac EHV4/1	Fort Dodge (USA)	EHV4, EHV1, EIV-1 and EIV-2, inactivated	Respiratory disease
Fluvac Innovator 5	Fort Dodge (USA)	EEV and WEE, EIV, EHV1 and EHV4 and tetanus	Respiratory disease
Pneumabort K +1B	Fort Dodge (USA)	EHV1, inactivated	Abortion and respiratory disease
Prestige	Intervet (USA)	EHV1 and EHV4, inactivated	Respiratory disease
Prestige II	Intervet (USA)	EHV1, EHV4, EIV-1 and EIV-2, inactivated	Respiratory disease
Prestige V	Intervet (USA)	EHV1, EHV4, EIV-1, EIV-2, EEEV, WEEV and tetanus, inactivated	Respiratory disease plus
Equigard-Flu	Boehringer Ingelheim (USA)	EHV1, EHV4, EIV-1 and EIV-2, inactivated	Respiratory disease
Double-E FT EHV	Fort Dodge (USA)	EHV1, EHV4, EIV-1, EIV-2, EEEV, WEEV and tetanus, inactivated	Respiratory disease plus
Prodigy	Intervet (USA)	EHV1, inactivated	Abortion
Resequin	Intervet (Europe)	EHV1 and EHV4, inactivated	Respiratory disease
Resequin Plus	Intervet (Europe)	EHV1, EHV4, EIV-1 and EIV-2, inactivated	Respiratory disease
Rhinomune	Pfizer (USA)	EHV1, modified live RacH strain	Respiratory disease
Rhino-Flu	Pfizer (USA)	EHV1, modified live, EIV-1 and EIV-2, inactivated	Respiratory disease
Prevaccinol	Intervet (Germany)	EHV1, modified live, RacH strain	Respiratory disease
Duvaxyn EHV1/4	Pfizer (USA)	EHV1 and EHV4, inactivated	Respiratory disease

[a]Abbreviations: EIV, equine influenza virus types 1 (H7N7) and 2 (H3N8); EEEV, Eastern equine encephalitis virus; WEEV, Western equine encephalitis virus

[b]All vaccines administered parenterally, twice 3–4 weeks apart and 6 monthly boosters (respiratory disease claim) and for each single pregnancy at the fifth, seventh and ninth months of gestation (abortion claim). Pneumabort K is no longer on the market. Modified from Patel and Heldens (2005)

ponies showed gD- and gC-specific antibody responses. However, following challenge infection, vaccinated ponies showed clinical signs of disease, indicating EHV1 DNA vaccination-induced limited immune responses and protection (Soboll et al. 2006).

Live Attenuated Vaccines Immunization with a live attenuated virus is expected to stimulate an immune response similar to those induced by infection. Two principal types of EHV1 mutants have been used as live attenuated EHV1 vaccines, namely, thymidine kinase-negative (TK-) and temperature-sensitive (Ts) mutants.

A Ts mutant of EHV1 (clone 147) was derived from a German abortion isolate of EHV1 (strain M8). On intranasal immunization with this attenuated vaccine, ponies developed mild or no clinical signs and had an increased level of VN antibody 6 weeks after the inoculation. After challenge infection, vaccinated ponies developed only mild clinical signs of disease and shed virus, but none of them developed a cell-associated viraemia (Patel et al. 2003). However, there are safety concerns of virus shedding and cell-associated viraemia after vaccination with this vaccine (Ellis et al. 1997).

There are two currently licensed modified live vaccines (MLV): one is based on RacH strain, which has been passaged 256 times on primary swine kidney cells, resulting in genomic alterations, gp2 gene sequence alteration and deletion of IR6 gene. The resultant attenuated RacH gene afforded protective immune response and safety in equines. Another MLV is derived from Kentucky KyA strain that was modified by passage in murine L-M cells, resulting in deletion of gene 1 and 2 and also deletion of Us region deleting gE and gI (Rosas et al. 2006).

The introduction of bacterial artificial chromosome (BAC) technology has facilitated the rapid construction of recombinant attenuated equine herpesviruses (Akhmedzhanov et al. 2017; Azab et al. 2011; Tischer and Kaufer 2012). BACs have been generated and stably maintained in *E. coli*, in which genetic mutations such as point mutations, deletions and insertions can be easily introduced by different mutagenesis methods including RecA- and Red/ET-mediated recombinations. Such mutations can be introduced in BACs for generating attenuated live EHVs for use as vaccine candidates. Functions of different genes like IR6, gE, gI and gp2 have been studied in detail for the attenuation of EHV1 employing BAC technology (Tsujimura et al. 2006, 2009). ICAR-NRCE has also ventured into developing BAC of indigenous isolate of EHV1 and has developed deletion mutant for gE, gI and IR6 which are being studied further for developing modified live vaccine.

Vectored Vaccine Recombinant poxviruses have been widely used for vaccination and poxviruses derived from canarypox or fowlpox virus are commercially available. A modified vaccinia Ankara (MVA) vaccine coding for EHV1 gC was evaluated in hamsters in combination with a DNA vaccination coding for the same protein, which induced both humoral and cellular immune responses, including proliferation and cytotoxic T-lymphocyte (CTL) activity (Huemer et al. 2000). The immunizations of ponies with canarypox-based constructs coding for

gB, gC and gD glycoproteins of the Kentucky strain of EHV1 resulted in marked reduction of virus shedding on challenge with EHV1 (Minke et al. 2006).

An immediate-early (IE) gene of EHV1 has been identified as a potent stimulator of virus-specific CTL responses in ponies. A recombinant poxvirus vector (vaccinia-derived NYVAC strain) or a recombinant modified vaccinia Ankara (rMVA) coding for the IE protein has been used for vaccination in horses in two different studies. Multiple immunizations increased CTL activity and IFNγ synthesis specific for EHV1 compared with unvaccinated ponies. Vaccination conferred significant clinical protection and a significant reduction in EHV1 viraemia (Paillot et al. 2008, Soboll et al. 2010).

4.11 Conclusion and Future Directions

EHVs are the most common infections among horses worldwide; however, their diagnosis, treatment and prevention are very challenging, partly because of the complexity of the virus-host interactions. Unraveling these complexities in EHV biology is important for the development of rational disease control programmes. Prevention and control efforts are also hampered by the fact that many EHV infections occur early in life followed by a life-long latency. Accurate laboratory diagnosis relies on well-coordinated efforts of equine practitioners, horse owners, breeders, diagnostic laboratory personnel and researchers working for refinement of diagnostic approaches. Collection of appropriate specimen at right time and transport to the laboratory are very critical steps in the diagnosis. It is estimated that only in 20% cases do right specimens reach to diagnostic laboratories in perfect condition to facilitate diagnosis. Emphasis needs to be laid on training and infrastructure for sample collection, processing and transport to laboratories. Despite development of molecular approaches for diagnosis, serological diagnosis is still the preferred method for detection of equine herpesvirus infections. The cross-reactivity remains a problem in commonly employed serological assays, and further refinement in diagnostic tools for definite discriminative diagnosis is anticipated.

The development of neurological disease due to EHV1 infection is likely to be multi-factorial. Although there is a strong association between EHM and the G_{2254} mutation, this nucleotide substitution is not the only determinant of neurological disease. DNA polymerase is only one out of six proteins involved in 'elongation complex' of DNA replication machinery. Substitutions occurring in the ORF of any one of these proteins could have a considerable impact on neuropathogenicity. This is an area of research that needs further investigation. Comparative whole genome sequencing of neuropathogenic EHV1 strains from different geographical locations might decipher other markers related to neuropathogenicity. The latently infected animals with EHV1 and/or EHV4 are the major source of persistence of virus in horse population. The virus activity depends on a delicate balance of constraining and activating factors, and minor disturbances that upset this balance seem sufficient to lead the virus towards production of lytic infection. It would be important

to understand the molecular mechanisms for establishment and maintenance of equine herpesvirus latency and reactivation, delineating the role of viral regulatory genes and miRNAs. Further, EHV vaccinations are ineffective in controlling latent infection. For a successful equine herpesvirus control programme, it would be desirable that tools and techniques are developed to prevent or control establishment of latent infection in equine herds.

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Avian Infectious Laryngotracheitis

5

Palanivelu Munuswamy, Asok Kumar Mariappan, Kuldeep Dhama, and Maddula Ramakoti Reddy

Abstract

Infectious laryngotracheitis (ILT), caused by *Iltovirus* or gallid herpesvirus I (GaHV-1) of *Alphaherpesvirinae*, is an acute highly contagious viral respiratory disease of chickens. The disease is characterized by sneezing, expectoration of blood-mixed mucus, severe haemorrhagic tracheitis and conjunctivitis amidst mortality of up to 70% in its acute form, and a milder form shows varying degrees of catarrhal tracheitis, sinusitis and conjunctivitis with relatively low morbidity and occasional mortality. ILT has been associated with colossal economic loss to the poultry industry due to high mortality, reduced egg production, poor growth and expenses spent on vaccination, biosecurity management and treatment to counteract secondary infections. Vaccination and strict biosecurity measures are the principal components in controlling disease. Live attenuated and recombinant viral vector vaccines are commercially available. The live attenuated tissue culture or chicken embryo origin vaccines significantly reduce the mortality; however, these vaccines retain or regain their virulence after bird-to-bird passage leading to outbreaks simulating classical disease in unprotected flocks. The recombinant viral vector vaccines are reported to be a safer alternative, but still they are not completely safe as they often fail to reduce shedding of the challenge virus posing threat of outbreaks. Now, several new strategies to improve the live attenuated as well as recombinant vector vaccines are being evaluated in order to have a better ILT vaccine that would have least residual virulence and mass application potentials.

P. Munuswamy (✉) · A. K. Mariappan · M. R. Reddy
Division of Pathology, ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Bareilly, Uttar Pradesh, India

K. Dhama
Avian Disease Section, Division of Pathology, ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Uttar Pradesh, India

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Infectious laryngotracheitis · Gallid herpesvirus 1 · Pathogenesis · Immunopathobiology · Diagnosis · Vaccine strategies

5.1 Prologue

Avian infectious laryngotracheitis (AILT) or infectious laryngotracheitis (ILT) is one of the acute contagious respiratory viral diseases of chickens caused by gallid herpesvirus I (GaHV-1) which belongs to the genus *Iltovirus*, within the subfamily *Alphaherpesvirinae* of *Herpesviridae* family. This virus is commonly recognized as infectious laryngotracheitis virus (ILTV) (Davison et al. 2009) worldwide. This was the first major avian viral disease for which an effective vaccine was developed. Although chickens are considered to be the primary target host (Bagust et al. 1986), natural disease has been reported in peafowls and pheasants (Crawshaw and Boycott 1982; Hanson and Bagust 1991). Other species, including closely related Galliformes, are refractory to infection, and birds such as crows, ducks, pigeons, sparrows and starlings seem to be resistant (Guy and Garcia 2008). In turkeys, experimentally induced infection has been reported, but the infection was associated with an age-related resistance (Winterfield and So 1968). All the ages are susceptible, as early as 3-week-old birds, but the most characteristic signs and lesions of disease are seen in adult chickens. In chickens, two main forms of ILT have been described under field conditions which include the severe acute or epizootic form characterized by significant respiratory distress, sneezing, expectoration of blood-mixed mucus, severe haemorrhagic tracheitis and conjunctivitis accompanied by high mortality reaching up to 70%, (ranging from 5% to 70%) and a milder form characterized by mild to moderate catarrhal tracheitis, sinusitis, conjunctivitis, relatively low morbidity and occasional mortality which usually ranges between 0.1 and 2% (Ou and Giambrone 2012).

ILT can cause colossal economic loss to the poultry industry due to markedly high mortality in its acute form, reduced egg production, poor growth and expenses spent on vaccination and biosecurity measures and also on therapy to counteract secondary infection by other avian pathogens (Guy and Bagust 2003; Jones 2010; Guy and Garcia 2008; García et al. 2013). It is one of the OIE-listed diseases which need mandatory notification. Control of ILTV infection relies on regular vaccination and strict biosecurity measures. Several types of vaccines, such as live attenuated vaccines produced by sequential passages in tissue cultures (tissue culture origin, TCO) or embryonated chicken eggs (chicken embryo origin, CEO) and recombinant/mutant vaccines, have been used to control outbreaks; however, the attenuated vaccines retain or regain their virulence after natural bird-to-bird passage leading to a milder vaccinal laryngotracheitis (VLT) or outbreaks simulating classical ILT in unprotected flocks (Piccirillo et al. 2016).

5.2 Etiology: Virus-Structure/Genome Organization and Types/Variants

Infectious laryngotracheitis virus (ILTV) is a member of the genus *Iltovirus*, within the subfamily *Alphaherpesvirinae*, under *Herpesviridae* family (Thureen and Keeler 2006; Davison 2010). Under electron microscopy, ILTV particles exhibit orphology typical of herpes virions which consists of a DNA-containing core within an icosahedral capsid closely surrounded by a proteinaceous tegument layer and an outer envelope anchored with viral glycoproteins (Cruickshank et al. 1963; Watrach et al. 1963; Roizman and Pellett 2001). The size of the viral capsid is about 100 nm in diameter, and the complete viral particle size is within the range of 200 to 350 nm (Granzow et al. 2001). The virus genome is made up of a linear dsDNA of about 150–155 kb which comprises a unique long (UL) and unique short (US) region and an inverted internal (IR) repeat and a terminal (TR) repeat. The GC (guanine and cytosine) content of ILTV genome is 48.2%, and the entire ILTV genome encodes at least 80 predicted open reading frames (ORFs). Out of these ORFs, nearly 65 ORFs are located within the UL region and nine within the US region, while the inverted repeats contain only three genes (ICP4, US10 and ORFs 4/3) (Leib et al. 1987; Andreasen et al. 1990; Johnson et al. 1991; Piccirillo et al. 2016) (Fig. 5.1). Among these 80 ORFs, 63 ORFs display homologies to genes of herpes simplex virus-1 (HSV-1), the prototypic alphaherpesvirus, with respect to position of genome and overall structure of the deduced translation products. Eleven of the highly conserved ORFs encode for glycoproteins gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL and gM which are homologues to HSV-1. Between the UL2 and UL3 regions, the ILTV genome contains an additional ORF (UL3.5) which is not present in HSV genomes,

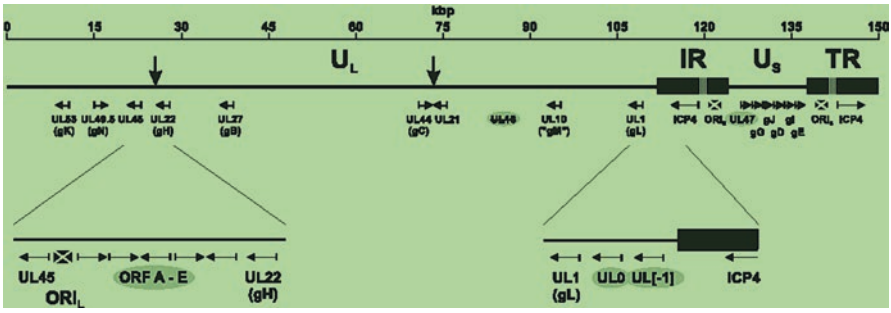

Fig. 5.1 Map of the dsDNA genome of ILTV which is around 155 kb in size. It consists of long and short unique regions (UL, US) with inverted and terminal repeats (IR, TR) flanking the US region. The IR and TR regions contain variable copy numbers of direct repeat elements (vertical lines). Compared to most other alphaherpesvirus genomes, a part of the UL region is inverted (bordered by vertical arrows). Origins of DNA replication (ORIL, ORIS) and selected genes (horizontal arrows), including those of conserved glycoproteins (gB to gN), are indicated. Enlarged sections show two clusters of *Iltovirus*-specific genes (ORF A–E, UL0, UL(-1)). Other specific features of ILTV are the translocation of UL47 to the US region and the absence of a UL16 homologue (highlighted). (Adapted from Fuchs et al. 2007)

but conserved in most other mammalian and avian alphaherpesviruses (Fuchs and Mettenleiter 1996). Further, the ILTV genome displays other differentiating features such as the absence of a UL16 or its homologue which is otherwise conserved in all herpesvirus subfamilies (Roizman and Knipe 2001) and localization of a homologue of UL47, the major tegument protein, between the US3 and US4 genes within the US region instead of being located within the UL genome region (McGeoch et al. 1988; Wild et al. 1996). Besides this translocation of major tegument protein, the genome of ILTV also shows a large internal inversion of a conserved gene cluster within the UL region (Ziemann et al. 1998). This inversion includes the UL22 to UL44 genes which is similar to a previously described inversion of the UL27 to UL44 gene region of pseudorabies virus (PRV) genome (Fuchs et al. 2007). Recently, an inversion within the UL genome region identical to that of ILTV and a translocation of UL47 have been found in the genome of psittacid herpesvirus 1 (PsHV-1), the etiology of Pacheco's disease in parrots (Thureen and Keeler 2006).

Among the avian alphaherpesviruses, the genome of PsHV-1 has been identified to be in close cognate with that of ILTV till date because several ORFs in PsHV-1 do possess homologous genes of ILTV which were earlier considered to be present only in ILTV. Besides several poorly conserved genes in the US region and IR/TR sequences, a cluster of five ORFs designated as ORF-A to ORF-E specific for ILTV and PsHV-1 are found to be localized in the UL region (Fuchs et al. 2007); however, these genes share no significant homology with any other viral or cellular genes. Two ILTV-specific genes designated as UL0 and UL(-1) show marked similarities in the deduced amino acid sequences, suggesting that these genes presumably might have resulted from a duplication event. Interestingly, the PsHV-1 genome seems to contain only UL(-1) but not UL0 (Thureen and Keeler 2006). Due to these unique features, the ILTV and PsHV-1 genome align in a separate branch within other herpesvirus phylogenetic tree. ILTV has been considered as the only member of *Iltovirus* genus within the *Alphaherpesvirinae* (Davison et al. 2005), but sequence similarities between ILTV and PsHV-1 clearly indicate that these two viruses could possibly have a common genetic lineage, and hence the PsHV-1 should also be included in *Iltovirus* genus (Fuchs et al. 2007). Comparative analysis of ILTV genome with other alphaherpesviruses reveals marked homology with several genes of herpes simplex virus (HSV) and pseudorabies virus (PRV). The homologous genes which are shared between ILTV and HSV include glycoprotein B (gB) (Kongsuwan et al. 1991; Griffin 1991; Poulsen et al. 1991), glycoprotein C (gC) (Kingsley et al. 1994), glycoprotein D (gD) (Johnson et al. 1995b), ICP4 (Johnson et al. 1995b), ICP27 (Johnson et al. 1995a), glycoprotein K (gK) and DNA helicase complex (Kongsuwan et al. 1993), while the gene glycoprotein X (gX) of ILTV is homologous to that of pseudorabies virus (PRV) (Kongsuwan et al. 1993). All these genes were found to be located in collinear positions with respect to the genome of prototype HSV-1, and analysis of these genes at the level of amino acid identity confirms that ILTV is an ancient member of *Alphaherpesvirinae* of *Herpesviridae* family. Like other mammalian alphaherpesviruses, ILTV has three origins of DNA replication which include OriL positioned within the UL

genome region and two copies of OriS located within the internal repeat (IR) and terminal repeat (TR) sequences (Ziemann et al. 1998). ILT viruses having properties of increased cytolytic infection and high transmission rates can persist in poultry flocks and increase the probability of genetic recombination with other co-circulating viruses. One such example is the emergence of multiple GaHV-1/ILTV virulent strains in Australia due to nucleic acid recombination between Australian vaccine strains (SA2 and A20) and the European Serva vaccine strain (Lee et al. 2012).

Molecular analysis of DNA isolated from Australian and European vaccine ILT viruses had helped to decipher the recombination sites within their genome. Similarly in the United States, analysis of different ILTV isolates by PCR-RFLP revealed nine groups which were segregated in three core clusters. The first cluster comprised of group I, II and III ILT viruses which included the USDA reference strain, TCO vaccine strain and isolates from commercial poultry, respectively, in each group. The second cluster comprised of group IV (the CEO vaccine strain and isolates from commercial chicken) and group V (isolates from commercial chicken). The third cluster comprised of group VI (isolates from commercial chicken) and groups VII, VIII and IX containing isolates from backyard chickens. Phylogenetic analysis of these ILT viruses revealed that the viruses most frequently associated with VLT/ILT outbreaks in the United States were closely related to the CEO vaccines, which have been identified as group V, and a second genetic lineage that is distant from both CEO and TCO vaccines, designated as group VI (Spatz et al. 2012). Complete genome sequencing of ILTV reveals that several regions within the ILTV genome possess more than one sequence, which in some cases exhibited considerable differences.

5.3 Epidemiology

ILT was described for the first time in 1925 (May and Tittsler 1925), and since then the disease has been reported in several countries where it remains as an endemic disease (Hidalgo 2003; Chacon et al. 2010). The continuing trend towards high flock density, shorter production cycles, rearing of multiage and multipurpose group of poultry within limited geographical area and compromised vaccination strategies and lack of biosecurity measures are the important factors which possibly contributed to the increased ILT outbreaks worldwide (García et al. 2013). ILT has been reported from several countries in Asia (China, Georgia, India, Japan, Lebanon, Sabah, Sarawak, Myanmar, Philippines, Taiwan and Uzbekistan), Africa (Cameroon, Uganda), North America (Canada, New Brunswick, Ontario, Mexico, Delaware, Georgia, Maryland, North Carolina, Pennsylvania and Virginia), Central America and Caribbean (Costa Rica, Trinidad and Tobago), South America (Argentina, Brazil, Chile, Peru, Suriname and Uruguay), Europe (Austria, Belgium, Denmark, Germany, Italy, Moldova, Norway, Netherlands, Poland, Sweden, Switzerland and United Kingdom) and Oceania (Australia, Cook Islands, French Polynesia, Guam, Kiribati and New Zealand) (Hidalgo 2003; Chacón and Ferreira 2009; OIE 1999).

From India, ILT was reported for the first time by Singh et al. in 1964 and then serological evidence for ILT has been recorded in several poultry flocks in Central India and Rajasthan during the 1970s (Sharma and Malik 1970). After the 1970s, no case of ILT was documented from India, and the epidemiological status and disease magnitude in Indian chicken population were unknown for more than four decades. Recently, cases of ILT with the history of bloody expectoration from nares, severe respiratory distress characterized by pump handle type of respiration, varying intensities of conjunctivitis and mortality of up to 80% have been reported by poultry farmers and field veterinarians in major poultry belts in India (Gowthaman et al. 2014). Although ILT is prevalent in Indian poultry population, it is underreported or not reported due to various policy issues. The recent reports on ILT outbreaks in India include a field outbreak that occurred in layer farms in Namakkal District, Tamil Nadu, in 2012 (Srinivasan et al. 2012) and a few more reports in the subsequent years (Puvarajan et al. 2013; Sivaseelan et al. 2014; Gowthaman et al. 2014; Surajit et al. 2016). Chickens are considered to be most susceptible natural host of ILTV in which the virulent or reactivated vaccine virus can cause typical characteristic signs and lesions of the disease. ILT affects all age groups of chicken starting from 8 days to 4 years of age (Kingsbury and Jungherr 1958; Jordan 1966; Linares et al. 1994); however, birds over 8 days of age are reported to be highly susceptible (Zavala 2008). GaHV-1/ILTV infections have been reported in pheasants, pheasant-chicken hybrids and peafowls since the early description of the disease in chicken (Crawshaw and Boycott 1982; Kernohan 1931), and experimental induction of respiratory disease in young turkeys by ILTV infection revealed substantial differences in the infectivity and pathogenicity (Winterfield and So 1968). Subclinical ILTV infection in ducks (Yamada et al. 1980) and a clinical form of ILT in a turkey (*Meleagris gallopavo*) have also been reported (Portz et al. 2008). In experimental setup, embryonating eggs of chickens and turkeys were highly susceptible to ILTV and duck eggs were less susceptible, while embryonating eggs from guinea fowl and pigeons were resistant to ILTV infection (Jordan et al. 1967).

5.4 Transmission

The disease is transmitted by horizontal route via contact with the upper respiratory tract and conjunctival mucosa. Primary virus multiplication takes place in the epithelial cells of tracheal mucosa, where it causes mild to severe inflammation characterized by exudation of serous to mucoid discharge, dyspnoea and cough (Coppo et al. 2013). Ingestion of contaminated feed and water can also be a mode of infection; however, the ingested virus needs to pass through the nasal epithelium for effective multiplication and infection (Robertson and Egerton 1981). The ILT virus can be indirectly transmitted via contaminated equipment including the water pipeline, clothing and shoes, litter and manure as well as infected carcasses (Zavala 2008) and mechanically transmitted by darkling beetles, scavenging birds, vermin or dogs in the absence of adequate biosecurity measures (Kingsbury and Jungherr

1958; Jordan 1966). Wind can also increase the spread of disease in endemic areas (Johnson et al. 2005). In intensive commercial production setup, ILTV is well controlled by vaccination, but the virus may persist in backyard and fancier chickens (Bagust and Guy 1997). From acutely infected birds, direct bird-to-bird transmission is rampant than through contact with latently infected or recovered carrier birds. Chickens recovered from the clinical disease or those which have been vaccinated with live attenuated vaccines carry the virus in tracheal and trigeminal ganglions during the course of acute cytolytic infection and develop latency (Bagust 1986). The latent virus can be reactivated either spontaneously after a specified time period, may be after 10–15 weeks, or when the latently infected birds are exposed to stress conditions, including environmental, hormonal and production stress (Hughes et al. 1989). Once the virus has been reactivated, the virus multiplies in latently infected cells and sheds infectious viruses to the predilection site, trachea and conjunctival mucosa, leading to direct bird-to-bird transmission and spread of disease to susceptible birds (Hughes et al. 1991). Mixing of vaccinated and non-vaccinated naive chickens is of particular importance with respect to direct transmission. No vertical transmission occurs, and transmission of virus through the egg shell has not been demonstrated. No typical viraemia has been reported during ILTV infection; however, viral DNA has been detected in different organs including the liver (Bagust et al. 1986), caecal tonsils and cloaca (Oldoni et al. 2009) in experimentally inoculated chickens which indicates systemic spread of the virus. The systemic spread of the virus to non-respiratory sites has been attributed to the ability of ILTV to infect leucocytes (Chang et al. 1973), particularly the macrophages (Calnek et al. 1986; Oldoni et al. 2009). Apart from these factors, lack of biosecurity measures in the farm premises is an important factor that initiates ILTV transmission and often associated to disease outbreaks (Davison et al. 2005). The source of outbreak related to wild virulent virus could possibly be the backyard poultry flocks and long-lived birds (parent stocks/breeders or layers) which have been regularly vaccinated (Zavala 2008) or vaccine strains which have gained virulence after natural recombination events in the target host (Guy et al. 1991).

5.5 Pathogenesis

The ILT virus enters the upper respiratory tract and conjunctival mucosa via aerosol route or by ingestion and establishes initial infection. ILTV primarily multiplies in the epithelial cells lining the upper respiratory tract including the nasal sinuses, nasolacrimal duct, larynx, trachea and epithelium of conjunctival mucous membrane resulting in severe mucosal epithelial damage and necrosis due to cytolytic infection with associated local acute inflammatory reaction. The virus titre at the primary multiplication peaks between 4 and 6 days post-infection, and the virus can be detected in the TRG as early as 2 days during acute phase of cytolytic infection (Bagust et al. 1986; Kirkpatrick et al. 2006a; Oldoni et al. 2009). Although vaccine strains usually are of low virulence, serial passages in natural target host can

produce highly virulent virus within six to ten passages (Guy et al. 1990; Guy et al. 1991). Experimental infection using field as well as vaccine strains of ILTV leads to latency in the TRG which is followed by reactivation and spontaneous shedding of virus for a prolonged period of time (Bagust 1986; Hughes et al. 1987, 1991; Williams et al. 1992).

5.6 Virus Replication

The pattern of virus replication in the case of ILTV appears to be similar to that of human alphaherpesvirus such as HSV-1 with little differences. As the first step, the envelope glycoproteins of ILTV interact with certain host receptors, yet not completely identified, which leads to viral attachment to the target host cells followed by viral envelope-plasma membrane fusion. After virus-cell fusion, nucleocapsid of the virus is released into the cytoplasm and soon transported to the nuclear membrane and then into the nucleus through nuclear pores. Once the dsDNA reaches the nucleus, transcriptional events start leading to viral DNA replication. Since the replication pattern of ILTV closely resembles that of HSV-1, the replication of HSV-1 is briefly reviewed for better understanding of replication events. In HSV-1 replication, the gene expressions in infected cells are immediate-early (IE), early (E) and late (L) transcripts depending upon the order of gene expressions and their dependence on pre-transcription or replication (Roizman et al. 2007), while in ILTV replication, the transcription kinetics of ILTV genes are not strictly maintained and appeared "leaky" when compared to HSV-1 replication because most of the ILTV gene transcripts could be detected in infected cells within 1 hour post-infection, and hence the ILTV replication pattern is reported to be more complex than HSV-1. Therefore, the ILTV transcripts were categorized into immediate-early (IE), early (E), early-late (E/L) and late (L) depending upon the level of expression of different genes and transcription. In ILTV, transcription of more than 74 genes including several enzymes and DNA-binding proteins involved in the regulation of replication was already confirmed; however, these genes are not only involved in replication events but also encode for structural proteins such as capsid, tegument and envelope glycoproteins (Mahmoudian et al. 2011). Subsequent to DNA replication, the viral concatemeric DNA is cleaved into different unit lengths and packaged into nucleocapsids. The nucleocapsid then acquires an envelope when it migrates through nuclear membrane lamellae and then passes through the endoplasmic reticulum and accumulates within cytoplasmic vacuoles. The capsid in cytoplasm associates with the tegument and is re-enveloped during a second phase of budding in the trans-Golgi region. Virions mature in the cytoplasm and the matured enveloped virions are released by either cell lysis or exocytosis (Guo et al. 1993; Mettenleiter 2002). Other unique uncommon features frequently observed during ILTV replication in infected cells include formation of tubular structures and large vacuoles containing virions in the cytoplasm. Besides fewer complete virions, several light (L) particles consisting of tegument and envelope are also being found in the infected cells;

however, these L particles lack nucleocapsids which might be responsible for the low viral titres in tissue culture (Fuchs et al. 2007).

The ILTV has tissue tropism towards the epithelia of the respiratory tract and eyes, although the virus can be detected in various other organs. Within the respiratory system, the laryngeal and tracheal lining epithelial cells are always affected, while other mucous membranes, respiratory sinuses, air sacs and lung tissue may or may not be affected depending upon the route, infective dose and sequelae of infection. Irrespective of the route of infection, the most active viral replication occurs within the tracheal epithelium (Hanson and Bagust 1991) than any other tissue. After infection by the virulent virus, the initial clinical signs begin within 6–12 days under natural field conditions, while under experimental infections involving delivery of virus directly into the trachea or conjunctival mucosa, the onset of clinical signs commences within 2–4 days post-infection. After acute infection, the surviving chickens usually recover from ILT within 7–10 days or may show continued clinical signs which is usually milder. From 10 days onwards until 4–5 weeks after tracheal infection, active ILTV infectivity is considerably reduced and latency is established in sensory nerve ganglions of trachea as well as in TRG. Infection of the conjunctival epithelium occurs regularly in both naturally infected and experimental infections, via spread of contaminated exudates from sick birds or direct instillation of the virus into the eyes, respectively. After local replication in the conjunctiva, the virus reaches the larynx and tracheal tissues including the lining epithelia and sensory nerve ganglions which innervate the trachea and establishes infection. From conjunctival or respiratory tissues, the virus may be spread to other organs by infected macrophages. Occurrence of viraemia during ILTV infection has not been completely understood, although avian macrophages are reported to support virus multiplication in vitro (Von Bulow and Klasen 1983).

5.7 Clinical Signs and Pathology

The clinical signs during lytic infection include mild to severe serous or catarrhal or suppurative conjunctivitis characterized by increased serous or mucoid or mucopurulent ocular discharges, frequent coughing, gasping, dyspnoea with pump hand type of respiration and mortality in severe cases (Coppo et al. 2013; Gowthaman et al. 2014). Other associated signs include decrease in egg production and weight gain. The enzootic or acute form shows high morbidity which can range from 90 to 100% with variable mortality between 5% and 70% (Devlin et al. 2011; Guy and Garcia 2008). During peracute to acute infection, the internal core temperature rises and the fever peaks between 4 and 6 days post-infection. Total leucocyte count during acute infection shows mild to marked lymphopenia and heterophilia (Chang et al. 1997). Gross pathology includes abnormal exudation of serous or mucoid or mucopurulent discharges from conjunctival mucosa and/or tracheal mucosa. In peracute and acute cases, there may be haemorrhagic exudate or even fibrino-diphtheritic membrane in the upper respiratory tract (Guy and Garcia 2008; Tripathy and Reed 1998). Microscopic lesions in acute form of ILT are

characterized by formation of multinucleated syncytial cells with or without intranuclear eosinophilic inclusion bodies which could be detected from 3 days post-infection. The tracheal lumen often contains fibrino-haemorrhagic exudates, necrotic denuded mucosal epithelium with or without syncytia formation and inflammatory cells predominated by heterophils and lymphocytes. Oedema and inflammatory cell infiltration predominated by heterophils and lymphoid cells can be observed in the tracheal lamina propria of acutely dead birds (Guy and Garcia 2008; Guy et al. 1990; Tripathy and Reed 1998; Coppo et al. 2013). These host inflammatory responses are grossly exhibited as catarrhal or serofibrinous or haemorrhagic or fibrinopurulent tracheitis, conjunctivitis and rarely tracheo-bronchitis and airsacculitis, which lead to naming the manifestations as infectious laryngotracheitis (Guy and Bagust 2003).

During this lytic phase of infection, within 2–3 days, the virus invades the trigeminal nerve and establishes latent infection in trigeminal ganglion (TRG) that may remain life-long. The onset of the ILTV latency starts during late acute phase of infection or immediate post-acute phase of infection, which range between 2 and 10 days after tracheal exposure. The ILTV latency is not readily detectable during the first two months after infection; however, from around 3 months onwards throughout the lifespan of the infected chicken, reactivation of latent virus occurs with sporadic shedding of infectious virus into the upper respiratory tract (Bagust et al. 1986; Hughes et al. 1987, 1991). The exact route of infection from the trachea to TRG as well as reinfection of the trachea from TRG is not completely understood although the phenomenon of neural migration of the virus through the trigeminal nerve can be strongly inferred, as this sensory nerve innervates the upper respiratory tract, eyes and mouth (Bubien-Waluszewska 1981). ILTV latency has also been detected in other nerve ganglions which are located on the course of the trachea. Some stress factors such as sudden introduction of other birds and production stress that occurs on the onset of egg laying can cause reactivation of virus at the site of latency, replication of infectious virions and excretion of infectious viruses to primary predilection sites (Hughes et al. 1989; Hughes et al. 1991; Williams et al. 1992; Fuchs et al. 2007; Coppo et al. 2011; Coppo et al. 2013). Recent experimental studies detected presence of the virus in other organs such as the tongue, lung, heart, liver, spleen, thymus, kidneys, pancreas, proventriculus and small and large intestines including caecum, caecal tonsils and bursa of Fabricius and also the brain (Oldoni et al. 2009; Zhao et al. 2014; Parra et al. 2016). Establishment of latency in TRG bypasses the immune clearance by the host leading to persistence of ILTV in the infected or recovered chicken flocks. The establishment of ILTV latency in TRG has been studied by detecting putative latency-associated transcripts (LATs) within the infected cell protein 4 (ICP4) which is a major transcriptional activator (Johnson et al. 1995a), and two microRNAs (miRNAs) map to ICP4 protein. The LATs are reported to downregulate ICP4 expression by cleaving the ICP4 transcript, which plays a crucial role in maintaining the balance between lytic and latent phases as well as mediates viral reactivation (Coppo et al. 2013).

5.8 Immunopathobiology

Infectious laryngotracheitis virus infection in chickens induces a variety of innate and adaptive immune responses which include the production of neutralizing antibodies and induction of an effective and protective cell-mediated immunity. The ILTV-specific neutralizing antibodies can be detected in the serum within 5–7 days of tracheal infection, and the antibody titre peaks at 21 days post-infection and then diminishes over the next several months and persists at low titre for a year or more. Mucosal antibodies, IgG and IgA, could be detected in tracheal secretions and washings from 7 days post-infection onwards (Bagust 1986), but the titre reaches plateau within 10–28 days. Although virus-neutralizing antibodies are detected after ILTV infection, these antibodies are reported to confer very low protective immunity. In experimentally bursectomized birds, although no ILTV-specific antibodies could be detected, the birds were protected against ILTV reinfection, signifying the role of cell-mediated immune responses against ILT (Fuchs et al. 2007). Primary ILT vaccination partially protects against ILTV challenge by 3–4 days post-exposure, and near-complete protection commences after 7 days post-vaccination (Jordan 1981). The primary immunized birds are highly protected against virulent ILTV for a period of 15 to 20 weeks, and thereafter varying degrees of immune protection occur within the immunized flock over the next year. Booster vaccination with live attenuated viruses may or may not help to maintain the protection levels due to the fact that the infectivity of the new attenuated virus may get inactivated at the portal of entry itself (Jordan 1981). Several laboratory research and field studies have independently confirmed that immune protection to ILTV challenge is independent of serum antibody levels, and hence the local cell-mediated immune response in the trachea as well as the conjunctiva chiefly acts as the effector mechanism of protection from ILTV infection. The ILTV latency may be induced and maintained by immune-mediated mechanisms because the ILTV latency is usually established between 1 and 2 weeks after tracheal exposure during which period there is a markedly detectable host immune response against the ILTV infection. Latency is the biological survival mechanism for several herpesviruses and retroviruses in birds which permits the virus to evade immune surveillance by host and escape from being cleared from the body. As immunity diminishes due to various stress factors (Hughes et al. 1989), the latent virus gets reactivated, actively replicates and causes cytolytic infection leading to disease outbreaks even in the vaccinated flocks. In contrast to other alphaherpesvirus latent infections of mammals, experimental immunosuppression caused by immunosuppressive drugs such as corticosteroids or cyclophosphamide (Bagust 1986; Hughes et al. 1989) is not associated with reactivation of latent ILTV in chickens, and the other exogenous or endogenous factors which precisely reactivate the latent ILTV in TRG need to be elucidated.

ILTV evokes an intense inflammatory response in the tracheal and conjunctival tissues, which is crucial in controlling virus replication and regulating pathogenesis, and also directs the subsequent adaptive immune responses to infection by secreting

an array of chemokines and cytokines (Tripathy and Reed 1998). At the site of inflammation, the infiltrating cells and inflammatory exudates significantly impact tracheal aperture leading to respiratory distress of the host. Between 0 and 72 h after experimental infection, there is a mild infiltration of a small number of lymphocytes and heterophils in the lamina propria particularly in areas where syncytia are present. Between days 3 and 5 post-infection, there is severe oedema in the lamina propria and in the underlying tissues, with numerous macrophages and lymphocytes and marked number of histiocytes and plasma cells (Guy and Garcia 2008) throughout the lamina propria. Devlin et al. (2010) have detected clusters of CD4+ and widely scattered CD8+ lymphocytes in the tracheal mucosa by immunohistochemistry and reported that the types of inflammatory cells recruited to the site of infection appear to influence the outcome of infection and possibly regulate the balance of the adaptive response. These findings and the variations in the susceptibility of different chicken lines to ILTV infection and ability to mount a protective immune response suggest that the innate immune factors are crucial to the outcome of ILTV infection (Poulsen et al. 1998). However, the exact role of innate immune response in counteracting ILTV infection in chicken is not completely understood, although transcriptional profiling of host cells is now helping to understand post-infection events which occur during host-pathogen interaction (Lee et al. 2012).

The envelope glycoproteins of ILTV appear to be the most immunogenic antigens capable of eliciting humoral as well as cell-mediated immune responses in chicken (York and Fahey 1990). As mentioned earlier, the cell-mediated immunity is correlated with protection against ILT than humoral immunity (Fahey and York 1990; Honda et al. 1994). Although passive transfer of antibody to offspring has been demonstrated (Hayles et al. 1976), there were no significant differences in the protective immunity level between chicks hatched from immunoglobulin-transferred and control parents (Jordan 1981). Similarly, the mucosal antibodies, IgA and IgG, were also not protective (Fahey and York 1990). Evaluation of immunity between bursectomized and non-bursectomized control birds revealed no differences, but the thymectomized chicken showed much lower protection against ILTV challenge than non-thymectomized control group (Fahey et al. 1983; Honda et al. 1994) which further supports the role of antibody-independent resistance to ILTV infection (Coppo et al. 2013). Among surface glycoproteins, glycoprotein G (gG), a secreted glycoprotein not anchored to viral envelope, is reported to facilitate entry of the virus (Tran et al. 2000) and cell-to-cell spread (Nakamichi et al. 2002) and also function as a broad-spectrum viral chemokine-binding protein (vCKBP) (Bryant et al. 2003). Glycoprotein G (gG) is conserved among most alphaherpesviruses, including ILTV, and reported to play an important role in immune evasion in several poxviruses (Alcami et al. 1998; Alcami and Koszinowski 2000; Coppo et al. 2013, 2018) and murine gammaherpesvirus 68 (MHV-68) (Parry et al. 2000; van Berkel et al. 2000). The secreted gG binds to chemokines of the subfamilies C, CC and CXC and prevents the interaction between chemokines and their respective receptors. It also blocks binding of chemokine to glycosaminoglycans, which is necessary for in vivo chemokine activity (Bryant et al. 2003). The vCKBP of ILTV (gG), during early stages of infection, induces innate immune

responses by recruiting particular subsets of immune cells (Devlin et al. 2010). Administration of cytokine-adjuvanted ILT vaccine resulted in greater CD4+ to CD8+ T-cell ratios, emphasizing the significant role of Th1 cells in the containment of ILTV infection (Chen et al. 2011).

5.9 Diagnostics: Conventional and Modern Techniques

5.9.1 Pathological Diagnosis

Acute form of ILT can be diagnosed based on the characteristic clinical signs, which include nasal discharge, conjunctivitis, gasping, hand pump type of respiration, bloody mucous expectoration and dyspnoea and postmortem lesions such as catarrhal to haemorrhagic tracheitis, fibrinopurulent to caseous exudates or plugs in the larynx and trachea, with mortality ranging from 5% to 70%. The mild form of disease shows mild conjunctivitis, catarrhal tracheitis and reduction in egg production, with less than 2% mortality. Histopathological detection of multinucleated syncytial cells with intranuclear eosinophilic inclusion bodies (Fig. 5.2) and presence of inflammatory cells consisting of heterophils, lymphocytes and macrophages in the larynx, trachea and conjunctival mucosa are diagnostic features of ILTV infection (Guy et al. 1990; Tripathy and Reed 1998; Guy and Garcia 2008).

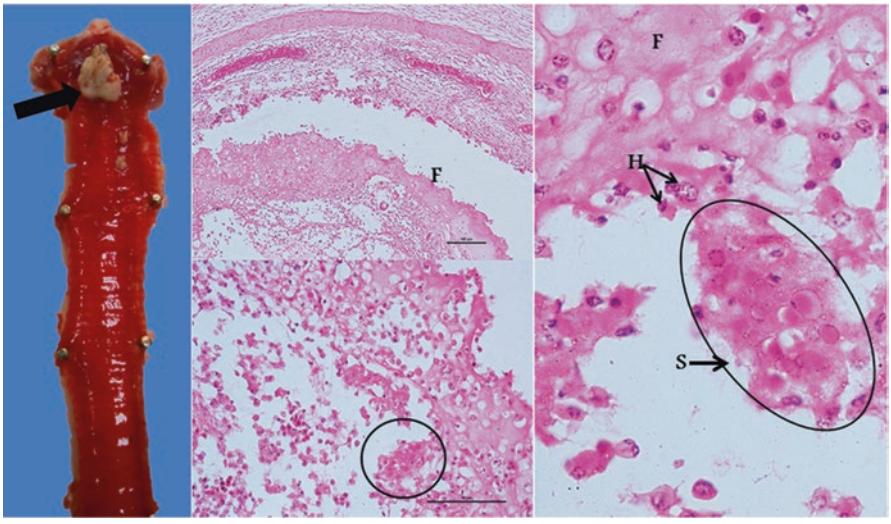

Fig. 5.2 Gross pathology: chicken died of acute ILT showing caseous plug in the larynx (bold arrow) and severely haemorrhagic tracheal mucosa. Histopathology: section of the trachea showing fibrino-haemorrhagic (F) exudates in the lumen, multinucleated syncytia (S) with intranuclear eosinophilic inclusion bodies and heterophils (H)

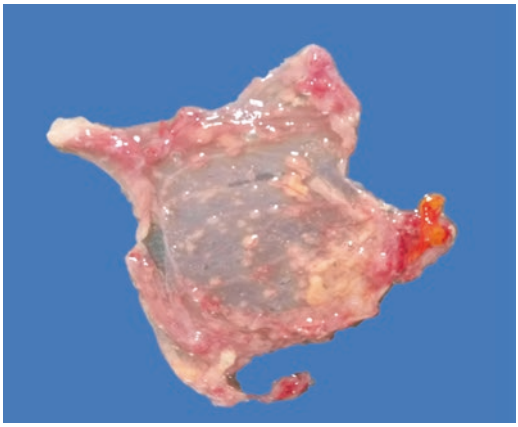

Fig. 5.3 Chorioallantoic membrane (CAM) of chicken embryo infected by ILTV showing severe thickening and multiple greyish plaques to round nodular pock-like lesions (third passage, 3 dpi)

5.9.2 Isolation and Identification

Embryonating chicken eggs (ECE) and cell lines of avian origin are routinely used for isolation of infectious laryngotracheitis virus. When the virus-infected tissue suspension is inoculated on dropped chorioallantoic membrane (CAM) of embryonating chicken eggs, the virus induces opaque plaques with a depressed centre to variably sized pock-like lesions on CAM (Fig. 5.3). As early as 2 days after inoculation, opaque plaques can be observed in ILTV-infected CAM, and death of embryos occurs between 2 and 8 days depending upon virulence and level of passages in homologous host. Generally, the survival time of infected embryos increases with additional passages in homologous host and thus the virus more efficiently propagates. The cell lines used to propagate ILTV include chicken embryo lung cells, chicken embryo liver (CEL) cells, chicken embryo kidney (CEK) cells, adult chicken kidney (CK) cells as well as chicken embryo fibroblast (CEF) cell line. Among these cell lines, the CEL and CK cells are the most preferred cell lines. Although the chicken embryo fibroblast (CEF) cell line is not suitable for primary virus isolation, it has been occasionally used to propagate cell-associated strains of ILTV but with limited success (Thureen and Keeler 2006; Portz et al. 2008). The cytopathic effect (CPE) consisting of increased refractiveness, cellular swelling, displacement of chromatin, rounding of the nucleoli and formation of multinucleated giant cells (syncytia) can be observed in cell culture as early as 4–6 h post-infection. As early as 12 h post-infection, intranuclear inclusion bodies can be detected within syncytia as well as in few isolated cells, and the number of cells with viral inclusions peaks at about 30–36 h post-infection. Large cytoplasmic vesicles are often noticed in CEK cell lines infected with ILTV, which become basophilic mass as the infected cells degenerate (Reynolds et al. 1968). Avian leucocyte cultures obtained from chicken buffy coats are also reported to permit the ILTV replication in which the infected cells reveal nuclear clumping and syncytia formation within 72 h post-infection (Chang et al. 1977).

A continuous cell line, LMH (leghorn male hepatoma) cell line, derived from a chemically induced chicken liver tumor permits ILTV replication if the virus has at least once been adopted in LMH cell line, but it is not suitable for primary isolation (Schnitzlein et al. 1994). However, the LMH cell lines are widely employed in the construction of recombinant ILTV and to study genome of ILTV (Fuchs et al. 2007). Other cell lines such as QT35 or IQ1A, a quail-origin cell line, and Vero cells from African green monkeys do permit limited replication, but the virus titre is reported to be very low and thus not desired. Other systems routinely used to study host-pathogen interaction and for diagnosis are tracheal organ culture (TOC) and conjunctival organ cultures (COC) obtained from chicken embryos or day-old chicks. TOC has commonly been used for diagnosis and host-pathogen interaction and pathogenesis studies of several avian pathogens including ILTV for a long time (Bagust 1986; Jones and Hennion 2008; Reemers et al. 2009; Zhang et al. 2012), and the viability of TOCs is analysed by assessing the ciliary beating and removal of latex beads after infection (Anderton et al. 2004). Similarly, COC have also been used to study host-virus interactions (Darbyshire et al. 1976); the cells infected by ILTV are identified by characteristic cytopathic effect and immunofluorescence staining using ILTV-specific antibodies.

5.9.3 Immunodiagnosis

For serological diagnosis, agar-gel immunodiffusion, immunofluorescence, enzyme-linked immunosorbent assays using different glycoproteins and virus neutralization tests using ILTV-specific antibodies are being routinely used. Monoclonal and polyclonal antibodies against ILTV proteins and gC, gG, gE, gJ and other viral glycoproteins can be used for immunofluorescence, immunohistochemistry, immunoelectron microscopy, radio-immuno-precipitations and western blot assays (Fuchs et al. 2007).

5.9.4 Molecular Diagnosis

Polymerase chain reaction (PCR) or quantitative real-time PCR is the preferred molecular assay for confirmation and quantification of viral load in biological samples (Guy et al. 1992; Williams et al. 1992; Scholz et al. 1994; Abbas and Andreasen 1996; Creelan et al. 2006; Fuchs et al. 2007; Kirkpatrick et al. 2006b; Zhao et al. 2014; OIE Terrestrial Manual 2014; Roy et al. 2015). Differentiation of wild ILTV and vaccine strains is based on restriction length polymorphism (RFLP) profiles of the complete viral genome (Kotiw et al. 1982; Leib et al. 1986; Andreasen et al. 1990; Keeler et al. 1993; Oldoni and Garcia 2007); however, amplification of different regions (thymidine kinase; glycoproteins G, E, X and C; infected cell polypeptide 4 (ICP4); and other glycoprotein coding regions of the ILTV) by PCR followed by RFLP analysis has also been used in characterizing and differentiating ILTV strains (Craig et al. 2017). The advances in DNA sequencing technologies enabled

differentiation of wild and vaccine virus isolates for epidemiological and phylogenetic studies, and probe-based assay such as TaqMan single-nucleotide polymorphism genotyping assay has also been used to study the infectious laryngotracheitis virus recombination events in the natural host (Loncoman et al. 2017). The commonly used oligonucleotide primer and probe sequences in conventional as well as quantitative real-time PCR protocol are presented in Table 5.1.

Collectively, on the basis of gross and microscopic findings, detection of viral antigens in tissues by immunohistochemistry, identification of the virus particles by electron microscopy, detection of cytopathic features in cultured cells or studying embryopathology in chicken embryos, diagnosis of ILT can be made; however, molecular diagnostic tests such as conventional PCR and quantitative real-time PCR are routinely used in confirming the aetiology and estimating virus load. These molecular methods have significant diagnostic value but do not discriminate between viable and non-viable virions (Menendez et al. 2014). Hence, the PCR results need to be interpreted carefully, as positive results indicate only presence of virus nucleic acid but not active infection.

5.10 Prevention and Control: Vaccines, Antivirals and Other Measures

Infectious laryngotracheitis was the first major avian viral disease for which an effective vaccine was developed. The first method of ILT vaccination in poultry was application of tracheal scrapings collected from ILTV-infected chickens into the vent/cloaca using a brush (Gibbs 1934). Thereafter, several types of vaccines including killed vaccine and live attenuated and recombinant vaccines were produced and tested. The live attenuated chicken embryo origin (CEO) and tissue culture origin (TCO) vaccines were the first-generation ILT vaccines attenuated by sequential passages in embryonated eggs or in tissue cultures (Samberg et al. 1971). Later during the 1950s to 1960s, live attenuated ILT vaccines named as Cover and Hudson strains were first discovered in the United States and are widely used even today. These Hudson and Cover strains were commonly categorized as chicken embryo origin (CEO) vaccines, although the method of embryo inoculation for virus propagation was slightly differed between the two vaccines. During the late 1950s, Cover and Benton identified a field ILTV strain of lower virulence which was capable of protecting chicken against challenge ILT virus infection via intra-tracheal inoculation. This low virulent strain was named as CEO Cover strain vaccine (Benton et al. 1958). A decade later, an attenuated ILT vaccine designated as Hudson strain was introduced for intranasal, ocular or intra-tracheal route of administration. Gelenczei and Marty in 1964 have developed the first tissue culture origin (TCO) modified ILT vaccine by subjecting the virulent ASL L-6 strain of ILT virus over a hundred consecutive passages in primary chicken cell cultures. This first TCO vaccine was found to confer protective immunity after ocular or intranasal application (Gelenczei and Marty 1964). Two years later, an Australian field isolate of ILTV was attenuated in chicken embryos by serial passages which resulted in CEO SA2 vaccine strain.

Table 5.1 Conventional and quantitative Real-Time PCR for detection, preliminary typing and quantification of ILTV

Oligonucleotide sequences: 5′ to 3′ direction	T_a	Target gene	Amp. Size	References
Conventional Polymerase Chain Reaction (PCR) for detection and preliminary typing of ILTV				
F-5′-CTGGGCTAAATCATCCAAGACATCA-3′	60 °C	Thymidine kinase (TK)	2.24 kbp	OIE Terrestrial manual (2014)
R-5′-GCTCTCTCGAGTAAGAATGAGTACA-3′				
F-5′-ACTGATAGCTTTTCGTACAGCACG-3′	60 °C	Infected cell polypeptide 1 (ICP4-1)	688 bp	OIE Terrestrial manual (2014)
R-5′-CATCGGGACATTCTCCAGGTAGCA-3′				
F-5′-CTTCAGACTCCAGCTCATCTG-3′	60 °C	Infected cell polypeptide 2 (ICP4-2)	635 bp	OIE Terrestrial manual (2014)
R-5′-AGTCATGCGTCTATGGCGTTGAC-3′				
F-5′-CGTATACCATCCTACAGACGGCA3′	60 °C	Glycoprotein E (gE)	540 bp	Parra et al. (2018)
R-5′-CGTACAATGGTTCGGTCTTGGA-3′				
Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) for detection and quantification of ILTV				
F-5′-CAGTATCTGGCATCGCCTCAT-3′	60 °C	Glycoprotein B (gB)	148 bp	Zhao et al. (2014)
R-5′-CCTGGGAACAGAACCTGAACT-3′				
(Probe: 5′-FAM-CTAACCCGTTCG CCGCACTCG-BHQ-1-3′				
F-5′-CCTTGCGTTTGAATTTTTCTGT-3′	60 °C	Glycoprotein C (gC)	103 bp	OIE Terrestrial manual (2014)
R-5′-TTCGTGGGTTAGAGGTCTGT-3′				
(Probe: 5′-FAMCAGCTCGGTGACCCCATTCTABHQ1-3′				
F-5′-ACGCACATGCCCTCGAA-3′	60 °C	Glycoprotein E (gE)	56 bp	Parra et al. (2018)
R-5′-GGTCCGGGACTGCCAATTA-3′				

Similarly, an isolate obtained from field ILT outbreaks in Israel was attenuated by continuous passages on the chorioallantoic membrane (CAM) of chicken embryos, and this attenuated strain was recognized as CEO Samberg strain intended for vaccination through intra-ocular or vent-brush application methods. By 1983, further attenuation of the Australian CEO SA2 vaccine virus in chicken embryo cell culture yielded the A20 vaccine strain (Kirkpatrick et al. 2006a). During the same time period, European Serva vaccine strain was also introduced for mass vaccination. Live attenuated ILT vaccines were originally approved for application in individual bird by either ocular, intranasal, intra-tracheal or cloacal routes; however with the expansion of the poultry industry, mass application via drinking water or by vaccine spray became a rapid, easy and cost-effective method to accomplish wider vaccine coverage within a short time period. Among several variants of ILT vaccines, TCO vaccine was the only ILT vaccine endorsed for individual application by eye-drop immunization (Gelenczei and Marty 1964). Due to the increased risk of latent infection and economic losses, mostly the layers and breeders are vaccinated against ILT in the United States; however on the other hand, broilers are vaccinated typically during the threat of outbreaks but not on routine basis. With respect to the broiler industry, zone or blanket vaccination with the CEO vaccine had been an effective approach in curbing large outbreaks of ILT (Zavala 2008). These attenuated vaccines significantly reduced mortalities, particularly the chicken embryo origin (CEO) vaccines, and have shown to limit outbreaks of the disease. These attenuated vaccines are administered individually by eye drop in precious stocks or via aerosol spray or drinking water for mass application. Although live attenuated vaccines are efficacious, they retain marked residual virulence, which can be further exalted during bird-to-bird passages in natural target host (Fulton et al. 2000; Han and Kim 2003; Rodríguez-Avila et al. 2008). Thus, several outbreaks of ILT in unprotected flocks were attributed to revertant vaccine strains leading to vaccinal laryngotracheitis (VLT) (Piccirillo et al. 2016). Despite the shortcomings of live attenuated viruses, the CEO attenuated ILT vaccines are still the most commonly used vaccines to control ILT globally as these live attenuated vaccines induce instantaneous and effective immunity and can readily be administered through drinking water or spray when an outbreak is forecasted (Zavala 2008).

The second-generation ILT vaccines, such as the recombinant viral vector vaccines including fowlpox virus (FPV) and herpesvirus of turkeys (HVT) vectors expressing one or several ILTV immunogenic proteins, were first introduced in the early twentieth century. These vectored vaccines are considered safer than attenuated vaccines as they do not regain virulence, and they have been reported to improve bird's production performance and ameliorate clinical signs of ILT (Bublot et al. 2006; Islam et al. 2008; Esaki et al. 2013); however, these vaccines failed to reduce shedding of the challenge virus, resulting in increased likelihood of outbreaks. Live viruses attenuated by deletion of virulence genes or by selection of viral subpopulations with low virulence are being continuously evaluated as potential vaccine candidates (Boettger and Keeler 2004; Devlin et al. 2008, 2010; Garcia 2015, 2017; Garcia et al. 2016). Also new alternative viral vectors such as the LaSota strain of Newcastle disease virus (NDV) that expresses ILTV glycoproteins

have been reported to confer immunity against wild-type NDV and ILTV (Basavarajappa et al. 2014; Zhao et al. 2014). Similarly, modified very virulent Marek's disease virus (vvMDV) that expresses ILTV glycoproteins has been reported to protect against Marek's disease (MD) and ILT; however, these vaccine candidates are being evaluated for practical utility (Gimeno et al. 2011, 2015; Gimeno 2016). The FPV vector vaccine containing glycoprotein B and UL32 genes of ILTV has been used for wing web vaccination of breeders and subcutaneous vaccination of 1-day-old commercial layers in the United States (Davison et al. 2006). Two HVT vector vaccines, one containing ILTV glycoproteins I and D and another containing ILTV glycoprotein B, are commercially available (Esaki et al. 2013). These recombinant HVT- or FPV-vectored vaccines are bivalent vaccines which confer protective immunity against ILTV and Marek's disease (MD) (Gimeno et al. 2015) or ILT and fowlpox (Swayne et al. 1997) depending upon the vector backbone used. Vector vaccines were initially approved for subcutaneous and transcutaneous application, but later they were registered also for in ovo vaccination (Zavala 2008; Johnson et al. 2010; Williams and Zedek 2010; Vagnozzi et al. 2012; Esaki et al. 2013). Utilizing a bacterial artificial chromosome (BAC), genes which encode glycoprotein B (gB) or glycoprotein J (gJ) of ILTV were introduced into meq gene-deficient very virulent MDV (vvMDV) to create the BACDMEQ-gB and BACDMEQ-gJ recombinant strains (Silva et al. 2010; Gimeno et al. 2015) and were tested if they could be used as bivalent vaccines against MD and ILT. The BACDMEQ-gB recombinant vaccine had conferred immunity and protected the birds, after subcutaneous vaccination at one day of age followed by virus challenge at 28 days of age, which was comparable to the immunity conferred by a commercial ILT-HVT-vectored vaccine; however, the BACDMEQ-gJ virus did not protect adequately when it was administered alone, but the enhanced protection was observed when administered in combination with BACDMEQ-gB (Garcia 2017).

The widely used modified live vaccines are capable of establishing latent infections which may be followed by intermittent reactivation and shedding. Serial passage in natural host can lead to reversion to virulence, and vaccinal strains of the virus have been implicated in many outbreaks of ILT. Hence, modified live vaccines should not be used in regions where ILTV is not reported. Critical care must be taken in vaccine storage as well as application to ensure that each bird receives an adequate dose of vaccine. Comparison of protection induced by vector vaccines and live modified vaccines revealed that the vector vaccines applied in ovo as well as through subcutaneous routes have provided partial protection with and partial reduction in clinical signs and virus replication in the trachea. Among recombinant vaccines, the HVT-LT vaccine has been reported to be more efficacious than the FPV-LT vaccine (Vagnozzi et al. 2012), but their limited utility in mass vaccination has been a major constraint. Continuous efforts are being put to improve the quality of live attenuated as well as vectored ILT vaccines to have more stable ILT viruses that can maintain attenuation without insidious reactivation. Despite the adequate immunity conferred by recombinant viral vector vaccines, their slower onset of immunity, reduced or lack of local cell-mediated immunity in the upper respiratory

tract and limited utility in mass vaccination programmes make the recombinant vector vaccines less effective than the live attenuated ILT vaccines (Garcia 2017).

Prior to development of viral vector vaccines, CEO vaccine was the only vaccine available for broilers (Zavala 2008). With the recent advancement in biotechnology, three vector vaccines were developed which had led to the diversification of ILT vaccination programmes. As the broiler industry produces a wide range of birds, i.e. varying sizes of meat-type chicken, use of combinations of live attenuated CEO and recombinant vector vaccines is a routine practice in several developed nations. In situations where outbreaks of ILT cannot be contained only with recombinant viral vector vaccines, the poultry industries will switch to vaccination with live attenuated CEO vaccine. In highly endemic areas, poultry industries utilize recombinant vector products prior to or when an ILT outbreak is anticipated. In other instances, the susceptible populations are first immunized with CEO vaccine and then subsequently with a recombinant vector vaccine to transition out of CEO, while some industries use only CEO vaccine (Garcia 2015). As the weaknesses and strengths of the current ILTV live attenuated and recombinant vector vaccines are being uncovered, the alternative vaccination strategies such as in ovo vaccination using recombinant vector vaccines or mutant vaccine, vaccination at hatch or post-hatch and spray vaccination with a NDV-ILTV recombinant vector vaccine or attenuated CEO vaccine strains should be considered, particularly when vaccinating broiler birds in endemic areas. Layers can be vaccinated with HVT, MDV, FPV and NDV recombinant vector vaccines or mutant vaccine at one day of age followed by vaccination with attenuated CEO viral subpopulations (Garcia 2017).

5.11 Husbandry Methods and Good Practice

Maintenance of strict biosecurity measures is a prerequisite to prevent the introduction of ILTV onto poultry production sites, and site quarantine and disinfection procedures should be placed in practice to prevent the introduction of virus on fomites such as clothing and personnel, vehicles, feed and equipment (Menendez et al. 2014). The poultry housing area should be maintained in such a way to prevent ingress of wild birds, rodents and dogs. Proper record should be maintained to prevent the possibility of vaccinated and non-vaccinated birds being mixed. Contact between commercial poultry and backyard or susceptible fancier birds should be avoided. In the event of an outbreak, the carcasses should be disposed of immediately by burning or burying. Cooperation between the local government and poultry industry can facilitate effective control of ILT outbreaks by promoting rapid diagnosis, timely vaccination and initiation of biosecurity measures and movement controls to minimize further spread.

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Marek's Disease Virus

6

Asok Kumar Mariappan, Palanivelu Munuswamy, Maddula Ramakoti Reddy, Shambhu Dayal Singh, and Kuldeep Dhama

Abstract

Marek's disease (MD) is caused by an oncogenic alphaherpesvirus, a common lymphoproliferative inducing agent usually characterized by mononuclear cellular infiltrates, mostly T-cell lymphomas in various visceral organs and peripheral nerves. The genome is linear and made up of double-stranded DNA molecules of nearly 160–180 kb in size. This was first reported by Dr. József Marek in the year 1907. Various pathotypes exist, and pathotyping is generally done based on the pathology the particular isolate induces in vaccinated and unvaccinated chickens and on their ability to overcome the effects of vaccination. Several avian species including both domesticated and wild birds are susceptible to Marek's disease, and genetic susceptibility/resistance to MD is well characterized in chickens. The disease is highly contagious, and the transmission occurs mainly by the airborne route. The host responds to MDV infection by mounting both innate and adaptive immune mechanisms. The incidence of Marek's disease is variable depending upon the pathotype and host susceptibility. Nerve lesions and visceral lymphomas are the prime pathologic changes noticed in MD. In the field, diagnosis is primarily based on the clinical signs and postmortem lesions. Apart from the above methods, virus isolation, identification of various viral markers in tissues, genomic detection assays (PCR, qPCR, nested PCR), and antibody detection (ELISA) aid in diagnosis of MD. Some of the strains used for vaccination are HVT, SB-1, and CVI988. Vaccination against MDV using these strains offers good protection. Despite effective vaccination regime, MD continues to be a threat to the industry due to the evolution of newer pathotypes. Thus, genetic resistance and strict biosecurity measures will be very critical adjuncts to vaccination in controlling the disease.

A. K. Mariappan (✉) · P. Munuswamy · M. R. Reddy · S. D. Singh
Division of Pathology, ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Bareilly, Uttar Pradesh, India

K. Dhama
Avian Disease Section, Division of Pathology, ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Uttar Pradesh, India

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6.1 Prologue

Marek's disease (MD) is caused by an oncogenic alphaherpesvirus, a common lymphoproliferative inducing agent usually characterized by mononuclear cellular infiltrates, mostly T-cell lymphomas in various visceral organs and peripheral nerves. The disease causes strong immunosuppression and neurological disorders, leading directly to death and/or health implications in susceptible domesticated and wild avian species.

In the year 1907, the maiden report of the disease was made by a distinguished veterinary pathologist Dr. József Marek working at the Royal Hungarian Veterinary School in Budapest through his publication entitled *Multiple Nervenentziindung (Polyneuritis) beiHiihnern* (Marek 1907), wherein he described thickening of the sacral plexuses in four adult cockerels leading to paralysis of the wings and legs. Microscopically, the nerves of the affected birds showed infiltration by mononuclear cells that lead him to name the disease as a "neuritis interstitialis/polyneuritis." After a span of 14 years, Kaupp in the USA (1921) and Van Der Walle and Winkler-Junius (1924) in the Netherlands described and reported similar disease conditions affecting the central and peripheral nervous system; thus, the terms "neurolymphomatosis gallinarum, fowl paralysis, and range paralysis" were given. Until 1929, it was thought that the disease only affects peripheral nerves and spinal ganglia, until Pappenheimer et al. (1929) reported that the disease not only affects nerves but also causes lymphoid tumors in the ovary and other visceral organs, thus deciphering its lymphoproliferative nature. Jungherr and colleagues in 1941 recommended that the term lymphomatosis be further sectioned into visceral, neural, and ocular forms based on the system affected. Between 1950s and 1960s, the disease was divided into two entities, viz., acute and classical forms, wherein the term acute Marek's disease was used to describe visceral lymphomas and classical Marek's disease for nervous lymphomas (Biggs 1966).

6.2 Taxonomy of MDV

MDV belongs to Group I (dsDNA) (order, *Herpesvirales*; family, *Herpesviridae*; subfamily, *Alphaherpesvirinae*; genus, *Mardivirus*; species, Gallid alphaherpesvirus 2. Other relevant species apart from Gallid alphaherpesvirus 2 in *Mardivirus* are Gallid herpesvirus 3 (serotype 2) and Meleagris herpesvirus 1 (serotype 3/herpesvirus of turkey, HVT). Gallid alphaherpesvirus 2 causes oncogenic Marek's disease, and Gallid herpesvirus 3 and Meleagris herpesvirus 1 are found to be nononcogenic species and were isolated from apparently healthy chickens and turkeys, respectively.

Pathotyping of serotype 1 Gallid alphaherpesvirus 2 is done on the basis of their virulence, and they are further divided into different pathotypes, as mild MDV (m MDV), virulent MDV (v MDV), very virulent MDV(vv MDV), and very virulent plus MDV (vv+ MDV) strains (Sun et al. 2017).

6.3 Physicochemical Properties of MDV

Genome is linear and made up of double-stranded DNA molecules of nearly 160–180 kb in size. The buoyant density of the MDV genome in neutral CsCl is 1.706 g/mL with guanine plus cytosine (G+C) ratio ranges from 43.9% to 53.6% (Izumiya et al. 2001). Pulse-field gel electrophoresis used to obtain pure viral DNA as the density of viral DNA is close to that of chicken DNA (646). MDV is infective at pH 7, but there is loss of infectivity at mild changes above and below pH 7, but, at pH 3 and 11, there is a complete loss of infectivity. Storage temperature plays a major role in the maintenance of virus titer wherein the virus is stable at −65 °C for minimum 7 months, but loses its titer when stored for 7 months at −20 °C. Infectivity is lost when stored at 4 °C in 2 weeks, 25 °C in 4 days, 37 °C in 18 h, 56 °C in 30 min, and 60 °C in 10 min. Lyophilization in the presence of glutamate, phosphate, sucrose, albumin, and EDTA causes no loss of titer. The survival of virus remains unaffected even up to four repeated cycles of freezing and thawing and even short cycles of sonic vibration. Cell-free MDV is found sensitive to solvent like ether and fixative like formalin (Nazerian 1973). Interestingly, feather materials and poultry dander infected with virus retain their infectivity for many months even at room temperature (Hlozanek et al. 1973).

6.4 Genomic Organization

The genomic structure is typical for alphaherpesviruses with a unique long sequence (UL) and a unique short sequence (US) that are flanked by sets of inverted repeat sequences: the terminal repeat long (TRL), internal repeat long (IRL), internal repeat short (IRS), and terminal repeat short (TRS), respectively (Fig. 6.1) (Davidson and Nair 2004). MDV genome also contains host cell telomere-like sequences (Kishi et al. 1991), which assists in the favored integration of the MDV genome adjoining to the telomeres present in the host cellular DNA especially in the latently infected cells (Delecluse et al. 1993). The genome of MDV 1 includes 97 single genes and 114 open reading frames (ORF) including the same genes in duplicates in repeat regions. Genome also contains retroviral-like sequences in the genome

Fig. 6.1 Genomic organization of Marek's disease virus

especially in serotype I which is absent in other serotypes which plays a role in transcriptional regulation (Schat and Nair 2008). Complete genome sequences of serotype 1 strains, viz., Md5, GA, BAC clone of Md11, and BAC clone of CV1988, are available in GenBank, which can be retrieved using accession numbers AF147806, AF147806, AY510475, and DQ530348, respectively (Hirai 2001). There exists a very meager difference in the genome of the above sequences, and these changes are mostly limited to the numbers of the direct repeats in the repeat regions of the genome (Schat and Nair 2008). The two serotype 1 strains vMDV GA and vvMDV Md5 strains showed little structural differences (Hirai 2001). The unique long sequence (UL) regions of GA and Md5 are very comparable in length, whereas the unique short sequence (US) regions in GA was found quite longer than Md5 (Schat and Nair 2008). The difference in the genome is due to the presence of one additional copy of small ORF2 (SORF2) in GA and a second small ORF2-like gene in Md5. The SORF1 is truncated in Md5 and is located in the repeats. The vaccine strain CV1988 BAC contains 14 copies of the 132 bp repeat, which is the reason behind the viral attenuation (Silva and Gimeno, 2006). There exist differences in the two of the three promoter regions of ICP4 genes between the GA RB-1B and CV1988 strains, which leads to the difference in the transcription level of ICP4 genes between these strains. Complete analysis of the genomes of 13 strains of varying virulence of the terminal repeat long (TRL) and internal repeat long (IRL) regions showed several single-nucleotide polymorphisms (SNPs) which enables to differentiate non-attenuated and attenuated strains (Spatz and Silva 2006). In conclusion, based on cross-hybridization studies, the genomes for all the three different serotypes are found to be collinear (Igarashi et al. 1987). Further, all these three serotypes vary significantly in their restriction endonuclease digestion patterns (Silva and Barnett 1991).

6.5 Spread

The disease is highly contagious, and the transmission occurs by the airborne route by direct or indirect contact to other susceptible hosts. The infective material is dander/feather follicle epithelium consisting of fully infective enveloped virus. The shedding of the infected material/dander occurs 2–4 weeks post infection, and shedding can continue all the way through the bird's life span. The infectivity of the virus associated with feather debris and dander remains for months. Once the virus enters into a susceptible chicken flock, the infection spreads quickly from bird to bird despite vaccination. Indirect transmission by darkling beetles (*Alphitobius diaperinus*) also plays a minor role in transmission apart airborne mode of transmission. Vertical transmission of MDV through the egg is not evident. Several factors influence the spread of Marek's disease within a flock, viz., the level of initial exposure, the concentration of susceptible birds, handling, change of housing, and vaccination, and females tend to develop more tumors.

Table 6.1 Various pathotypes and their respective reference strains of MDV

Pathotypes	Reference strains
Virulent (V)	JM/102W, 571A, 596A, 617A
Very virulent (VV)	Md5, RB1B, 549A, 587A, 595, 643P, 653A
Very virulent plus (VV+)	584A, 610A, 645, 648A, 648B, 651, 660A, 776

6.6 Pathotyping

Pathotyping of MDV isolates is done based on the pathology the particular isolate induces in vaccinated and unvaccinated chickens and also based on their ability to overcome the effects of vaccination. This method of pathotyping described as Avian Diseases and Oncology Laboratory assay (ADOL assay) was developed at the United States Department of Agriculture (USDA). In short, ADOL assay measures the ability of the isolate to induce lymphoproliferative lesions in chickens that are vaccinated. Based on the ADOL assay, the isolates can be classified as mild MDV (m MDV), virulent MDV (v MDV), very virulent MDV(vv MDV), and very virulent plus MDV (vv+ MDV) strains (Witter et al. 2005). Table 6.1 mentions various reference pathotypes of MDV.

6.7 Susceptible Hosts

Several avian species including both domesticated and wild birds are susceptible to Marek's disease. The species include chickens (*Gallus gallus*), domestic Japanese quail (*Coturnix japonica*), European common quail (*Coturnix coturnix*), domestic turkey (*Meleagris gallopavo*), common buzzard (*Buteo buteo*), red jungle fowl (*Gallus gallus bankiva*), sparrow hawk (*Accipiter nisus*), Ceylon jungle fowl (*Gallus gallus lafayettii*), mallard (*Anas platyrhynchos*), little owl (*Athene noctua*), mute swan (*Cynusolor*), eagle owl (*Bubo bubo*), and domestic goose (*Anser anser*).

6.8 Genetic Resistance and Susceptibility to MD

Genetic makeup of the birds especially the alleles that are blood group linked precipitates the severity of the disease. Other gallinacious species, wild turkeys, and birds that belong to other orders resist infection due to their unique genetic resistance to MDV infection. The chicken genotype proven was associated with susceptibility and resistance to Marek's disease. Owing to its genetic susceptibility/resistance to MD infection, genetic approaches to control MD are being attempted. Thus, poultry breeders have incorporated MD resistance in the selection programs for breeder stocks. Moreover, genetic resistance could be surpassed by challenge with highly virulent MDVs. MD resistance is seen in chickens expressing the B21 allele, which is linked with the major histocompatibility complex class I genes, for

whose mechanism is not understood still. Contrary to the above findings, expression of the major histocompatibility complex class I allele (B2) is seen in line 61 and line 71, but line 61 is resistant to MD and line 71 is susceptible to MD (Burgess et al. 2001).

6.9 Virus Replication and Pathogenesis

Airborne route is the primary route of MDV transmission by both direct and indirect contacts between chickens. Dander consisting of feather follicular epithelial cells with fully infectious virus serves as a potential source of contamination to the environment and to other susceptible hosts (Schat and Nair 2008). The pathogenesis of MD in a susceptible host occurs in four distinct phases: (a) early cytolytic infection, (b) latent infection, (c) late cytolytic infection/immunosuppression phase, and (d) transformation phase (Calnek 1986, Schat 2000) (Fig. 6.2). Following inhalation of the virus, the virus could be detected in the spleen as early as 2 days and then get released via the feather follicle epithelium to the environment in the form of dander at around 2 weeks. The virus-encoded gB glycoprotein expressed by MDV binds to heparin sulfate of the host cell surface and is found to be the potential cellular receptor molecule for MDV entry (Lee et al. 2001).

Post inhalation of MD virus, early cytolytic phase occurs in B cells of the bursa of Fabricius, spleen, and thymus. Following the early cytolytic phase, latent infection starts wherein the MDV becomes latent in activated T cells at 6–7 days post MDV infection, and the virus spreads throughout the entire body by the MDV-infected lymphocytes resulting in cell-associated viremia (Silva et al. 2003). The virus is disseminated to various organs including feather follicles. Then, late cytolytic infection occurs in the feather follicular epithelium, which disseminates infectious cell-free virus to the environment via feather follicle debris and dander. Few latently infected T cells consequently are transformed, leading all the way to the development of lymphoma in peripheral nerves and visceral organs (Schat 2004, Schermuly et al. 2015). The principal target cells that undergo transformation in MDV infection are the CD4+ T cells. Apart from CD4+ T cells, the virus has the ability to transform CD8+ T cells.

6.10 Genes Specific to MDV 1

Various proteins are expressed during specific phases of the infection as mentioned in the pathogenesis. The viral proteins and their potential roles are given in Table 6.2.

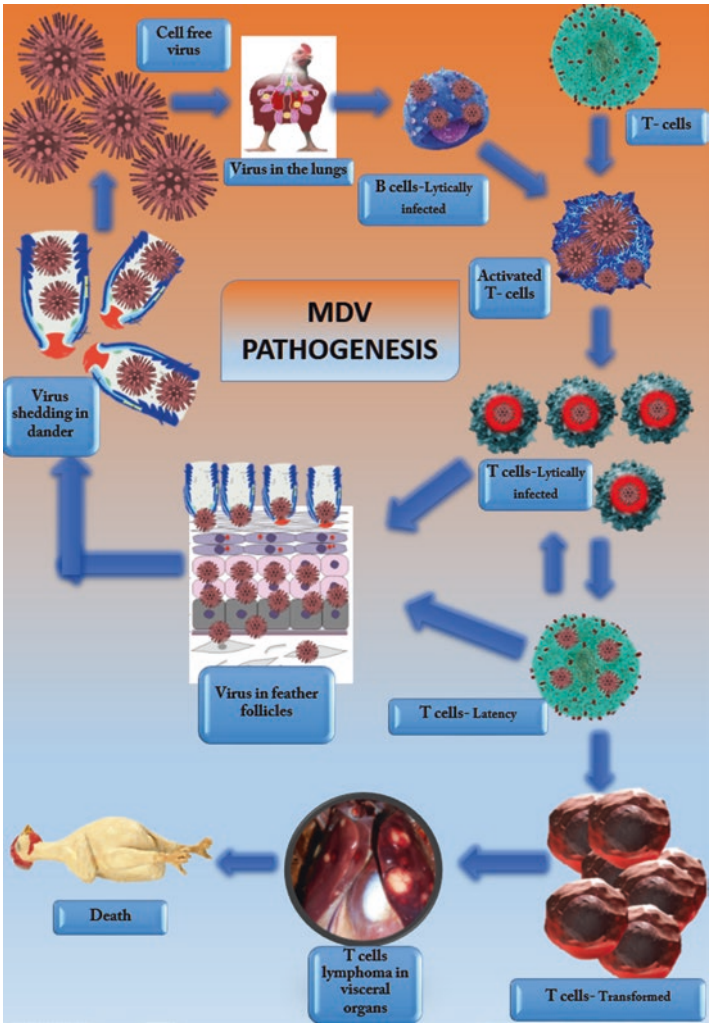

Fig. 6.2 Pathogenesis of Marek's disease virus

Table 6.2 Genes specific to MDV and their functions (Boodhoo et al. 2016)

MDV gene (protein)	Function	Infection stage
MDV003/078 (vIL-8)	Recruitment of immune system cells to site of viral replication	Lytic replication
MDV010 (vLIP)	Forms covalent bonds with lipids Lytic replication	Lytic replication
MDV011/012	Downregulates cell surface and in turn immune evasion	Lytic replication
MDV012 ORF012	Necessary for both in vivo and in vitro viral growth	–
MDV040 (gB)	Facilitates viral fusion with host membrane	Lytic replication
MDV052/053 UL39/40 (RR)	Necessary for both in vivo and in vitro replication	Lytic replication
MDV073 (pp38)	Early protein expressed during cytolytic infection	Lytic replication
MDV092 (Us3p)	Serine/threonine protein kinase that phosphorylates pp38	Lytic replication
MDV084/100 (ICP4)	Viral gene transactivation function	Lytic replication
MDV001a (vTR)	Required for integration of viral genome into host DNA for immune evasion, neoplastic transformation, and viremia	Latency
MDV006 (pp14)	Neurovirulence factor required for PNS neuropathy	Latency
MDV062 (VP22)	Tegument protein essential for viral replication and modulates host cell cycle	Latency
MDV057 (gC/UL44)	Type 1 transmembrane protein required for horizontal transmission/shedding from feather follicle epithelium	Feather follicle shedding
MDV005/076 (MEQ)	Viral oncogenic protein involved in T-cell neoplastic	Neoplastic transformation
MDV029(pUL17)	Colocalizes with VP5 and VP13/14 tegument protein and essential for in vivo viral growth, capsid maturation, and DNA packaging	Neoplastic transformation

6.11 Immunobiology

6.11.1 The Innate Immune Response to MDV

The host responds to MDV infection by mounting both innate and adaptive immune mechanisms, in turn controlling the MDV infection. Interferon production plays a major protective role against MDV infection and found to be the chief mode of innate immune response. MDV is taken up by the antigen-presenting cells like dendritic cells or macrophages present within the respiratory system, and further the virus is recognized by TLR21 leading to a cascade of events by stimulation and expression of type I interferons. An in vitro model proved that interferons could control MDV replication by reducing the plaque formation. Contrary to the mammalian counterparts where type I interferons stimulate NK cells and enhance its cytotoxic function causing reduction in viral replication, chicken interferon-α does

not increase natural killer cell cytotoxicity. Apart from inhibition of viral replication, type I IFNs are shown to be involved in stimulating the latency infection of MDV. IFN-γ induces nitric oxide production leading to inhibitory effects on MDV replication.

Dendritic cells are the proposed immune cells that mount both innate and adaptive immunities against MDV exposure in the hosts. They initiate adaptive immunity by presenting MDV antigens to both MHC class I and II molecules. Macrophages, on the other hand, play a vital role in controlling viral replication and MDV-induced tumor formation. Thus, macrophages are directly involved in the inhibition of viral replication and tumor development in Marek's disease by their potent phagocytic ability. It has proven that MDV replication inhibited effectively by macrophages collected from MDV-infected chickens than macrophages obtained from noninfected chickens. Production of inducible nitric oxide (iNOS) is the prime mechanism by which macrophages inhibit MDV infection progressing to tumor formation. Experimental evidences prove that nitric oxide production occurs in various organs like the spleen, brain, and lungs of MDV-infected chickens. In vivo experimentation of iNOS inhibition in chickens increased viral load proving the pivotal role of macrophages in MDV pathogenesis. Another main function of macrophages is tumoricidal activity of virus-transformed tumor cells. Macrophages from tumor tissues of MDV-infected chickens have comparable functional abilities of tumor-associated macrophages that could suppress T-cell proliferation, factors that promote in vitro tumor growth and factors that cause immunosuppression (Boodhoo et al. 2016).

Natural killer (NK) cells have functions like production of IFN-γ with potent antiviral activity and sensing of virus-infected cells and tumor cells through downregulation of cell surface markers such as MHC I. NK cells are associated with MD resistance as MDV-susceptible chickens have greater cytotoxic capacity than the susceptible chickens.

6.11.2 The Adaptive Immune Response to MDV

As far as MDV infection is concerned, protective immunity is not conferred by antibodies and thus do not play a crucial role in the MDV infection. Among all antibodies raised against MDV glycoproteins by chicken, anti-glycoprotein B neutralizing antibodies block the entry of the virus into the host cells and thus play a protective role against MDV infection in chickens. Studies proved that maternal antibodies deferred both clinical signs and tumor development, but, on the other side, maternal antibody neutralizes the vaccine virus by interfering with the live replicating vaccine strains.

Cell-mediated immunity (CMI) plays a key role in combating the intracellular cell-associated virus and also offers protection against MDV after vaccination. T cells are efficient in controlling replication of virus but proved inefficient in controlling tumor growth. Infected chickens produce CD8+ T cells against various viral proteins, viz., gB, Meq, pp38, and ICP4. Apart from CD8+ T-cell activation by viral

proteins, they also act on cytotoxic T cells to release perforins and granzymes. The activated T cells express Marek's disease tumor-associated surface antigens (MATSA) on its surface but once reported to be MD specific but later proved that MATSA does not exclusively express on the transformed T cells. CD30 molecule, a co-stimulatory molecule, was found to be one of the MATSA antigens and had been identified with pleiotropic effects. CD30 molecule plays a major role in MDV pathogenesis by its involvement in apoptosis, cytotoxicity, T-cell activation, and regulation of T-cell migration. Certain pathways like programmed death-1 (PD1), programmed death-ligand (PD-L1), and cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) get upregulated in chicken immune system cells during MDV infection. In MD pathogenesis, there is increased expression of PD-1 and CTLA-4 in the early cytolytic phase, while, at the latent phase, there is increased expression of PD-L1. In the tumorigenic phase, expression increases in both PD-1 and PD-L1 (Boodhoo et al. 2016).

6.12 Clinical Signs

The first indication of infection is the development of several nonspecific signs like weight loss, pallor, reduced feed intake, and diarrhea. The birds suffering from Marek's disease exhibit various clinical signs. Some chickens die without showing any clinical signs. In neurolymphomatosis, most of the affected birds show varying degrees of paralysis, and asymmetric progressive paralysis of one or more of the extremities, drooping of wings, torticollis of the neck, dilatation of the crop, and/or gasping in case of vagal nerve involvement can also be seen (Singh et al. 2012). Paralyzed birds develop anorexia and die because the birds cannot reach feed and water. The eyes of birds with ocular lymphomatosis (gray eye) lose its ability to accommodate light intensity and blindness occurs. In the cutaneous form of MD, broilers are condemned at slaughter, predominantly due to the presence of numerous cutaneous nodules/tumors. Acute lymphomatosis do not show paralytic signs, and the tumors in the internal organs cause significant damage to the immune system leading to suboptimal performance and clinical outbreaks of other diseases such as coccidiosis, worm infestations, and Gumboro disease.

6.13 Gross and Microscopical Lesions

6.13.1 Gross Pathology

Nerve lesions and visceral lymphomas are the prime pathologic changes noticed in MD. Peripheral nerves enlarge in the affected birds. The brain does not show any gross changes, but the spinal ganglia may show gross enlargements. Almost all nerves and plexi are involved, but prominent lesions are seen in sciatic and brachial nerves (Pappenheimer et al. 1929; Payne and Biggs 1967). Almost all visceral

organs show gross changes. Affected nerves show localized to diffuse swelling, grayish/yellowish discoloration, loss of cross-striations, and edematous changes. Lesions may be either unilateral or bilateral; thus, both sides of the nerves are to be examined to detect changes. Birds affected by more virulent pathotype of the virus succumb to visceral lymphomas. Lymphomas occur in almost all visceral organs, viz., the testis, ovary, lung, heart, mesentery, kidney, liver, spleen, bursa, thymus, adrenal gland, pancreas, proventriculus, intestine, iris, skeletal muscle, and skin (Benton and Cover 1957; Smith et al. 1974; Haesendonck et al. 2015). Lymphomas in visceral organs appear as focal nodules to diffuse enlargements. Cut surface of the nodules is white/gray in color and firm in consistency with a smooth surface. Cutaneous lymphoma usually associated with feather follicles leads to condemnation in broiler chickens. Cutaneous lymphoma involves multifocal feather follicles or all follicles, which later coalesce. Later, in extreme cases, these nodules dry and become scab-like with brownish to blackish crust formation (Benton and Cover 1957). A term "Alabama red leg" is given for erythematous lesions involving the shank integument caused by highly virulent pathotype of the MDV in broiler chickens. Figure 6.3 shows common gross lesions**.**

6.13.2 Microscopic Pathology

In the case of peripheral nerves, two distinct types of microscopical lesions are recognized (Payne and Biggs 1967). Type A lesion, which is neoplastic form, nerves are infiltrated with neoplastic cells consisting of pleomorphic lymphocytes with demyelination and Schwann cell proliferation. Type B lesion is of inflammatory type and characterized by diffuse infiltration with small lymphocytes, macrophages, and plasma cells, interfascicular edema, demyelination, and Schwann cell proliferation. In the brain, varying degrees of encephalitis are noticed. Lesions are characterized by endotheliosis of blood vessels, perivascular infiltration of lymphocytes, and macrophages covering the entire parenchyma succeeded by severe infiltrations of large lymphocytes and glial cells (Pappenheimer et al. 1929). Secondary demyelination occurs due to severe lymphoblastic infiltration and extensive vacuolations in the parenchyma. Lymphomas in visceral organs are proliferative in nature and consist of small and medium lymphocytes, lymphoblasts, reticular cells, plasma cells, and macrophages (Payne and Biggs 1967; Purchase and Biggs 1967). Macrophages are primarily seen in slow-growing tumors. The gross presentation of lesions varies in different visceral organs that are affected, but various cellular components of tumors are similar in all organs. In cutaneous lymphoma, lesions are mostly inflammatory and less lymphomatous. Lymphomas surrounding the infected feather follicles with the presence of compact aggregates of proliferating lymphocytes, plasma cells, and histiocytes are seen (Moriguchi et al. 1982). Feather pulp lesions are useful for antemortem diagnosis with lymphomatous lesions surrounding feather follicles with intranuclear inclusions in the feather follicle epithelium (Schat and Nair, 2008). The microscopic lesions seen are represented in Fig. 6.4.

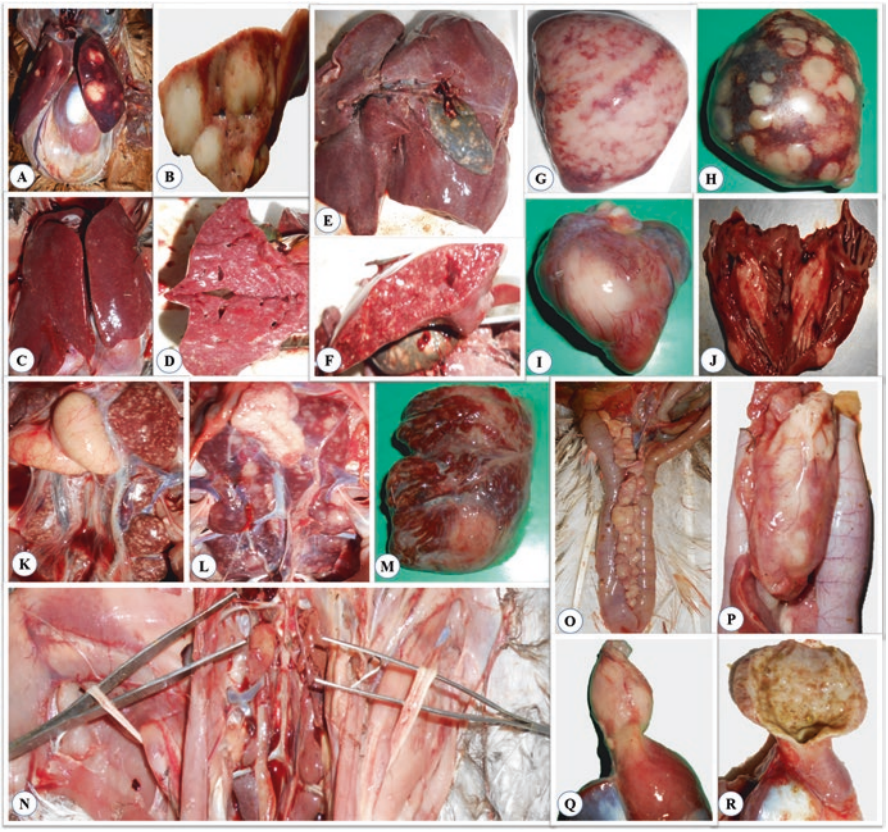

Fig. 6.3 Gross lesions in MD. (**a**) A liver showing numerous distinct tumorous nodules over the surface. (**b**) A liver cut surface showing distinct tumor nodules. (**c**) A liver showing diffuse swelling with indistinct gray-colored miliary foci. (**d**) A liver cut surface showing indistinct gray-colored miliary foci present throughout the parenchyma. (**e** and **f**) A gall bladder with numerous pale grayish tumor nodules on the wall. (**g**) A spleen showing swelling and diffuse grayish discoloration with lymphoid infiltration. (**h**) A spleen embedded with solid tumor nodules. (I) A heart with the presence of tumor nodule over the epicardium. (**j**) A heart with the presence of tumor nodule over the endocardium. (**k** and **l**) A kidney with the presence of grayish tumorous nodules over the entire lobes. (**m**) A lung with the presence of tumor nodules embedded in the parenchyma. (**n**) Unilateral enlargement of the sciatic nerve. (**o** and **p**) A pancreas with the presence of nodular tumor (single and multiple) attached to the parenchyma. (**q** and **r**) A proventriculus showing thickened walls

6.14 Morbidity and Mortality

The incidence of Marek's disease is variable depending upon the pathotype and host susceptibility. Not all the birds that develop signs may succumb to clinical disease; few may recover (Biggs and Payne 1967), but, in general, mortality is nearly equal to morbidity. Prior to vaccination practices, mortality ranged

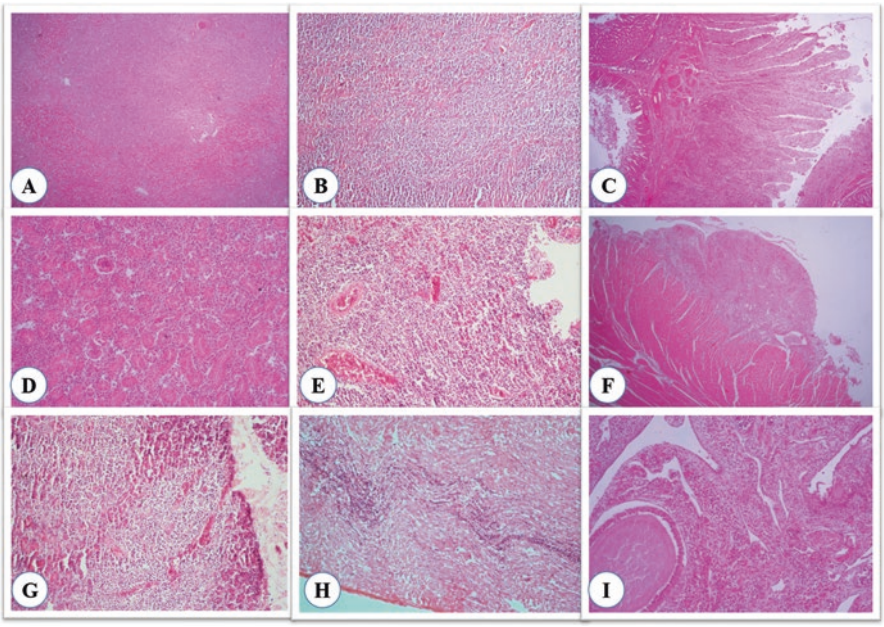

Fig. 6.4 Microscopic lesions in MD showing multifocal to diffuse areas of pleomorphic lymphoid cell infiltration in (**a**) the liver, (**b**) spleen, (**c**) proventriculus, (**d**) kidney, (**e**) lung, (**f**) heart, (**g**) pancreas, (**h**) nerve, (**i**) ovary (H&E, X10)

between 30% and occasionally as high as 60%. Presently, due to stringent vaccination practices, mortality reduced less than 5% in most of the countries. Broiler industry succumbs to severe economic losses despite vaccination not due to mortality but due to condemnations.

6.15 Diagnosis of MD

In the field, diagnosis is done primarily based on the clinical signs, postmortem lesions like thickened peripheral nerves, visceral lymphoid tumors, iris/pupillary changes, and atrophy of the bursa and the thymus. Apart from conventional diagnostic techniques, various techniques are used to arrive at an apt and final diagnosis. Test methods available for the diagnosis of Marek's disease and their purpose are depicted in Table 6.3.

6.15.1 Virus Isolation

Common materials used for the virus isolation are white blood cells isolated from blood samples, suspensions made from visceral lymphoma and splenocytes. Blood lymphocytes or single-cell lymphoid organ suspensions are the preferred source of

Table 6.3 Test methods available for the diagnosis of Marek's disease and their purpose

Purpose	Method					
	Agent identification				Detection of immune response	
	Histopathology	Virus isolation	Antigen detection	Real-time qPCR	AGID	IFA
Population freedom from infection	n/a	n/a	n/a	n/a	n/a	n/a
Individual animal freedom from infection prior to movement	n/a	n/a	n/a	n/a	n/	n/a
Contribute to eradication policies	n/a	n/a	n/a	n/a	n/a	n/a
Confirmation of clinical cases	+++	+	+	++	–	–
Prevalence of infection surveillance	+	–	–	+	+	+
Immune status in individual animals or populations post-vaccination	–	–	–	–	–	+

the virus from infected chickens. Chicken kidney cells (CKC) and duck embryo fibroblasts (DEF) cultures are the two preferred cell culture systems for initial isolation of serotype 1 MDV, whereas serotypes 2 and 3 grow well in CEF. The first attempt of MDV was first isolated in CKC and DEF cultures (Churchill and Biggs 1967, Solomon et al. 1968). Later, isolation of MDV has been carried out in MD-derived tumor transplant cells (JMCT), chicken embryonal fibroblasts (CEF), chicken embryonal liver (CEL) cell, chicken embryonal lung cell, and chicken embryonal kidney cell (CEKC) (Schat 2005). Characteristic cytopathic effects (CPE) caused by MDV include focal degeneration of infected cells owing to direct cell-to-cell transfer of the virus. The spreading of the virus occurs concentrically, so that the cells peel off with enlarged, rounded cells, surrounded by healthy tissue. Infected cells become enlarged, rounded, and highly refractile, which in due course detach from the surface. Typical plaque formation develops in inoculated cultures within 3–12 days. These plaques induced by all three serotypes are distinguished morphologically and by immunofluorescent staining.

6.15.2 Viral Markers in Tissues

Detection of the MDV antigen "Meq" is a suitable marker in the diagnosis of MD, as induced only by MDV-induced tumors and not in avian leukosis or reticuloendotheliosis induced.

6.15.3 Viral Antigen Detection

MD viral antigen is demonstrated in the feather tip by the use of monoclonal antibody by employing agar gel immunodiffusion (AGID) (Scholten et al. 1990). Birds infected with Marek's disease virus express viral oncoprotein "Meq" in tissues; a dot-ELISA technique that could detect Meq protein in tissues was developed which was found to have better specificity than conventional polymerase chain reaction (Kumar et al. 2016).

6.15.4 Genomic Detection Assays

Polymerase chain reaction (PCR) and the real-time quantitative PCR (qPCR) allow the distinction between vaccine and field isolates (Handberg et al. 2001). Polymerase chain reaction (PCR)-based diagnosis from the lungs, liver, spleen, kidneys, skin, pancreas, and ovary was standardized. Serotype-specific PCRs play a vital role in differentiating serotype 1 pathogenic herpesviruses from serotype 3 HVT vaccine strain (Handberg et al. 2001). Nested PCR not only detects Meq gene of MDV in infected chickens but also could aid in differentiating highly virulent pathotypes from attenuated/vaccine MDV strain (Murata et al. 2007a, b). Apart from simple and nested PCR, quantitative real-time PCR can differentially quantify CVI988/Rispens virus vaccine strain and virulent RB-1B strain in chicken tissues, in turn understanding vaccinal efficiency (Baigent et al. 2016). MDV genome copies are quantified by real-time quantitative PCR (qPCR) (Islam et al. 2004; Baigent et al. 2005; Abdul-Careem et al. 2006). Loop-mediated isothermal amplification (LAMP) targeting the meq gene was proved to be a rapid method of diagnosis especially from feather follicles (Wei et al. 2012; Angamuthu et al. 2011). Another variation of quantitative real-time PCR is the SYBR green duplex q-PCR assay targeting the Eco-Q protein gene (meq) that could detect and quantify viral loads and distribution patterns of the virus in various organs (Zhang et al. 2015).

6.15.5 Antibody Detection

Agar gel immunodiffusion (AGID) is the most commonly used test to detect antibody in infected chickens. Detection of antibodies to MDV was described using enzyme-linked immunosorbent assay (ELISA) (Cheng et al. 1984; Zelnik 2004). An antigen-detecting ELISA for detecting the MDV antigens in feather tips of infected chickens with better sensitivity than AGID was devised (Davidson et al. 1986).

6.16 Differential Diagnosis

Several criteria have been used by Gimeno et al. (2005) to differentially diagnose different lymphoma-inducing viral agents, viz., Marek's disease virus (MDV), avian leukosis virus (ALV), and reticuloendotheliosis virus (REV). Viral-induced lymphomas

can be differentiated based on various criteria, viz., age affected, signs showed by affected bird, incidence of the disease, different organ involvements, cytology of the lymphoma, and type of cells affected (Table 6.4). Meq oncoprotein was seen consistently expressed in the MDV-induced lymphomas, and it was found to be directly proportionate to the MDV genome copy number detected in the MD tumors; however, other proteins including pp38 expression were inconsistent. Meq expression was negative in retrovirus-induced lymphomas; in addition, there was a low copy number of meq gene in MDV 1-vaccinated group/MDV latently infected cells.

Table 6.4 Differentiating features of viral-induced lymphomas (OIE 2017)

Feature	Marek's disease	Lymphoid leukosis	Reticuloendotheliosis
Age	Any age. Usually 6 weeks or older	Not under 16 weeks	Not under 16 weeks
Signs	Frequently paralysis	Nonspecific	Nonspecific
Incidence	Frequently above 5% in unvaccinated flocks. Rare in vaccinated flocks	Rarely above 5%	Rare
Macroscopic lesions			
Neural involvement	Frequent	Absent	Infrequent
Bursa of Fabricius	Diffuse enlargement	Nodular tumours	Nodular tumours
Tumours in the skin, muscle, and proventriculus, "gray eye"	May be present	Usually absent	Usually absent
Microscopic lesions			
Neural	Yes	No	Infrequent
Liver tumours	Often perivascular	Focal or diffuse	Focal
Spleen	Diffuse	Often focal	Focal or diffuse
Bursa of Fabricius	Interfollicular tumor and/or atrophy of follicles	Intrafollicular tumor	Intrafollicular tumor
Central nervous system	Yes	No	No
Lymphoid proliferation in the skin and feather follicles	Yes	No	No
Cytology of tumors	Pleomorphic lymphoid cells, including lymphoblasts; small, medium, and large lymphocytes; and reticulum cells Rarely can be only lymphoblasts	Lymphoblasts	Lymphoblasts
Category of neoplastic lymphoid cell	T cell	B cell	B cell

6.17 MDV Vaccination

Vaccines available commercially are all based on three viral serotypes and recombinant DNA vaccines that are capable of protecting chickens against MD. The maiden MD vaccine was developed by Churchill et al. (1969) using an oncogenic HPRS-16 strain of serotype 1 MDV. HPRS-16 strain was attenuated by serial passages using CKC cultures to generate a vaccine. Later the HPRS-16 strain was replaced by herpesvirus of turkey strain (HVT strain, FC-126) as a potent vaccine candidate (Okazaki et al. 1970). The economic losses due to MD have successfully dropped after the introduction of HVT. HVT vaccines are generally used alone or used in combination with SB-1 strain, which is a serotype 2 strain (Morrow and Fehler 2004). SB-1 strain is the naturally avirulent isolates of serotype 2 (Schat and Calnek 1978). Generally, bivalent vaccines are used incorporating both HVT and SB-1 to take advantages of the synergistic activity between serotype 2 and serotype 3 (Witter 1982).

Serotypes 1 and 2 vaccines are generally available as cell-associated products (Nair 2005). An attenuated MDV CVI988 strain is the most protecting vaccine strain that is available currently and used in several chicken-rearing countries (Davidson and Nair 2004). MD vaccines are generally administered in ovo at the day of embryonation or as day 1 vaccination. Apart from MDV CVI988 strain, R2/23 strain, an attenuated serotype 1 strain, was also reported in the 1990s and Md11 in the USA, although it is now considered less protective than CVI988 (Witter 1991). However, with a growing evidence of vaccination failures and the emergence of new and more virulent strains (Fig. 6.5), Marek's disease poses a continuous risk to the poultry population and causes substantial economic losses. The main drawback of MD vaccination is the inability of the Marek's disease vaccines to induce a "sterile immunity" in the vaccinated host (Nair 2005) which causes the virulent virus strains to replicate even inside the vaccinated host and cause shedding of virus particles into the environment which acts as a source of infection to the other

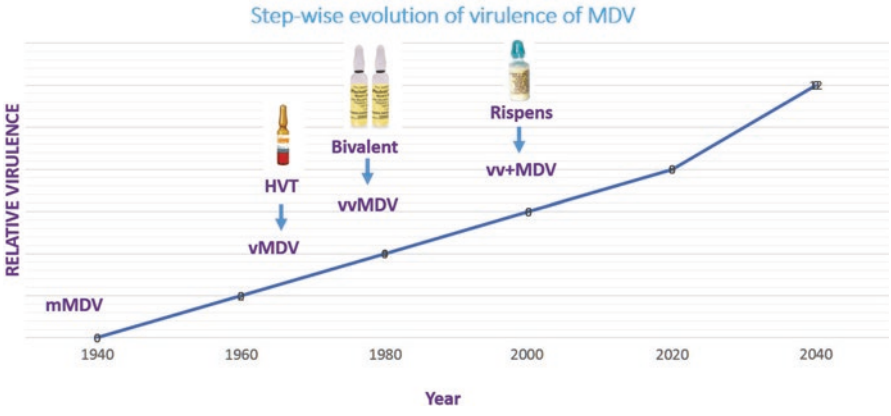

Fig. 6.5 Relationship between the virulence increase and the introduction of different vaccines

susceptible hosts. Meq gene-deleted mutant vvMDV, a genetically modified vaccine for MD, showed effective protection in the vaccinated birds by controlling the formation of tumors (Lupiani et al. 2004; Li et al. 2011).

Recombinant fowl poxvirus (rFPV) and herpesvirus of turkey strain vaccines incorporated with various MDV genes showed some protective efficacy (Ross et al. 1993; Nazerian et al. 1996; Hirai and Sakaguchi 2001; Lee et al. 2005), and rFPV vaccines were also found to be effective in chickens with maternal antibodies against MDV (Lee et al. 2003). Avian myelomonocytic growth factor gene has been incorporated as a source of cytokine in rFPV, and its inclusion was found to improve vaccine efficacy against MDV (Djeraba et al. 2002). Apart from these conventional vaccines, pBAC20-engineered DNA vaccine conferred partial protection against challenge with virulent MDV (Tischer et al. 2002). Several candidate vaccines have been developed in the past, but none proved more efficacious than CV1988 vaccines (Witter and Kreager 2004).

6.18 Biosecurity in Farms Against Marek's Disease

There are two hazard points to be taken cared of to control the MDV: (i) reducing the initial viral load on a farm by preventing the entry of MDV into the poultry rearing premises and (ii) preventing the exit of MDV to the environment from the affected flock (Schat and Nair 2008). Reducing the initial viral load on a farm can be possible if practicing all-in-all-out systems, avoiding multiage flocks, etc. Measures to avoid the exit of MDV to the environment from the affected flock include air management and the disposal of dead/infected chicken carcasses and manure. Biofilters are used to decrease the viral load level in the effluent air from affected poultry farms. Carcasses should be disposed of by either burning or burial. Composting of the manures has shown to be an effective method to discard both carcasses and manure (Schat and Nair 2008).

6.19 Future Perspectives

Marek's disease is an important lymphoproliferative/neoplastic disease of poultry and can be effectively controlled by vaccination. MD serves as a potential model system to study vaccinology, viral oncogenesis, and tumor immunology. Despite effective vaccines being used to prevent this disease, the virus continues to evolve into more virulent pathotypes and, in turn, causes a serious health threat to the poultry industry; thus, it is very much necessary to develop viable vaccine strategies that could prevent the generation of newer pathotypes. Thus, natural genetic resistance of the birds against MD should be taken into account while breeding which would help in preventing newer pathotype emergence. Apart from the conventional vaccination regime, strict biosecurity measures are needed on the farms, which is a critical adjunct to vaccination in controlling the disease.

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Conflict of Interest There is no conflict of interest.

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Camelpox Virus

7

Bidhan Chandra Bera, Thachamvally Riyesh, Sanjay Barua, and Raj Kumar Singh

Abstract

Camelpox is a highly contagious skin disease of camelids caused by camelpox virus (CMLV), a member of genus *Orthopoxvirus* within the family *Poxviridae*. The disease is often manifested as a mild local skin infection and sometimes in the severe form with systemic involvement. The disease is enzootic in the camel-rearing areas of arid and semiarid regions of the world and causes economic loss in terms of morbidity, mortality, loss of weight, and reduction in milk and wool production. The CMLV infection is transmitted mostly by direct contact and aerosol route. The disease gained attention globally in the recent past due to its close similarity with the causative agent of smallpox (variola virus) and irrefutable incidences of few zoonotic infections in humans. Like many other poxviruses, the CMLV has a large DNA genome, capable of encoding genes responsible for replication, host range, immunomodulation, virulence, and other functions. Despite the presence of a myriad of host range genes, the host tropism of camelpox virus is very limited. Both live attenuated and inactivated vaccines are available to combat the disease in camels; however, no vaccine has been developed till date for use in humans. Few antiviral agents have been shown to be effective against CMLV; however, their use is very limited in field outbreaks. The research on CMLV is gaining global interest due to CMLV zoonosis especially in the context of naive human population to poxvirus immunity. The present chapter enlightens the brief overview of background, history, incidence, and prevalence of the disease, immunobiology, diagnostics, risk factors, transmission, and prevention and control of camelpox.

B. C. Bera · T. Riyesh · S. Barua
National Centre for Veterinary Type Cultures, ICAR-National Research Centre on Equine, Hisar, Haryana, India

R. K. Singh (✉)
ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Uttar Pradesh, India

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Camelpox virus · CMLV · Camel · Orthopoxvirus · OPV · Zoonosis

7.1 Prologue

Camelpox is a highly contagious viral skin disease of camelids, which occurs throughout the camel-rearing areas of the world. The disease mainly occurs in Old World camelids (*Camelus dromedarius and C. bactrianus*) of Africa, the Middle East, and Asia, except dromedary camel in Australia and tylopoda (llama and related species) in South America. The disease affects all animals irrespective of age, breed, and sex; however, young camels (2–3 years old) are found to be more susceptible to the infection. Clinical lesions are confined to the skin with rare systemic infections. The disease is characterized by fever, enlarged lymph nodes, and papular pustular eruptions on the skin. In the systemic form, pock lesions are found in the mucous membranes of the mouth and respiratory and digestive tracts. The disease causes a considerable economic impact in terms of morbidity, mortality, loss of weight, and reduction in milk, meat, and wool production. The disease also results in the imposition of trade restrictions on camels and their by-products. The morbidity rate is variable depending on the circulating viral strains. The mortality rate in adult animals is between 10% (Higgins et al. 1992) and 28% (Jezek et al. 1983) and in young animals between 25% and 100% (Mayer and Czerny 1990). The infection generally occurs by direct contact between infected and susceptible animals or indirectly via a contaminated environment through inhalation or skin abrasions.

The etiology of the disease is camelpox virus (CMLV), a member of the genus *Orthopoxvirus* (OPV) and the subfamily *Chordopoxvirinae* in the family *Poxviridae*. Other important members of the genus (i.e., *Orthopoxvirus*) include human pathogens such as variola (smallpox), monkeypox, cowpox, and vaccinia viruses (Moss 2013). The CMLV is similar to the prototypic VACV with respect to size, shape, structure, physicochemical properties, and replication mechanisms. Like other OPVs, CMLVs are large, and average virion size is $217.6 \pm 18.7 \times 293.15 \pm 18.8$ nm (Erster et al. 2018). CMLV is secreted in milk, saliva, and ocular and nasal discharges, and the virus can survive in dried scabs for at least 4 months. The genome of CMLV consists of a linear double-stranded DNA with hairpin loops at the terminal portion (Moss 2007). The full-length genome sequences of three CMLV strains CMLV-CMS, CMLV-M96, and 0408151v have been unveiled, and the genome size has been found to range from 202 to 205.7kbp (Afonso et al. 2002; Gubser and Smith 2002). The detailed analysis of genome sequence and phylogeny has revealed the close relationship of CMLV with the variola virus (VARV), the causative agent of dreadful "smallpox" disease, which had been eradicated in 1980 (Afonso et al. 2002; Gubser and Smith 2002). The in vitro growth characteristics in respect to pock formation on chorioallantoic membrane (CAM), growth in cells, and low or absence of pathogenicity in various animal models also reiterate that CMLV has strong similarities to VARV (Baxby 1972, 1974; Baxby et al. 1975). More focused

studies on genomic, biological features and pathogenesis of CMLV are getting the impetus for better understanding of the biology of VARV.

CMLV is highly host-specific and does not infect other animal species. However, jumping of CMLV in spillover hosts like human and causing infections have been documented in literature. Earlier reports had described mild skin lesions in humans associated with camelpox virus infections (Jezek et al. 1983; Lesse 1909). Recently, the conclusive reports of CMLV zoonosis have been reported from India (Bera et al. 2011) and Sudan (Khalafalla and Abdelazim 2017). The human infections with CMLV have been confirmed on the basis of clinical and epidemiological features coupled with serological tests and molecular characterization of the causative agent. Hence, CMLV can act as an occupational public health hazard. The emergence of CMLV zoonosis certainly points toward a declining immunity against OPVs in humans, which could be of serious public health concern. Due to the frequent outbreaks of CMLV and other poxvirus diseases in animals and their transmission to spillover hosts including humans, focused attention is required on understanding the ecology and epidemiology of the disease, the identification of potential reservoirs of these poxviruses, the transmission chain, and the immune response upon natural infection, improved diagnostics, and new-generation vaccines in order to control the disease in camels and zoonotic infection in the near future.

7.2 History

Poxviruses are the best known and most feared viruses of humans and animals. Viruses of the family *Poxviridae* are very large in size (220–450 nm × 140–260 nm) and have a double-stranded DNA genome of size ranging from 130 to 375 kbp. Only members of the subfamily *Chordopoxvirinae* are capable of infecting vertebrate species. As per the latest ICTV classification (2017), the subfamily contains 13 genera (including two unassigned genera), of which genera *Orthopoxvirus* (OPV), *Parapoxvirus*, and *Yatapoxvirus* contain viruses with zoonotic potential and gained much attention recently. The most prominent species of the genus *Orthopoxvirus* (OPV) is variola virus (VARV), the causative agent of smallpox, which was a threat for humans worldwide for centuries. In addition to VARV, the genus OPV also contains many other viruses with or without zoonotic potential (Table 7.1). The infection caused by other OPVs didn't get much attention till the eradication of smallpox, because smallpox was the most dreaded viruses among all these members. Eradication of human smallpox in the late 1970 and subsequent cessation of small vaccination resulted in a human population with waning antibodies against poxviruses. This resulted in an increased incidence of human cases of cowpox virus in Europe, monkeypox in Africa, and bovine vaccinia virus infection in India and Brazil. Since then, the knowledge on poxviruses is increasing at an incredible speed, and many poxvirus diseases are getting much attention among researchers and public health authorities.

Camelpox is one such disease, which got major attention in the early 1970s although the outbreak was reported a long time back, for the first time, from India

Table 7.1 Classification of *Orthopoxvirus* and their host range

Family: *Poxviridae*		
Subfamily: *Chordopoxvirinae*		
Genus	*Species*	Host
Orthopoxvirus	*Camelpox virus*	Camel, human
	Cowpox virus	Cattle, human, cats, dogs, rodents, nonhuman primates, elephant, rhinoceros, gerbils
	Ectromelia virus	Laboratory mice, wild mice, wild rodents
	Monkeypox virus	Monkeys, apes, human, rope squirrel (*Funisciurus anerythrus*), sooty mangabey (*Cercocebus atys*)
	Raccoonpox virus	Raccoons (*Procyon lotor*)
	Skunkpox virus	Skunk (*Mephitis mephitis*)
	Taterapox virus	African gerbil (*Tatera kempi*)
	Vaccinia virus	Human, cattle, buffalo, rodents
	Variola virus	Human
	Volepox virus	Vole (*Microtus californicus*) Pinyon mouse (*Peromyscus truei*)

(Lesse 1909). Since then, the disease has continuously been reported from many countries. For a long period, the disease has been recognized as a generalized pox disease of camels, and the causative virus (CMLV) was first isolated by cultivation in chick embryos in 1970 (Sadykov 1970). Later, CMLV was also isolated in tissue culture in 1972 (Ramyar and Hessami 1972). In the late 1970, CMLV was considered as "smallpox-like" member of the genus *Orthopoxvirus*, due to its similarities with VARV in the form of culture characteristics, narrow host range, and even serological cross-reactivity (Baxby 1972; Baxby et al. 1975; Davies et al. 1975). The speculation of similarities among CMLV and VARV was further supported by findings of in vivo experiment, where camels infected with VARV strain EA8 were protected against challenge with an infective dose of CMLV (Baxby et al. 1975). This raised a great concern among those involved in the global smallpox eradication campaign. However, 20 years later, the genome characterization studies by restriction fragment length polymorphism analysis using *HindIII* enzyme confirmed that CMLV was a separate member of OPV genus (Pfeffer et al. 1996; Renner-Muller et al. 1995). Subsequently, the full-genome sequence of CMLV strains depicted that CMLV is closest to VARV, sharing several genes involved in basic replication and host-related functions, and, probably, they may share a common ancestor (Afonso et al. 2002; Gubser et al. 2007a, b). A recent isolate of CMLV from Israel was found to be genetically different from the currently annotated camelpox isolates; however, complete genome sequencing is essential to arrive at a conclusion (Erster et al. 2018).

7.3 Incidences and Prevalence

Camelpox is prevalent in almost every camel-rearing regions of the world. Since the first report of the disease from India in 1909, regular incidences of CMLV infections have been reported from many countries of the world with large number of recorded outbreak being reported after the year 1972. The disease has been reported from Middle East (Bahrain, Iraq, Iran, Israel, Oman, Saudi Arabia, United Arab Emirates, and Yemen), Asia, (India, Afghanistan, Pakistan, Kazakhstan, and Turkmenistan), Africa (Algeria, Egypt, Ethiopia, Kenya, Mauritania, Morocco, Niger, Somalia, Sudan, and Syria), and southern part of Russia (Wernery and Kaaden 2002; Duraffour et al. 2011; Erster et al. 2018). The incidence of disease outbreaks in different countries/regions has been depicted in Table 7.2. It is interesting to mention that camelpox has never been reported from Australia in spite of the presence of natural populations of dromedary camels and camel farming (Wernery and Kaaden 2002). Similarly, disease has not been described in llama and related species (New World camelids) of South America (Duraffour et al. 2011)).

The overall epidemiological data of camelpox is limited. Camelpox outbreaks are often temporal in nature due to the movement of camels for grazing which results in mixing of the infected camels. Generally, young camels under the age of 4 years and pregnant females are more susceptible to the disease. The mean morbidity rates of the disease can be as high as 100%, while the mean mortality rates may range from 0% to 15%, and the case fatality rates may vary from 0% to 25% (Alhendi et al. 1994; Abu Elzein et al. 1999; Duraffour et al. 2011). The prevalence studies of this disease at Jazon region of Saudi Arabia during the period 2003–2004 revealed that mortality was higher in camels of less than 1 year of age (83%) followed by camels of less than 1–4 and above 4 years of age (8.3% each) (Ommer Dafalla and Abdelhamid Elfadil 2007). Among the endemic Southeast Asian countries (India, Pakistan, and Afghanistan), majority of the outbreaks are reported from India. CMLV outbreaks have regularly been reported from the North Central regions – the primary camel-rearing parts of the country (Chauhan and Kaushik 1987; Khanna et al. 1996; Marodam et al. 2006; Balamurugan et al. 2008, 2009; Bhanuprakash et al. 2010, Bera et al. 2011; Dahiya et al. 2017). Though the disease is endemic in India, sporadic outbreaks occur during the rainy season. Other countries like Ethiopia (Gelaye et al. 2016), Sudan (Khalafalla and Abdelazim 2017), and Israel (Erster et al. 2018) also witnessed camelpox outbreak in recent times.

Although CMLV is highly species-specific in infecting camels, the reports of human infections associated with camelpox have raised a debate on zoonotic nature of the virus. The first case of human camelpox was described in Somalia in a 40-year-old camel herder (Kriz 1982); however, the association of CMLV in the affected individual could not be confirmed (Kriz 1982; Jezek et al. 1983). The first indisputable proof of zoonotic CMLV infections in three human cases associated with camelpox outbreaks (2009) in dromedary camels has been recently reported from India (Bera et al. 2011). Subsequently, another report described the conclusive human case of CMLV infection related to the camelpox outbreaks (2014) in dromedary camels in Sudan (Khalafalla and Abdelazim 2017). The increasing incidences

Table 7.2 Some of the outbreaks of camelpox in different regions of the world

Continent/ country/region	Outbreaks reported	Reference
Middle East		
Bahrain	1992	Higgins et al. (1992)
Iran	1972, 2014	Baxby (1972); Mosadeghhesari et al. (2014)
Iraq	1977, 2001, 2007, 2013	Falluji et al. (1979); Gatie (2016)
Saudi Arabia	1986,1994, 1999, 2003, 2004, 2009	Hafez et al. (1992); Alhendi et al. (1994); Abu Elzein et al. (1999); Ommer Dafalla and Abdelhamid Elfadil (2007); Yousif and Al-Naeem (2011)
United Arab Emirates	1995, 1993, 1994	Renner-Muller et al. (1995); Pfeffer et al. (1996)
Israel	2016	Erster et al. (2018)
Asia		
India	1909, 1987, 1996, 2006, 2008, 2009, 2011, 2014, 2016	Lesse (1909); Chauhan and Kaushik (1987); Khanna et al. (1996); Marodam et al. (2006); Balamurugan et al. (2008, 2009); Bhanuprakash et al. (2010); Bera et al. (2011); Dahiya et al. (2017); Narnaware et al. (2018)
Pakistan	1997, 2010	Khan (2010)
Kazakhstan	1978	Tantawi et al. (1978)
Turkmenistan	1978	Tantawi et al. (1978)
Africa		
Egypt	1974, 2009	Tantawi et al. (1974); Mahmoud et al. (2012)
Ethiopia	2001, 2011, 2012, 2013, 2014	Tefera and Gebreah (2001); Ayelet et al. (2013); Gelaye et al. (2016)
Kenya	1975, 1992	Davies et al. (1975); Gitao (1997)
Libya	1996	Azwai et al. (1996)
Mauritania	1989	Nguyen-Ba et al. (1989)
Morocco	2000	El-Harrak and Loutfi (2000)
Niger	1989	Nguyen-Ba et al. (1989)
Somalia	1982	Kriz (1982)
Syria	2005	Al-Zi'abi et al. (2007)
Sudan	1992, 1994, 1998, 2013, 2014	Khalafalla et al. (1998); Khalafalla and Mohamed (1998); Motalab and Ahmed (2014); Khalafalla and Abdelazim (2017)

of human CMLV infections raise the concern of camelpox zoonosis and public health safety especially in the context of reduction of cohort immunity in human population against OPVs after the cessation of smallpox vaccination. Further, antibody prevalence rates of 6% and 10% have been observed in sheep and goat, respectively, in Saudi Arabia, suggesting potential adaptation of camelpox in hosts other than camel in enzootic areas (Housawi 2007; Duraffour et al. 2011).

7.4 Immunobiology

The immune response to CMLV is mediated through both humoral and cell-mediated immune arms of the immune system. At early stage of infection, the host innate immune response tries to prevent the infection, while the acquired immune response is mounted. Various host defense components like complement, interferon, NK, and inflammatory cells play an important role to control the early infection. However, within a few days of infection, the poxvirus-specific antibodies are generated to control the infection. Simultaneously, a cell-mediated immune response is also generated through the production of poxvirus-specific cytotoxic T cells (CTLs), which kill the virus-infected cells resulting in the clearance of the poxvirus infection. Like many other *Orthopoxvirus*, CMLV infections also produce long-lasting immunity in recovered animals. For successful transmission and multiplication in host cells, viruses require the ability to evade or subvert the innate and acquired host immune responses. Poxviruses encode multiple classes of immunomodulatory genes capable of inhibiting diverse immune mechanism of host cells such as apoptosis, complement activation, activity of natural killer (NK) cells and CTLs, and production of interferons and inflammatory cytokines. Immune-modulation mechanisms of VACV and CPXV have been well described, but reports on immune-modulation mechanisms of CMLV are limited. However, complete genome sequencing of CMLV has brought additional knowledge on immunomodulatory proteins encoded in its genome. The functions of some of these proteins have been studied experimentally. The ORF CMLV-CMS-007 encodes a virus Golgi antiapoptotic protein (v-GAAP), which inhibits apoptosis (Gubser et al. 2007a). CMLV also expresses novel virulence factor, the Schlafen-like encoded by ORF CMLV-CMS-226 affecting the host immune response to infection (Gubser et al. 2007a, b). The gene 252 of CMLV-CMS encodes a protein capable of binding to IFN-α and blocks its activity (Alcami et al. 2000; Montanuy et al. 2011; Symons et al. 1995). The ORF CMLV-CMS-233 encodes an IFN-γ receptor which binds to host IFN-γ and prevents its interaction with cellular receptor (Alcami and Smith 2002). The ORFs CMLV-CMS-002 and 265 encode soluble virus tumor necrosis factor receptor II CrmB (vTNFR); however, its action has not been studied in detail (Alcami et al. 1999). The virus chemokine-binding protein (vCKBP) encoded by CMLV-CMS-001 and 266 has been found to bind with CC chemokines and affect their interaction with cellular receptors (Alcami et al. 1998). In addition, CMLV also contains genes which have high sequence similarity with immunomodulatory genes present in other poxvirus, hence assumed to have a similar biological activity/function. The products of gene CMLV-CMS-115 have been shown to prevent the action of IFN-γ on infected cells. The products of ORF CMLV-CMS-115 has been shown to prevent activation of PKR (dsRNA-dependent protein kinase), thereby decreasing the production of IFN (Perdiguero and Esteban 2009). Further, ORF CMLV-CMS-258 encodes a serine proteinase inhibitor 1 (SPI-1), affecting host range, and

ORF CMLV-CMS-258 encodes serine proteinase inhibitor 2 (SPI-2 or CrmA), having antiapoptotic activity and blocking IL-1β and IL-18 processing. CMLV also encodes a complement-binding protein by gene CMLV-CMS-115 which inhibits complement-mediated neutralization and lysis. The genes CMLV-CMS-031 and CMLV-CMS-033 encode virokine/NFkB inhibitor and serine proteinase inhibitor 3 (SPI-3), having antiapoptotic and anti-inflammatory functions, respectively. IL-1β inhibitor of CMLV (encoded by three genes, CMLV-CMS-243, CMLV-CMS-244, and CMLV-CMS-246) has been found to inhibit IL-1β, a potent pro-inflammatory cytokine involved in inflammation.

Although there is lack of information on the expression profile of different cytokines and other immune effector cells, recent studies on the pathogenesis of CMLV in mice model have described the increased level of CD11b + F4/80+ macrophages in the spleen, CD11c + CD8α + in the lymphoid, and CD11c + CD11b + in the myeloid dendritic cell in lymph nodes and interleukin (IL)-6 and IL-18 in the sera of CMLV-infected mice (Duraffour et al. 2011). This indicates the need of detailed investigation of immune effector cells and cytokine profile in camels upon CMLV infection for the development of disease control strategies.

7.5 Risk Factors

Several risk factors are involved in outbreaks of camelpox in susceptible camel population throughout the world. The most predisposing factors for disease outbreaks include introduction of new animals into the herd, common watering, contact with common animal handlers, age of animals (higher incidence in animals less than 4 years old), and rainy season (Khalafalla and Ali 2007). Further, movement of animal herds also facilitates the spread of disease. The transmission of disease occurs mainly by direct contact between infected and susceptible animals or indirectly via contaminated environment. Infection generally occurs through the skin abrasions or via aerosol through inhalation route (Wernery and Kaaden 2002). The feeding of animals on thorny plants sometimes results in abrasion on the skin which provides an easy access for the virus entry, leading to infection. Moreover, poor nutrition and the absence of maternal antibodies along with immunological immaturity in young animals result in infection and heavy mortality (Buller and Palumbo 1991; Kriz 1982). After initial multiplication at local sites, the virus is reported to reach into most of the body secretions, viz., milk, saliva, and nasal and ocular discharges (Ramyar and Hessami 1972). The virus remains alive for at least 4 months in the dried scabs; hence, the environment containing scabs is highly risky for susceptible animals (OIE manual). The disease has got a seasonal trend with higher incidence during the rainy season, possibly associated with higher activity of arthropod vectors involved in transmission. The isolation of CMLV from the tick *Hyalomma dromedarii* further supports this statement (Pfeffer et al. 1996). CMLV has got a limited host range, and outbreak of the disease has not been reported in other animals although antibodies to CMLV have been reported in sheep (6%) and goat (10%) (Housawi 2007). Hence, the possibility of sheep and goat being

asymptomatic carriers of CMLV cannot be excluded. The virulence of infecting strain of the virus also determines the severity of infection and, based on this infection, may range from mild skin lesions to serious systemic infection (Wernery and Kaaden 2002). Upon close contact with infected animals, the human can also be infected with CMLV, and further these infected herdsmen may act as a source of infection to animals (Bera et al. 2011; Khalafalla et al. 2015).

7.6 Transmission

The CMLV is spread by three modes of transmission through direct contact, indirect contact, and insect vectors. The direct transmission of the virus occurs through contacts with sick animals either by inhalation or through skin abrasion. In indirect transmission, a camel becomes infected after contact with the contaminated environment. Infected camels shed the virus through scab materials and secretions like milk, saliva, and ocular and nasal discharges (Ramyar and Hessami 1972) to the environment. The virus particles in the dried scabs may survive for 4 months and contaminate the environment (Elliot and Tuppurainen 2008). Then, the infected environment becomes the source of infection for susceptible animals (Khalafalla and Ali 2007). The possibility of transmission of the disease via an arthropod vector has also been suspected. The CMLV has been isolated from camel ticks (*Hyalomma dromedarii*) recovered from animals with generalized camelpox (Wernery et al. 1997). Thus, it was speculated that the ticks can play a role in spreading the disease from camel to camel. This theory is further supported by the findings of the various studies, which demonstrated that incidences of camelpox infections increase immediately following heavy rains, during which the camel tick population also increases greatly (Wernery and Kaaden 1995). However, whether ticks transmit the disease biologically or mechanically could not be confirmed. Further studies are needed to ensure the involvement of arthropods in the transmission of CMLV (Duraffour et al. 2011).

Transmission of CMLV in unnatural host like humans was also conclusively confirmed in 2009 from India (Bera et al. 2011) (Fig. 7.1) and in 2014 from Sudan (Khalafalla and Abdelazim 2017). The camel herders showed skin infections confined in hands and fingers as reported from India, whereas lesions were found in the

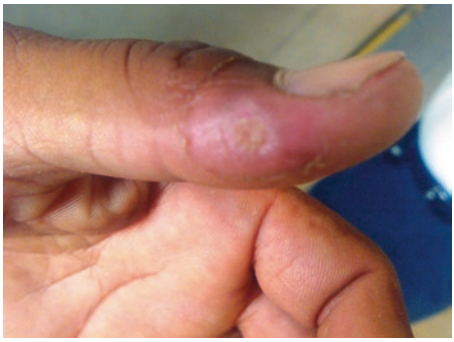

Fig. 7.1 Pock lesion in the finger of animal handler infected with camelpox virus showing typical scab formation

arms, hands, legs, back, and abdomen of camel herder in Sudan. Although earlier few human cases associated with camelpox infection have been reported, no other cases have been verified. It is assumed that CMLV is transmitted to humans via direct contact between infected camels and their human handlers.

7.7 Diagnostics

The presumptive diagnosis of camelpox infection can be made on the basis of clinical symptoms and lesions in the affected animals. However, the disease has to be differentially diagnosed from other poxvirus diseases (contagious ecthyma and papillomatosis), which produce lesions similar to camelpox infection. Several diagnostic methods are available for the detection of camelpox virus, and it is always better to use more than one test for confirmatory diagnosis. Various tests such as cell culture isolation, polymerase chain reaction (PCR) assays, real-time PCR, transmission electron microscopy (TEM), immunohistochemistry, and demonstration of neutralizing antibodies are employed for diagnosis of camelpox.

7.7.1 Clinical Symptoms and Lesions

Typical pock lesions can be seen in the affected camels. The lesions initially appear as papules, further progressing through vesicle, pustules, and finally scab formation (Fig. 7.2). During these period, animal exhibits lymphadenopathy, salivation, pyrexia, lacrimation, and nasal discharge. The lesions initially appear on the skin of the head, eyelids, nostrils, muzzle, and ears. After the development of primary skin lesions, the virus spread to local lymph nodes, and leukocyte-associated viremia occurs. The lesions further extend to the neck, limbs, genitalia, mammary glands, and perineum. In the generalized form, the lesions can be seen all over the body, and, in systemic infection, mucous membranes of the mouth and respiratory and digestive tracts get affected. Although CMLV infection is of benign nature in adult camels, mortality occurs in young animals. Diarrhea and anorexia occur if the

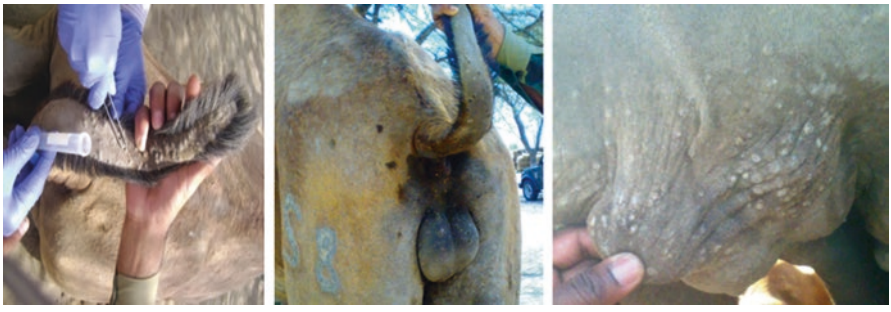

Fig. 7.2 Pock-like lesions on the skin of camel and collection of scabs for laboratory diagnosis

digestive tract is affected. Abortions and stillbirth are observed in pregnant animals, and lactating camel shows reduction in milk yield. Complete healing of skin lesions may take up to 4–6 weeks.

7.7.2 Virus Isolation

CMLV can be propagated in a wide range of cell lines and primary cultures as well as in specific pathogen-free embryonated chicken eggs. The virus can be isolated in 10–13-day-old-specific pathogen-free embryonated chicken eggs by chorioallantoic membrane (CAM) route of inoculation. The inoculated eggs exhibit opaque white proliferative pock lesions (Chauhan and Kaushik 1987; Erster et al. 2018; Marodam et al. 2006) upon incubation at 37 °C for 5 days. The pocks can be excised from the harvested CAM and used for further passaging.

The cell lines, viz., Vero, GMK-AH1, BSC-1, HeLa, and WISH, and primary cultures like lamb testicle, lamb kidney, camel embryonic kidney, fetal dromedary skin cell (Dubca), calf kidney, and chicken embryo fibroblast have been successfully used for propagation of CMLV (Bhanuprakash et al. 2010; Tantawi et al. 1974). However, the CMLV easily replicates in Vero, MA-104, or Dubca cells; hence, these cell lines are generally preferred for primary isolation of CMLV from clinical samples (Pfeffer et al. 1998). While attempting for virus isolation from clinical samples, initial three blind passages in cell lines should be monitored for 7–8 days for cytopathic effects (CPE). After adaptation, the virus produces typical CPE at 3–5 days post infection in the infected cells. The characteristic cytopathic changes include cell rounding, ballooning of cells, vacuolation, syncytia and multinucleated giant cell formation, and degenerative changes (Fig. 7.3). The virus also produces intracytoplasmic eosinophilic inclusion bodies in infected cells which can be demonstrated by hematoxylin and eosin staining (Marodam et al. 2006; Pfeffer et al. 1996).

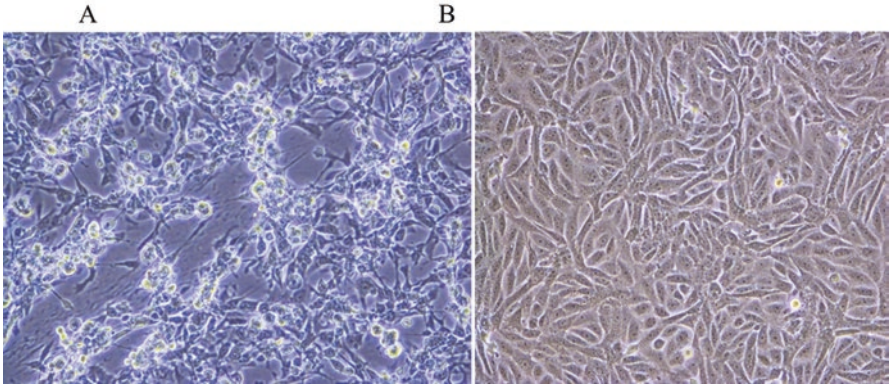

Fig. 7.3 (**a**) Cytopathic effect of CMLV in Vero cells (20X): cell rounding, syncytia formation, cell detachment, and degenerative changes. (**b**) Control healthy Vero cells (20X)

7.7.3 Electron Microscopy

Transmission electron microscopy is employed for direct visualization of viruses in clinical samples as well as in cell culture supernatant. CMLV can be observed as brick-shaped enveloped virus with its outer surface bearing irregularly arranged tubular proteins, which can easily be distinguished from ovoid-shaped parapoxviruses. The diameter of virus particle ranges from 217.6 ± 18.7 × 293.15 ± 18.8 nm (Erster et al. 2018). The requirement of high concentration of virus in the sample and inability to distinguish CMLV from other OPVs are some of the drawbacks of transmission electron microscopy-based diagnosis.

7.7.4 Serological Test

A wide range of serological tests are available to identify camelpox virus infection of which serum neutralization test is most confirmatory. Polyclonal antibody-based fluorescent antibody test (FAT) and immunoperoxidase test (IPT) can detect the presence of CMLV-specific antibodies; however, there is chance of cross-fluorescence between virus and antisera of other members of the OPV group. However, CMLV can be differentially diagnosed from other related viruses such as capripox, avipox, and parapox viruses using FAT and IPT (Bera et al. 2011; Davies et al. 1975). ELISA has also been employed for detection of CMLV antibodies using semi-purified and purified CMLV antigen. Usage of purified CMLV antigen yielded better results in the form of low background signal with the negative control camel sera (Davies et al. 1975; Munz et al. 1986). The western blot analysis was also used for identification of CMLV-specific protein banding pattern during the early period (Azwai et al. 1996; Pfeffer et al. 1996). However, most of these conventional serological tests are time-consuming and less sensitive. So they are not preferred for primary diagnosis but highly useful in secondary confirmatory diagnosis and retrospective epidemiological studies (Balamurugan et al. 2013).

7.7.5 Molecular Methods

Various molecular tests such as polymerase chain reaction (PCR), PCR-RFLP (PCR-restriction fragment length polymorphism), loop-mediated isothermal amplification (LAMP), and real-time PCR have been developed for rapid and differential diagnosis of CMLV infection. Further, recent advances in sequencing technology particularly next-generation sequencing (NGS) techniques has opened up new vista in the field of disease diagnosis. The NGS is being employed for fast detection of infectious agent along with simultaneous identification of multiple viruses in clinical samples within hours.

7.7.5.1 Polymerase Chain Reaction (PCR)

The currently used conventional PCR assays detect CMLV-specific genomic regions by amplification of various genes like A-type inclusion body (ATI), hemagglutinin (HA), ankyrin repeat protein (C18L) genes, etc. (Balamurugan et al. 2009; Meyer et al. 1997; Ropp et al. 1995). However, in field condition, mixed infections with related viruses are also common. This situation demands a single diagnostic test capable of detecting multiple infectious agents simultaneously rather than detecting each pathogen individually. To overcome this problem, duplex, multiplex, and pan-pox universal PCR assays have also been developed recently (Balamurugan et al. 2009; Li et al. 2010; Khalafalla et al. 2015). The newly developed multiplex PCR is capable of simultaneously detecting three important viruses of camel, i.e., camelpox virus (CMLV), parapox virus, and papilloma virus (Khalafalla et al. 2015). The pan-pox universal PCR assay developed by Li et al. is able to detect most of the members of the family *Poxviridae* (Li et al. 2010). Further, a high-throughput pan-orthopoxvirus detection assay has been developed employing PCR amplification of helicase and polymerase genes followed by electrospray ionization mass spectrometry (PCR/ESI-MS) for rapid identification of each species of OPVs from clinical sample (Eshoo et al. 2009).

7.7.5.2 PCR-Restriction Fragment Length Polymorphism (PCR-RFLP)

This technique employs amplification of specific region(s) by conventional PCR followed by restriction enzyme digestion of PCR products. The difference in band pattern after restriction enzyme digestion helps in specific detection of each virus. The HA and A36R gene-based PCR-RFLP techniques have been developed for specific and differential detection of CMLV from other OPVs (Huemer et al. 2008; Ropp et al. 1995). However, this assay is time-consuming and not being used in most of the diagnostic laboratories nowadays.

7.7.5.3 Loop-Mediated Isothermal Amplification (LAMP)

A highly specific loop-mediated isothermal amplification (LAMP) assay based on C18L gene was developed recently for rapid detection of CMLV (Venkatesan et al. 2012). This assay is proposed to be highly useful in less equipped rural diagnostics laboratory settings of developing countries; however, its large-scale evaluation is undergoing.

7.7.5.4 Real-Time PCR (qPCR)

The real-time PCR has facilitated the detection of minute quantities of viral nucleic acids in a fast and specific manner. In addition, this assay also provide the option to quantify and to genotype the target nucleic acid reliably. Different qPCR-based assays have been developed for specific and differential detection of CMLV. The fluorescence resonance energy transfer (FRET)-based qPCR targeting A13L and rpo18 and viral early transcription factor (VETF) genes have been developed for simultaneous and differential detection of CMLV and other OPVs (Nitsche et al.

2004; Panning et al. 2004). Recently, a SYBR green (Balamurugan et al. 2009) and a TaqMan hydrolysis probe-based qPCR (Venkatesan et al. 2012) assays targeting the ankyrin repeat protein (C18L) gene have also been developed for specific detection of CMLV. The universal TaqMan qPCR assay based on HA and DNA polymerase-E9L genes enables rapid detection of pan-orthopoxviruses (Kulesh et al. 2004).

7.7.5.5 Next-Generation Sequencing (NGS)

The high-throughput sequencing technologies like next-generation sequencing (NGS) using a number of different modern sequencing platforms, viz., Illumina, Roche 454, ion torrent (Proton)/PGM, and SOLiD, enable rapid sequencing-based detection of viruses in clinical samples (Barzon et al. 2011). These sequencing platforms have advantages of sequencing of viral genomes from clinical specimen having mixture of genetic materials and less quantity of virus without needing prior sequence information. Further direct sequencing helps in preventing sequence alteration during in vitro passaging of the virus. The NGS-based diagnostics are not being routinely used in diagnostic laboratories due to the involvement of more cost; however, these techniques are useful for the detection of mixed and unknown infections.

7.8 Prevention and Control

Camelpox can be controlled by reducing exposure to the virus, improving sanitation, and eliminating the vector involved in transmission, immunoprophylaxis, and treatment. To curtail the spread of disease, infected animals should be contained in a separate place and be provided with separate feeding and watering facilities. Care should be taken for young and pregnant animals not to come in contact with infected animals. Personal hygiene of herdsmen and veterinarians is of importance in lowering transmission of CMLV. The personnel caring/treating the infected animal should not be allowed to enter the premises where healthy animals are kept. Newly brought animal should be kept in quarantine for approximately 15–21 days period. Movement of animals should be restricted during outbreak period, and any animal fair should be banned. The vector control is another approach in preventing the transmission of CMLV especially controlling the tick population during rainy season. Tick control with acaricides is only a realistic option for well-managed livestock production facilities; however, this approach has become less reliable, because acaricides are expensive and resistance has developed against many of them. Further, due to the resemblance of CMLV with VARV at molecular level and possibility of CMLV zoonosis, there is a need to follow strict biosecurity and biosafety measures for controlling this transboundary and emerging disease.

7.8.1 Vaccination

Vaccination is the best way to control any viral diseases. Regular vaccination should be practiced in camel herds, particularly before the onset of monsoon, so that animals will have sufficient antibody level before vector population become active. Camelpox vaccines have been developed as prophylactic measures to contain the spread of camelpox in enzootic countries. The CMLV vaccine development was initiated after the eradication of smallpox worldwide. The usage of VACV for the control of OPV infection in animals was not recommended during that period because of the possibility of transmission of VACV to non-vaccinated humans from vaccinated contact animals (Hafez et al. 1992). Therefore, researchers began to focus on developing vaccine against camelpox using CMLV strains that have limited host range (i.e., camels). Both live attenuated and inactivated vaccines are available to control camelpox. Efficacy and safety of three live attenuated (Jouf-78, VD47/25, and Ducapox (298/89)/Ducapox^{R}) and one inactivated vaccines (CMLV-T8/CAMELPOX^{R}) have been evaluated thoroughly and are being used for controlling camelpox in different countries (Hafez et al. 1992; Nguyen-Ba et al. 1996; Wernery and Zachariah 1999; Harrak and Loutfi 2000). The live attenuated CMLV Jouf-78 vaccine is being used in Saudi Arabia and has been found to be protective in the field at a dose rate of 10^3 to 10^4 $TCID_{50}$ when given intradermally or subcutaneously (Hafez et al. 1992). The cell culture-based live attenuated CMLV VD47/25 vaccine was found to be innocuous in camels at a dose of $10^{4.7}$ $TCID_{50}$, by subcutaneous route, and is being used in Mauritania (Nguyen Ba et al. 1996). The third live attenuated vaccine Ducapox^{R} (standing for Dubai CAmelPOX vaccine) has been in use in United Arab Emirates since 1994 with great success. Although there are reports on 6 years of protection upon vaccination, the study was conducted on a very limited number of animals (Wernery and Zachariah 1999). The starting age for vaccination is 6 months. Although a single dose is enough to sustain protection for at least 1 year, booster dose is recommended in 6–9-month-old camels to avoid any vaccine breakdown because of maternal antibody (Khalafalla and Dirdiri 2003). Ducapox^{R} vaccine is commercially produced in South Africa. The inactivated vaccine against camelpox is derived from CMLV strain T8 isolated from Morocco in 1984. The vaccine is found to be safe and efficacious in young and adult camels. For efficient protection, a second injection is required 1 month post primary vaccination, which is followed by annual booster (Harrak and Loutfi 2000). Recently, a live cell culture attenuated camelpox vaccine has been reported from India, and the thermostability of this vaccine has also been evaluated using various stabilizers which will help in their use in dry and hot camel-rearing regions (Prabhu et al. 2014).

7.8.2 Antiviral Therapy

Antiviral agents inhibit viral replication at the cellular level, interrupting one or more steps involved in the life cycle of the virus. These agents have a limited spectrum of activity, and most of them interrupt host cell function and are toxic to cells at various degrees. Postexposure therapeutic approaches for camelpox infection have got limited use in adult animals; however, this can be useful in young animals which are more susceptible to infection. Only few antiviral agents have been evaluated for their potential use against CMLV in both in vitro and in vivo studies (Duraffour et al. 2007; Smee et al. 2002). The drug molecules found to be effective against CMLV include cidofovir (acyclic nucleoside phosphonate family) and its derivates like CMX001, HPMP-5-azacytozine, and HPMPDAP. In a study conducted in athymic nude mice, cidofovir was found to give 100% protection from morbidity when administered intraperitoneally (once per day for 3 days at 50 mg/kg) as well as topically as 1% cidofovir cream (once per day for 5 days). Another cidofovir analogue HPMP-5-azacytozine has also shown anti-CMLV effect in in vitro studies; however, more detailed study is required to confirm its antiviral activity. The efficacy of the drug CMX001 (lipid derivative of cidofovir) has not been evaluated against CMLV, although the EC_{50} of this drug has been calculated for CPXV (0.6 μM) and VACV (0.8 μM) in human foreskin fibroblasts. Hence, it is assumed that CMX001 will also be active against CMLV within the EC_{50} range (0.6 μM–0.8 μM) reported for CPXV and VACV. The drug ST-246 which was found to be highly effective against OPV infection both in vivo and in vitro has also been evaluated for anti-CMLV activity in vitro. The drug molecule exhibited EC_{50} values of 0.01 μM, 0.05 μM, and 0.08 μM in the cell lines Vero, PHK, and HEL, respectively (Duraffour et al. 2007; Yang et al. 2005).

Despite all these adaptive measures, the success of any prevention and control policy depends on the implementation of an adequate surveillance system. The presence of antibodies to CMLV in sheep and goat and spillover infection to humans reminds us that adaptation of CMLV to other animal species should not be neglected. The role of arthropod vectors in the transmission of camelpox needs to be clearly ascertained by screening tick population collected from goats, sheep, and cattle from countries endemic for the disease. Limited geographical presence of the disease (arid and semiarid regions), host restriction of CMLV, and availability of suitable vaccines make this disease a better candidate for eradication campaign in the near future in similar line with other diseases.

7.9 Conclusion and Future Prospects

In recent times, the increasing number of OPV infections among animals and humans in the different parts of world warrants the focus on stringent research on CMLV. Although the disease was considered inconsequential until recent times, the recent incidences of zoonotic infections in Asian and African countries have brought the disease into limelight. Many researchers believe that two strains of CMLV exist

(virulent one causing systemic infection and less virulent strain causing mild skin lesions); however, more detailed study on genome-level information is required to confirm this hypothesis. The genome of CMLV encodes many gene homologues to those in VARV, VACV, CPXV, and MPXV; however, whether all these genes with putative immunoregulatory functions are functionally active or not have to be ascertained. Unlike other OPV, the pathogenesis of CMLV has not been studied in detail, which is highly required to answer the questions on tissue tropism, limited host range, and spread from the initial site of infection, counter host immune responses, etc. It is also unknown whether any reservoir host exists for CMLV. The presence of antibodies to CMLV in sheep and goat and spillover infection to humans reminds us that adaptation of CMLV to other animal species should not be neglected. Further, the role of arthropod vector in disease transmission has also to be confirmed. For the control of the disease, vaccines have been developed; however, new-generation vaccines for long-term immunity are to be developed. With the present antiviral treatment being a costly affair, cheaper broad-spectrum alternative antivirals may be explored for developing effective therapeutics for disease management. Limited geographical presence of disease (arid and semiarid regions), host restriction of CMLV, and availability of suitable vaccines make this disease a better candidate for eradication campaign in the near future in similar line with other diseases. Usage of vaccines which produce long-lasting immunity (considering the 40–50 years life span of camels) and inclusion of even wild (non-domestic) and Bactrian camels (existing in some Asian countries) in vaccination program with proper health approaches may help in the eradication of the disease.

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Conflict of Interest There is no conflict of interest.

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Fowlpox Virus

8

P. Raja

Abstract

Poxviruses are ubiquitous in nature and dispersed globally among domestic and wild birds. A total of 230 species have been reported with poxvirus infection from terrestrial and marine environment. Fowlpox virus (FPV) is a major economically important virus especially in the layer birds which cause drop in egg production and heavy mortality. It belongs to genus *Avipoxvirus* (APV), subfamily *Chordopoxvirinae*, of the family *Poxviridae*. The genus *Avipoxvirus* (APV) consists of a cluster of poxvirus that infects fowl, turkey, pigeon and many wild birds. However, their pathogenicity, host specificity and degree of relationship may vary. The cutaneous or diphtheritic form or both occurred in naturally infected birds. Host specificity is considered one of the important criteria for differentiation of APVs. Till now the precise figure of existing strains and variants of APV is not clearly defined due to emergence of new strains from different avian species. Nowadays, the turkey and pigeon pox also gained much economic importance due to their virulence nature. Even though the APV is strongly host specific, there are several reports on FPV infection on turkey that reveals the fact that FAV is emerging pathogen of turkey. The FPV is highly infectious for chickens and turkeys, rarely for pigeons and not for ducks and canaries. The turkey pox virus is virulent to ducks. In the current scenario, the disease has emerged as a new risk to poultry industry and several incidences also documented in vaccinated poultry flocks worldwide. The pathogenicity of the field FPV strains is higher than vaccine strains and studies reveal that these field FPV strains are integrated with reticuloendotheliosis provirus in their genome. Due to altered genome, the pathogenicity of the field FPV is higher and could not be protected by vaccine virus.

P. Raja (✉)
Department of Animal Biotechnology, Madras Veterinary College, TANUVAS, Chennai, India

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8.1 Prologue

Fowlpox is a slow-spreading virus disease of birds. Its DNA virus belongs to genus *Avipoxvirus* (APV). APVs are the largest as well as composite viruses which belong to subfamily *Chordopoxvirinae* of the *Poxviridae* family. It infects and causes severe diseases in most of the avian species (including poultry, pet, wild and endangered species of birds) which results in financial losses to the poultry flocks (Bailey et al. 2002; Deem et al. 2012). The disease condition is well distinguished by discrete, nodular, proliferative lesions in the skin and diphtheric membranes of the upper respiratory tract and oesophagus of infected birds (Bollinger 1873). The infection with APVs will cause poor growth, lower feed conversion ratio and tremendous reduction in egg yields. Its incidence is variable in different geographical locations due to different climatic conditions, management practice of poultry rearing and the use of vaccination. The APV infection was reported in more than 232 species of 23 orders of birds (Bolte et al. 1999).

The APVs are large ovate-shaped enveloped viruses and their genome composed of approximately 260–365 kb size of a double-stranded DNA molecule. It can replicate rapidly in the cell culture and produces the cytopathic effect (CPE) after 4–6 days of infection (Tripathy et al. 2000; Tulman et al. 2004). It can also replicate in the chorioallantoic membrane (CAM) of embryonated chicken eggs and results in proliferative pock lesions (either focal or diffuse type). APVs produce intracytoplasmic inclusion bodies in membranous epithelial tissues and mononuclear cells of susceptible birds which are similar to other chordopoxviruses. Fowlpox (FP) is globally distributed and the higher incidences are being reported in tropical and subtropical countries (Beytut and Haligur 2007). Flock mortality is very low in cutaneous form but in diphtheritic form it will be high. In adult birds, the morbidity is usually very high and the mortality rate varies from 5% to 10% with reduction in egg production to 50%. However, the current molecular understanding of APV is confined to FPV and canarypox virus (CNPV) due to availability of the complete genome sequence (Afonso et al. 2000; Tulman et al. 2004; Manarolla et al. 2010).

Classification of field strains disclosed that the reticuloendotheliosis provirus (REV) is fused with APV genomes (Singh et al. 2000: García et al. 2003; Tadese et al. 2003). The very early expression of this acquired retroviral gene could modify the pathogenic properties of the host and result in the evolving of new virus for which the vaccines available in the field could not offer adequate protection. This chapter will brief about the current knowledge on history, incidence and prevalence, immunobiology, diagnosis, risk factors, transmission and prevention of Fowlpox disease.

8.2 History

The APV is one among the earliest avian diseases because of its easy recognition of obvious external lesions. Bollinger (1873) and Borrel (1904) initially correlated the lesions and the structure of inclusion bodies in APV infection. In 1930, Woodruff and Goodpasture reported that the avian poxvirus was associated with the inclusion bodies. During the mid-twentieth century, APV was isolated from cell culture and chorioallantoic membrane (CAM) of embryonated chicken eggs (Cunningham 1966).

The *Avipoxvirus* shares more biological characteristics with the other poxviruses. The poxviruses were classified on the basis of nature of infecting the wide host range, growth characteristics on the cell culture or appearance of the pock lesions (either diffuse or focal type) on the CAM of embryonated chicken eggs and the clinical manifestations in different hosts rather than the molecular basis of identification and characterization which may provide accurate virus identification. The International Committee on Taxonomy of Viruses classified the *Avipoxvirus* worldwide and these strains differ in virulence and host specificity (Fauquet et al. 2005). The *Poxviridae* family has two subfamilies – (i) *Chordopoxvirinae* (for vertebrate host) and (ii) *Entomopoxvirinae* (for insects). The *Chordopoxvirinae* subfamily is further divided into nine genera, i.e. *Parapoxvirus*, *Capripoxvirus*, *Yatapoxvirus*, *Suipoxvirus*, *Orthopoxvirus*, *Molluscipoxvirus*, *Avipoxvirus*, *Leporipoxvirus* and *Cepvidpoxvirus*. Again there are 25 numbers of different members of the genus *Avipoxvirus* and their host spectra were also reported (Weli and Tryland 2011).

Despite the regular vaccination in endemic areas, the disease shows a tendency to persist for a longer period (Fatunmbi and Read 1996). The disease has been evolved as a new risk to poultry industry and several incidences have been reported even in vaccinated flocks (Lawson et al. 2012). The diphtheric form of infection has produced high mortality in vaccinated flocks (Kim and Tripathy 2001; Bányai et al. 2015). There are several reports by various researchers on the pathogenicity of field virus which is higher than vaccine strains (Tripathy et al. 1974, 1975; Tripathy and Hanson 1978; Singh et al. 2000).

8.3 Structure and Genome Organization of APVs

In 1981, Carter and Cherville described the structure of avipoxviruses and they reported several morphological, biochemical and physiochemical features with other poxviruses. The virus particles are in the size of 270 × 350 nm which is made up of centrally located electron dense core and two lateral bodies. In negative staining, the membrane shows an outer coat made up of random arrangement of tubules.

Avipoxviruses consist of a linear double-stranded DNA molecule with lower G + C content (30–40%). The APV genome is flanked by two identical inverted terminal repeats (ITRs) which are covalently linked by hairpin loops with several open reading frames (ORF) at the central region. The region also contains several homologous genes which play a vital role in viral transcription and modification, and proteins are involved

in the assembly of intracellular mature and extracellular enveloped virions (Afonso et al. 2000). The genes located in this central region have typical functions and are in comparatively conserved regions among poxviruses (Tulman et al. 2004).

Until now, only few APVs were completely sequenced. They are FWPV US (FP-challenge virus, USA) and FWPV-FP9 (tissue culture attenuated European virus) strains and a CNPV virulent strain (ATCC VR-111). Comparison between the FWPV and FP9 strains revealed 118 differences in the genome and they are mostly of deletion (26 of 1–9334 bp) and insertion (15 of 1–108 bp) type of mutations, followed by substitution and termination or frameshift mutations. At the same time, comparison between the FWPV and CNPV strains revealed higher amino acid identity, nucleotide sequence rearrangements and deletion and insertion type of mutations. However, the genome of CNPV is of 80–100 kb greater than the FWPV genomes (Laidlaw and Skinner 2004). The CNPV strain showed a broader tissue tropism compared to FWPV (Sadasiv et al. 1985). These divergences are mainly observed in the terminal non-conserved regions (Tulman et al. 2004; Ruiz-Martoanez et al. 2016). However, to compare the genetic diversity of different avipoxviruses, the 4b core protein gene (p4b) which encodes for the protein (75.2 kDa) is used (Ghalyanchilangeroudi et al. 2018). The open reading frames 175 and 176 of FWPV strain are orthologues of conserved vaccinia virus ORF A11R and A12L, which encode a non-structural protein involved in virion formation and a 25 kDa core protein involved in morphogenesis, respectively.

There are several specific genes which are associated with phenotypic differences in poxviruses. The FWPV strain encodes for five serpin homologues (fpv010, fpv040, fpv044, fpv204 and fpv251) and two homologues of cellular nerve growth factor (fpv072, fpv076) which may be involved in early innate immune responses and important for viral infection. It also encodes for a gene (fpv073) which involved in inhibition of inflammation (Afonso et al. 2000). The penguin pox virus, canary pox virus and pigeon pox virus may contain one or more of these anti-inflammatory genes which may be responsible for their phenotype on CAM.

The genes which are in the non-conserved regions are highly prone to mutation and recombination which are responsible for host range, immunomodulation and pathogenesis (Seet et al. 2003). The poxvirus is highly resistant to desiccation and can survive in the extreme environment for long periods.

8.4 Incidence and Prevalence

The periodic re-emergence of FPV due to change in epidemiological distribution, antigenicity, virulence of fowlpox virus stains even after vaccination in worldwide (Singh et al. 2000). In India, 1981, six different strains of FPV were isolated from vaccinated flocks (Sharma et al. 1981). A diphtheric form of APV infection without cutaneous lesions is reported in chicken layer flocks in Egypt (Aly 1995). The APV outbreak from 45 flocks in NSW (New South Wales), QLD (Queensland) and WA (West Australia) has been reported. The NSW and QLD isolates were

isolated from the flocks vaccinated with M strain fowlpox vaccine (Diallo et al. 1998). Apart from this, FPV48 and FPV52 were also isolated from wild birds. Singh et al. (2000) reported APV outbreak from vaccinated chickens. In a natural outbreak of APV infection in Uttar Pradesh, the overall morbidity and mortality were 2.34% and 2.11%, respectively. The gross lesions included proliferative wartlike lesions on the comb and wattles in 10% of the affected birds. In 90% of cases, the lesions were characterized by the formation of a necrotic mass in the trachea, which blocked the airways (Shukla et al. 2000). In Australia, several field strains have been isolated from APV-infected commercial poultry flocks that had been previously vaccinated with live vaccine (Jianning 2002).

Concurrent infection of chickens with infectious laryngotracheitis virus (ILTV), along with fowlpox virus (APV), has also been reported (Tadese et al. 2007). Silva et al. (2009) reported APV outbreak in chickens reared under backyard in two villages of Bahia, USA. The APV infection in 1-month-old chickens with gross lesion in the skin had been reported in Grenada, West Indies (Arathy et al. 2010). Senties-Cue et al. (2010) also reported an unusual form of cutaneous fowlpox outbreak in 8-week-old broilers in California. They reported round, longitudinal and proliferative scratch-associated lesions were found only in feathered areas and the head, legs, feet and toes did not have any lesions. Fowlpox virus (FPV) infection was confirmed by amplification of the 4b core protein gene, enveloped gene of REV genome and 5' ITR region of FPV by PCR in 15 birds in a flock of 37 (Biswas et al. 2011). Hess et al. (2011) reported the occurrence of an APV infection in turkeys. The disease was distinguished by severe epithelial and proliferative lesions on the head and neck regions. Pawar et al. (2012) amplified the fpv167 and fpv140 genes from eight Indian wild birds. Sawale et al. (2012) observed numerous black to brown-coloured nodular growths on the wattle and comb with varied proportion among the birds during their study in seven farms. The mortality rate ranged from negligible to the high value of 1.5%. Layer birds showed drop of production nearly 20%. Based on gross, histopathological and isolation study, the disease was identified as fowlpox. Characterization of fowlpox field isolates revealed that PCR combined with restriction endonuclease analysis with EcoR V and Mse I enzymes showed similar profile patterns among the different field isolates from different parts of West Bengal (Roy et al. 2013). El-Mahdy et al. (2014) performed molecular detection of field poxvirus from suspected farms by PCR targeting thymidine kinase (TK) gene in Egypt.

8.5 Impact of APV Infection on Wild Bird Population

Until now only very few reported are available about mortality rates in free flying wild birds due to natural avipoxvirus infection. In most of the wild birds, the APV infections are characterized as very mild and self-limiting and will be cured on their own. At the same time, the APV severity and mortality rates were similar in pheasants, quail and wild turkeys as well as chickens.

8.6 Immunopathology

8.6.1 Pathogenesis

If APV enters into the host epithelium, within a short period of time (even as early as 1 h), it can penetrate to cell membranes and then uncoat the viral capsid and release the viral nucleic acid for synthesis of a new virus (Arhelger et al. 1962). The biosynthesis of a new virus particle was occurring in two distinct phases in the host dermal epithelial cell. During the first phase, the host response starts in the first 72 h against APVs, accomplished by the formation of new infectious virus particles after 72–96 h of postinfection. At 36–48 h of postinfection, the host DNA synthesis occurred followed by epithelial cell hyperplasia and sharp declining of host DNA at 60 h. The second phase consists of long latent period in which the incomplete membranes will surround the viroplasm within the cytoplasm (Cheever and Randall 1968). The viroplasmic particles become condensed and accomplished by an additional outer membrane to make an incomplete virions. Further, these incomplete virions will move into vacuoles of the inclusion bodies and gain a membrane coat (Cheville 1966). Finally, the viruses come out of the cells by a budding process, which results in another outer membrane that is gained from the cell membrane. During this process, a classical inclusion body (Bollinger body) can be observed via light microscopy.

After the entry into an avian host, the overall initial incubation period was varied depending on the poxvirus strain and host species. The complete duration of the disease is different, and in the case of chickens the avian pox infection persists for 4 weeks (Tripathy and Reed 2013; Manarolla et al. 2010), and in the case of wild birds the incubation period was varied and it may extend up to 1 month (Kirmse 1969). The APVs cause localized proliferative lesions in epithelial cells. The infected cells become hyperplastic and hypertrophic as they increase in the rate of multiplication in the basal germinal layer of epithelial cells. The inclusion body (hypertrophy with acidophilic intracytoplasmic granules) seems to be matured cells in germinal layers of the epithelium. The accumulation of infected epithelial cells will form pock lesions and persist for different hosts with varied incubation periods (Karstad 1971). Diphtheritic form of infection is commonly identified in wild bird pox infections. The pock lesions on the mucous membranes appear as white, opaque, nodules which will increase in shorter duration, often coalescing to form a yellowish, cheesy, necrotic material that will give the appearance of a pseudomembrane (Cunningham 1972). This condition is further exacerbated by entry of secondary bacterial infections and that it may cause respiratory distress.

8.7 Host Immunity

The birds which are recovered from natural infections or that have been vaccinated against APV will evoke immune response to reinfection for the same virus strain (Fenner 1968). This type of immunity is widely cell-mediated immune responses, although antibodies can also play a vital role. Until now the transovarial type of

transmission of immunity for APV infection has not been reported, and the virus strains were isolated from a specific host species which are varying in the degree of infectivity to other species. Commonly, most of the poxviruses of wild birds are not pathogenic in nature for chickens (Tripathy et al. 2000). APV strains from one host species can provide reciprocal immunity to other host species, and cross immunity is also reported for various strains of avian pox.

8.8 Diagnosis

8.8.1 Clinical Signs

The APV infections are reported primarily in two different forms: (i) *cutaneous form*, a common form which affects the skin, in which distinct, wartlike, proliferative epithelial skin lesions were observed (Fig. 8.1), and (ii) *diphtheritic form*, a less common form of pox virus infection in which moist, necrotic lesions developed on the mucous membranes of the upper respiratory tract, mouth and oesophagus (Fig. 8.2). However, a third form of pox infection is also reported as systemic infection very rare in wild birds (Tripathy and Reed 2013). After infection with avian poxvirus, the adult birds will recover rapidly but young birds are more severely affected. The individual birds which are affected by avian pox infections may suffer, decline in number and become permanently blind forever. In advanced cases, lesions are reported on both mucous membranes and the skin of affected birds. Lesions of the mucous membranes, particularly mouth, oesophagus and upper respiratory air passages, will block the air passage and result in high mortality. Usually chickens with diphtheritic form of pox infections have higher mortality rates when compared to cutaneous pox.

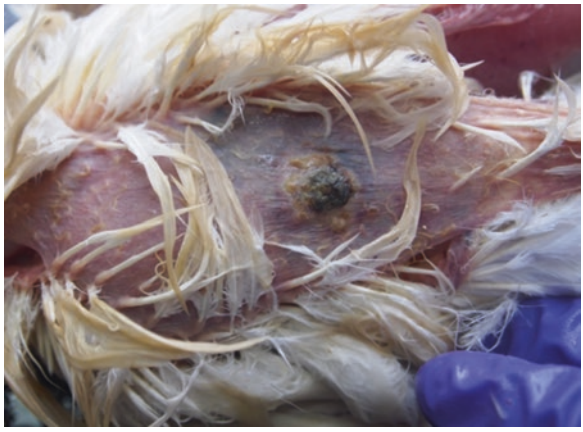

Fig. 8.1 The affected bird shows prominent necroproliferative cutaneous lesion over the skin – cutaneous pox

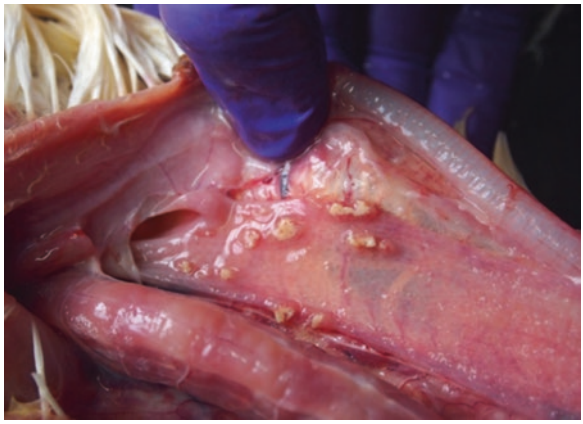

Fig. 8.2 Pseudomembrane formation over the upper oesophagus – wet pox

8.9 Gross and Histopathological Study

The field avipoxvirus inoculated in embryonated chicken eggs reveals the presence of pox lesion on CAM and intracytoplasmic inclusion body (Tripathy et al. 2000; Singh et al. 2000). The biological studies of APV infection revealed the gross lesions like crusts and nodule formation on the comb, eyelids, oral mucosa, legs and toes of affected birds. In histopathological examination, eosinophilic intracytoplasmic inclusion bodies were observed in epithelial cells. Apart from this, in the inclusion body, several pox virions are identified by electron microscopy (Yoshikkawa and Alam 2002). The cutaneous form of APV infection in vaccinated flocks with pox lesions on the skin and proliferative and necrotic lesions with eosinophilic cytoplasmic inclusions (Bollinger bodies) has been reported (Kikuyasu et al. 2006; Sawale et al. 2012). Transmission electron microscopy study reveals the characteristic viral particles of pox virus in the infected CAM and tissues (Prukner-Radovcić et al. 2006; Hess et al. 2011). The immunohistochemistry study of APV infection reveals the different developmental stages of inclusion bodies in experimentally infected CAM (Haligur et al. 2009; Offerman et al. 2013). Figure 8.3a, b depicted the eosinophilic intracytoplasmic inclusion bodies on epithelial cells.

8.10 Isolation and Identification of Virus

8.10.1 Virus Isolation

The isolation of fowlpox virus is done by the inoculation of tissue suspension or diphtheritic lesion via chorioallantoic membranes in 9- to 12-day-old embryonated chicken eggs which are then examined for the presence of focal white pock lesions or generalized thickening of the CAMs after 5–7 days (Tripathy and Reed 2013). The adaptation of virus strains into cell cultures is prerequisite for the formation of plaque. Primary chicken embryo fibroblasts, chicken embryo kidney cells, chicken embryo dermis

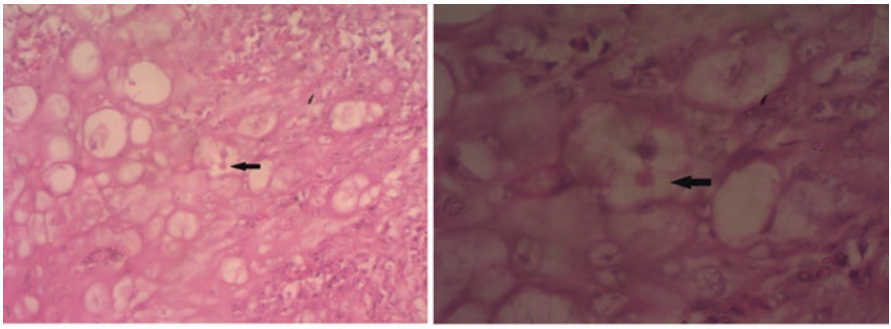

Fig. 8.3 (**a**, **b**) H&E staining of chicken affected with fowlpox virus shows eosinophilic intracytoplasmic inclusion bodies on epithelial cells (40× and 100×)

cells or the permanent quail cell line QT-35 is commonly used to adaptation and propagation of fowlpox virus (Ghildyal et al. 1989; Schnitzlein et al. 1988).

8.11 Immunological Methods

8.11.1 Agar Gel Immunodiffusion Test

The antigenic relationships between different pox viruses using embryonated egg-adapted fowlpox, pigeon pox and canary pox viruses were studied by agar gel precipitation tests, and the results revealed that the two lines of precipitation were formed when antisera against FPV were diffused against homologous antigen and one line of precipitation was formed when antisera raised against duck pox virus, pigeon pox virus or canary pox virus were diffused against either homologous or heterologous antigen (Uppal and Nilakantan 1970). The identity of the virus by AGPT as clear precipitation lines was observed with positive fowlpox antiserum (Tamador et al. 2001). Avipox infection in quails was reported later using AGPT (Gulbahar 2005). Survey of antibody status to APV in free-range chickens was done by testing 229 sera samples and 52 sera samples were found to be positive for FPV antibody from Nigeria (Adebajo et al. 2012).

8.11.2 Counterimmunoelectrophoresis

The counterimmunoelectrophoresis (CIE) technique was used for detection of APV antibodies and found to be a rapid and very sensitive technique (Sarma and Sharma 1987). The immunoelectrophoretic pattern of local strains of FPV reveals the identity of field and vaccine strains by CIE (Das et al. 1990; Tamador et al. 2001).

8.11.3 Haemagglutination Test

The haemagglutination test and haemagglutination inhibition test were carried out with avian pox virus using RBCs from different species of animals (Mathew 1967). The passive haemagglutination (PHA) test was performed using tanned horse RBCs coated with partially purified APV viral antigen for titration of antibodies against APV. However, the precipitating antibodies appear late compared to AGPT (Tripathy et al. 1970). The HA pattern of pigeon pox and fowlpox viruses reveals the heat labile haemagglutinin in CAM and allantoic fluid which was first detected 96 hours after infection (Narain et al. 1979). The PHA is used for monitoring the immune response of chicks following vaccination with APV and the level of IgG concentration was higher after vaccination than control birds (Saini et al. 1990).

8.11.4 Fluorescent Antibody Test

The specific intracytoplasmic fluorescence in infected cells was identified by direct or indirect immunofluorescence tests. Usually the indirect immunofluorescence test is a common method and the antibody against fowlpox virus reacted with the antigen, and a secondary (FITC) labelled antibody raised against chicken will be used.

8.11.5 Molecular Tools for Detection and Characterization of APV

Usually the APVs are identified and characterized by several molecular tools such as PCR, PCR-RFLP (restriction fragment length polymorphism), hybridization techniques and sequencing based on 4b core protein gene methods. PCR-based methods are more sensitive and specifically detect pox virus genome with higher sensitivity compared to other immunological techniques and culturing methods. The cloned genomic fragments were used as nucleic acid probes for detection of fowlpox (Fatunmbi et al. 1995). The partial P4b core protein gene of 578 base pairs (bp) of APV is commonly used for diagnosis due to their highly conserved nature among all the pox viruses (Luschow et al. 2004). Several researchers reported the PCR-based identification of FPV using the specific primers. This technique is useful even for a small amount of viral DNA (Lee and Lee 1997; Fallavena et al. 2002; Gholami-Ahangaran et al. 2014).

Several workers reported the use of PCR-RFLP analysis to differentiate field and vaccine strains of fowlpox virus (Ghildyal et al. 1989; Schnitzlein et al. 1988). This technique is also useful to distinguish fowlpox infection from other avian pathogens (Abdallah and Hassanin 2013). The genomes of FWPV and quail pox virus isolates were digested with three different restriction enzymes (BamHI, EcoRI and HindIII) and results reveal the differences in the restriction profile patterns between these isolates. Unique antigens were detected when immunogenic proteins of different pox

virus isolates (FWPVs, quail pox viruses, juncopox virus and pigeon pox virus) were identified by immunoblotting technique (Ghildyal et al. 1989; Schnitzlein et al. 1988). However, the genetic differences between the quail pox and FWPV might be responsible for differences in immunogenicity and antigenicity properties.

8.12 Phylogenetic Classification

To understand the host specificity, variation in the genome of different APVs and their virulence, it is essential to classify them based on their phylogenetic relationships. Since only a very few strains of APV complete genome sequences are available (FWPV and CNPV), it is not possible to classify them based on their complete genome sequence analysis. In most of the studies several researchers (Luschow et al. 2004; Weli et al. 2004; Jarmin et al. 2006) reported use of either genus or species-specific PCR primers to amplify various target regions or genes of APV. The P4b locus was the most common target region used for classification of APV by PCR (Fasaei et al. 2014). The APVs phylogenetically classified based on three different genes including P4b revealed that penguin pox virus was most closely related to turkey, ostrich and pigeon pox virus (Carulei et al. 2009; Offerman et al. 2013).

Phylogenetic studies have demonstrated that the majority of avian poxvirus isolates clustered into three major clades such as *Fowlpox virus* (clade A), *Canarypox virus* (clade B) and *Psittacinepox virus* (clade C). Clade A consists of seven subclades, namely, subclade A1 to A7. Subclade A1 is composed of viruses which are isolated from domestic fowl from different geographic locations (order Galliformes). Subclade A2 is composed of viruses which are isolated from rock doves (order Columbiformes) from Europe, Korea and North America. Subclade A3 is composed of albatross virus, falcon virus and seabird virus from the Pacific coast and Atlantic Ocean. Subclade A4 is composed of viruses from peregrine falcon and red-footed falcon from Hungary and the United Arab Emirates, respectively. The subclade A5 is composed of isolates from Anseriformes from the USA, sharing a common ancestor with subclade A1. However, the subclades A6 and A7 share a common ancestor with subclades A2 and A3. Subclade A6 is composed of viruses from mourning doves (order Columbiformes) and geese from North America. At the same time, the subclade A7 is composed of viruses from Accipitriformes from the USA and Europe. Clade B is composed of three subclades, namely, subclades B1 to B3. The subclade B1 is composed of viruses isolated from Passeriformes of worldwide distribution. However, this subclade is further divided into three clusters. The subclade B2 is composed of viruses from starlings and mynas. The subclade B3 is composed of viruses from a wide range of different wild bird species (Gyuranecz et al. 2013; Fasaei et al. 2014).

The phylogenetic analysis revealed that majority of the APV strains clustered either CNPV or FWPV, while another study with same locus from psittacine birds revealed third cluster. However, Amano et al. (1999) reported that the thymidine kinase (TK) gene of CNPV was highly diverged from FWPV and orthologue amino acid similarity between CNPV and FWPV based on P4b gene was only 64.2%.

8.13 Risk Factors

There are many factors which may involve in the incidence and prevalence of APV infections. Four important factors are determined as the primary causes of an APV outbreak. The most significant factors are host density and susceptibility and presence of numerous vectors in that particular time. In temperate regions, the vectors are not active due to winter, and in summer and early rainfall season the infections are more common (Tripathy 1993). However, the avian pox is more common throughout the year in warmer regions of the world (Akey et al. 1981).

8.14 Transmission

Poxvirus infection is transmitted in different routes. However, the poxvirus cannot penetrate the normal unbroken skin; small abrasions are sufficient to cause infection. The common route of transmission is by biting insects (mosquitoes, mites, midges and flies). The biting insects act as mechanical vectors, transferring virus from the infected population into susceptible populations. Transmission of virus can also be occurred by direct contact or with contaminated objects. Aerosol transmission is very rarely reported.

8.15 Prevention and Control

The challenges faced by poultry farmers all over the globe in controlling APV required low-cost vaccine and treatment. Several researchers reported the recombinant and live modified vaccines have been further developed and used to avert APV infections in avian species worldwide to control the APV outbreaks (Wang et al. 2006; Singh et al. 2000). The use of live APV or pigeon pox virus vaccine is very effective and used to prevent the disease outbreaks in commercial poultry farming (Boyle et al. 2004).

The birds can be vaccinated with any age and route of administration. It is important to check the birds for "vaccination take" after 7 to 10 days post-vaccination by observing the swelling and scab formation at the site of injection. Several live pox virus vaccines are available in the market for use in commercial flocks. In the case of chicken and pigeons, wing-stick method of vaccination is followed with the help of two-needle applicator, and in the case of turkeys thigh-stick method of vaccination is followed.

8.16 Role of REV in Vaccine Failure

In spite of this, over the past few decades, several incidences of APV have been reported in vaccinated flocks, which indicates that vaccines were not fruitful to prevent the disease. The vaccine used against APV infection was contaminated with REV genome and among the broiler chickens it caused lymphoma in the

United States (Fadly et al. 1996). There are several reports on the REV genome that is integrated with DNA of vaccines as well as field FWPV isolates (Singh et al. 2000, 2003; García et al. 2003; Kim and Tripathy 2001). The size of the integrated fragments varied and at the same time the integration site is constant. Two different types of REV integrated sequences are found in the APV genome which is in the size of long terminal repeats (LTRs) which ranged from 200 to 600 bp and the near-full-length provirus of about 800 bp. Most of the vaccine strains carry only an LTR sequence and most field isolates carry the near-full-length provirus. Singh et al. (2000) confirmed the insertion of REV LTR sequence in various lengths in the genome of two commercial APV vaccine strains and four field isolates; meanwhile there are several reports about the source of REV infection due to contamination of APV vaccine with REV genome and herpes virus vaccines against turkeys (Fadly et al. 1996; Bagust and Dennett 1977; Bendheim 1973; Jackson et al. 1977). Reticuloendotheliosis is a tumorigenic and immunosuppressive disease in nature. REV strains are able to cause diseases which are distinguished by chronic lymphoma and a runting-stunting syndrome in avian species (Witter and Fadly 2003; Payne 1998). The presence of REV genome in APV vaccines and the failure of vaccine to give proper protection against field APVs are of major concern to the poultry industry, which are highlighting the need for research to produce advanced vaccines.

8.17 Use of Avipoxviruses as Vaccine Vectors

The APVs are isolated from a different host but only a few isolates have been characterized, developed as vaccine for the commercial poultry flocks. The APV has more possibilities to use as vectors because (i) they are replicating in the cytoplasm of host by its own enzyme. (ii) APV genome has several consequences sequences to use as vaccine vectors (iii) can able to insert larger genome size of foreign DNA or multiple genes (iv) inability to complete the entire replication cycle in non-avian host (v) the antisera against Orthopoxviruses do not neutralize APV. (vi) The avipoxviruses (FWPV and CNPV) can be used multiple times without compromising the potency and do not elicit high levels of neutralizing antibodies (Weli and Tryland 2011).

Recently, some veterinary APV-vectored vaccines are licensed for commercial use in North America, South America and Europe. Some of the APV-vectored vaccines are available in the market against several animal infections like canine distemper virus, rabies virus, feline leukaemia virus, avian and equine influenza virus and West Nile virus. The APV-vectored vaccine is produced both cell-mediated and humoral immunity. Some of the APV-vectored vaccines are in the clinical trials for human use. Unlike other viral infections, there is no specific treatment for avian poxvirus in birds. However, it is recommended to apply externally iodine-glycerin combination on proliferating skin lesions with vitamin A injection which will help to improve the healing process and antibiotics to control secondary bacterial infections.

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Conflict of Interest There is no conflict of interest.

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Swinepox Virus

9

M. A. Ramakrishnan and D. Ashokkumar

Abstract

Swinepox is one of the major virus diseases of pigs caused by the swinepox virus in the genus *Suipoxvirus*. The disease mainly appeared in the young piglets which are reared under poor sanitary conditions. Although the disease has been reported from various countries, very few works have been carried out maybe due to self-limiting nature of the disease. The main mode of the transmission is through mechanical vector – the pig louse *Haematopinus suis*. Like all other poxviruses, swinepox virus also replicates in the cytoplasm. The genome of the virus is ~146 kbp in size which encodes 150 proteins. The lesions caused by swinepox need to be differentiated from vaccinia virus (multispecies-infecting virus) which produces similar clinical conditions in pigs. The swinepox virus has been used as a delivery vector for several bacterial and virus vaccines. Currently, no vaccine is available to control the disease and management and hygeinic practices only can reduce the incidence of the disease.

Keywords

Swinepox virus · Genome · Pathology · Immunology · Epidemiology · Diagnosis · Control

9.1 Prologue

The pig farming in developing countries has been recommended as the best source of income generation, capital storage, employment generation and improving the economic status of the weaker section of society. Pigs are mostly reared under the

M. A. Ramakrishnan (✉) · D. Ashokkumar
Division of Virology, ICAR-Indian Veterinary Research Institute, Mukteswar, Uttarakhand, India

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poor sanitary condition and this leads to infections of animals with various pathogens including viruses (e.g. swinepox, classical swine fever, foot and mouth disease, rotavirus), bacteria (e.g. tuberculosis) and parasites (e.g. *Trichinella spiralis* infestation) which results in a decline in a pig population (Malik et al. 2014). Among these, swinepox is one of the major concerns and the disease has been reported from different swine-raising countries with considerable economic losses to the farmers.

9.2 History

The first description of swinepox (SWP) was presented by Spinola in Europe in 1842 (Spinola 1842). The viral aetiology was confirmed by the reproduction of the diseases by infection of healthy pigs with blood and papules (filtered material) of infected pigs (Poenaru 1913). In the USA from Iowa state the disease was reported in 1929 as a contagious malignant pustular skin disease (McNutt et al. 1929). Then, the occurrence of the disease was reported from several countries including Morocco, Japan, England, Italy, Hungary, Kenya, India, Austria, Papua New Guinea, Nigeria, Argentina, Brazil and Belgium. The isolation of swinepox virus (SWPV) in primary culture of porcine origin was first reported in 1960 (Kasza et al. 1960). Later, Garg and Meyer (1972) demonstrated the cultivation of SWPV in PK-15 cell line (Garg and Meyer 1972). The first genetic information of SWPV was obtained by sequencing of thymidine kinase (TK) gene (ORF of 181 amino acid; ~ 20.6 kDa) (Feller et al. 1991). In 2002, the whole genome sequencing of SWPV was made available (Afonso et al. 2002). PCR-based diagnostic assay was established for the detection of SWPV in 2011 (Medaglia et al. 2011).

9.3 Aetiology

Swinepox virus (SWPV) is the only member of the genus *Suipoxvirus*, one of ten genera of the subfamily *Chordopoxvirinae*, family *Poxviridae* (Delhon et al. 2007). The SWPV is brick shaped with a dimension of 300 × 250 × 200 nm and the genome is surrounded by an outer protein coat. The SWPV genome consists of linear double-stranded DNA of ~146 kb with conserved central coding regions (106 genes) bounded by two identical inverted terminal repeats (ITR; ~ 3.7 kbp) which are covalently closed at their extremities. Swinepox virus is host-restricted and affects swine only, though nonproductive experimental infection is demonstrated in rabbits (Datt 1964). Genetic analyses of SWPV revealed that *Suipoxvirus* is clustered with the genera *Yatapoxvirus*, *Leporipoxvirus*, *Capripoxvirus* and *Cervidpoxvirus* (Afonso et al. 2002; Oliveira et al. 2017). The SWPV is falling under high A-T containing poxvirus with 72.5% A-T content. Overall, the SWPV contains 150 ORFs (putative genes encode proteins of 53–1959 amino acids), of which 146 are poxvirus homologues (Afonso et al. 2002). Most of the genes in central core (SPV021 to SPV125) are involved in basic replicative functions (viral transcription, DNA replication and virion assembly and maturation) whereas the terminal regions are likely involved in immune responses, apoptosis, tissue tropism, viral virulence and host range (Afonso et al. 2002). In general, genes located in the

terminal regions are oriented toward the ends, whereas the genes located in the central regions are oriented in both directions. In the terminal region (~13 kbp), the SWPV shows more uniqueness by the absence of multiple poxvirus gene homologues and sharing less nucleotide and amino acid identity with other pox viruses. Therefore, these regions likely play a role in virulence and host range. Four short ORFs (63–73 amino acids), viz. 018, 019, 020 and 026, are unique for SWPV and may play a role in pathogenesis in pigs (Afonso et al. 2002; Delhon et al. 2007). Like all other poxviruses SWPV also replicates in the cytoplasm. Being an enveloped virus, the SWPV is sensitive to the ether. Due to the large genome, the SWPV has been used as a delivery vector for various bacterial and viral pathogens (Lin et al. 2012; Xu et al. 2013; Yuan et al. 2017, 2018).

9.4 Epidemiology

Swinepox is an acute disease characterized by typical poxviral eruptive dermatitis. Animals up to 3 months of age are the most susceptible to clinical disease, while adults usually develop a mild, self-limiting form of the disease (Delhon et al. 2012). Swinepox only infects pigs especially piglets at the age of 3–6 weeks but does not infect other species (Datt 1964). Swinepox was first reported in Europe by Spinola in 1842 (Spinola 1842) and in Papua New Guinea in 1974 (Copland 1974). Swinepox outbreak occurred in Nigeria in 1981 (Olufemi et al. 1981). Sporadic cases of congenital swinepox were detected in the Netherlands; virus was isolated and identified by *Hind III* restriction endonuclease digestion (Borst et al. 1990). In 1991, swinepox was diagnosed in two backyard pig herds located in the towns of Kununurra and Wyndham in north Western Australia; young piglets were more severely affected, adult animals developed pustules (Jubb et al. 1992). Between 1976 and 2001, four outbreaks of swinepox occurred in Brazil (Medaglia et al. 2011). The other group of researchers reported five outbreaks in backyard pigs in Rio Grande do Norte state of northeastern Brazil (Olinda et al. 2016). SWPV outbreaks have been reported from Italy since 2002 with infection in different breeds (Mariano et al. 2015). The existence of swinepox was realized as early as the 1990s (Mohanty et al. 1989; Verma 1987; Verma and Rai 1989). In 2007, swinepox outbreak was reported from Punjab (Mittal et al. 2011). Two outbreaks were reported in Haryana – one occurred in Sonipat district in August 2007 and second in Rohtak district in September 2013 (Jindal et al. 2015; Riyesh et al. 2016). We have confirmed swinepox outbreak in Uttar Pradesh (2013, 2015 and 2016) and in Haryana (2015) (unpublished). Recently, SWPV outbreaks reported from Assam (Bora et al. 2017).

The main mode of transmission is through the pig louse *Haematopinus suis* which serves as a mechanical vector; however, direct animal contact and congenital transmission are also reported (Delhon et al. 2012; Mittal et al. 2011; Thibault et al. 1998). Flies and mosquitoes have been implicated as mechanical vectors (Delhon et al. 2007). Swinepox virus sheds from nasal and oral secretions and from lesions. Swinepox virus is present in infected epithelium and in dry scabs produced in the later stages of infection. Abraded skin can serve as the route of entry for the virus (Delhon et al. 2012). Vertical transmission is indicated by sporadic cases of congenital infection resulting in stillborn foetuses with generalized lesions (Borst et al. 1990; Paton et al. 1990). The virus may survive for years in the dried form

and persists in the affected herds. In our study also, we could observe the recurrence of swinepox in an organized swine farm in Uttar Pradesh, India.

9.5 Clinical Signs and Lesions

In general, a severe form of the disease mainly appeared in young piglets and mild self- limiting disease in adults (Cheville 1966). The lesions can start as maculae around 2 days postinfection and progress to papulae. The other clinical signs include a transient rise in temperature and loss of appetite also seen (Kasza et al. 1960). A true vesicle stage is absent or transient (Kasza and Griesemer 1962). After a week, the lesions are replaced by crusts, which ultimately shed, leaving discoloured spots (Kasza and Griesemer 1962). The infected animals usually recovered within a month of post-exposure (Bratke et al. 2013). Suckling piglets may develop a generalized disease with lesions all over the body. Mechanical transmission by pig louse, *Haematopinus suis*, results in lesions on the lower parts of the body, including the udder and vulva. Rarely, the transmission may occur through flies and mosquitoes and in these cases, lesions may appear on the dorsal parts of the body, including the snout and ears. The infected pigs may exhibit conjunctivitis, unilateral or bilateral keratitis and/or panophthalmia. The kerato-conjunctivitis without cutaneous eruption lesion has also been observed in piglets (Delhon et al. 2007, 2012). In our study, the swinepox diseases occurred in both Landrace cross and local nondescript pigs and the lesions occurred throughout the body (Fig. 9.1).

9.6 Pathogenesis

The virus enters mainly through the skin either via skin abrasions or the bite of the lice – *H. suis*. After entry, the virus preferentially replicates in epidermal keratinocytes of the *stratum spinosum* (spinous layer) (Meyer and Conroy 1972) or the

Fig. 9.1 Lesions of swinepox seen in the infected herd

basal layer of the skin (Teppema and De Boer 1975). The virus multiplies and produces lesions in the skin in the form of papules which progress to macules, pustules and then scab. In several occasions, the secondary bacterial infections cause an abscess. In SWPV infection, animals develop a non-cell-associated viraemia whereas in other poxviruses it is usually cell-associated. The virus could not be isolated from the blood of infected animals (Paton et al. 1990). In an experimental infection of gnotobiotic piglets with intravenous inoculation of SWPV, animals developed skin lesions over the body surfaces but no visible lesion could be observed in internal organs (Meyer and Conroy 1972; Paton et al. 1990).

9.7 Immunity

Studies on immunological response against SWPV infection are scanty. Generally, it is believed that CMI played a major role in poxviral infection and protection. Research on other poxviruses reveals that both arms of immune response, viz. CMI and humoral responses, are elicited upon poxvirus infection which includes cytokines, chemokines, interferons, etc. (Smith and Kotwal 2002). Decreased mitogen and SWPV-induced proliferative responses have been observed in peripheral blood mononuclear cells from experimentally infected swine (Kasza et al. 1960; Meyer and Conroy 1972; Williams et al. 1989).

9.8 Pathology

The histopathological changes caused by SWPV are very similar to those of other poxviruses. The readily visible histological change includes hydropic degeneration of *stratum spinosum* and keratinocytes (Paton et al. 1990). Compared to other poxviral infections, hyperplasia of epidermal cells is not well delineated in SWPV infection (Delhon et al. 2007). The cytoplasm of infected cells became enlarged and contains various types of inclusion bodies (Teppema and De Boer 1975) whereas the nucleus exhibits margination of chromatin and a large, central "vacuole". Electron microscopy examination of skin lesions shows large brick-shaped virus particles in various stages of maturity. The incomplete virus may be seen in cells located in an intermediate position in the skin lesion (Delhon et al. 2007, 2012).

9.9 Diagnosis

Generally, the diagnosis of swinepox is based on the clinical signs/lesions, demonstration of typical poxvirus morphology in the electron microscopy and the demonstration of cytoplasmic inclusion bodies under light microscopy (Kasza et al. 1960). The typical swinepox lesions present on the skin of the abdomen, ears, snout, vulva and back. Secondary bacterial infections lead to more severe lesions and formation

of a local abscess. The differential diagnosis of swinepox includes vaccinia virus infection (cause very similar lesion that of swinepox), vesicular diseases, pityriasis-rosea, parakeratosis, parasitic skin disorders, allergic skin reactions, ringworm, staphylococcal or streptococcal epidermitis and cutaneous erysipelas. The viral antigen may be confirmed using immunofluorescence and electron microscopy (Massung and Moyer 1991; Teppema and De Boer 1975). The presence of intracytoplasmic inclusion bodies along with central nuclear clearing in affected epithelial cells is pathognomonic for swinepox (Riyesh et al. 2016).

The virus may be isolated by using primary cells or cell lines of swine origin by several blind passages. The characteristics CPE include cell rounding, a detachment of cells, nuclear vacuolation, intracytoplasmic inclusion bodies, cytoplasmic stranding and cell death (Riyesh et al. 2016). For the serological diagnosis, the agar gel immunodiffusion test or counterimmunoelectrophoresis test can be used (De Boer 1975). Recently, singleplex and duplex PCR assay has been used for the diagnosis and differentiation of SWPV (Medaglia et al. 2011, 2015). Medaglia et al. (2011) used a combination of primers targeting genes which conserved in different poxviruses, viz. viral late gene transcription factor 3, DNA polymerase gene and DNA topoisomerase gene. In order to differentiate from vaccinia virus infection, haemagglutinin gene of orthopox virus was targeted. Later, the same group of researchers came up with a duplex PCR for the detection and differentiation of SWPV (ORF149) from vaccinia virus (HA gene). Riyesh et al. (2016) targeted a unique SWPV gene for PCR amplification (Riyesh et al. 2016). In our lab, we have developed a non-enzymatic fast and simple DNA extraction method and ORF18–ORF20 gene-based PCR for the specific detection of SWPV.

9.10 Control Measures

Currently, no commercial vaccines are available to control the swinepox. However, recently in China, three SWPV deletion mutants (Δ003, Δ010 and ΔTK) were constructed and evaluated for their safety and immunogenicity. Two out of three constructs were found to be safe for piglets and the mutant viruses elicited both humoral and immune responses (Yuan et al. 2018). The secondary bacterial infection should be controlled by proper antibiotic treatment. The affected pigs should be isolated from uninfected animals. Maintaining hygienic measures in the farm premises is foremost important in controlling the swinepox. Periodical treatment of animals with ectoparasiticides will significantly reduce the lice infestation

The SWPV is gaining attention for developing recombinant vector-based vaccines for bacterial and viral vaccines. However, information on host-pathogen interaction, genomic characterization and vaccine development are lacking. In the

coming years it is expected that more research opportunities and funds will be provided for SWPV research.

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Porcine Circovirus

10

G. Saikumar and Tareni Das

Abstract

Porcine circovirus (PCV) infections associated with post-weaning multisystemic wasting syndrome (PMWS) are characterized by weight loss, respiratory distress, jaundice, etc. Although PCV2 infection is ubiquitous, the prevalence of clinical disease is lower and the most common form is PCV2 subclinical infection. Recently, a novel porcine circovirus (PCV3) has been identified in pigs in the USA that is associated with porcine dermatitis nephropathy syndrome, acute myocarditis and multisystemic inflammation, etc. Genetic heterogeneity of PCV2 has been studied in Indian pig population. Different genotypes like PCV2a-2D, PCV2b-1C, PCV2d and recombinant strain between PCV2a-2C and PCV2b-1C are reported from different studies. PCV2 has been discovered in human faeces, human vaccines and beef. But its pathogenicity in humans is not clear. PCV detection is based on common golden standard techniques including nucleic acid and antigen detection in the tissues, in situ hybridization (ISH) and immunohistochemistry (IHC) using monoclonal or polyclonal antibody against PCV2, respectively. The commercial vaccines available are effective in reducing the severity of clinical diseases and improving production parameters. Recently, antiviral compounds have also shown promising results against PCV2. This chapter summarizes aetiology, epidemiology, transmission, immunopathobiology, diagnosis, prevention and control of porcine circovirus.

Keywords

Porcine circovirus · PCV · Porcine kidney cell line · Post-weaning multisystemic wasting syndrome · Aetiology · Epidemiology · Transmission · Immunopathobiology · Diagnosis · Prevention and control

G. Saikumar (✉) · T. Das
Division of Pathology, ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Uttar Pradesh, India

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10.1 Prologue

Porcine circovirus (PCV) is a small non-enveloped icosahedral virus containing a circular single-stranded DNA genome. It was first recognized in 1974 as a contaminant in porcine kidney cell line (PK-15) without any cytopathic effect (Tischer et al. 1974). Under experimental condition, this virus did not produce any aliment in pigs (Tischer et al. 1986). Later on in 1991, a novel PCV associated with a sporadic disease called as post-weaning multisystemic wasting syndrome (PMWS), characterized by weight loss, respiratory distress, jaundice, etc., was reported from Saskatchewan, Canada (Harding 1996; Clark 1997). The variant which was associated with PMWS was designated as PCV2 and the non-pathogenic one as PCV1 (Meehan et al. 1998). Retrospective study revealed that PCV2 has been circulating in pig population more than a decade (Jacobsen et al. 2009). As clinical disease was reproduced experimentally when PCV2 was inoculated along with other infectious or non-infectious agent, it was considered as necessary but not sufficient factor to develop clinical disease (Allan et al. 1999; Ellis 2003).

The PCV2 was found associated with a number of clinical diseases which were collectively termed as porcine circovirus disease (PCVD) and porcine circovirus associated diseases (PCVAD) in Europe and North America, respectively (Segalés et al. 2005; Opriessnig et al. 2007). PCVAD was recognized as a globally emerging disease, having huge impact on swine industry causing severe economic loss. Although PCV2 infection is ubiquitous, the prevalence of clinical disease is lower and the most common form is PCV2 subclinical infection (PCV2-SI) (Segalés 2012). The economic impact of PMWS and PCV2-SI for the pig industry, England for the year 2008, prior to introduction of vaccination was estimated to be £52.8 million and £88 million per year, respectively, during epidemic period (Alarcon et al. 2013). In 2016, a novel porcine circovirus (PCV3) was identified in pigs in the USA associated with porcine dermatitis nephropathy syndrome, acute myocarditis and multisystemic inflammation, etc. (Phan et al. 2016; Palinski et al. 2017). PCV3 is not strongly genetically related to other PCV and it has been observed that PCV3 has emerged over the last 50 years (Saraiva et al. 2018). The current paper summarizes aetiology, epidemiology, transmission, immunopathobiology, diagnosis, prevention and control of porcine circovirus.

10.2 Virus

PCV1, PCV2 and PCV3 belong to genus *Circovirus* under family *Circoviridae*. They are small, non-enveloped, icosahedral viruses containing single-stranded negative-sense circular DNA genome organized in a typical ambisense pattern. The genome sizes for PCV1, PCV2 and PCV3 are 1759 nucleotides, 1767–1768 nucleotides and 2000 nucleotides, respectively. PCV1 and PCV2 contain eleven predicted open reading frames (ORF), out of which ORF1, ORF5, ORF7 and ORF10 are encoded by positive strand and transcribed clockwise and others (ORF2, ORF3, ORF4, ORF6, ORF8, ORF9 and ORF11) are encoded by negative strand or

complementary strand and transcribed anticlockwise (Hamel et al. 1998; He et al. 2013). ORF1 and ORF2 are major open reading frames which are oriented in opposite direction. ORF1 encodes for replication protein and ORF2 for capsid protein which is having immunodominant antigenic epitopes. ORF1 of PCV1 and PCV2 share 83% nucleotide identity and 86% amino acid identity whereas ORF2 of PCV1 and PCV2 share 67% nucleotide and 65% amino acid sequence identity. The rep protein of PCV3 shared 55% identity to rep protein of bat and 48% identity to rep protein of PCV2, and the cap protein of PCV3 shared 35% amino acid identity to bat circoviruses and 24–26% amino acid identity to PCV1 and PCV2, respectively (Phan et al. 2016). ORF3 is one of the major proteins of PCV and also most variable protein with 60% amino acid identity between PCV1 and PCV2. ORF3 region is embedded within ORF1 region and oriented in opposite direction. The ORF3 protein of PCV2 is involved in apoptosis. The fourth one ORF4 has been suggested to be located in ORF3 region and transcribed in the same direction. The PCV1 and PCV2 have 83% predicted amino acid identity for ORF4. It plays an important role in suppressing caspase activity and regulating CD4+ and CD8+ lymphocyte population (Hamel et al. 1998; He et al. 2013). The multiplication of PCV is speculated to involve rolling circle method (Allan and Ellis 2000; Mankertz et al. 2004; Phan et al. 2016). The viruses mainly rely on host-encoded protein because of their limited encoding capacity. PCV2 requires actively multiplying cells for replication (Tischer et al. 1987; Mankertz et al. 2004).

PCV2 has been divided into PCV2 group 1 and PCV2 group 2 with genome sizes 1767 nucleotides and 1768 nucleotides, respectively (Olvera et al. 2007). North American laboratories considered grouping into North American isolate, PCV2a, which comes under PCV2 group 2 and European-like isolates, PCV2b, which is under PCV2 group 1 (Gagnon et al. 2007). PCV2 strains were divided into three different genotypes (PCV2a, PCV2b and PCV2c) based on proportion of different nucleotide sites in ORF2 (Segalés et al. 2008). PCV2a was subdivided in four clusters (2A to 2D) and PCV2b into three clusters (1A to 1C) (Olvera et al. 2007). PCV2a was predominant genotype in pig population worldwide since 2000. PCV2b has been present in Europe and Asia since 1997 and PCV2c was identified in archived serum samples from Denmark (Dupont et al. 2008). Later on, in 2009, two new genotypes PCV2d and PCV2e were detected (Wang et al. 2009; Zhai et al. 2011; Xiao et al. 2015). Emergence of new recombinant genotype PCV1/2a, having ORF1 of PCV1 and ORF2 of PCV2, has been reported in Canada with very low prevalence rate in 2008 (Gagnon et al. 2010). In Northeast India, emergence of PCV2a and PCV2b has been reported (Bhattacharjee et al. 2015). A novel genotype PCV2f was reported in three out of 23 PCV2 strains identified from the archived pig tissue samples collected between 1996 and 1999 in China (Bao et al. 2018). After introduction of PCV2 vaccination in the USA in 2006, PCV2d-2 is found to be predominant genotype in the USA which is a second genotypic shift after a major genotypic shift in mid-2000 replacing PCV2a with PCV2b (Xiao et al. 2016). Recently, PCV3 has also been reported from different parts of the world such as the USA (Phan et al. 2016), China (Shen et al. 2017), Korea (Kwon et al. 2017), Europe (Stadejek et al. 2017), etc.

10.3 Epidemiology

First, PCV1 was encountered in un-inoculated PK-15 cell line ATCC-CCL 33. Later on, PCV1 was recovered from pig foetal materials, wasting syndrome in France, wild boar, human rotavirus vaccine, etc. PCV1 infection is spread worldwide. Serum antibodies to PCV1 have been demonstrated in pigs in Germany, Canada, New Zealand, Great Britain and Northern Ireland. Much of the seropositivity might be due to cross-reactivity to PCV2 antigen in indirect immunofluorescence or indirect immunoperoxidase assays (Allan et al. 2012). But actually in field cases prevalence of PCV1 is very low (Ellis et al. 2000). Also serum antibodies to PCV1 have been detected in other species like humans (30.2%), mice (12–69%) and cattle (35%) in Germany by IIF and ELISA which need further confirmation (Tischer et al. 1995).

PCV2 is having a widespread geographical distribution and is one of the emerging swine pathogens. After its first discovery in Canadian weaning piglet in 1991, it has been subsequently reported from Spain, France, Hungary, UK, Brazil, Uruguay, Oceania, Australia, Caribbean, Cuba, Israel, South Africa, Uganda, Korea, China, etc. (Afolabi et al. 2017). The virus is ubiquitous in swine population reaching seroprevalence up to 100%. PCV2 has also been reported from different parts of India from different clinical cases of swine. In an organized swine farm, located in the state of Uttar Pradesh (North India) with a history of mummification, stillbirth, neonatal mortality and decreased litter size, 12 out of 70 litters examined were found positive for PCV2 (Sharma and Saikumar 2010).

Genetic heterogeneity of PCV2 has been studied in Indian pig population. Different genotypes like PCV2a-2D, PCV2b-1C, PCV2d and recombinant strain between PCV2a-2C and PCV2b-1C were reported (Anoopraj et al. 2015). There is also report of infection of PCV2 in a piggery unit from Southern India, Tamil Nadu having high incidence of stillbirth and neonatal mortality (Kumar et al. 2014; Karuppannan et al. 2016). PCV2 has been reported in Assam. Out of 54 stillbirth and mummified foetuses collected from pig farms in Kamrup districts of Assam during 2013–2014, 16.6% samples were PCV2 positive and 7.4% both porcine parvovirus (PPV) and PCV2 positive (Pegu et al. 2017). There is also report of high seroprevalence of PCV2 (80.8% in 2014, 79.1% in 2015, 96.2% in 2016) by antibody ELISA in 11 districts of Meghalaya, India, out of 1899 serum samples collected from different age groups of pigs (Mukherjee et al. 2018b). In another study, out of 249 serum samples collected between 2014 and 2016 from Meghalaya, India, PCV2 antibodies were detected in 83.93% suspected serum by ELISA and in 62.25% by PCR. Also 18.94% (36/190) tissue samples were positive for PCV2. Molecular characterization indicated prevalence of PCV2a, PCV2b-1c, PCV2d and recombination genotype of PCV2a and PCV2b in India (Mukherjee et al. 2018a).

In Canada and in Costa Rica, antibodies to PCV2 were detected in 82.4% out of 386 serum samples and 14.2% out of 322 serum samples collected from normal healthy pigs (Liu et al. 2002). In Poland, PCV2 was detected in 75.6% cases out of 312 tested wild boar tonsil samples by real-time PCR (Fabisiak et al. 2012). Between

2013 and 2015, prevalence of PCV2 is reported to be significantly higher in Eastern China (87.3%) than Western China (47.6%) (Yuzhang et al. 2016). In Thailand, during 2009–2015, PCV2 was detected in 306 (44.09%) samples collected from 56 farms (80%) with PCV2d as the predominant genotype (Nattrat et al. 2017).

PCV2 infection is characterized by high prevalence and low morbidity. PCV2 is an essential factor required for the development of PCV-associated diseases. As PCV2 is detected in healthy pigs, the presence of other triggering factors is essential for the outcome of PCV2 infection. They are mainly classified into four groups, i.e. viral factors, host factors, housing and management factors, co-infections and immunomodulation.

10.3.1 Viral Factors

Virus is ubiquitous in nature and it is present in most of the pig farms across the world. All the infected pigs do not develop disease. Some are severely diseased while others are healthy. The disease occurs in different clinical manifestations. But no significant difference is observed in virus genome involved in different clinical conditions and also between affected and unaffected farms (Grierson et al. 2004). Also no PCV2 molecular markers for virulence have been identified between affected and unaffected farms (De Boisseson et al. 2004). Virulence can also be altered with minor change in genome as PCV2 was attenuated after 120 time's serial passage in cell culture resulting in two amino acid mutations in capsid protein (Fenaux et al. 2004). Genotypic shift is associated with more severe diseases. It was observed after PCV2b replaced PCV2a in mid-2000. After PCV2 vaccination, PCV2d is found to be predominant genotype in the USA during 2014–2016 because of its greater ability to multiply under vaccination pressure (Xiao et al. 2016).

10.3.2 Host Factor

All breeds of pigs are susceptible to infection. But in field condition, it has been observed that certain genetic lines of pigs are more or less susceptible to disease as compared to others. Experimentally, it was found that Landrace breeds were more susceptible to PCVAD than Duroc and Large White pigs (Opriessnig et al. 2006a). Under field condition, lower mortality was observed in pure Pietrain or Large White and Pietrain cross as compared to Large White and Duroc cross pigs (Lopez-Soria et al. 2011). High titre of maternal antibody is protective as compared to low titre maternal antibody. Host variation in onset of adaptive immune response also affects the susceptibility and outcome of disease. Age-related susceptibility to PCV2 was also observed which is directly related to maternal antibody level. In the USA, under field condition and experimental conditions, 12–16-week pigs are more affected with PCVAD in comparison to 2–7-week-old pigs (Shen et al. 2012).

10.3.3 Housing Management Factors

It has been observed that various factors like on farm management, housing, vaccination schedule, husbandry condition, biosecurity and hygiene practices are strongly related to PCVAD (Grau-Roma et al. 2011; Rose et al. 2012). Housing like large pen in weaning facility; common pit between different fattening rooms and proximity to other pig farms; hygiene and husbandry practices like sows in poor condition, early weaning, pig mixing, purchase of replacement gilts, short empty period in nursery and farrowing sector; use of farm boar for semen collection; vaccination schedule like vaccination of gilts against PRRS; use of separate vaccine like *Erysipelothrix rhusiopathiae* and PPV in gilts; etc., are found to be risk factors for development of PCVAD whereas vaccination against atrophic rhinitis is protective. Biosecurity measures like shower facility were also found effective. Some of the practices modify the course of PCV favouring early infection while others trigger virus replication directly or indirectly (Andraud et al. 2009).

10.3.4 Co-infections

A number of pathogens like virus, bacteria and mycoplasma infection along with PCV2 are able to increase incidence of PCV2 or exacerbate the disease. Most potential triggering pathogens are PRRSV, PPV and *Mycoplasma hyopneumoniae* (Allan et al. 2000; Opriessnig et al. 2004; Ha et al. 2008). Some other pathogens are also found as a result of immunosuppression or by chance as co-circulating pathogens. Other important pathogens which are associated with PCV2 are swine influenza, hepatitis E virus, pseudorabies virus, classical swine fever virus, porcine enterovirus, corona virus, *Actinobacillus pleuropneumoniae*, *Pasteurella multocida*, *Haemophilus parasuis*, *E. coli*, *Cryptosporidium parvum*, *Chlamydia* species, *Aspergillus* species, *Candida albicans*, etc. (Kim et al. 2003a; Grau-Roma et al. 2011). The speculation is that co-infections may enhance replication of PCV2 and their accumulation in immune cells or interfere with clearance of virus by changing cytokine profile (Opriessnig and Halbur 2012; Segalés et al. 2013).

10.3.5 Immunomodulation

Immune stimulation may enhance PCV2 infection and disease outcome. It was observed that gnotobiotic piglets immunized with keyhole limpet hemocyanin emulsified in incomplete Freund's adjuvant or commercial bacterin containing mineral oil adjuvant developed PMWS following vaccination (Krakowka et al. 2007). Also severe microscopic lesions and virus load were observed in PCV2-infected colostrum-deprived piglets vaccinated with commercially available modified live PRRS vaccine as compared to non-vaccinated piglets (Allan et al. 2007). Potentiation of PCV2 replication after immunostimulation may be due to massive activation of certain cells like macrophages in lymphoid system. The effect of immune

suppression on PCVAD has also been studied. Injection of cyclosporine and dexamethasone before PCV2 infection resulted in increased PCV2 replication and granulomatous lymphadenitis as compared to control (Krakowka et al. 2002; Kawashima et al. 2003).

Recently, a novel porcine circovirus categorized as PCV3 has been identified. In 2015, this novel circovirus, PCV3, was detected in the USA from three pigs showing cardiac lesions and multi-organ inflammation through metagenomic analysis (Phan et al. 2016). Again PCV3 was reported from a case of PDNS and reproductive failure in the USA through metagenomic sequencing (Palinski et al. 2017). In 2016, PCV3 was detected for the first time in China from pyretic and pneumonic piglets (Shen et al. 2017). In China, PCV3 was identified in 24 out of 35 farms and suspected to be an important factor in reproductive failure (Ku et al. 2017). PCV3 was detected in 132 out of 222 cases without any clinical infection signs from Shandong province, China (Zheng et al. 2017). In Korea, 44.2% (159/360) and 72.6% (53/73) PCV3 prevalence is reported at individual and farm level, respectively, using pen side oral fluid sample (Kwon et al. 2017). In Europe, PCV3 was detected in 12 out of 14 Polish farms and in PCV3-positive farms, PCV3 was detected in 5.9% to 65% serum pools by real-time PCR out of total 1050 serum samples collected between 2014 and 2017 (Stadejek et al. 2017). PCV3 has also been described in Italy (Faccini et al. 2017), Sweden (Ye et al. 2018), Northern Ireland and England (Collins et al. 2017).

10.4 Transmission

Porcine circoviruses are transmitted in different modes. PCV2 can be secreted in various secretions and excretions of the body like nasal, tonsilar, bronchial and ocular secretions, faeces, urine, semen, saliva and milk of both clinically affected and infected but apparently healthy animals. Clinically affected animals shed more virus than infected but clinically healthy animals.

Direct contact with infected animals is the more efficient route of virus transmission. The oronasal route is the most likely route of transmission. Also naive pigs fed orally with uncooked tissues of viraemic pigs resulted in infection. Indirect transmission also occurs when pigs were kept in adjacent pens. Porcine circoviruses are shed in milk of infected sow and can be transmitted to offspring by oral route through milk. PCV1 and PCV2 were detected in the colostrums collected from 33 sows in Japan by virus isolation and PCR (Shibata et al. 2006). In an experimental study, sows infected with PCV2 intranasally at 93 days of gestation shed virus from the first day of lactation to 27th day of lactation. But it is not clear whether virus is secreted as free or cell-associated form as virus multiplies in macrophages of mammary gland, but most viruses are detected in cell-free portion or whey portion of milk (Ha et al. 2009).

There is also possibility of airborne transmission which cannot be ruled out. High concentration of PCV2 DNA up to 10^7 genome copies per cubic millimetre of air was detected by quantitative PCR in the Canadian swine confinement building but its infectivity was not studied (Verreault et al. 2010).

House flies also act as vectors for transmission of PCV2. The flies have on farm potential to carry and transmit PCV because of their close association with pigs and the environment. It was studied in a pig farm where identical PCV2b DNA was isolated both from faeces of weaner and nursery pigs and house flies. These insects form a measure of environmental contamination at a pig farm site by PCV2 (Blunt et al. 2011). Also mosquitoes like *Culex* species can serve as mechanical vectors for transmission of PCV2 (Yang et al. 2012).

Cross-species transmission of circoviruses has also been reported. USA beef contained circoviruses which showed 99% similarity to PCV2b (Li et al. 2011). In China, cross-species transmission between the pig and buffalo was also reported recently (Zhai et al. 2017). In China, 6.15% prevalence of PCV2 in goats was reported with PCV2d genotypes (Wang et al. 2018). PCV2 was also reported in rat samples in swine farms. In China, 31.6% (30/95) rat samples were found positive for PCV2 by PCR (Zhai et al. 2016). PCV2 has been discovered in human faeces, human vaccines and beef. But its pathogenicity in human is not clear. There are various possible routes of PCV infection in humans such as ingestion or contact with pork products, ingestion of undressed beef or raw milk, drinking raw water, vaccine contamination, airborne transmission, vector transmission, xenotransplant infection, etc. (Zhai et al. 2012).

Vertical transmission has also been reported both in natural condition and experimental condition. Transplacental transmission results in reproductive abnormalities. Following intranasal infection of pregnant sows 3 weeks prior to farrowing resulted in PCV2-infected, aborted and live-born piglets (Park et al. 2005). Experimentally in utero inoculation of swine foetuses with PCV2 at late stage of gestation resulted in stillborn, mummified and weak-born piglets at farrowing. Also antibodies to PCV2 were found in the sera and thoracic fluids of both abnormal and normal pre-suckle piglets (Johnson et al. 2002). PCV2 can replicate in embryos before 21 days of pregnancy and might result in embryonic death in most of the cases (Mateusen et al. 2007).

PCV2 has been demonstrated in the semen samples. In an experiment, mature boars were infected intranasally with PCV2. The presence of PCV2 antibodies was detected in the serum from 11 dpi to 90 dpi by IFA and PCV2 DNA was detected in serum from 4 dpi to 35 dpi by PCR and nested PCR and in semen from 5 dpi to 47 dpi (Larochelle et al. 2000). In Korea, 13, 26 and 11 were positive for PCV2 by conventional PCR, by nested PCR and by virus isolation, respectively, out of total 98 semen samples collected from 1-year-old boars in 49 herds and also greater amount of DNA was detected in the seminal fluid and non-sperm fraction (Kim et al. 2003b). Persistently infected boars may continue to shed virus in the semen up to 71 weeks and it did not affect percentage of morphologically normal and live sperms (McIntosh et al. 2006). PCV2 antigen was also demonstrated in the cytoplasm of a macrophage and fibroblasts of seminal vesicles with PCV2 DNA in the semen of an 11-month-old boar having history of illness and infertility by nested PCR (Opriessnig et al. 2006b). PCV2 can be transmitted via artificial insemination with semen spiked with PCV2. It was observed in an experiment where PCV2-free mature female pigs were artificially inseminated with PCV2 DNA-negative semen

spiked with PCV2a and PCV2b. Viraemia was observed in both groups in comparison to control group, those artificially inseminated with PCV2 DNA-negative semen. Sows inseminated with PCV2a-spiked semen failed to become pregnant and sows inseminated with PCV2b semen gave birth to viraemic live-born piglets, PCV2-infected stillbirth and mummified foetuses (Madson et al. 2008).

10.5 Immunopathogenesis

Porcine circoviruses can multiply in different types of cells like epithelial cells, mononuclear cells, endothelial cells, fibrocytes, etc. PCV attaches with host cells through interaction with host cell glycosaminoglycans like heparin sulphate and chondroitin sulphate B, and it enters epithelial cells by actin and Rho-GTPase and monocytic cells by clathrin-mediated endocytosis (Misinzo et al. 2005, 2006, 2009). Acidic medium, i.e. endosome-lysosomal acidification, is required for PCV2 multiplication (Misinzo et al. 2008). After entry into cells, PCV2 DNA enters into the nucleus with the help of many cellular factors and multiplies there by rolling circle model. PCV DNA replication is initiated during S phase of growth as many required cellular enzymes are expressed during this phase (Tischer et al. 1987). HSP 70 and HSP 27 positively regulate DNA replication whereas cyclin A and HMG co-A reductase negatively regulate replication (Liu et al. 2013, 2014; Tang et al. 2013; Huang et al. 2014). The ubiquitin proteasomal system (UPS) is required for effective PCV2 replication, viral protein expression and RNA transcription in a cell cycle-dependent manner (Cheng et al. 2014). The ORF3 causes degradation of regulator of G protein signalling 16 (RGS 16) through ubiquitin proteasomal system (UPS) and enhances translocation of nuclear factor kappa B into the nucleus through extracellular signalling pathways (ERP 1/2) resulting in increased virus proliferation and increased IL-6 and IL-8 mRNA transcript leading to more inflammatory response around host cells during the early stage of PCV2 infection (Choi et al. 2015). Progeny viruses begin to appear after 30 h postinfection.

PCV infection enhances cellular oxidative stress which further influences its replication in PK 15 cells. Malondialdehyde concentration is increased and glutathione and superoxide dismutase concentration is found to be decreased in 48 h after PCV infection (Chen et al. 2013). Factors like ochratoxin which increase oxidative stress may enhance PCV multiplication (Gan et al. 2015).

PCV2 infection results in host immunosuppression, characterized by lymphoid depletion. Lymphoid depletion affected B cells, T cells and NK cells. Virus replication is enhanced in actively dividing lymphocytes than resting lymphocytes (Yu et al. 2007, 2009). The virus multiplies in lymphoblastoid cells and causes its lysis (Rodríguez-Cariño et al. 2011). Lymphoid depletion is also induced by apoptosis which could be either due to activation of caspase 3 and caspase 8 by ORF3 protein or by increased p53 or by activation of NF-κβ or by Fas-Fas ligand activation (Liu et al. 2006, 2007; Chang et al. 2007). Apoptosis signal-regulating kinase-1 (ASK-1) also plays a vital role in regulating PCV2-induced apoptosis (Wei et al. 2013). Decreased proliferative activity in lymphoid cell could also be a major cause of

lymphoid depletion (Mandrioli et al. 2004). PCV2 can induce anti-apoptotic response during the early stage of viral infection through activation of phosphatidylinositol 3-kinase/Akt pathways resulting in suppression of premature apoptosis for improved virus growth after infection (Wei et al. 2012).

PCV2 is most commonly associated with monocytic cells and dendritic cells (DC). Virus can persist in DC for days without any signs of apoptosis or modulation of cell surface marker. Virus uses DC for its transmission and spread (Vincent et al. 2003). The presence of PCV2 in macrophages suppresses its microbicidal activity by inhibiting production of o_2^- and H_2O_2. Also alveolar macrophage infected with PCV2 produces high amount of TNF-α, IL-8, granulocyte colony-stimulating factor (CSF), neutrophil chemotactic factor, monocyte chemotactic protein-I, etc. (Chang et al. 2006). CpG oligodeoxyribonucleotide present in PCV2 genome inhibits production of IFN-α and TNF-α by plasmacytoid DC; as a result co-stimulatory potential to myeloid DC and responses induced after ligand stimulation of TLR7 and TLR9 are negatively impaired (Vincent et al. 2007). PCV2 dsDNA also prevents actin polymerization and endocytosis in DC, thus strongly suppressing basic innate immune response and increasing secondary infection (Balmelli et al. 2011).

Increased expression of IL-10 is also reported in peripheral blood mononuclear cells and lymphoid tissues of animals infected with PCV2 (Doster et al. 2010). Higher IL-10 expression is observed in bystander cells as compared to infected cells as a result of paracrine effect. IL-10-mediated immune suppression may result in pathology associated with PCV2 infection. PCV2 also strongly induces IL-1β and IL-8 expression in both naive and PCV2-infected pigs suggestive towards chronic inflammation. But PMWS-affected pigs produce less IL-2, IL-4 and IFN-γ as compared to healthy ones which may be due to lymphoid depletion (Darwich et al. 2003a, b; Darwich and Mateu 2012).

10.6 Clinical Features of PCVAD

10.6.1 PCV2 Systemic Disease (PCV2-SD)

It is clinically characterized by weight loss, respiratory distress, diarrhoea, pale skin, jaundice, enlarged subcutaneous lymph nodes, etc. (Harding and Clark 1997). Morbidity is 4–30% commonly, occasionally 50–60%, and mortality is 4–20% (Segalés and Domingo 2002).

10.6.2 PCV2 Lung Disease (PCV-LD)

It is characterized by respiratory distress and dyspnoea. It also plays an important role in porcine respiratory disease complex (PRDC) along with other pathogens like *Mycoplasma hyopneumoniae*, swine influenza, PRRS virus, etc., in 8- to 26-week-old pigs which is characterized by decreased feed efficiency and growth rate, anorexia, fever, cough and dyspnoea (Anoopraj et al. 2014; Kim et al. 2003a).

10.6.3 PCV2 Enteric Disease (PCV2-ED)

It is characterized by enteritis. Most of the PCV2 enteric disease cases in fields are from 8- to 16-week age group pigs (Kim et al. 2004).

10.6.4 PCV2 Reproductive Disease (PCV2-RD)

The clinical manifestations of PCV2-RD include abortions, stillbirths, mummification and pre-weaning mortality (West et al. 1999). PCV2-RD is rare in field condition which may be due to its high seroprevalence in adult pigs and absence of clinical disease in breeding stocks (Pensaert et al. 2004). The affected herds include gilt startups and new populations. PCV2 also replicates in an embryo and may lead to embryonic death and return to oestrus. In a small proportion of embryos, PCV2 did not have any detrimental effect on their development before 21 days of pregnancy (Mateusen et al. 2007).

10.6.5 PCV2-PDNS

The most important clinical sign is the presence of irregular red to purple macules and papules in the skin of hind legs and perineal region mostly. Later on lesions become covered by dark-coloured crust, which may fade leaving scar tissue. Other clinical signs are lethargy and fever. It affects nursery, growing and adult pigs with prevalence below 1% and mortality 100% in pigs older than 3 months and 50% in younger age groups (Segalés 2012; Segalés et al. 1998; Drolet et al. 1999).

10.6.6 PCV2 Subclinical Infection (PCV2-SI)

It is the most common form. It is characterized by decreased average daily weight gain without any overt clinical signs. Vaccination is helpful in the improvement of productive parameters (Young et al. 2011; Segalés 2012). PCV2 infection may be limited to 1–2 lymph nodes with necrotizing lymphadenitis in clinically healthy pigs.

Initially, PCV2 was found to be associated with type II congenital tremor resulting from myelin deficiency in the USA (Stevenson et al. 2001). But in further study, PCV2 was not found to be associated with congenital tremor in 40 pigs from the UK, Sweden, Spain and Ireland (Kennedy et al. 2003).

PCV2 was also found to be involved in per acute syndrome called as acute pulmonary oedema (APE) affecting nursery and younger finisher pigs. The condition is characterized by rapid onset of respiratory distress leading to death. Often animals are found dead without any obvious clinical signs. The mortality is around 20% in the affected group. PCV2 multiplies in the endothelial cells and mononuclear cells of lungs of younger animals without any protective antibody level, and pulmonary

oedema occurs as a result of damage to endothelial cells and release of cytokines from mononuclear cells (Cino-Ozuna et al. 2011; Segalés 2012).

10.7 Pathology of PCVAD

In PCV2-SD, the primary gross lesions observed are rough hair coat, prominent backbone, oversized head, enlarged lymph nodes, non-collapsible and mottled tan lungs, white spots in the kidney, discoloured liver, catarrhal enteritis, less commonly gastric ulceration and spleen infarcts. Microscopically, lymphohistiocytic to granulomatous inflammatory lesions were observed in lymphoid tissues mainly the tonsil, spleen, Payer's patches and one to more lymph nodes, lung, liver, kidney, heart and intestine. The characteristic lesions in lymphoid tissue include lymphoid depletion and histiocytic replacement both in follicular and parafollicular area. Multinucleated giant cells are frequently seen. Macrophages containing sharply demarcated spherical basophilic intracytoplasmic botryoid inclusion bodies may also be observed. Scoring system for lesion severity has also been described (Opriessnig et al. 2007). Necrotizing lymphadenitis has also been described in at least one lymph node of 10% pigs suffering from PCV2-SD which may be due to hypertrophy and hyperplasia of the endothelium of blood vessels and subsequent thrombosis of blood vessels (Segalés et al. 2004; Galindo-Cardiel et al. 2011). Other microscopic lesions are lymphohistiocytic or granulomatous interstitial pneumonia with rare peribronchiolar fibrosis, mild to severe necrotizing bronchiolitis in the lung, interstitial lymphoplasmocytic or granulomatous or mixed-type nephritis, granulomatous enteritis, lymphohistiocytic hepatitis with disorganization of hepatic cords along with apoptosis and perilobular fibrosis in the liver, etc. Lymphohistiocytic and plasmacytic vasculitis has also been observed. Acute necrotizing myocarditis or chronic fibrosing myocarditis along with chronic vasculitis has been reported in PCV2-affected cases (Opriessnig et al. 2006c). Brain lesions are of rare occurrence in PCV2-SD, but lymphohistiocytic vasculitis associated with haemorrhage or lymphohistiocytic meningitis in the cerebellum; degeneration and necrosis of grey and white matter have been reported (Correa et al. 2007; Seeliger et al. 2007; Segalés 2012).

In PCV2-LD, non-collapsible and tan-mottled lungs are observed. Histopathologically, lymphohistiocytic to granulomatous interstitial pneumonia, peribronchiolar fibroplasias and mild to severe necrotizing and ulcerative bronchiolitis are found (Opriessnig et al. 2007).

The lesions in PCV2-ED resemble subacute to chronic ileitis associated with *Lawsonia intracellularis*. The intestinal mucosa is thickened and mesenteric lymph nodes are enlarged. Histopathologically, granulomatous enteritis and characteristic lesions in Payer's patches are observed (Jensen et al. 2006).

In PCV2-RD, foetal mummification, stillbirths and abortions are observed. Grossly the foetuses are oedematous. Ascites, hydrothorax and hydropericardium are observed. In a foetal heart, cardiac hypertrophy with multifocal areas of discolouration in the myocardium is commonly present. Foetal liver is congested and

enlarged due to chronic passive congestion. In a foetus, the heart is the target organ for PCV2. Gross lesions are present when foetuses are infected at 57 days of gestation but no gross lesions are observed in foetuses when sows are infected at 75 and 92 days of gestation (Sánchez et al. 2001). Microscopically, non-suppurative to necrotizing or fibrosing myocarditis, chronic venous congestion in the liver and mild pneumonia are observed.

PDNS-affected pigs show necrotizing skin lesions and enlarged tan, waxy-appearing kidneys with petechial haemorrhage. Microscopically, systemic necrotizing vasculitis, fibrino-necrotizing glomeruli nephritis and non-purulent interstitial nephritis are prominent lesions which are suggestive of type III hypersensitivity reaction. Mild to moderate lymphoid depletion is also observed in lymphoid tissue (Rosell et al. 2000). In recovered animals, chronic fibrous interstitial nephritis and glomerulosclerosis are observed. In PCV2-SI cases, necrotizing lymphadenitis is present without apparent clinical signs (Kim and Chae 2005). In APE cases, hydrothorax, non-collapsible lung and interstitial oedema are observed. Microscopically, interstitial oedema, alveolar oedema, mononuclear infiltration in the interstitial septa, fibrinoid necrosis of blood vessel and lymphoid depletion in lymphoid tissues are present (Cino-Ozuna et al. 2011).

Recently, PCV3 has been isolated from different pathological conditions like porcine dermatitis and nephropathy syndrome, reproductive failure, cardiac and multisystemic inflammation, pyretic and pneumonic piglets, etc., from different parts of the world.

10.8 Diagnostics

The first step towards diagnosis of PCVAD is assessment of clinical signs. But in PCV2-SI, no clinical signs are observed. The second step is histopathology. The PCVAD cannot be confirmed without microscopic evaluation of complete set of tissue samples and demonstration of PCV2 antigen or nucleic acid in that lesion. In PCV2-SD, characteristic histopathological lesions are severe lymphoid depletion and proliferation of histiocytes in the lymphoid tissues. Multinucleated giant cells and botryoid inclusions in the macrophages are also characteristics but not observed in all the cases.

The most common golden standard techniques to detect PCV2 nucleic acid and antigen in the tissue are in situ hybridization (ISH) and immunohistochemistry (IHC) using monoclonal or polyclonal antibody against PCV2, respectively. PCV2 is usually demonstrated in the cytoplasm of histiocytes, multinucleated giant cells, other monocytic or macrophage lineage and dendritic cells and sporadically in the cytoplasm of renal and respiratory epithelial cells, vascular endothelium, acinar and ductular cells of the pancreas, lymphocytes and in the nucleus of macrophage or giant cells, smooth muscle cells, hepatocytes, enterocytes and pancreatic cells (Segalés and Domingo 2002). Both IHC and ISH were able to detect PCV2 in tissues that were stored in formalin for up to 6 months (McNeilly et al. 1999). Non-radioactive digoxigenin probes targeting ORF1 region of PCV1 and ORF2 of PCV2

are also in use to differentiate two viruses in formalin-fixed paraffin-embedded tissue samples (Kim and Chae 2001). Double ISH using double labelling like digoxigenin and biotinylated probes is used to detect PPV and PCV2 simultaneously in tissue samples, respectively (Kim and Chae 2002). The IHC is more rapid and less expensive than ISH for routine use in laboratory (Sorden et al. 1999). A strong correlation has been observed between the amount of PCV2 and severity of microscopic lesions (Rosell et al. 1999).

Other techniques for detection of PCV2 nucleic acid are PCR and real-time PCR. PCR is the most sensitive technique. Since PCV is ubiquitous in nature, detection of nucleic acid by PCR cannot rule out the actual presence or absence of disease. PCR is used to study the routes of virus excretion and to evaluate PCV status in semen. There are different types of PCR which are in use to detect and quantify PCV nucleic acids. Multiplex PCR is used to detect and differentiate between PCV1 and PCV2 (Larochelle et al. 1999). Multiplex nested PCR assays have been described for simultaneous detection of PCV1, PCV2 and PPV (Kim and Chae 2003). Multiplex PCR for simultaneous detection of six swine viruses such as CSFV, PRRSV, PCV2, JEV, PPV and porcine pseudorabies virus is a very useful approach for clinical diagnosis in mixed infection (Xu et al. 2012). Real-time PCR assays both SYBR Green and TaqMan-based assays have been developed for accurate and rapid quantification of PCV in serum and tissue samples for early detection of disease as it is a more sensitive method, for correlating virus load with extent of disease, for post-vaccination tracking of viral load in different tissues and for evaluating potency of developmental vaccine (Zhao et al. 2010; Nan-Chang et al. 2010). A severe PCV2-associated lesion was found to be associated with more than or equal to 10^7 (Brunborg et al. 2004). Multiplex real-time PCR is in use for differentiation of various PCV2 genotypes (Gagnon et al. 2008). An ORF2-based PCR has also been used for differentiation between PCV1 and PCV2 and for differentiation of various PCV2 genotypes, respectively (Fenaux et al. 2000; Hamel et al. 2000). Recently, duplex nanoparticle-based PCR has been developed for detection of PCV2 and PCV3 (Zhang et al. 2018).

Virus isolation can be carried out in suitable cell culture system. PCV can multiply in PK 15 cells without causing any gross cytopathic effect (CPE). Multiplication of PCV is found to be increased several times after glucosamine treatment by enabling entry of PCV genome into cell nucleus (Tischer et al. 1987). As no CPE is observed, virus replication is usually detected by PCR or IHC or IFT. As virus isolation is a time-consuming procedure, it is not routinely used for PCV diagnosis.

Sequencing and phylogenetic analysis are very useful for analyzing genetic variation among PCV from different geographical regions for study of molecular epidemiology and molecular evolution (Olvera et al. 2007; Cortey and Segalés 2012; Anoopraj et al. 2015; Palinski et al. 2017).

Various serological tests for detection of antibodies to PCV have been developed. These tests have limited use in diagnosis of diseases associated with PCV because of its ubiquitous nature and similar seroconversion pattern between diseased and clinically healthy animals. However, they can be used as management tools for breeding herds, for determination of timing of infection on the basis of IgM

and IgG antibody levels and for determination of passive antibody level for formulation of future control strategies. Serological tests include indirect fluorescent antibody assay (IFA), serum virus neutralization assay, IgM immunoperoxidase monolayer assay, indirect ELISA, competitive ELISA, etc. (Walker et al. 2000; Opriessnig et al. 2007; Patterson et al. 2008).

10.9 Prevention and Control

Vaccination with safe and effective vaccine is traditionally considered to be the most effective method for preventing viral diseases. Currently available commercial vaccines have shown effectiveness in reducing severity of clinical diseases and improving production parameters. The duration of protection period is limited and complete virus eradication has also not been achieved (Afolabi et al. 2017). Vaccination of breeding boar decreases viraemia, systemic viral load and subsequently shedding of virus in semen, which is helpful in controlling vertical transmission of virus through semen. Vaccination of breeding sow also decreases systemic virus load in sow, decreases quantity of virus transferred to progeny during gestation and pre-weaning period and increases neutralizing antibody against PCV2 in colostrum. And passive immunity in the form of maternal antibody decreases pre-weaning mortality and improves average daily weight gain in the offspring. Vaccines administered to growing pigs have shown to decrease virus load and mortality and increase growth performances. Vaccination in the presence of maternal antibody has resulted in decreased vaccine efficacy. But in another study, vaccination in the presence of maternal antibody has been found to be effective (Fort et al. 2008; Opriessnig et al. 2008; Beach and Meng 2012).

Most of commercially available vaccines are based either on whole inactivated PCV2a virus or on its immunogenic Cap protein. The Circovac vaccine (Merial) is composed of inactivated whole PCV2a virus and mineral oil adjuvant; recommended for use in piglets more than 3 weeks age intramuscularly with single dose and for healthy breeding age female pigs, two injections at 3–4 weeks interval before breeding followed by one booster dose 2–4 weeks before farrowing are recommended. Subunit vaccines based on recombinant capsid protein of PCV2a are available which include Ingelvac CircoFLEX (Boehringer Ingelheim Vetmedica Inc.) with carbomer adjuvant, Porcilis PCV (Schering-Plough, Merck) with mineral oil adjuvant and Circumvent PCV (Intervet, Merck) with Microsol Diluvac Forte. They are recommended for piglets above 3 weeks of age. The recent one, FosteraTM PCV (Pfizer), with killed chimeric PCV1 and PCV2a virus and SL-CD aqueous adjuvant is recommended for use in piglets above 3 weeks of age (Beach and Meng 2012; Afolabi et al. 2017).

Vaccination of pigs with PCV2a leads to emergence of vaccine escape strain PCV2b worldwide with severe occurrence of clinical disease (Carman et al. 2008). Although cross protection is observed between PCV2a and PCV2b, vaccine based on genotype PCV2b is more effective against PCV2b than vaccine based on PCV2a (Opriessnig et al. 2013; 2014a, b). Recently, PCV2d genotype has replaced PCV2b

genotype which is the most prevalent genotype (Xiao et al. 2016). It has also been observed that vaccine with PCV2a can confer adequate cross protection against clinical disease with PCV2d genotype (Opriessnig et al. 2017). In China, commercial vaccines based on inactivated PCV2b (DBN-SX07 strain, WH strain and ZJ/C strain) and PCV2d (SH strain) are in use and licensed for various age groups of pigs (Zhai et al. 2014).

As PCVAD is a multifactorial disease, vaccination along with other interventional strategies is required for effective control of disease. Good management practices like limiting pig to pig contact; decreasing stress, good hygiene, good nutrition and changing genetic background of pigs; use of disinfectant in buildings and transport vehicles; control of coinfection with use of vaccine, antimicrobial, bacterins and anti-inflammatory drugs; and control of other potential factors that induce immune stimulation are essential for controlling disease (Opriessnig et al. 2007).

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Chicken Infectious Anaemia Virus

11

V. Gowthaman

Abstract

Chicken infectious anaemia virus (CIAV) is an immunosuppressive and vertically transmitted disease of poultry, prevalent worldwide. It is caused by the smallest DNA virus known as chicken anaemia virus (CAV), which belongs to family *Circoviridae* that is classified under genus *Gyrovirus*. The genome contains single-stranded, negative-sense, circular DNA, which consists of 2300 nucleotides. Chicken is the only host for CAV and the disease is transmitted by vertical and horizontal routes. The disease is commonly noticed in 3 weeks of age. CAV is relatively tolerant to commonly used chemical disinfectants and also found resistant of exposure to pH 3, lipid solvents like ether or chloroform and not inactivated by heating at 70 °C for 1 h. Hence, it makes the virus extremely difficult to eradicate from commercial poultry facilities. The characteristics consist of weakness, retarded growth, gangrenous dermatitis, atrophy of thymus and bone marrow leading to severe anaemia and immunosuppression. The economic loss occurs mainly due to vaccine failures, secondary complications and subclinical disease. Laboratory diagnosis of CAV can be achieved by the demonstration of CAV antibody or antigens by paired sera sampling, ELISA test and polymerase chain reaction technique (PCR). The virus could be isolated by using yolk sac route in embryonated chicken eggs, in MDCC-MSB1 cells or in SPF chicks. Since CAV is highly resistant, adaptation of stringent biosecurity measures is required to control the infection. Apart from biosecurity, keeping the other immunosuppressive viral diseases under control by proper vaccination is also required. The breeders should be vaccinated between 8 and 16 weeks of age with a live vaccine; MDA titre of >8 log2 is required to limit the vertical transmission.

V. Gowthaman (✉)
Poultry Disease Diagnosis and Surveillance Laboratory, Veterinary College and Research Institute Campus, Tamil Nadu Veterinary and Animal Sciences University, Namakkal, India

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Keywords
Chicken anaemia virus · Blue wing disease · Gangrenous dermatitis syndrome

11.1 Prologue

Chicken infectious anaemia virus (CIAV) is an immunosuppressive, highly contagious and vertically transmitted disease of poultry, prevalent worldwide especially in the countries where intensive production is practised (Kaffashi et al. 2017; Li et al. 2017; Erfan et al. 2018). The disease is known by different names, such as blue wing disease, anaemia dermatitis syndrome, haemorrhagic syndrome and infectious anaemia syndrome (Pope 1991). The disease was first reported in Japan during 1979 by Yuasa (Yuasa et al. 1979, Krishan et al. 2016), from the young chicks with the characteristic signs of weakness, retarded growth, gangrenous dermatitis, atrophy of thymus and bone marrow leading to severe anaemia and immunosuppression. The economic loss occurs mainly due to vaccine failures, secondary complications and subclinical disease (Oluwayelu 2010).

11.2 Aetiology

Chicken anaemia virus (CAV) belongs to family *Circoviridae* that is classified under the genus *Gyrovirus*. It is a non-enveloped, icosahedral virus which has a diameter of 23–25 nm (Miller and Schat 2004). The genome contains single-stranded, negative-sense, circular DNA, which consists of 2300 nucleotides.

The viral proteins, namely, VP1, VP2 and VP3, are transcribed from a single major transcript (2.0 kb) from three overlapping reading frames. VP1 is the major structural protein that comprises the viral capsid. Although the CAV genome is well conserved, the VP1 gene shows more variability with a hypervariable region between amino acids 139 and 151. Previous studies of Renshaw et al. (1996) revealed that amino acids at positions 139 and 144 play a major role in virus replication and spread in cell cultures. Apart from that amino acid at position 394 also could be a major genetic determinant of virulence (Yamaguchi et al. 2001). VP2 is a scaffolding protein with phosphatase activity and might play a role in capsid formation such as a scaffold or chaperone for protein folding of VP1 (Lai et al. 2017, 2018). VP3 is a non-structural protein that is responsible for apoptosis, playing a vital role in pathogenesis (Noteborn 2004). The VP1 and VP2 are the important targets of virus-neutralising antibodies (Noteborn et al. 1992). The virus has two serotypes (Kim et al. 2010) with several genetic groups (Islam et al. 2002).

11.3 Epidemiology

Among different bird species the chicken is the only host for CAV (Chat 2003). The disease is transmitted by vertical and horizontal routes of exposure (McNulty 1991). Though all the ages are susceptible, the clinical disease is usually noticed in young chicks of 10–14 days old which is acquired vertically. The susceptibility decreases rapidly in immunologically intact chicks at first 1–3 weeks of age (Rosenberger and Cloud 1989). Aged chickens do not develop clinical signs, although they are susceptible. Maternal antibodies towards CAV are present in majority of the young chicks which protect them from infection (von Bülow et al. 1986); thereafter it gradually decreases by 3 weeks of age, and most of the flocks become seropositive by 8–12 weeks, probably acquired by horizontal infection, which occurs subclinically which is exhibited by poor vaccine responses and susceptibility to secondary infections (McNulty et al. 1988). Since majority of the breeders seroconvert due to vaccination at the point of lay or natural exposure, the clinical disease caused by CAV is not common. However, commercial layer and breeder flocks seroconvert during their lifetime, while broiler flocks might remain serologically negative until processing (Tor 2016). As a non-enveloped virus CAV is relatively tolerant to commonly used chemical disinfectants such as amphoteric soap, quaternary ammonium compound and ortho-dichlorobenzene. The virus also found resistant of exposure to pH 3, lipid solvents like ether or chloroform and also not inactivated by heating at 70 °C for 1 h (Dhama et al. 2008). Hence it makes the virus extremely difficult to eradicate from commercial poultry facilities, which leads to the episodes of continuous infection and subsequent seroconversion in susceptible birds leading to endemicity of the CAV. The economic losses due to CAV infections result from reduced growth parameters, high mortality, vaccine failures and exacerbation of the symptoms of other diseases (McNulty 1991). McIlroy et al. (1992) reported a net income loss of about 18.5% due to low body weight at processing and high mortality around 3 weeks of age in 15 broiler flocks.

11.4 Vaccine Contamination

CAV is a significant extraneous pathogen that contaminates the avian virus vaccines. The vaccine contamination occurs vertically by embryonated chicken eggs, despite the use of CAV-free specific pathogen-free (SPF) eggs (Su et al. 2018). Previous studies suggested that the epidemiology of CAV in Brazilian and Argentinian poultry flocks might have been influenced by contamination of live vaccines with CAV (Marin et al. 2013). This type of contamination results in impaired immune response that interferes with vaccination programmes. Sometimes vaccine contamination of CAV leads to severe anaemia, retarded growth, vaccine failures and secondary infections. Hence the CAV contamination should be eliminated by testing of SPF embryonating eggs and examining the vaccines for possible contamination before release (Amer et al. 2011).

11.5 Pathogenesis

After entry of the virus through vertical or horizontal route, CAV infects the lymphoid and erythroid tissues mainly the thymus, spleen and bone marrow of susceptible chickens (Hoop and Reece 1991; Smyth et al. 1993; Adair 2000). CAV mainly targets progenitor cells of lymphoid and erythroid lineages, namely, precursor T lymphocytes, reticular cells and haemocytoblasts (Jeurissen et al. 1992; Smyth et al. 1993; Wani et al. 2015). It causes downregulation of ChT1 and T-cell receptor b that leads to transient depletion of thymocytes in the thymus (Giotis et al. 2015). CAV adversely affects the functions of lymphocytes and macrophages by decreasing the lymphocyte transformation responses, T-cell growth factor production and interferon production. During the initial period of infection, it depresses T-helper cell responses resulting in poor antibody response (Otaki et al. 1988). This always leads to severe immunosuppression with increased susceptibility to a wide range of bacterial and viral pathogens and also vaccine failures (Rosenberger and Cloud 1989; Cloud et al. 1992). It also destroys the bone marrow erythroblastoid cells (haematopoietic precursors) (Adair 2000) leading to severe anaemia and haemorrhagic syndrome. Apart from infection and immune and haematopoietic systems, the virus can also persist in intrathecal cells of the ovaries and to a lesser degree in the infundibulum of the oviduct in females and also vas deferens in males.

11.6 Clinical Signs and Lesions

Chicken infectious anaemia infections manifested either clinical or subclinical forms causing a mortality up to 60% (Engström and Luthman 1984). In clinical CAV the early signs start at the end of the second week of age. The affected birds show stunted growth, weakness, ruffled feathers, depression, anorexia, paleness (Fig. 11.1a), anaemia with low haematocrit values and watery blood. In acute form of the disease, the mortality reaches peak within a week (Engström and Luthman 1984; Yuasa et al. 1987). The affected birds exhibit focal skin lesions characterised by oedematous, reddish blue gangrenous areas releasing a serosanguinous exudate in the head, wings (Fig. 11.1b), sides of the thorax, abdomen, thigh region (Fig. 11.1c) and feet (Fig. 11.1d) (Weikel et al. 1986; Goryo et al. 1987; Chettle et al. 1989; Gowthaman et al. 2012, Krishan et al. 2016). These clinical conditions are defined as haemorrhagic anaemia syndrome, anaemia-dermatitis syndrome and blue wing disease. In few flocks, a second, smaller peak of mortality has been observed after 2 weeks of first due to horizontal spread of the disease in seronegative birds (Bisgaard 1983; Engström and Luthman 1984). This kind of mortality is due to secondary or mixed infections of CAV and other agents, such as fowl adenovirus, Marek's disease virus and infectious bursal disease virus, or from secondary bacterial infection of the skin lesions like *Clostridium perfringens*, *Staphylococcus* and *E. coli*. The pathological lesions are characterised by pale and anaemic carcass including visceral organs (Fig. 11.1e); subcutaneous haemorrhages with accumulation of gelatinous and oedematous fluid in the subcutis of the affected skin; atrophy

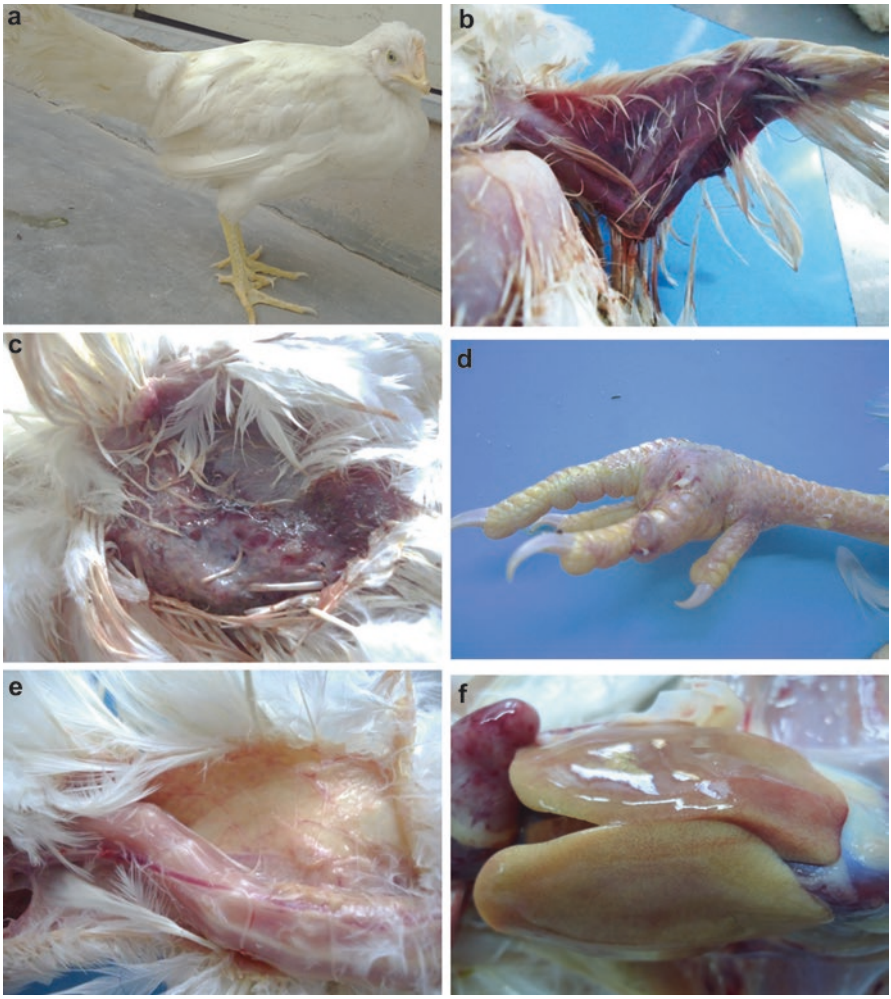

Fig. 11.1 (**a**) The affected bird exhibits stunted growth, weakness, ruffled feathers, depression, and paleness. (**b–d**) Skin lesions in the affected birds characterized by edematous, reddish blue gangrenous areas releasing a serosanguinous exudate. (**e**) Atrophy of thymus is a typical post mortem finding in CAV. (**f**) The CAV affected bird exhibit pale liver

of the thymus, spleen and bone marrow; petechial to ecchymotic haemorrhages in the thigh and breast muscles; and haemorrhages between the proventriculus and gizzard junction (Gowthaman et al. 2012). Histologically, severe depletion and atrophy of lymphoid cells are seen in the thymus (Fig. 11.1f), spleen, bone marrow and bursa of Fabricius. Apart from this, intranuclear inclusions of CAV are also noticed in macrophages of the thymus cortex and haemocytoblasts of the bone marrow (Pope 1991). In subclinical CAV poor FCR, low body weight, increased mortality and condemnations are observed due to secondary infections (McNulty 1991).

11.7 Diagnosis

A tentative diagnosis of CAV can be made based on the history with the clinical signs and pathological findings indicative of anaemia dermatitis syndrome. However, laboratory conformation is highly mandatory to prove the CAV infection in the affected flocks. Laboratory diagnosis can be achieved by the demonstration of CAV antibody or antigens in the affected chicken. The antibodies of CAV could be confirmed by paired sera sampling (McIlroy et al. 1992) using ELISA test. The presence of CAV antigens can be demonstrated by immunostaining (McNulty et al. 1988) or detecting CAV DNA by dot-blot hybridisation (Todd et al. 1992) from the thymus, bone marrow and spleen. The CAV could be isolated by using yolk sac route in embryonated chicken eggs, in MDCC-MSB1 cells or in SPF chicks (McNulty 1989). However, this virus isolation is not followed in most of the diagnostic laboratories since the procedure is time consuming, expansive and also difficult in growing CAV in common chicken cell cultures, except specific T and B lymphoblastoid cell cultures (Yuasa et al. 1979). Therefore, polymerase chain reaction (PCR) has been widely used to demonstrate viral DNA in tissues of the affected flocks (Tham and Stanislawek 1992) because it allows the testing of a large number of samples. Further the virus can be typed and classified by restriction endonuclease analysis or sequencing (Gowthaman et al. 2016). Recently, LAMP method has been developed for detecting of chicken anaemia virus with high sensitivity and specificity (Song et al. 2018).

11.8 Control

The CAV is generally present as a ubiquitous pathogen in the poultry-rearing environment. Since it is highly resistant to inactivation by most of the disinfectants, adaptation of stringent biosecurity measures is required to control the infection in poultry farm premises (Rosenberger and Cloud 1989). Apart from biosecurity, the other immunosuppressive pathogens like Marek's disease virus, infectious bursal disease virus and fowl adenovirus can increase the susceptibility to CAV manyfolds (Markowski-Grimsrud and Schat 2003). Hence, it highly necessities to keep the other immunosuppressive viral diseases under control by proper vaccination. The breeders should be vaccinated between 8 and 16 weeks of age with a live vaccine; a MDA titre of >8 log2 is required to limit the vertical transmission. The clinical infection of CAV can be controlled by ensuring transfer of sufficient level maternal antibodies to the progeny by vaccination of breeders and use of immunomodulatory agents, herbal extracts and protein supplements (Krishan et al. 2015a, b; Latheef et al. 2017). The higher the level of maternal antibody, the higher the level of protection against clinical CAV infection (Fussell 1998).

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Conflict of Interest There is no conflict of interest.

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Canine Parvovirus

12

Mithilesh Singh, Vishal Chander, and Sukdeb Nandi

Abstract

Canine parvovirus-2 (CPV-2) is one of the key pathogens of dogs causing an acute disease which is characterized by haemorrhagic enteritis, vomition and myocarditis. The dogs of any age group may be invariably affected by CPV; however, the disease is mainly fatal in pups. The genetic variation amongst CPV-2 resulted into emergence of five new genotypes (CPV-2a, CPV-2b, new CPV-2a, new CPV-2b and CPV-2c) that differ in amino acid sequences over their capsid VP2 protein. Various epidemiological surveys indicate that these newly evolved CPV variants are prevalent in different geographic regions and have completely replaced the original genotype. Despite the availability of a live modified CPV vaccine, CPV cases are regularly reported, which could be due to the appearance of these new antigenic variants. However, interference by maternal-derived antibodies is one of the key factors behind CPV vaccination failure. The present chapter is focused on the general introduction about CPV, evolution of its antigenic variants and the disease pathogenesis. The various diagnostic methods from conventional to latest one are discussed in detail with their merits and demerits. In addition, the currently available strategies adopted for prevention and control of CPV infection along with various risk factors are discussed in length.

M. Singh (✉)
Immunology Section, ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Bareilly, Uttar Pradesh, India

V. Chander
Virology Laboratory, Centre for Animal Disease Research and Diagnosis (CADRAD), ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Uttar Pradesh, India

S. Nandi
Centre for Animal Disease Research and Diagnosis (CADRAD), ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Uttar Pradesh, India

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Antibodies · Antigenic variants · Canine parvovirus · Diagnosis · Dogs · Risk factors · Vaccine · VP2

12.1 Prologue

Canine parvovirus-2 (CPV-2) is an important enteropathogen-causing agent of acute haemorrhagic enteritis and myocarditis in dogs. The virus is very sturdy, highly contagious causing extremely fatal disease. The virus was first reported in 1977 and over time it has contracted a large number of dogs around the world with extremely high morbidity (100%) but low mortality of 10% in adult dogs and 91% in pups (Appel et al. 1979). It is believed that CPV originated as a host range variant from feline panleukopenia virus (FPV), which includes a direct mutation from FPV, a mutation from a FPV vaccine virus and the adaptation to the new dog host via non-domestic carnivores, like mink and foxes. The disease has two prominent clinical forms depending on the age of dogs: (i) severe enteritis associated with vomition and diarrhoea reported in all age group dogs (Appel et al. 1979; Woods et al. 1980) and (ii) myocarditis and subsequent heart failure reported in very young pups of less than 3 months of age. There is another closely related virus, namely, canine parvovirus 1 or canine minute virus (CnMV). CnMV is a completely different member under genus *Parvovirus* which had not been associated with natural disease until 1992. This *Parvovirus* genus may cause pneumonia, myocarditis and enteritis in very young pups or transplacental infections in pregnant dams associated with embryo resorptions and foetal death (Carmichael et al. 1994). Martella et al. (2006) have reported about 30 confirmed cases of CPV-1 from the USA, Sweden, Germany and Italy. The canine parvovirus infections have emerged to be a problem in dogs in spite of a number of potent and efficacious live as well as inactivated vaccines available to be used in the dogs. So, it is the prime time to create awareness amongst the dog owners, pet lovers, Kennel club owners, pet shop owners and defence personnel about the disease in order to prevent and control the disease in a more effective and efficient manner.

12.2 History

In early 1978, entire canine population in the world had been collapsed with haemorrhagic gastroenteritis and high mortality. It was found to be a viral agent and designated as CPV-2 to differentiate it from CPV-1, a relatively non-pathogenic virus. In 1980 another mutant of CPV-2 was reported in the USA and termed as CPV-2a. Subsequently it has been reported in Japan, Belgium, France, Denmark and Australia due to strong positive selection and strong epidemiological advantage over CPV-2 and became the most common and dominant virus in many other carnivores (Parrish et al. 1988). There is six-amino acid difference between CPV-2 and

CPV-2a mutants. These are 87 (Met to Leu), 300 (Ala to Gly) and 305 (Asp to Tyr) of VP2 protein and responsible for replication in cats. Other changes in VP2 gene are 101 (Ile to Thr), 297 (Ser to Ala) and 555 (Val to Ile).

CPV-2b and CPV-2c After the emergence of CPV-2a, two more antigenic variants CPV-2b and CPV-2c were detected in 1984 in the USA and in 2000 in Italy, respectively (Buonavoglia et al. 2001). The differentiation of CPV-2a, CPV-2b and CPV-2c is based on the amino acid present at 426 residues of VP2 protein [CPV-2a (Asn), CPV-2b (Asp) and CPV-2c (Glu)] and affects the major antigenic region (epitope A) located at threefold spike in the VP2 protein. There is another change at 555 positions (Ile to Val) of VP2 protein between CPV-2a and CPV-2b. The substitution of VP2 residues at 555 represented a reversion to or retention of the sequence of original CPV-2. Thus CPV-2b and CPV-2c differ from CPV-2a only at 426 residues of VP2 protein. Presently CPV-2c is the most dominant strain in Italy, Germany, Uruguay and Argentina (Decaro and Buonavoglia 2012). Another mutant 300 (Gly to Asp) of CPV-2a and CPV-2b has been reported in leopard, in cats in Vietnam in 1999 (Ikeda et al. 2000) and in a few dogs in Vietnam and Korea (Kang et al. 2008). CPV-2c has become the dominant strain throughout the world and has been reported from Europe, North and South America, Africa and part of Asia (Decaro et al. 2005a, b, 2007; Kapil et al. 2007; Nandi et al. 2010; Touihri et al. 2009). In Asia, CPV-2c has been reported in Vietnam, India and China (Nandi et al. 2009; Nakamura et al. 2004; Geng et al. 2015) but not in Korea, Taiwan, Iraq, Turkey, Russia and Japan. Further a substitution at 324 Ile frequently confirmed in Korean and Chinese strains (Zhang et al. 2010; Jeoung et al. 2008) has also been reported in Japan (Soma et al. 2013). The residues 324 and 323 located at threefold spike regulate the host range along with residues 101, 300 and 426 (Hueffer et al. 2003). Another important feature of CPV-2c is that this mutant does not readily transmit in the dogs where CPV-2b prevails but causes severe clinical signs particularly in adult dogs. Further, in addition to this CPV-2a with substitution at residues 413 (Asp to Asn), 418 (Ile to Thr), 435 (Pro to Ser) and 440 (Thr to Ala) have been reported in Korea (Zhang et al. 2010; Jeoung et al. 2008; Kang et al. 2008), Europe (Decaro et al. 2009a) and the USA (Kapil et al. 2007). The VP2 protein residue 297 is located in the middle of epitope B and mutation (substitution) at this position influences the changes in antigenicity of CPV variants (Truyen 2006). Although the change T440A has been reported worldwide (Kapil et al. 2007; Kang et al. 2008; Decaro et al. 2009b), its implications are not clearly understood. Most of the changes in the VP2 protein lies between residues 267 and 498 and plays an important role in the evolvement of new variants due to its presence on the capsid surface and is liable to undergo mutation.

Further new CPV-2a and new CPV-2b have also been reported due to non-synonymous substitution at 297 residues located close to epitope B (Ser to Ala) of the VP2 protein without affecting the antigenicity of those variants (Ohshima et al. 2008). The mutation at 440 (Thr to Ala) located in the G-H loop and at 324 (Tyr to Ile) of VP2 protein has also been reported in the same isolates and may influence the antigenic structure and host range, respectively (Mittal et al. 2014). The mutant 300

Asp (CPV2a/2b) detected in domestic or wild felids in Southern Asia as well as in raccoons indicates the adaptation of the mutant to replicate in these hosts (Ikeda et al. 2000).

Nowadays three antigenic variants can infect a variety of different hosts and have a worldwide distribution. CPV-2 is considered to be a host range variant derived from FPV via wild carnivores and gained the ability to infect dogs. The phylogenetic analysis of VP2 protein of raccoon parvovirus (RPV) and bobcat parvovirus revealed the intermediate location between CPV-2 and CPV-2a strains. Hence, RPV might have played an important role in the evolution of CPV-2a and related strains which regained the ability to infect cats, a property that is absent in CPV-2. The CPV-2a-specific residues at 87 and 101 might be acquired during evolution of the virus in raccoons while the changes in 300 and 305 were acquired in canine hosts. Other wild animals such as jackals, coyotes, foxes and wolves also played a pivotal role in the evolution of CPV-2 and related strains. One of the most interesting facts about the CPV-2 evolution is the high rate of nucleotide substitutions associated with infection of canine population (Pereira et al. 2007).

12.3 Incidence and Prevalence of Disease

Canine parvovirus infection has been spread around the world in dogs and other members of *Canidae* family. Incidence of parvoviral infection is higher in animal shelters, pet stores and breeding kennels. CPV affects dogs of any age group; however, younger pups between 6 weeks and 4 months are more susceptible to severe infection (Appel et al. 1979). All breeds of dogs are susceptible (Hueffer et al. 2003). The pure breeds, namely, English Springer, Doberman Pinschers, Rottweilers, Spaniels and German Shepherd, are highly susceptible to the disease but there is exception such as Toy Poodles and Cocker Spaniels which are less susceptible (Houston et al. 1996). CPV affects only dogs and cannot be transmitted to humans or other species. The disease is acute in nature and ailing dogs which survived the first 4 days of illness usually recover rapidly and become lifelong immune to the disease. Most puppies die in the absence of medical interventions. A large proportion of infected dogs usually do not show the clinical manifestations of the disease but they may shed the virus in faeces which are a source of infection to other susceptible dogs (Stann et al. 1984).

Molecular epidemiology of CPV revealed that different geographical locations of the world are facing canine parvovirus enteritis outbreaks caused by different CPV-2 variants in recent years. In Europe all strains with widespread presence of CPV-2c have been reported from Germany (Decaro et al. 2013), Poland (Majer-Dziedzic et al. 2011) and Portugal (Miranda and Thompson 2016). Unlike other European countries, in Greece, Hungary and Bulgaria, CPV-2a remains the most common variant with sporadic presence of CPV-2b

and CPV-2c (Ntafis et al. 2010; Csagola et al. 2014). Calderón et al. (2015) have reported presence of all antigenic variants but CPV-2c is the most dominant strain in South American countries like Ecuador (Aldaz et al. 2013), Brazil (Pinto et al. 2012) and Argentina. In North American continent like in the USA and Canada, all antigenic variants are circulating but CPV-2b is the dominant one (Hong et al. 2007), while the current dominant viral variant in Mexico is CPV-2c (Pedroza-Roldan et al. 2015). In Africa, both CPV-2a and 2b are present in South Africa but only CPV-2a in Nigeria (Dogonyaro et al. 2013) and in Tunisia all types of antigenic variants were reported (Touihri et al. 2009). New Zealand had reported only CPV-2a (Ohneiser et al. 2015) whereas in Australia CPV-2a was the most prevalent strain with sporadic presence of CPV-2b (Meers et al. 2007). In Asia, China and Taiwan have reported all types of CPV-2 variants, while Korea (South) (Yoon et al. 2009), Japan (Soma et al. 2013), Thailand (Phromnoi et al. 2010), Iraq (Ahmed et al. 2012) and Turkey (Timurkan and Oguzoglu 2015) reported both CPV-2a and CPV-2b strains.

In India canine parvovirus-2 was first reported by Ramadass and Khadher (1982). Different canine parvovirus variants (CPV-2a, CPV-2b, new CPV-2a, new CPV-2b and CPV-2c) have been reported from dogs of almost all the states of India (Chinchkar et al. 2006; Mohan et al. 2010; Kumar and Nandi 2010; Srinivas et al. 2013; Mittal et al. 2014; Thomas et al. 2014). However, only single report was documented about the presence of CPV-2c strain (Nandi et al. 2010). Presently, new CPV-2a has surpassed the earlier circulating strain of CPV-2b or new CPV-2b and has been established as a predominant strain of CPV-2 in the canine population of India (Srinivas et al. 2013; Mittal et al. 2014; Thomas et al. 2017a).

12.4 Diagnosis

The precise diagnosis of the canine parvoviral enteritis not only aids in proper treatment and recovery of affected dogs but also helps in isolation of affected dogs from other susceptible healthy dogs. Clinical diagnosis in CPV-affected dogs can be inconclusive because of the similarities in clinical signs with several other diseases. Therefore, suspected cases with typical clinical signs must always be verified with reliable laboratory tests (Table 12.1). The diseases that should be differentially diagnosed include virus-induced enteritis (canine distemper, coronaviral and adenoviral enteritis), bacterial as well as parasitic gastroenteritis (e.g. *Ancylostoma* spp., *Trichuris vulpis*, *Uncinaria* spp.), some systemic disturbances with secondary GI involvement (e.g. hepatic disease, pancreatitis, renal failure, etc.), coagulopathy (e.g. thrombocytopenia, etc.), neoplasia and severe GI ulceration or perforation (Greene and Decaro 2012).

Table 12.1 Merits and demerits of the present methods/tests employed for CPV diagnosis

S. no.	Diagnostic test	Detection principle	Sensitivity	Specificity	Time	Cost	Labour
1.	***Conventional methods***						
	Virus isolation	Infectivity assay	Poor	Excellent	Days	Inexpensive	High
	Electron microscopy	Viral particle	Poor	Good	Weeks	Expensive	High
	Haemagglutination test (HA)	Viral protein	Good	Poor	Hours	Inexpensive	Moderate
2.	*Immunodiagnostic methods*						
	Counter immunoelectrophoresis	Viral protein	Poor	Good	Hours	Inexpensive	Moderate
	Fluorescent antibody test	Infectivity assay	Good	Good	Days	Expensive	High
	Agglutination Test	Viral protein	Good	Poor	Hours	Inexpensive	Low
	Enzyme-linked immunosorbent assay (ELISA)	Viral protein	Good	Excellent	Hours/ days	Expensive	Moderate
	Immunochromatographic assays	Viral protein	Good	Excellent	Minutes	Inexpensive	Low
	Dot blot/Dot-ELISA	Viral protein	Good	Good	Hours	Inexpensive	Moderate

3.	***Molecular diagnostic methods***						
	Polymerase chain reaction (PCR)	CPV-VP2 gene	Excellent	Excellent	Hours	Inexpensive	Moderate
	Nucleic acid hybridization/dot blot	CPV-VP2 gene			Days	Expensive	Moderate
	In situ hybridization assay	CPV-VP2 gene			Days	Expensive	High
	Real-time polymerase chain reaction (RT-PCR)	CPV-VP2 gene (CPV detection and typing)			Hours	Expensive	Moderate
	Multiplex amplification refractory mutation system PCR (ARMS-PCR)	CPV-VP2 gene (CPV detection and typing)			Hours	Inexpensive	Moderate
	Peptide nucleic acid-based (PNA) array	CPV-VP2 gene (CPV detection and typing)			Days	Expensive	Moderate
	Loop-mediated isothermal amplification (LAMP) assay	CPV-VP2 gene			Hours	Inexpensive	Low
	Insulated isothermal PCR method	CPV-VP2 gene			Hours	Expensive	Low
	Polymerase spiral reaction (PSR)	CPV-VP2 gene (CPV detection and typing)			Hours	Inexpensive	Low
	Oligonucleotide sequencing	CPV-VP2 gene/whole genome			Days	Expensive	Moderate
4.	*Biosensors*	Viral protein	Excellent	Excellent	Hours	Expensive	Low

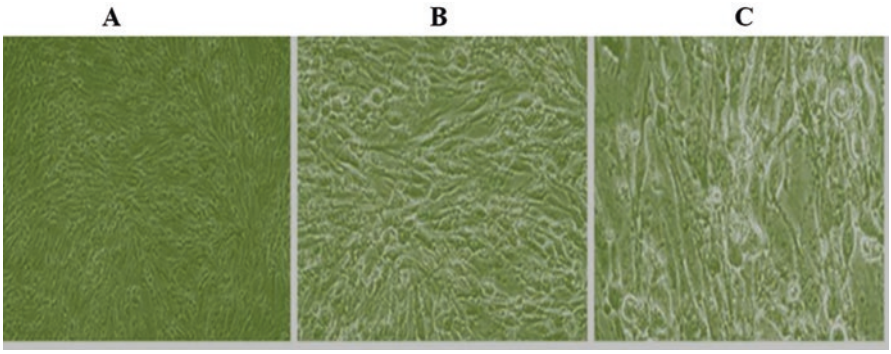

Fig. 12.1 CPV-2-infected A-72 cells showing cytopathic effect at 3rd passage level; cells showing increase in granularity, rounding and detachment, (**a**) 10x view, (**b**) 20x view and (**c**) 40x

12.5 Laboratory Diagnosis

12.5.1 Conventional Methods

Virus Isolation A number of cell lines like CRFK (Crandell Rees feline kidney), MDCK (Madin-Darby canine kidney) and A-72 support the replication of CPV (Appel et al. 1979). The A-72 cell line is an established cell line originated from canine subcutaneous tumour, most relevant for routine canine parvovirus growth and isolation (Carmichael et al. 1980; Verma et al. 2016). The virus causes cytopathic effect in infected cell lines which is appreciable only after third passage in the form of cell rounding, granulation and aggregation (Fig. 12.1). However, this method is quite laborious and time consuming as it requires minimum of 5–10 days incubation for the proper growth of CPV and it needs further confirmation by other laboratory tests (Desario et al. 2005).

Electron Microscopy The virions can be directly demonstrated in faeces by negative staining through electron microscopy. Immunoelectron microscopy can be performed by using CPV-specific antibodies which may improve the specificity of the test (Burtonboy et al. 1979). However, need of a sophisticated and costly instrument (electron microscope) renders it unsuitable for routine diagnosis.

Haemagglutination Test (HA) The ability of CPV to agglutinate pig, cat or rhesus monkey red blood cells at 4 °C is exploited for performing HA test. This test is usually done in 'U'- or 'V'-shaped microtitre plates. In general, mat and button formation indicates the presence and absence of haemagglutination, respectively (Fig. 12.2). The reciprocal of the highest dilution of virus displaying complete agglutination of erythrocytes (mat formation) is denoted as HA titre of

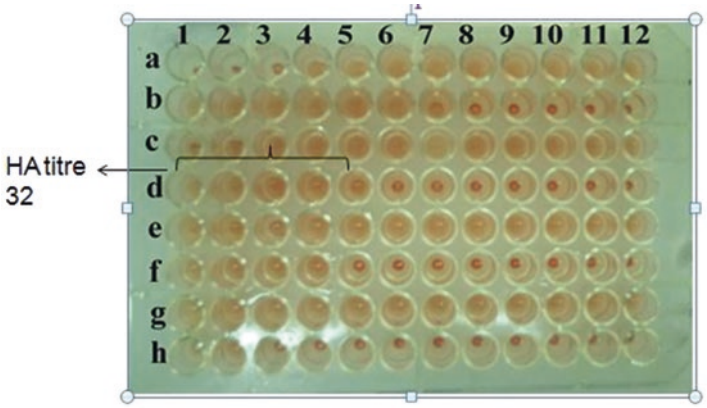

Fig. 12.2 Haemagglutination test; mat formation in d1-d5 wells (HA = 32) hence HA positive, d6-d12 wells showing button formation hence HA negative

that particular sample. The HA titre less than 1:32 is usually considered as non-specific or negative for CPV (Carmichael et al. 1980; Muthuraj et al. 2016).

12.5.2 Immunodiagnostic Assays

Counter-Immunoelectrophoresis (CIEP) This is a laboratory-based technique in which application of electric current facilitates the rapid migration of antigen and antibody towards each other resulting into the formation of precipitation line quicker than simple diffusion reaction. Appearance of a sharp precipitation line is indicative of the binding of antigen with specific antibodies which is considered as positive result. Deepa and Saseendrannath (2000) have successfully employed this technique to know the prevalence of CPV infection in clinically suspected dogs.

Fluorescent Antibody Test (FAT) This is also a laboratory-based test in which an antibody tagged with fluorescent dye is employed for detection of specific antigen. Like many other immunodiagnostic methods FAT may also be categorized into either direct or indirect fluorescent tests depending upon the tagging of fluorescent dye either with primary or secondary antibody. Gray et al. (2012) have demonstrated the usefulness of FAT in diagnosis of CPV infection.

Agglutination Test The test is simply based on antigen-antibody interactions employing specific antigen or antibody. Latex agglutination test (LAT) is one of the commonest agglutination tests frequently used for disease diagnosis under field conditions. It is a very simple and rapid test based on the agglutination property of polystyrene beads which are coated with either specific antigen or antibody on their

surface. Earlier, anti-CPV monoclonal and polyclonal antibody-based LAT has been developed to detect canine parvovirus in faeces of affected dogs (Sanekata et al. 1996; Subhashini et al. 1997). Bodeus and coworkers (1988) have developed a modified form of LAT using latex beads coated with anti-CPV monoclonal antibodies for both qualitative and quantitative evaluation of CPV in suspected dog faeces. Alternatively, Singh et al. (1998) have reported the coagglutination test (COAT) for CPV detection using anti-canine parvovirus serum raised in dogs coated with protein-A containing *Staphylococcus aureus* Cowan I strain. Recently, Thomas and coworkers have developed a recombinant VP2 protein-based LAT for determination of immune status in dogs against CPV-2 (Thomas et al. 2017b). Besides LAT, a slide agglutination inhibition test was designed by Marulappa and Kapil (2009) to detect the presence of CPV-specific antibodies by exploiting the agglutination property of canine parvovirus towards porcine RBCs.

Enzyme-Linked Immunosorbent Assay (ELISA) ELISA is an enzyme-based immunoassay involving antigen-antibody reactions which enables screening of a large number of samples at a time. Recombinant truncated VP2 protein-based indirect ELISA was developed by Shi et al. (2012) to detect and quantify antibodies against canine parvovirus. To determine the threshold of maternal antibody interference, ELISA was developed based on full-length recombinant VP2 protein expressed in baculovirus expression system by Elia et al. (2012). Kumar et al. (2010) developed a novel polyclonal antibody-based antigen capture ELISA using rabbit anti-CPV hyperimmune sera as capture antibody and guinea pig anti-CPV hyperimmune sera as detector antibody. Chicken egg yolk-derived IgY-based ELISA has been developed for detection of both canine parvovirus antigen and antibodies by Pokorova et al. (2000). Various commercial ELISA kits are currently available for CPV antigen and antibody detection; however any indigenous ELISA kit is not yet available in India.

Immunochromatographic Assays Immunochromatographic assays also known as lateral flow assays are simple strip-based devices intended to detect the presence of a target analyte in test samples without the need for any specialized and costly equipment. Colloidal gold nanoparticles are frequently employed in synthesis of the probe (conjugate) in most of these strip-based points of care assays. Whole assembly includes a total of five components: sample pad, conjugate pad, nitrocellulose membrane, absorbent pad and a plastic support. These tests have become the most common test for routine parvovirus diagnosis in puppies. A number of lateral flow assay-based commercial kits are available for rapid detection of both canine parvovirus antigen in faeces and antibodies in serum, e.g. Scan Vet Parvo (Intas), SNAP canine parvovirus antigen test kit (Idexx), VetScan Canine Parvovirus Rapid Test (Abaxis), ImmunoRun (Biogal), CPV Ab Kit (Bionote), etc. The advantages associated with these kits include its field application and prompt results within 10–15 min. Recently, Sharma et al. (2018) have designed an immunochromatography test based on recombinant VP2 protein for rapid detection of CPV under field conditions (Fig. 12.3).

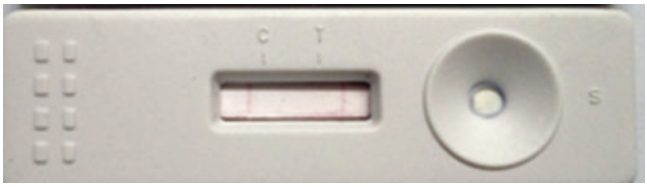

Fig. 12.3 Immunochromatographic assay showing coloured line at both 'T' and 'C' mark which is indicative of positive reaction for CPV

Dot Blot/Dot-ELISA It is an immunological test useful in diagnosis of canine parvovirus infection especially under field conditions. This involves charging of test antigen on to a nitrocellulose or PVDF membrane followed by detection using specific antibody against the antigen and a secondary antibody labelled with enzyme that gives colour when an insoluble substrate is added. Dot-ELISA has been developed earlier by Joshi et al. (2001) for detection of CPV-2 using hyper-immune sera raised against whole virus. Park et al. (2007) have developed a Dot-ELISA based on polyclonal sera specific to full-length recombinant VP2 protein of CPV which was found to be more sensitive than monoclonal antibody-based Dot-ELISA. Commercial dot ELISA kits are also available for assessing IgM response against canine parvovirus after vaccination or infection (e.g. ImmunoComb, Biogal, Israel).

12.5.3 Molecular Diagnostic Assays

Polymerase Chain Reaction (PCR) PCR can detect viral nucleic acid and is also more sensitive than traditional and immunodiagnostic techniques (Desario et al. 2005; Thomas et al. 2014). Canine parvovirus mutants can be differentiated by utilizing strain-specific primer or by employing nested PCR or simply PCR followed by restriction enzyme analysis (Pereira et al. 2000; Kumar et al. 2010). Oligonucleotide sequencing of specific gene amplified product is routinely applied to screen different CPV strains. However, healthy vaccinated animals may excrete modified live virus in low titres in their faeces following vaccination for a short period of time which can give false positive results in PCR. In such cases it is essential to differentially detect vaccine virus with CPV field strains by suitable molecular assays (Fig. 12.4).

Nucleic Acid Hybridization/Dot Blot In this technique, the nitrocellulose paper or nylon membrane is charged with the extracted viral genomic DNA from suspected samples and subsequently allowed for hybridization with CPV-specific biotin or radiolabelled probe. Appearance of colour onto the nitrocellulose paper and band in the X-ray film in case of non-radio- and radiolabelled probe, respectively, is indicative of positive reaction (Cho et al. 2004).

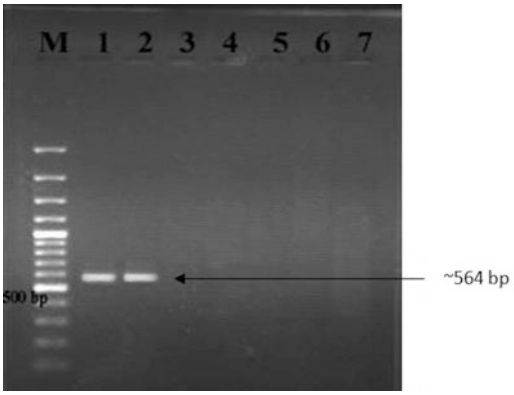

Fig. 12.4 Agarose gel electrophoresis of PCR-amplified partial VP2 gene of CPV-2. Lane M, 100 bp plus DNA ladder; lane 1 and 2 showing PCR-positive faecal samples as amplified product of 564 bp size

In Situ Hybridization Assay This is also a probe-based method employed for detection and tracking of CPV nucleic acid in affected dog tissue specimens. It requires longer incubation time for development of signals as this method utilizes isotopic-labelled probe (Nho et al. 1997).

Real-Time Polymerase Chain Reaction (RT-PCR) Real-time PCR can be employed to quantitate canine parvovirus in faeces using either TaqMan probe technology or SYBR Green method (Decaro et al. 2005c). Real-time PCR using minor groove binding probe can also be a valuable tool to differentiate vaccine strain from wild CPV strains (Decaro et al. 2006).

Multiplex Real-Time PCR This molecular technique involves use of multiple (usually two to four) fluorogenic oligoprobes for the differentiation of several amplicons simultaneously. The VP2 gene-based multiplex real-time PCR has been validated earlier for simultaneous detection of CPV, FPV and PPV. Recently, this has also been used to detect and quantify CPV as well as typing of its three antigenic variants (Decaro et al. 2007).

Multiplex Amplification Refractory Mutation System PCR (ARMS-PCR) Amplification refractory mutation system PCR (ARMS-PCR) is a novel technique to simultaneously detect and type the known point mutations/single nucleotide polymorphism based on variable size of PCR-amplified products specific to a particular allele (Newton et al. 1988). This is a single tube approach in which two pairs of primers are employed (two inner and two outer specific primers corresponding to individual allele type) without any post-PCR processing like restriction enzyme digestion (PCR-RFLP) or sequencing which reduces both time and cost involved in SNP typing. ARMS-PCR is a well-known technique frequently employed for phenotypic association and single nucleotide polymorphism (SNP)

studies. Recently, this technique has been successfully employed for CPV detection and its antigenic typing (Chander et al. 2016).

Peptide Nucleic Acid-Based (PNA) Array Peptide nucleic acids (PNA) are generally considered as a stable nucleic acid analogue. It contains a pseudo-peptide skeleton in place of sugar phosphate backbone which is chemically and biologically highly stable. The efficiency of hybridization of PNAs towards cRNAs or cDNAs is relatively more than DNA. This is possible maybe due to electrically neutral nature of PNA backbone. Newly developed PNA-DNA hybridization assay was found to be highly sensitive and specific in comparison to TaqMan-based real-time PCR (a gold standard method) for differentiation of various CPV antigenic types (An et al. 2012).

Loop-Mediated Isothermal Amplification Assay (LAMP Assay) Loop-mediated isothermal amplification (LAMP) assay is a very sensitive and rapid technique used for amplification of DNA and thereby pathogen detection. This technique utilizes DNA polymerase with autocycling strand displacement activity by simple boiling at constant temperature (60–65 °C) in water bath for 1 hr. Generally, two sets of primers recognizing four to six different regions of target DNA are essential for amplification of target gene. LAMP has field application as there is no need for any thermocycler to carry out the target gene amplification. The amplification of VP2 gene of CPV-2 under constant temperature conditions followed by visual detection was developed by Cho et al. (2006) with a relative sensitivity of 100% and relative specificity of 76.9%. LAMP assay in conjunction with lateral flow dipstick (LFD) and LAMP-ELISA are other variants of this technique recently reported with an objective to detect CPV visually (Sun et al. 2014).

Insulated Isothermal PCR Method This is a convection-based method which utilizes a hydrolysis probe and runs in a commercial device. The reaction mixture is sequentially allowed to pass in an automatic manner through variable temperature zones in a capillary tube that is placed within the device to complete all three stages essential in PCR (denaturation, annealing and extension). The probe hydrolysis produces optical signals during the reaction which are recorded automatically on the screen as a positive or negative result within 1 hr. Wilkes et al. (2015) have reported the use of this method for on-site detection of CPV-2 and its antigenic variants.

Polymerase Spiral Reaction (PSR) This technique is a unique combination of conventional PCR (using only one pair of primers and one enzyme) and isothermal amplification techniques (constant temperature is needed for gene amplification similar to LAMP). The PSR is generally completed within 90 min and it does not require any thermocycler to carry out gene amplification. PSR is a very user-friendly

technique with high sensitivity and specificity. Usually, an exogenous sequence from an unrelated species or of botanical origin is incorporated at the 5′ end into the primer sequences used in PSR if a human or veterinary pathogen is targeted. Recently, PSR was employed successfully for detection of all CPV antigenic variants with tenfold higher sensitivity in comparison to conventional PCR (Gupta et al. 2017).

Oligonucleotide Sequencing This is the most common technique applied for antigenic typing of CPV variants with quite precision (Thomas et al. 2017a). The amplified PCR product either directly or as a cloned product can be sequenced with the help of automated DNA sequencing machine utilizing suitable primers. The final raw data is analysed using the suitable bioinformatics program. Either nucleotide or amino acid sequence data or even both could be simultaneously utilized to know the homology and evolutionary analysis of CPV-2 isolates from wider geographical locations.

12.5.4 Biosensor

Biosensor is basically an analytical device which captures the biological entities (DNA, RNA and protein or enzymes) and converts it to the detectable electrical signals. Biosensors have been widely used for accurate detection of pathogen/tumours, metabolic disorders and food bioanalysis, etc. Biosensors are highly specific and may detect the pathogen in seconds. Recently, a biosensor has been developed for the rapid and precise detection of CPV using quartz crystal microbalance (QCM) biosensor and ProLinker B. In comparison to PCR, QCM-based biosensor had shown 95% sensitivity and 98% specificity (Kim et al. 2015).

12.6 Immunobiology

The type of immunity (local/systemic) required for protection against CPV gastroenteritis was earlier not precisely known. Since there is a viraemic phase accompanied with intestinal infection, one or several types of antiviral immunity may be critically required. Generally, pattern recognition receptors (PRR) initiate the immune (innate) responses against many viral infections (including CPV infection) by recognition of specific viral elements and hence innate immunity is considered as the first line of defence (Janeway and Medzhitov 2002). The recognition and binding of viral ligands with PRR may finally result into the establishment of an antiviral state (Janeway and Medzhitov 2002; Raykov et al. 2013).

Systemic humoral immunity (antibodies) alone could control the disease by preventing viral spread to the gut and other secondary sites. However, antibodies located in the gut (coproantibodies) or a combination of coproantibodies and systemic humoral immunity may be essential for complete protection. The systemic

humoral antibodies may lessen the severity of CPV gastroenteritis by limiting the viraemia yet still allow CPV replication in the gut. Hence, in the absence of copro-antibodies the dog could conceivably become an inapparent carrier of this deadly enteric virus (Rice et al. 1982). All three classes of antibodies, namely, IgM, IgG and IgA, are normally synthesized and transported into the intestines of dogs suffering from canine parvoviral gastroenteritis. Hence, it could be speculated that effective immunoprophylactic measures against CPV must be focused towards the strategies which are designed to induce mainly local CPV antibody response (copro-antibodies) in the intestine of CPV-susceptible dogs. In general, natural CPV infection in puppies induces a rapid immune response and neutralizing antibodies could be detected within 3–5 days postinfection which increase rapidly to very high titres.

Maternal antibodies provide the specific systemic protection against CPV2 in puppies during first weeks of life. The vast majority of these maternal antibodies (90%) are transferred from dam to pups through colostrums while little amount (10%) may have transplacental origin. It is well known that an antibody titre of 80 or more (measured by the haemagglutination-inhibition assay) is indicative of protection against wild CPV challenge. Those pups born to seronegative bitches are susceptible at birth. However, the presence of maternal antibodies less than 80 in pups may interfere with successful immunization as the level of maternal antibodies for protection against wild-type virus is different than that which causes interference in successful immunization.

12.7 Risk Factors for Canine Parvovirus

Canine parvovirus infection may occur in any season but is more prevalent in the summer as the breeding season coincides with the number of puppies being born in this season and thus the susceptibility of the disease is comparatively more leading to higher risk. Dogs of certain breeds have been reported to be at higher risk for parvovirus enteritis which include American Pit Bull Terrier, Doberman Pinscher, Labrador Retriever and German Shepherd dog (Houston et al. 1996; Smith-Carr et al. 1997).

The antibodies are effective in prevention of the disease and therefore there is a correlation between the serum antibody titre and the immune status of the animal. In the case of the dog, antibodies are transferred to the young ones through the placenta and colostrum. This passive delivery of maternal antibodies plays a vital role in the safety of the puppies and at the same time are also regarded as one of the major reasons of vaccination failures (Waner et al. 1996). The puppies obtain greater part (about 90%) of the total maternally derived antibodies through colostrums as indicated by haemagglutination-inhibition (HI) antibody titres. In case the puppies do not acquire the colostrum, the amount of antibodies obtained through placenta may interfere with the vaccination and infection for quite a few weeks. The quantities of these antibodies depend on the titre of the dam and the litter size. It has been reported that immunity may persist throughout life in dogs that recover from natural infection (Buonavoglia et al. 1992). Other risk factors predisposing the

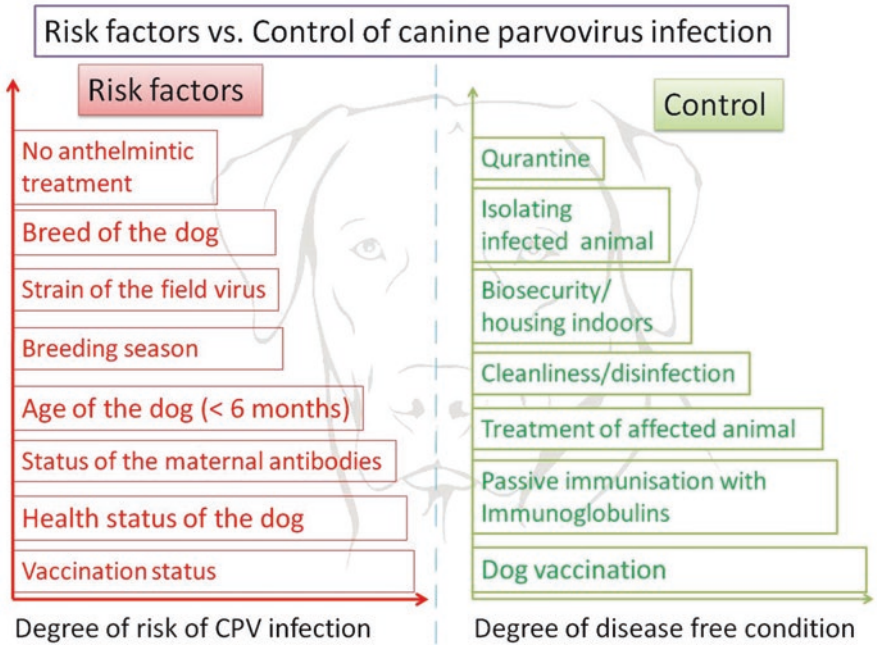

Fig. 12.5 Schematic presentation of the numerous risk factors leading to CPV infection along with preventive and control measures to check the spread of the disease

susceptibility for parvovirus enteritis include immunosuppression, stress, debilitation or malnutrition, simultaneous illness, incubation of disease prior to vaccination or any simultaneous surgery.

As the apparent health status is the most important especially to effectively respond to vaccination, such factors may possibly indeed add to reduced immunocompetency besides any particular factor leading to vaccine failure due to the interplay between the immunity of the animal and the pathogen. In addition the virulence properties of the pathogen and the dose or degree of exposure further affects the clinical development of disease (Kelman 2015). In a study, it was shown that if the last vaccination for puppies was carried out between 10 weeks and 14–16 weeks of age, then the risk of vaccination failure prior to CPV exposure was shown to significantly decline (Altman et al. 2017). In another study, it has been reported that if the primary vaccination is carried out at 4 weeks of age in pups with high maternally derived antibody levels, there may be decrease in the period of window of susceptibility with respect to parvoviral enteritis as an outcome the acquired seroconversion rates (De Cramer et al. 2011). Thus it may be inferred that the primary vaccination at 4 weeks and final booster shot at around 16 weeks of age provide complete protection against CPV infection in the puppies. It has also been reported many a times that showed that animals that had received anthelmintic treatment are relatively less liable to be affected by CPV infection than the non-treated animals (Fig. 12.5).

Sometimes, veterinarians will come across not just isolated cases but outbreaks of disease and further caution is essentially required to protect the patients that are not yet fully immunocompetent in such situations. Therefore it is important for the veterinarians as well as pet owners to be attentive of the risks of parvoviral enteritis and further the need for observation and make certain that every caution is taken to maintain the herd immunity and safety of puppies and dogs at this period, especially during outbreak situations.

12.8 Transmission

The virus is spread in the environment by vomitus, faeces and fomites. The susceptible animals get infected by oronasal exposure to the contaminated material. The incubation period may range from 3 to 14 days and is usually around 5–7 days. After 3–4 days of the animal exposure, the faecal shedding of the virus begins during preclinical phase and may prolong for more than 2 weeks. The virus is also shed in the faeces by subclinically infected animals, thus having a prolonged contagious phase. As the parvoviruses are stable and remain in the environment from months to years, stringent biosecurity measures should be taken to prevent the spread of the virus. Animals should be quarantined for 30 days (Montali et al. 1987) or more to completely immunize the animal by series of vaccinations as per the guidelines. The vaccination will cover the incubation period for parvovirus, in case the animal is earlier exposed. To further lessen the possibility of exposure, other animal species as cats and dogs susceptible to the parvovirus must not be merged in the shelter. It is best practice to check the immune status and test the animal for the presence of any parvovirus infection in the stool and serum for viraemia and shedding of the virus to other susceptible animals. In order to reduce the further transmission of the virus, cleanliness and disinfection should be maintained. Faecal material and soiled fomites as beddings, litter pans and dishes, etc., should be removed and kept in a separate trash bin.

Generally the disinfectants require prolonged contact time to be inactivated (Greene 2006). All the contaminated surfaces and materials in contact with the ill or infected animal should be cleaned and the disinfectant should be allowed to contact for 10–15 min in order to act effectively. As such the common disinfectants are not effective against parvoviruses, such as quaternary ammonium compounds (Kennedy et al. 1995) or alcohol, and therefore the efficient disinfection process requires the use of formaldehyde, glutaraldehyde or chlorine solutions (Scott 1980). The surfaces, tools and cage stuff should be regularly sanitized either with commercial or generic virucidal agents, including 0.175% sodium hypochlorite solution in case extraneous organic matter does not interfere and the exposure of the disinfectant should be more than 10 min (Scott 1980; McGavin 1987). Various other related disinfectants may be used for footbaths. Parvoviruses are inactivated by quick boiling, but the virus could stay alive for more than 7 h at 80 °C and several days at 56 °C (McGavin 1987). Parvoviruses are known to persist for months to years if not directly exposed to sunlight and disinfectant. Other disinfectants effective against

canine parvovirus include potassium peroxymonosulfate (Trifectant, Vetoquinol) and hydrogen peroxide products. The discarded and soiled material should be properly kept and sealed in the biohazard bags which should be sprayed with the disinfectant and appropriately discarded.

Further biosecurity protocols as not frequently taking the animals outdoors and housing them indoors check the spread of the disease. There is increase possibility of transmitting the infection from veterinary hospitals, breeding kennels, dog shelters and dog shows, etc.; thus crowding and transportation to other such places should be steer clear of except in unavoidable circumstances. Also areas such as parks are also common sources of infection for parvovirus; therefore young animals should be avoided for up to a month after booster dose of vaccination so as the animal becomes immunocompetent and does not catch the infection.

12.9 Prevention and Control

Vaccination is the most efficient way to protect the individual animal from the disease, reducing the clinical symptoms and the shedding of the virus during the course of infection (Day et al. 2016). The vaccines presently available against canine parvovirus include either inactivated adjuvanted or modified live vaccines (MLVs; attenuated). The MLVs replicate in the host animal and provide appropriate immune response and usually have less antigenic mass without the necessity of any adjuvants. All the vaccines for an infectious disease as parvovirus may not be similar in each circumstance. Many a times the status of the vaccine strain efficacy is questioned through various studies and reports; thus such information should always be updated. Amongst the potential causes of vaccine failure includes the animal having maternal antibodies reducing the response of the vaccine, or the animal was incubating the disease when vaccine was administered, and the animal was exposed to the field strain of the virus against which the vaccine is unable to protect (Fig. 12.5).

Amongst different types of vaccines available for canine parvovirus, the modified live vaccines (MLVs) are presently being used globally providing extended immunity for around 7 years, thus providing protection against both the disease and infection (Schultz et al. 2010). According to the vaccine guidelines group of the WSAVA (World Small Animal Veterinary Association), the primary vaccination is done generally at 6–8 weeks of age and subsequently every 2–4 weeks until 16 weeks of age or more (Day et al. 2016). For unvaccinated animals above the age of 16 weeks, two vaccinations at 2–4 weeks interval are commonly recommended, although one shot is also protective (Schultz et al. 2010). In the dog shelter or breeding kennels, strict vaccination program should be employed. Vaccinations for canine parvovirus in the form of multivalent disease vaccines could be initiated without delay, by 4 weeks of age, and the booster may be provided at a gap of 2 to 3 weeks until 20 weeks of age.

Presently commercially available CPV vaccines contain either CPV-2 or CPV-2b variants and are reported to protect against all natural CPV variants including CPV-2c (Siedek et al. 2011; Wilson et al. 2013). In spite of being appropriately

immunized, there are many cases of canine parvovirus infection in the young as well as adult animals (Decaro et al. 2008; Mittal et al. 2014).

Control of parvovirus infections is convenient only in confined conditions, because vaccination is the most effective method as to reduce the burden of diseased animals and shedding of the infective virus. The level of the environmental contamination by the infective virus should also be reduced to check the infection of the young animals which may have incomplete or partial immunity during the decrease of the maternal antibody titre. Intense sanitary and stringent isolation procedures are required to maintain a naive confined group of susceptible animals which is usually not possible. The significance of passive immunization could be adjudged in an animal whose immune condition is unidentified or unvaccinated or did not receive maternal antibody titre through colostrum (Greene 2006).

12.10 Treatment

Since parvoviral enteritis is a viral disease, therefore the supportive symptomatic therapy with fluids, antiemetics and antibiotics forms the basis of the treatment. The treatment is essentially required and the disease is almost fatal in case of no treatment. Antiemetics are indispensable to check vomition and a number of efficient antiemetics which include maropitant, metoclopramide and ondansetron or dolasetron are available for use.

As the parvovirus infection causes vomition and diarrhoea leading to dehydration and electrolyte imbalance, therefore intravenous fluid therapy is crucial. Caution should be taken to follow aseptic intravenous catheterization procedures and the intravenous catheters should be replaced after 72 h of use (Lobetti et al. 2002). Lactated Ringer solution is a fluid of choice as it is an isotonic balanced electrolyte solution. The rate of fluid management depends on the state of the animal. Animals having hypoalbuminemia, septic shock or vasculitis, response to isotonic crystalloid fluid administration can be insufficient and thus colloid therapy may be essential. In anaemic patients whole blood (20 mL/kg, within 4 h) may be indispensable. After correcting the hypoperfusion, dehydration is improved over 6 to 24 h. Regular monitoring is required to evaluate the fluid therapy management. Animals affected with parvoviral enteritis may develop hypokalaemia and hypoglycaemia due to continuing anorexia, vomition and diarrhoea, further leading to nausea, profound muscle weakness, gastrointestinal ileus, cardiac arrhythmias and polyuria (Goddard and Leisewitz 2010; Prittie 2004). Therefore potassium chloride may be administered along with the fluids after correcting hypoperfusion. Also as the blood glucose levels are frequently low due to inadequate hepatic function, sepsis and reduced feed intake, thus dextrose supplementation may be necessary. There has been minimal use of plasma infusion in fluid therapy due to complexity transfusion reactions; thus they are not recommended (Brown and Otto 2008).

Immunoglobulins are available against numerous canine infectious diseases including parvovirus as a passive therapy. These include the commercially available Canglob-P which includes purified hyperimmune immunoglobulins in the form of

liquid suspension to provide passive immunization to dogs against CPV infection, for both treatment and prophylactic use. This can be administered by intravenous, intramuscular or subcutaneous route. In cases with severe haemorrhagic diarrhoea, intravenous administration is recommended to provide an immediate onset of the passive immunity and also the deployment of immunoglobulins by this route is maximum. These immunoglobulins can really save the lives of young pups with severe parvoviral enteritis and showing no signs of any recovery right from the first shot when they are administered.

As the affected animals may develop sepsis related with the disruption of the mucosal barrier, pyrexia and neutropenia due to secondary bacterial infection, therefore parenteral therapy of wide-spectrum bactericidal antibiotics is crucial (Otto et al. 1997). Antibiotics ampicillin and cefoxitin alone or along with enrofloxacin are practical options which provide protection against numerous Gram-positive, Gram-negative and anaerobic organisms. Caution should be taken for standard doses and treatment not beyond 5 days during enrofloxacin administration as it is known to cause cartilage damage in young growing animals (Abrams-Ogg 2012; Prittie 2004). In comparatively improved hydration patients aminoglycosides may also be considered (Mylonakis et al. 2016). The antibiotics as other drugs should be parenterally administered as there may be poor absorption of oral preparations as a consequence of vomition and delayed gastric emptying. Further, as dehydration and hypovolemia may result in reduced absorption of subcutaneous drugs intravenous therapy is ideal (Li and Humm 2015).

To further improve upon the status of the affected animal during the course of treatment if the vomition subsides or diarrhoea still persists, the patient may be provided small, frequent meals of a low-fat, easily digestible diet. In the anorexic patient the feed can be administered with the help of a nasoesophageal tube as there is report that nasoesophageal feeding is well tolerated and aids in quick recovery (Mohr et al. 2003). Nasogastric tubes should be put with caution as to avoid aspiration of ingesta and further frequent refractory vomition. Additionally, feeding in such a way is favoured as it facilitates in improving intestinal mucosal integrity and thus reducing any bacterial translocation (Li and Humm 2015).

Although antiviral drugs are not of choice for treating CPV infection, oseltamivir, which is a neuraminidase inhibitor, has been used for the treatment (2 mg/kg, per os, for 5 days) and is shown to improve hematological parameters and body weight of the affected animals. Thus oseltamivir has shown indirect benefits such as reduction in bacterial translocation and lessening endotoxemia and sepsis (Savigny and Macintire 2010). Although another drug acyclovir was reported to have beneficial effects in a study in checking the CPV shedding and acting as therapeutic (Albaz et al. 2015).

For treating CPV infection in dogs, feline interferon (rFeIFN-ω) is licensed in Europe, Japan, Australia and New Zealand. In a study, rFeIFN-ω at 2.5 mU/kg q24h for 3 successive days was found to reduce mortality rates by 4.4-fold, although the mortality rate was as high as 50% in the placebo group of 94 clinical cases (de Mari et al. 2003). In another study, comparable results were shown with the usage of

1.0 mU/kg q24h for 3 successive days which was as efficient as the 2.5 mU/kg dose (Uchino et al. 2008).

Other drugs as gastric protectants, as histamine-2 receptor antagonists, such as ranitidine, cimetidine and famotidine, can be administered in patients having gastritis or esophagitis, although there is an issue of the change in gastric pH caused by the ranitidine (Dimmitt 1991). If vomition subsides, sucralfate may be given and in case of ulceration of upper GI tract omeprazole (or injectable esomeprazole) may be effective (Bersenas et al. 2005). Analgesic treatment due to abdominal pain arising recurrently because of severe enteritis may unfavourably influence the appetite (Kalli et al. 2010). Thus drugs like butorphanol or buprenorphine may be administered appropriately.

12.11 Conclusions and Future Prospects

Canine parvovirus infection leading to severe enteritis is an important cause of morbidity and mortality in young dogs below 6 months of age, even in the presence of efficacious modified live canine parvovirus vaccines. The area of the diagnosis, prevention and control approach for this deadly disease is continuously progressing and further improved strategies will certainly help in combating this sturdy pathogen and the disease. In coming time, the diagnosis and clinical management of CPV-affected dogs may be improved by developing some better investigative means to ascertain the markers of the disease for better outcome of the diseased patients. In addition, future studies may be taken up to throw the light towards cases of vaccination failures in different clinical situations as to whether these are directly related to the vaccine lot with low immunogenicity or the altered strain of the field virus infecting the animals. Furthermore, regular monitoring and surveillance studies are warranted to precisely determine the immune status of dogs in various locations, thus affecting the herd immunity as a whole, and the type of vaccine used and the schedule followed to exactly pinpoint the cause of the disease in any particular situation.

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Part II

RNA Viruses

Infectious Bursal Disease Virus (IBDV)

13

Maged Gomaa Hemida, Abdullah I. A. Al-Mubarak, Adel M. Abdelaziz, and Abdulazim M. Ibrahim

Abstract

Infectious bursal disease virus (IBDV) is one of the most important viral pathogens of chickens. This virus causes great economic losses to the poultry industry due to factors like high mortality rates and poor growth performance of the affected chickens. Despite the intensive application of different vaccines against IBDV, several outbreaks are still emerging in many parts across the globe. This chapter highlights some important basic and clinical information related to IBDV. Further, the pathological changes and the molecular pathogenesis of IBDV infection in chicken have been discussed. In addition, recent advances on the vaccine preparation and prophylaxis against the IBDV infection have been

M. G. Hemida (✉)
Department of Microbiology, College of Veterinary Medicine, King Faisal University, Al-Ahsa, Kingdom of Saudi Arabia

Department of Virology, Faculty of Veterinary Medicine, Kafrelsheikh University, Kafr El-Shaikh, Egypt
e-mail: mhemida@kfu.edu.sa

A. I. A. Al-Mubarak
Department of Microbiology, College of Veterinary Medicine, King Faisal University, Al-Ahsa, Kingdom of Saudi Arabia

A. M. Abdelaziz
Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt

The Veterinary Diagnostic Laboratory, Ministry of Environment, Water, and Agriculture, Riyadh, Saudi Arabia

A. M. Ibrahim
Department of Pathology, College of Veterinary Medicine, King Faisal University, Al-Ahsa, Kingdom of Saudi Arabia

Faculty of Veterinary Medicine, Department of Pathology, Suez Canal University, Ismailia, Egypt

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touched. There is a need for further research to find the most appropriate vaccine and control measures against the IBDV infection in chickens.

Keywords
IBDV · Chicken · Serotype 1 · Serotype 2 · *Birnaviridae* · Pathology · Pathogenesis · Virulence · Control · Recombinant · Subunit vaccine · DNA vaccine

13.1 Prologue

The infectious bursal disease (IBD) caused by infectious bursal disease virus (IBDV) is also known as "Gumboro disease." It is a highly contagious viral disease of chickens with a worldwide prevalence (Ganguly and Rastogi 2018). The clinical disease in birds is referred by various synonyms like infectious bursal disease, infectious bursitis, and infectious avian nephrosis (Castón et al. 2008). The virus targets the immune system, particularly the bursa of Fabricius. It destroys B lymphocytes resulting in immunosuppression, which further facilitates secondary infection and decreases efficacy of other vaccinations (Eterradossi and Saif 2013). The disease is usually associated with high mortality, especially in young chicks less than 3 weeks of age. It was first detected in 1962, in a small town in the Unites States—Gumboro (southern Delaware) (Khan et al. 2017).

13.2 Genome Structure and Organization of IBDV

The IBD virus belongs to the family *Birnaviridae*, genus *Avibirnavirus*. It is non-enveloped, hexagonal, 50–60 nm in diameter, with single-shelled icosahedral virus. The simple structure of the virus confers on its high resistance to environmental conditions, which adversely affects disease control (Van der Berg 2008). The viral genome is composed of two unrelated segments, A and B, encoding five major viral proteins (VP 1–5) (Maclachlan et al. 2017). Segment A, the larger segment, approximately 3.2 Kb, contains two largely overlapping open reading frames (ORF), A1 and A2. Larger ORF, A2, encodes VP2, VP3, and VP4, while A1 encodes VP5. Segment B, the smaller segment, encodes VP1. VP1 is an RNA-dependent polymerase responsible for viral replication and transcription. It is existing as a genome-linked protein (VPg) that circularizes segments A and B by tightly binding their ends (Maclachlan et al. 2017). VP2 and VP3 are structural viral proteins. VP2, an external capsid protein, is responsible for eliciting neutralizing antibodies, binding to cellular receptors, and determining the cellular tropism of the virus. In addition, it represents the molecular basis for antigenic variations. VP2 is folded into three main domains (basal, shell, and projections domains) (Mahgoub et al. 2012). VP3, an internal capsid protein, contains group-specific antigenic determinates and is associated with the genomic RNA. VP4, a

Table 13.1 The IBDV-encoded proteins and their functions

S. no.	Protein	Encoded by	Size (KDa)	Function	References
1	VP1	Segment B	97	RNA-dependent RNA polymerase	Macreadie and Azad (1993))
2	VP2	Segment A	54.4	Induces neutralizing antibodies	Qi et al. (2013)
				Responsible for antigenic variations	
				Virus virulence factor	
3	VP3	Segment A	32	Suppresses innate immune response	Mertens et al. (2015)
				Scaffold protein binds to the viral dsRNA and VP1	
4	VP4	Segment A	28	Viral protease cleaves polyprotein	Li et al. (2013)
				Suppresses innate immune response	
				Suppresses IFN-I expression	
5	VP5	Segment A	17	Apoptosis inducer	Lombardo et al. (2000)

viral protease, cleaves N-pVP2-VP4-VP3-C to VP2, VP3, and VP4 (Khan et al. 2017; Maclachlan et al. 2017). VP5 is a class II membrane, highly basic, cysteine-rich protein that possesses regulatory functions. Resembling other RNA viruses like reoviruses and influenza viruses, the genome terminals, 5′ and 3′, share a high degree of sequence identity between the segments (Ganguly and Rastogi 2018). The functions of the IBDV-encoded proteins are summarized in Table 13.1. IBD viruses have the ability to replicate in both chicken and mammalian cells; however, highly pathogenic strains cannot be easily cultivated. In chicken embryo cells, the multiplication cycle is approximately 10–36 h, whereas in Vero and BGM-70 cells, it takes a longer time, approximately 48 h (Eterradossi and Saif 2013). Two serotypes of IBD virus are described in literatures. Serotype 1 shows variation in pathogenicity, while serotype 2 is not associated with any diseases (Van der Berg 2008). There is no obvious cross-neutralization between the two strains in vitro, neither is there cross-protection in vivo (van den Berg et al. 2000). Regarding the virulence, serotype 1 has three antigenic groups—classic, variant, and very virulent strains. Classic (standard) viruses show worldwide distribution and can cause up to 10–50% mortalities in young chickens. Variant viruses were first discovered in the USA in broiler flocks that were properly vaccinated. They were characterized as antigenic drift of serotype 1. They did not induce any mortality but are reported to be associated with immunosuppression. vvIBDV have started to emerge in Europe in well-managed and vaccinated farms. They are highly virulent and are associated with mortalities ranging from 50% to 100%. They also occur in Asia, Africa, Caribbean islands, and South America and have been reported in California, USA (Van der Berg 2008; Maclachlan et al. 2017).

13.3 Classification of the IBDV

Three main parameters are used to classify the IBDV: pathology, antigenicity, and pathogenicity (Lim et al. 1999). According to pathogenicity, IBDV is classified into two main serotypes. Serotype 1 induces high mortality rates as well as bursal lesions in the affected chickens (Fig. 13.1). It is further classified according to virulence into four main groups: the very virulent, the virulent, the attenuated, and the variant strains (Lim et al. 1999). However, serotype 2 does not produce bursal lesions or cause mortality among the affected chickens (Lim et al. 1999). According to the antigenic properties of serotype 1, it is classified into six subtypes (Li et al. 2006). Meanwhile, the phylogenetic analysis based on the hv-VP2 revealed seven genotypes of this serotype (Michel and Jackwood 2017). In the Arabian Peninsula, both

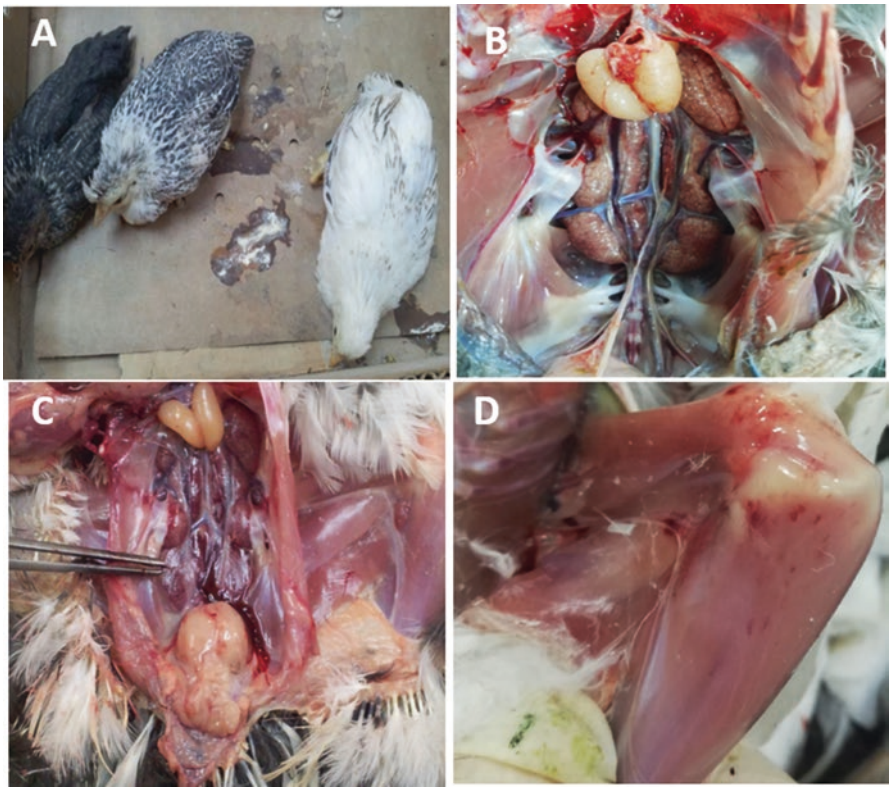

Fig. 13.1 Clinical signs and gross pathological lesions of IBDV-infected native chicken in Saudi Arabia. (**a**) Baladi native chicken infected with IBDV showing depression, ruffled feather, and diarrhea. (**b**) Baladi native chicken infected with IBDV showing large pale lobulated kidneys with distended ureters. Some recently reported dead birds. (**c**) Bursa of Fabricius of IBDV-infected chicken showing enlargement and hemorrhage. (**d**) Thigh muscle of IBDV-infected chicken showing pinpoint hemorrhage. All birds were IBDV positive confirmed by PCR using specific IBDV primers

genotypes 4 and 6 were detected in the UAE and Saudi Arabia, respectively (Lupini et al. 2016). Interestingly, genotype 4 isolated from UAE was quite similar to that isolated from chicken from Italy (Lupini et al. 2016).

13.4 Pathogenesis of IBDV

Four to five hours post-infection, the IBDV replicates within the macrophages and lymphocytes of the gut-associated lymphoid tissue in the duodenum, jejunum, and cecum. The virus travels through the portal circulation to the liver and then invades the bloodstream, as primary viremia, reaching BF within 11 h PI. In turn, BF releases a second wave of large numbers of the virus into the blood as secondary viremia following which the virus infects other organs such as the thymus and spleen (Eterradossi and Saif 2013; Maclachlan et al. 2017; Muller et al. 1979). Within the BF, the virus targets IgM-bearing B lymphocytes or non-immunoglobulin-bearing B lymphoblast, whereas stem cells and peripheral B cells are not affected. It has been shown that bursectomized chickens can survive the lethal infection without developing clinical signs (Sharma et al. 2000). The cortical and medullary regions within the BF suffer from extensive lymphoid destruction mediated through virus-inducing apoptosis, resulting in a dramatic drop in the humoral immunity of the affected chicken (Tanimura and Sharma 1998). Both CD4+ and CD8+ T cells, as well as the macrophages and the NK cells, infiltrate within the area of infection as early as day 1 PI. They may reach up to 65% of the cellular population of the BF (Withers et al. 2005). Activated T cells express upregulation of cytokine genes such as interferons (INF-γ), interleukin (IL-1β, IL6, IL12α, IL-12β, IL18), and TNF. These proinflammatory cytokines induce shock in birds, which become prostrated and reluctant to move. These cytokines may also promote cellular destruction. For example, INF-γ activates macrophages to produce nitric oxide (Mahgoub et al. 2012). Overall, IBDV infection induces immunosuppression that results in increased susceptibility to secondary infection and poor feed conversion and also decreases the efficacy of other vaccines.

13.5 Susceptibility

Chicken is the only known species known to develop the acute disease of IBD following infection with serotype 1 virus (Ingrao et al. 2013). The severity of the disease depends on various factors including age and breed of birds, virulence of the strain, and passive immunity of the infected bird (Van der Berg 2008). Three to six weeks is the typical age which correlates to the stage of maximum development of BF (Qin and Zheng 2017). Regarding the breeds, it has been shown that light breeds show high moralities compared to the heavy ones. Furthermore, layers are more susceptible than broilers (Bumstead et al. 1993; Tippenhauer et al. 2013). The presence of maternally derived antibodies (MDA) helps young chickens to overcome the infection until titers get lowered (Van der Berg 2008). Infection with serotype 2 does not produce any

clinical signs and gross or histologic alterations in chickens (Ismail et al. 1988). Neither serotype 1 nor serotype 2 can induce a clinical disease in turkeys (Jackwood et al. 1985). Other avian species including quail, pheasant, pigeon, duck, and guinea fowl do not develop IBD following natural or artificial infection (Mahgoub et al. 2012).

13.6 Transmission and Spreading

Infected chicken starts to shed the virus in their feces as early as 2 days PI, even before the first clinical sign, and shedding may last until day 21. Viral shedding during exhalation is not detected (Zhao et al. 2013). Birds are usually infected through the oral route with a possibility for aerosol infection (Jayasundara et al. 2017). Transmission occurs either by direct or indirect contact. As mentioned before, the virus can resist extreme environmental conditions and may remain viable for months in infected materials (bedding, premises, and staff clothes) or within vectors (mealworm and insects) (Benton et al. 1967; Howie and Thorsen 1981). Neither vertical transition nor true carrier state of recovering birds has been documented. The most likely mode of virus spread is the commercial trading of living birds or poultry meat. The diseases is classified as OIE list B disease (van den Berg et al. 2000).

13.7 Clinical Signs

Depending on the virulence of the strain, age and breed of the chicken, and the level of MDA, the severity of the clinical signs varies among flocks, localities, and countries. The disease occurs in two forms, acute and subclinical (Van der Berg 2008). The acute form usually starts suddenly following a short incubation period of 2–3 days and lasts about 7 days. The clinical manifestations include anorexia, depression, prostration, ruffled feathers, white watery diarrhea, and sudden death. Morbidity may reach up to 100%, whereas mortality ranges from 20% to 30%. Higher mortalities approaching 90–100% are recorded in certain areas of the world like Europe, when associated with very virulent strains (Chettle et al. 1989; Ivanyi and Morris 1976). Birds of age less than 3 weeks or infected with low virulent and variant stains usually show subclinical form of the disease. Subclinical disease is a mild form, characterized by immunosuppression and has been mainly reported from the United States. It is usually not associated with any apparent signs except for retardation of growth, secondary infection with opportunistic microbes, and ineffective vaccination programs (Jackwood et al. 2006; Kegne and Chanie 2014; Snyder 1990).

13.8 Gross Pathology

As mentioned earlier, BF is the primary target organ for the IBDV and shows typical gross pathological features. On day 3 PI, BF shows edematous changes and hemorrhage, resulting in increasing size and weight. This enlargement continues until it

reaches double size and weight in comparison to birds with similar age. The bursa returns to its normal size on day 5 PI, then begins to atrophy gradually until it reaches approximately one third of its original size (Eterradossi and Saif 2013). Gelatinous yellowish material may be seen covering the external surface of the bursa on the second and third day of infection. The bursal mucosa reveals multifocal areas of necrosis and hemorrhages (Mwenda et al. 2018).

Extra-bursal lesions include petechial and ecchymotic hemorrhages in the pectoral and thigh muscles and hepatosplenomegaly associated with grayish necrotic foci on their surfaces. Moreover, dehydrated birds have swollen kidneys with accumulation of urates, owing to obstruction of the ureters by the enlarged bursa. The intestinal lumen may contain large amount of mucous. Infrequently, the mucosa at the junction between gizzard and proventriculus show hemorrhages (Kegne and Chanie 2014).

13.9 Histopathology

The most prominent histopathologic lesion induced by IBDV is lymphocytolysis (degeneration and necrosis) of B lymphocytes in all lymphoid organs (BF, spleen, cecal tonsils, thymus, and bone marrow). As 98% of the lymphocyte population within BF is lymphocytes, the most striking lesions are expected to be observed in the bursa. Moreover, lesions in other lymphoid organs recover rapidly without sustained damage (Kegne and Chanie 2014; Abdul-Aziz and Fletcher 2016).

Necrosis of lymphocytes occurs as early as day 1 of infection, resulting in cellular and karyorrhectic debris, followed by an inflammatory response that is characterized by hyperemia, edema, heterophil infiltrations, and reticuloendothelial cell hyperplasia. On the 4th to 5th day of infection, the inflammation starts to diminish as evidenced by removal of debris by phagocytosis, necrosis of heterophils and plasma cells, as well as appearance of cystic cavities in the medullary areas of lymphoid follicles. As the disease advances, fibroplasia takes place in the interfollicular septa with atrophy of lymphoid follicles. Furthermore, the pseudostratified epithelium covering the mucosal folds becomes hyperplastic forming glandular structures with production of mucin globules (Eterradossi and Saif 2013; Yamazaki et al. 2017). Variant stains also provoke necrosis of lymphocytes but with a milder degree than classic strain and without obvious inflammatory reaction. On the other hand, virulent stains induce severe lymphocytolysis with hemorrhages in the bone marrow and thymus. Moreover, the virus has the ability to initiate apoptosis and reduce the number of plasma cells in Harderian glands in comparison to noninfected chickens (Abdul-Aziz and Fletcher 2016; Zakeri and Kashefi 2011).

13.10 Diagnosis

IBDV infection in chicken is one of the viruses that induce unique clinical signs and postmortem lesions. These phenomena make the clinical diagnosis of IBDV relatively reliable compared to other pathogens. The curve of the viral infection is

highly suggestive, characterized by a high peak of mortality among the affected chicken followed by rapid recovery in a week (Nunoya et al. 1992). The IBDV can be isolated on both the embryonated chicken eggs (ECE) as well as the various types of cell cultures. The 6–8 ECE is used to isolate the IBDV through yolk sac inoculation. Inoculation of IBDV specimens in the ECE induces several pathological changes on the embryos. Dwarfing, hemorrhage, and liver necrosis of the inoculated ECE have been reported in the case of IBDV serotype 1 infection (Mutinda et al. 2015). It is well known that IBDV serotype 1 causes death of some embryos during the first round of inoculation; however, serotype 2 does not produce any death among the inoculated ECE. However, the inoculated embryos tend to show dwarfing and pale yellow discoloration (Mutinda et al. 2015). The chicken embryo fibroblast from specific-pathogen-free (SPF) eggs may be used to isolate the IBDV from suspected specimens. The virus induces cytopathic effects on the inoculated cell culture in the form of small rounded cells (Mekuriaw et al. 2017). Using a combination of primers to detect both serotype 1 and 2 enhances the sensitivity of detection of the IBDV in clinical specimens (Le Nouen et al. 2006). The IBDV is classified according to pathogenicity into three types—variants, classical, and very virulent (Jackwood et al. 2018). The variant strains do not produce obvious clinical lesions in the ECE. However, they produce marked bursal lesions. The classical strains usually induce about 15–50% mortality as well as typical clinical signs. The virulent strains produce a wide range of mortality from 50% to 100% among the inoculated chickens (Jackwood et al. 2018). Histological diagnosis is also highly suggestive, especially the gross and microscopic lesion in the bursa of Fabricius (Fadly et al. 1976). However, the gold standard for IBDV diagnosis is the isolation and molecular detection of the viral nucleic acid by various techniques especially polymerase chain reaction (PCR). Sequencing of the viral nucleic acid is one of the new tools that can precisely identify the circulating strains, types, genotypes, as well as topotypes of the IBDV in certain regions (Banda et al. 2004). The combination of the restriction enzyme (RE) digestion and the amplified PCR products of the IBDV amplicons is one of the new trends in the classification of IBDV strains (Jackwood and Jackwood 1997). Although the RT-PCR/RE provide a good tool for the classification of IBDV strains, it may result in some confusing patterns, which are confusing in the classification of some IBDVs. Sequencing of certain regions across the IBDV VP2 gene is one of the most recent efficient methods in the classification of IBDV especially amino acids (279 N–284 T or 253 H–284 T) (Mundt 1999). Some serological techniques are used in the process of diagnosis of IBDV infection in birds such as the agar gel immunodiffusion test (AGID), the virus neutralization test (VN), and the enzyme-linked immunosorbent assay (ELISA) (De Herdt et al. 2005).

13.11 Preventive and Control Measures for the IBDV

Despite its emergence more than 50 years ago, the IBDV continues to pose great challenges to the poultry industry worldwide. It was believed that maternal antibodies could protect the newly hatched chickens against IBDV infection. This was an

ongoing assumption until the 1980s (Brown et al. 1994). However, the half-life time of the maternal antibodies in white leghorn chickens was about 3–8 days as previously described (Domanska et al. 2004). Interestingly, chickens that have medium level of maternal antibodies were found to be protected for at least 2 weeks, while chickens that had high level of maternal antibodies were found to be resistant to some field infections up to 4 weeks of age (Al-Natour et al. 2004). In case of outbreaks in certain farms or group of farms, strict biosecurity measures should be adopted to stop the virus from spreading to the neighboring farms (Rimi et al. 2017). The virus is extremely resistant to many physical and chemical agents. It can tolerate a wide range of pH ranging from 2 to 12 as well as some common disinfectants (Petek et al. 1973).

13.12 IBDV Vaccines

There are many types of IBDV vaccines—live attenuated, inactivated or killed, and the genetically engineered vaccines.

13.12.1 The Live Attenuated IBDV Vaccines

The live attenuated IBDV vaccines mimic the natural viral infection in birds and produce both humeral and cellular immunity. This type of vaccine replicates in the target organs, especially the bursa of Fabricius, and produces long-term immunity compared to other types of vaccines. However, this type of vaccine has many drawbacks including the risk of reversion to virulence and induction of typical IBDV infection (Thornton 1976). Most live attenuated vaccines are prepared from several strains of serotype 1. There are four types of these vaccines including the hot, intermediate plus, intermediate, and mild vaccines. Although the vaccine prepared from the hot strains produces potent immune response in chickens compared to the other mild and intermediate plus strains-based vaccine, it may also induce bursal lesions in some of the vaccinated chickens. This may also lead to immunosuppression in the vaccinated chickens (Mazariegos et al. 1990; Thangavelu et al. 1998). There are many other drawbacks and challenges for this type of vaccine such as the possibility of reassortment to produce new virulent virus. The neutralization of the maternal immunity is among the major concerns of this type of IBDV vaccines (Block et al. 2007). The intermediate plus and the intermediate IBDV vaccines produce detectable antibodies in sera of vaccinated chickens 14 and 28 days post-vaccination, respectively (Roh et al. 2016).

13.12.2 The Inactivated IBDV Vaccines

This type of IBDV vaccine is prepared from the whole inactivated virus. It can be administered only through the injection route. It should be injected in combination with other chemical substances called adjuvants. The optimum conditions of using

this type of vaccine are through "the prime post regime" in which the birds receive the attenuated vaccine which is then boosted with the inactivated vaccine (Roh et al. 2016). The inactivated vaccine is able to trigger the T-cell immune response against the IBDV (Rautenschlein et al. 2002). Priming layer chickens at 14–16 weeks of age with the live attenuated vaccines and then boosting them with the oil-inactivated vaccines at 22 weeks of age was successful in triggering high level of maternal antibodies in the newly hatched chickens against the IBDV (Eidson et al. 1980).

13.12.3 The Genetic Engineering-Based IBDV Vaccines

The technology of reverse genetics opened new windows and avenues for the development of novel recombinant vaccines for the IBDV. There are many approaches for the development of these new types of vaccines. These includes the development of mutant IBDV vaccines, the subunit vaccine, the DNA vaccine, and the vector-based vaccine. We will discuss briefly these types of vaccines in the following section.

13.13 The Attenuated Mutant IBDV

Site-directed mutagenesis is used to induce some active mutations in the IBDV VP2 genes of the very virulent strains of the virus. This resulted in an attenuated viral strain which induced good protective immunity against the IBDV challenge as well as field infection (Raue et al. 2004). The major concern about the safety of this vaccine is the reversion of virulence at some times after use. This triggers a great risk of using this kind of vaccines (Raue et al. 2004). There are two main candidates of this type of vaccines including the "intrasegmental chimeric vaccines" and the "interserotypic reassortant IBD vaccines." In the case of the intrasegmental vaccines, the coding sequence of the virulent strains from serotype 1 is replaced with that of the serotype 2 non-virulent strains. While in the case of the interserotypic vaccines, there is a reassortment induced between the virulent serotype 1 candidates and the non-virulent serotype 2 candidates. This resulted in the generation of a mild virus that can be used as vaccine against the IBDV (Schroder et al. 2000, 2001). Although these types of vaccines were studied experimentally, none of them is commercially available yet.

13.13.1 The IBDV Subunit Vaccines

The IBDV VP2 is the major player in the protective immunity against the virus. This protein triggers the production of antibodies against the virus. Several approaches are adopted to express this protein by various methods of expression and to use it as a potential vaccine; however, none of these approaches worked out (Bayliss et al. 1991; Oppling et al. 1991). Using the baculovirus expression system to express the empty IBDV capsid was successful in the production of specific IBDV antibodies

that protect the birds against the challenge by the virulent IBDV strains (Li et al. 2006). Generation of recombinant vaccine carrying the IBDV VP2 and the interleukin-2 is a promising vaccine candidate against IBDV (Li et al. 2006). Currently, there are three recombinant IBDV VP2 vaccines available in the market using the *E. coli*, baculovirus, and yeast expression systems (Pitcovski et al. 2003). The recombinant IBDV VP2-based protein possesses a great tool for the IBDV infections. It has the ability to distinguish between the field infected and the vaccinated birds (DIVA concept) (Muller et al. 2012).

13.13.2 The IBDV DNA Vaccines

The approach of introducing the naked DNA has many privileges in terms of the immune response. This allows the candidate vaccines to escape the effect of the neutralizing antibodies triggered by the antigenic epitopes in the regular vaccines. Several DNA approaches were tried for the IBDV. The cDNAs complementary to the IBDV polyprotein gave much more superior immune response than using the cDNA VP2 alone (Fodor et al. 1999). Remarkably, the approach of using the DNA vaccines even in ovo and in the day-old chickens as prime regime followed by inactivated vaccines in the newly hatched chickens is a promising trend in the IBDV vaccination (Oshop et al. 2003).

13.13.3 The IBDV Immune Complex Vaccines

This is a new trend in the field of IBDV vaccines. Simple collection of the hyperimmune sera from the IBDV naturally infected or vaccinated chickens and use of this immune complex in the vaccination of other birds. This approach was successful in the case of in ovo vaccination at day 18 of the embryonated egg incubation time as well as for the day-old chickens (Withers et al. 2005). This immune complex showed promising trend in protecting the chicken by vaccinating the birds by subcutaneous route at day old in the hatcheries (Ivan et al. 2005). The application of this immune complex vaccine gave more superior immune privilege to the vaccinated chickens in terms of protection against natural IBDV infection when compared to the live attenuated IBDV vaccines (Ivan et al. 2005). More recently, recombinant IBDV-neutralizing antibodies were used in the vaccination of birds against IBDV and showed very promising results (Ignjatovic et al. 2006).

13.13.4 The IBDV Live Viral Vector Vaccines

The strategy of vector-based vaccine is to insert certain genes or group of genes in another host then introduce to the body of the other host to produce immune response against both. There are many viral backbones used to express the VP2 gene of the IBDV such as the fowl poxvirus, the Newcastle diseases virus, the herpesvirus of

turkey, the adenovirus, and the bacteriophage T4 (Jackwood et al. 1985; Domanska et al. 2004; Thornton 1976). The herpesvirus of turkey (HVT) was used to express the VP2 gene of the IBDV to give immunity against both viruses. This vaccine is used in ovo and for the day-old chicken vaccination as well (Bublot et al. 2007). This type of vaccine has been developed and applied in many countries and has showed a promising trend in the protection of chickens against IBDV (Le Gros et al. 2009).

13.14 Conclusions and Future Prospective

IBDV infection continues to risk the poultry industry in many parts of the world despite the massive use of vaccines. There is a continuous emergence of new IBDV strains, which hampers the success of most currently used vaccines. Continuous monitoring of the emerging new IBDV genotypes is highly recommended. This is likely to have great benefits including the preparation of the homologue vaccine representing the actual circulating genotype and topotype in a certain area. This may also help in upgrading the laboratory techniques and diagnostic assays to match any changes in the genetic makeup of the IBDV strains. Development of a molecular-based monitoring system to follow up the immune status of the chicken population for the IBDV.

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Bovine Viral Diarrhea Virus

14

Niranjan Mishra and S. Kalaiyarasu

Abstract

Bovine viral diarrhea (BVD) is prevalent worldwide and causes high economic losses in cattle due to a variety of disease syndromes. BVD is caused by three bovine pestiviruses, bovine viral diarrhea virus 1 (BVDV-1), BVDV-2 and HoBi-like pestivirus (HoBiPeV) with considerable genetic and antigenic heterogeneity. Bovine pestiviruses belong to the *Pestivirus* genus within the *Flaviviridae* family that also comprises the genera *Flavivirus*, *Hepacivirus* and *Pegivirus*. As per the latest (10th) ICTV report, pestiviruses have been classified into 11 approved species, including bovine pestiviruses, which have been classified into species, *Pestivirus A*, *Pestivirus B* and *Pestivirus H*. The term BVDV in this chapter commonly refers to all the three bovine pestiviruses. The pathogenesis of BVDV infection is complex, with infection pre- and post-gestation leading to different outcomes. BVDVs are highly successful to persist and spread in their host populations due to their unique ability to produce persistent infection through evasion of adaptive immune response and innate immune response. Recent advances in diagnostic methods, nucleotide sequencing, and computer-assisted phylogenetic analyses have so far identified 21 BVDV-1 subtypes (BVDV-1a to BVDV-1u), 4 BVDV-2 subtypes (BVDV-2a to BVDV-2d) and 4 HoBiPeV subtypes (HoBiPeV-a to HoBiPeV-d). Providing acquired immune protection against BVDV is challenging due to the antigenic diversity among BVDV strains and ability of BVDV to infect the fetus. Both killed and live attenuated vaccines have been reported to be effective in the field, and recent advancements in molecular studies have helped toward future development of new-generation vaccines against BVD. However, over the years, vaccination alone has not resulted in the elimination of BVDV-related clinical disease or a significant reduction in BVDV losses. All successful BVDV control programs are based on identification and removal

N. Mishra (✉) · S. Kalaiyarasu
Indian Council of Agricultural Research-National Institute of High Security Animal Diseases, Bhopal, Madhya Pradesh, India

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of PI animals, movement controls, strict biosecurity and surveillance. To date, BVDV control programs without vaccination have been implemented successfully in Scandinavian countries, Austria and Switzerland, while control with vaccination has been used in Germany, Belgium, Ireland and Scotland. This chapter will focus on advances in research involving all aspects of BVDV with special emphasis on molecular biology, genetic and antigenic diversity, diagnosis, prevention and control besides discussion on future perspectives.

Keywords
Pestivirus · Bovine viral diarrhea virus · Persistent infection · BVDV-1 · BVDV-2 · HoBiPeV · Epidemiology · Diagnosis · Control · Cattle

14.1 Prologue

Bovine viral diarrhea (BVD) is one of the major economically important viral diseases of cattle and is prevalent in cattle populations worldwide. From its discovery in 1946 to the present date, without doubt BVD is one of the most complex infectious diseases encountered in veterinary medicine with regard to its pathogenesis, diagnosis, management and control. Bovine viral diarrhea virus (BVDV) causes BVD and mucosal disease (MD) and is highly complex, with respect to the heterogeneity in genetic and antigenic properties, host spectrum, host-virus interaction, virulence and immune response. Although its impact on cattle health and production remained underestimated for long time, the economic impact has recently been appreciated with control programs being implemented in several countries in Europe with an ultimate goal of BVD eradication.

Bovine viral diarrhea (BVD) was first reported in the USA in 1946 (Olafson et al. 1946) in association with epizootics of acute disease characterized by fever, leukopenia, reduced milk yield, high rates of abortion, diarrhea and erosive lesions of the digestive tract of cattle. The symptoms were similar to rinderpest (RP) with morbidity of 33–88% and mortality of 4–8%. In the 1950s, a special form of viral diarrhea called mucosal disease (MD), with hemorrhages and intestinal erosions, was reported in Iowa, USA (Ramsey and Chivers 1953). However, the relationship between the two illnesses could be established after many years, when it was clear that both BVD and MD were caused by BVDV.

Two biotypes of BVDV exist based on their effects on cell cultures, the noncytopathic (ncp) and cytopathic (cp). First isolation of ncp BVDV from BVD clinical cases was reported by Lee and Gillespie (1957), while cp BVDV was isolated first by Underdahl et al. (1957). Although BVD could be reproduced early experimentally, it took many years until the 1980s when MD could be experimentally reproduced, hypothesis of immune tolerance was proved, and mechanisms of persistent infection (PI) and pathogenesis of MD were established (Malmquist 1968; McClurkin et al. 1984; Brownlie et al. 1984). Only ncp BVDVs establish persistent infections, while cp BVDV is generated in PI animals by mutations in ncp BVDV and causes MD.

In 1987, BVD associated with thrombocytopenia, hemorrhage and high mortality was reported in US dairy herds (Perdrizet et al. 1987) followed by other reports from the USA and Canada (Bolin and Ridpath 1992; Pellerin et al. 1994). The causative agent involved in these outbreaks was identified as BVDV-2 and the classical BVDV strains identified earlier were termed as BVDV-1 (Ridpath et al. 1994). First identified in fetal bovine serum originating from Brazil in 2004, HoBi-like pestivirus has recently been recognized as a bovine pathogen in Europe, South America and Asia that causes clinical symptoms akin to the classical BVDV-1 or BVDV-2 infections (Schirrmeier et al. 2004; Liu et al. 2009; Bauermann et al. 2013; Mishra et al. 2014).

Animal health economics highlight the importance of the disease and play a major role in decision-making process regarding selection of control strategies. Although prevalence rates vary, BVDV is prevalent in most of the cattle populations. BVDV infection causes significant economic losses in cattle production and the economic impact varies within and between countries (Richter et al. 2017). Direct losses due to BVDV occur on account of increased morbidity and mortality in adult cattle due to acute infection, reduced milk yield, respiratory disorders, extended calving interval, reproductive disorders such as repeat breeding and abortions, congenital defects, increased neonatal mortality, non-thriving and death among young stock besides the costs for treatment and prevention (Houe 2003). BVDV-induced indirect losses arise due to implementation of control programs and trade restrictions.

BVDV can have devastating effects on the economy of individual dairy farmers which has been seen in the form of severe acute BVD outbreaks in many countries in North America and Europe, specifically in the USA, Canada and Germany. Significant differences exist in the virulence of BVDV strains, and both BVDV-1 and BVDV-2 have high or low virulent strains, and hence wide differences in economic impact due to BVD have been reported in various countries (Houe 1999). A recent study on direct financial losses due to BVDV infection in 15 countries around the world over the past 30 years has shown that direct financial losses due to BVDV were in the range of 0.50–687.80 USD per animal and the average direct losses were higher per dairy cow than per beef cow (Richter et al. 2017). Another study analyzed the data of 31 published studies undertaken around the world during 1991–2015 and has shown that the economic impact of BVD ranges from 0 to 552 GBP per cow per year and is based on outcome of the disease (Yarnall and Thrusfield 2017). Despite variation in calculation methods, calculation of economic losses has been a significant motivator for considering implementation of BVD control programs and mitigation activities in many countries in Europe.

The aim of this chapter is not only to summarize the available facts on BVDV but also to critically analyze the data and highlight recent advances on BVDV taxonomy, molecular biology, epidemiology, diagnosis and control.

14.2 Taxonomy

Bovine pestiviruses belong to the *Pestivirus* genus in the family *Flaviviridae* that also comprises the genera *Flavivirus*, *Hepacivirus* and *Pegivirus*. As per the ninth report of the International Committee on Taxonomy of Viruses (ICTV), the *Pestivirus* genus was comprised of four approved species, *Bovine viral diarrhea virus 1* (BVDV-1), *Bovine viral diarrhea virus 2* (BVDV-2), *classical swine fever virus* (CSFV), and *border disease virus* (BDV), and four tentative species, namely, Giraffe-1 pestivirus; Pronghorn antelope pestivirus; atypical bovine pestivirus, also termed as BVDV-3 or HoBi-like pestivirus; and Bungowannah virus (Simmonds et al. 2012). While several other pestiviruses have been proposed as additional species including the atypical porcine pestivirus (APPV), Linda virus, bat (*Rhinolophus affinis*) pestivirus and Norway rat pestivirus, there was no change in the taxonomy of *Pestivirus* genus by ICTV during 1999–2017.

Pestiviruses have several characteristics that differentiate them from other members of the *Flaviviridae* family. The two proteins unique to the *Pestivirus* genus are the E^{rns} envelope glycoprotein, which has RNase activity, and the nonstructural protease N^{pro}, which releases itself autocatalytically from the polyprotein. On the basis of genetic and antigenic characteristics, the four existing *Pestivirus* species have been demarcated using a range of criteria including complete coding nucleotide sequences that differ by more than 25%, displaying >10-fold differences in cross-neutralization titers, and may have differing or overlapping host range (Becher et al. 2003). Based on genetic analysis, several genotypes or subtypes within *Pestivirus* species have been proposed, but these subdivisions have not yet been officially approved.

The taxonomy of pestiviruses was problematic for long, since the earlier *Pestivirus* species names had been derived from names of virus isolates, which were based on host range and disease attributes. Hence, a new uniform naming system, analogous to that used for species belonging to *Pegivirus* and *Hepacivirus* genera of *Flaviviridae*, has been approved by ICTV recently for *Pestivirus* species with the format *Pestivirus X*, where *X* represents a different capital letter for each species without change in virus isolate names (Smith et al. 2017). The four existing species have been designated as *Pestivirus A*, which comprises of *Bovine viral diarrhea virus 1* (BVDV-1); *Pestivirus B*, which comprises of *Bovine viral diarrhea virus 2* (BVDV-2); *Pestivirus C*, which comprises of *Classical swine fever virus (CSFV)*; and *Pestivirus D*, comprising of *Border disease virus (BDV)* along with seven additional species. Atypical bovine pestivirus or Hobi-like pestivirus (HoBiPeV) or BVDV-3 belongs to the species *Pestivirus H*.

14.3 BVDV Structure

The BVDV virions are enveloped, spherical particles which are 40–60 nm diameter in size. The BVD virus particle is composed of a core region, consisting of the genomic RNA coated with structural capsid or core protein which is surrounded by a lipid envelope. The capsid is about 30 nm diameter and appears as an

electron-dense inner core (Horzinek et al. 1971). Cryo-electron microscopy of purified BVDV virions has recently shown that viral particles display an electron-dense capsid surrounded by a phospholipid bilayer with no visible spikes and most BVDV particles are 50 nm diameter in size and about 2% are 65 nm in size, suggesting some size flexibility during BVDV morphogenesis (Callens et al. 2016). However, whether BVDV capsid is icosahedral or not remains to be determined in future.

The lipid bilayer envelope is made up of three virus-encoded glycoproteins, E^{rns}, E1, and E2. The heavily glycosylated E^{rns} glycoprotein is loosely associated with the virus particle and is secreted in soluble form by infected cells, while E1 and E2 glycoproteins are integral membrane proteins and E1-E2 heterodimer is essential for BVDV entry. BVDV particles have a higher concentration of E^{rns} than E1 and E2. Due to the pleomorphic nature of envelope and association of BVDV particles with host cells, it is extremely difficult to achieve highly purified infectious particles by ultracentrifugation and identification by electron microscopy. The buoyant density of virion in sucrose is 1.134 gm/ml and molecular weight of the virion is estimated as 6.0×10^7 (Lindenbach et al. 2013). Similar to other enveloped viruses, BVDV is inactivated by organic solvents and detergents, but it is resistant to low pH unlike flaviviruses.

14.4 Genomic Organization

Similar to other pestiviruses, BVDV genome consists of a positive-sense single-stranded RNA of about 12.3 kb size. However, the size may vary up to 16.5 kb due to insertions, genomic duplications, and genomic recombination events (Becher et al. 1999). No subgenomic RNA is transcribed during BVDV replication and the plus strand genomic RNA only represents the viral mRNA and codes all viral proteins.

The genomic organization of BVDV is similar to all the recognized pestiviruses (Fig.14.1). The RNA genome codes for a polyprotein of about 3900 amino acids in 1 large open reading frame that is flanked by 5′- and 3′-untranslated regions (UTR) of about 400 and 200 nucleotides, respectively. The translated polyprotein is then processed by viral and cellular proteases resulting in 12–13 proteins, N^{pro}; C (capsid); the envelope proteins E^{rns}, E1, and E2; and nonstructural proteins p7, NS2, NS3, NS2–3, NS4A, NS4B, NS5A and NS5B (Lindenbach et al. 2013). The arrangement of proteins in the polyprotein is NH2–N^{pro}/C/E^{rns}/E1/E2/p7/NS2/NS3/NS4A/NS4B/NS5A/NS5B–COOH.

The 5′-UTR of BVDV has a stable stem loop structure (Ia hairpin), which is involved in both translation initiation and replication of the viral RNA (Grassmann et al. 2005). BVDV RNAs do not possess a 5′-cap and is not polyadenylated at 3′-end. The internal ribosomal entry site (IRES), located toward the end of 5′-UTR and first part of N^{pro}, directly recruits the small ribosomal subunit, positions it at the translational start site, and promotes start of translation even in the presence of a non-AUG codon (Poole et al. 1995; Pestova et al. 2008). The 3′-UTR has a conserved stem-loop structure, and the single-stranded domain at the 3′-end of the genomic RNA is necessary for efficient RNA replication. Significant variation

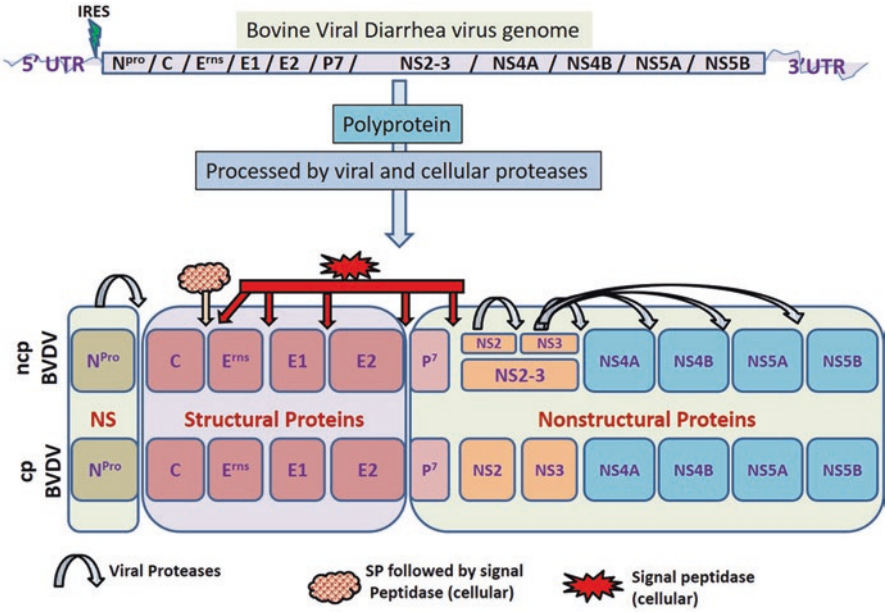

Fig. 14.1 Genome organization of BVDV and encoded proteins. The schematic representation of the genomic RNA (upper part) and the encoded proteins (below) is shown

exists in the length of 3′-UTR sequence among pestiviruses including BVDV. Although BVDV 3′-UTR is less conserved than 5′-UTR, it has both conserved and variable parts. BVDV-1 isolates have an eight-nucleotide repeat sequence (TGTATATA) in the variable part of 3′-UTR, while BVDV-2 isolates contain TGTAAATA repeat sequence.

Polyprotein processing occurs co-translationally and begins with the N-terminal autoprotease (N^{pro}) release. The N^{pro}, found only in pestiviruses, is responsible for cleavage at its own carboxy-terminus, thereby releasing amino-terminus of the C protein (Stark et al. 1993). The C/E^{rns} cleavage occurs by cellular signal peptidase (SPase) and signal peptide peptidase (SPPase), while processing at the E^{rns}/E1, E1/E2, E2/p7 and p7/NS2 borders is carried out by SPase (Rumenapf et al. 1993). The processing of NS2 and NS3 occurs by protease located within NS2, whereas all processing downstream of NS3 is done by NS3 protease. NS4A acts as a cofactor of the NS3 protease and is essential for NS4B/NS5A and the NS5A/NS5B site processing.

BVDV strains exist as two biotypes, non-cytopathic (ncp) and cytopathic (cp). Cytopathic viruses result following changes in the NS2/3 protein coding region leading to generation of NS2 and NS3 proteins. These genomic changes include point mutations, genomic duplications, and insertion of cellular mRNA sequences (Meyers and Thiel 1996; Kummerer and Meyers 2000). The genomic changes in the N^{pro}, capsid and NS4B have also been reported in some cp strains of BVDV.

14.5 Viral Structural and Nonstructural Proteins

With the exception of N^{pro}, the first coding region of the ORF encodes the structural proteins which are integral components of the viral particle. These include the capsid or core protein and three glycoproteins, E^{rns}, E1 and E2. BVDV capsid protein C is highly basic and binds RNA with low affinity. The E^{rns} glycoprotein, present on the virus particle and in virus-free supernatant or in the blood of infected cells, exhibits several unusual characteristics including its exceptional membrane anchor and RNase activity (Lindenbach et al. 2013). E^{rns} is highly glycosylated and commonly exists as disulfide-linked homodimers (Hulst and Moormann 2001). RNase activity of E^{rns} is responsible for both single-stranded and double-stranded RNA degradation, which is considered important in limiting the host innate immune response. However, the enzyme becomes active only in endoplasmic reticulum environment and when it gets separated from the viral genome by the lipid envelope.

The E1 and E2 glycoproteins are integral membrane proteins and interact to form disulfide-linked E1-E2 heterodimers which are responsible for BVDV entry and infectivity (Ronecker et al. 2008). The structure and function of E1 is not yet known and antibodies against E1 are not found in infected animals. However, it has recently been proposed that E1 contains the fusion peptide necessary for the fusion during entry. The 53 kDa BVDV E2 glycoprotein is responsible for virus attachment, generation of neutralizing antibodies, and host tropism (Weiland et al. 1990; Liang et al. 2003). BVDV E2 in general is 373 amino acids long and contains 17 cysteine residues in the homodimer form with four highly conserved N-glycosylation sites. BVDV E2 possesses three domains, domain I and II contain neutralizing epitopes that are exposed on the viral surface, while the domain III acts as an anchor.

The first nonstructural protein, N^{pro}, is an autoprotease and is unique to pestiviruses and comprises 168 amino acids in all the pestiviruses detected so far. In BVDV-infected cells, it inhibits interferon (IFN-1) production and thereby interferes with antiviral activity of the host cell. Although earlier studies showed that N^{pro} is a papain-like cysteine protease with Glu22-His49-Cys69 building a catalytic triad, a subsequent study classified it into C53 protease family (Rawlings et al. 2012). BVDV N^{pro} crystallographic study has revealed that a catalytic dyad of His49 and Cys69 is responsible for proteolytic activity of N^{pro} (Zogg et al. 2013). The p7 nonstructural protein, existing as free p7 or E2-p7, has ion channel activity and a role in assembly of infectious progeny virus.

The NS2 and NS3 proteins are found predominantly as the unprocessed NS2/3 in cells infected with ncp viruses and primarily as NS2 and NS3 in cells infected with cp viruses, but recent studies have shown that cleavage of NS2/3 is necessary for replication of ncp viruses very early in the infection (Meyers and Thiel 1996; Lackner et al. 2004; Mishra et al. 2010). The NS2 protease is essential for efficient NS2-3 cleavage, while cellular Jiv protein acts as an essential cofactor for ncp viruses (Tautz et al. 1996; Lackner et al. 2004). Insertions of cellular sequences or duplication of BVDV genomic sequences commonly occurs in NS2-3 region. The 80 kDa NS3 protein is immunogenic and possesses two distinct enzymatic activities, the

N-terminus serine protease domain, which along with cofactor NS4A is necessary for proteolytic cleavage of viral polyprotein beyond NS3 and virus viability, and the C-terminus RNA helicase and NTPase domain that participates in RNA replication.

The NS4A protein functions as a cofactor for the NS3 serine protease and has an important role in the morphogenesis of virions. NS4B is predicted as an integral membrane protein localized at intracellular membranes and is associated with RNA replication complex. NS5A co-localizes with membrane-bound NS4B and NS5B in the RNA replication complex and BVDV NS5A is tolerant to some deletions but its role in viral replication is not clear. The NS5B protein, containing GDD and NTPase functional motifs, acts as RNA-dependent RNA polymerase and functions as the major protein in genomic RNA replication, and C-terminus of BVDV NS5B is important for morphogenesis of virions.

14.6 BVDV Attachment and Entry

BVDV entry into bovine cells involves virion attachment to cellular receptors, internalization, and membrane fusion. Bovine CD46 has been reported to act as a cellular receptor for BVDV (Maurer et al. 2004), but it may not be sufficient and some unknown elements may be required for BVDV entry. For initiating the infection process, BVDV E1-E2 heterodimer binds the cellular receptor. BVDV entry into bovine cells occurs through clathrin-dependent endocytosis and endosomal fusion in a pH-dependent manner (Grummer et al. 2004; Krey et al. 2005), and a similar mechanism exists for entry into ovine cells (Mathapati et al. 2010).

Following uncoating and release of the genomic RNA, transcription and translation of viral proteins take place in cellular cytoplasm. However, the mechanisms of viral assembly and release from the cell following translation and maturation of viral proteins are not clear at present. BVDV virions are assembled in the endoplasmic reticulum (ER) along with final post-translational modifications. The viral proteins are maintained exclusively in the ER and Golgi bodies, hence are not displayed on the cell surface.

14.7 BVDV Replication

BVDV replication takes place in cellular cytoplasm. Following IRES-mediated initiation of translation, BVDV replication process begins with a positive strand replicase complex consisting of viral and cellular components formed at the 3′-terminus of the genome. The replicase complex catalyzes transcription of positive-sense RNA into full-length complementary negative-sense RNA, which then acts as template for synthesizing additional positive-sense RNA, using a semiconservative asymmetric replication model (Warrilow et al. 2000). In comparison to negative-sense RNA, a large amount of newly synthesized positive-sense RNA is generated. In this model, three virus-specific RNAs, a double-stranded replicative form (RF), a partially single-stranded and partially double-stranded replicative intermediate (RI)

and a single-stranded viral RNA are involved. The same positive-sense genomic RNA acts as a template for both replication and translation. Regulation of this process is mediated by secondary structure of IRES in the 5′-UTR. The budding of BVDV takes place in endoplasmic reticulum, where the RNA-core complex is packed in envelopes (Schmeiser et al. 2014). Following assembly, virions are released from the cell through the secretory pathway.

14.8 Genetic and Antigenic Diversity

BVDV strains show high genetic diversity, which results from accumulation of point mutations, homologous and non-homologous RNA recombination. Variation in evolutionary rates (5.9×10^{-4} to 9.3×10^{-3} substitutions/site/year) has been reported for BVDV-1. Mutations may lead to producing a population of viruses, called quasispecies, with each possessing a small number of nucleotide differences from the population mean. Like other RNA viruses, BVDV isolates exist as quasispecies in infected animals. A recent study has shown that similar to classical BVDVs, differences exist between the swarms circulating within HoBiPeV PI animals from the same inoculum suggesting involvement of host factors in the selection of genetic variants in PI animals (Weber et al. 2015). Besides the reports of homologous RNA recombination in BVDV-1 and BVDV-2, generation of cp BVDV variants with a variety of genomic alterations following non-homologous RNA recombination has been described. BVDV antigenic diversity happens by replication in vaccinated or previously infected animals, but a recent study has shown that antigenic changes may arise in absence of an immune response.

Genetic typing of BVDV strains is important for BVD epidemiology and control. Accurate genetic typing of BVDV has been obtained from the application of advanced molecular techniques such as reverse transcription polymerase chain reaction (RT-PCR), next-generation sequencing, and phylogenetic analysis. Although sequence analysis of the highly conserved 5′-UTR is mostly used for the classification of pestivirus isolates into species level, segregation into subtypes/subgenotypes within each species is more accurate by analysis of complete N^{pro} and E2 genes (Becher et al. 2003; Vilcek et al. 2001, 2004; Yesilbag et al. 2017). However, discrepancies in allocation of some BVDV isolates into subtypes have been reported either due to inconsistent use of different genomic regions or inconsistent use of methods for phylogenetic analysis. Additionally, antigenic similarity detected by monoclonal antibody (MAb) binding and cross-neutralization assays with homologous and heterologous antisera is used for determining antigenic diversity of BVDV strains (Paton et al. 1995; Becher et al. 2003; Dias et al. 2017).

So far, BVDV-1 has been segregated into 21 subtypes (1a–1u), while BVDV-2 has been segregated into 4 subtypes (2a–2d) and HoBiPeV has been segregated into 4 subtypes (a–d). Global distribution of BVDV subtypes is shown in Table 14.1. In India, all the three bovine pestivirus species, *Pestivirus A*, *Pestivirus B* and *Pestivirus H* and two genotypes/subtypes of BVDV-1 (1b, 1c), BVDV-2 (2a, 2b), and HoBiPeV (c, d) within these species have been detected so far (Mishra et al. 2011, 2014).

Table 14.1 Global distribution of BVDV subtypes

Continents	Country	BVDV-1																				BVDV-2				HoBiPeV			
		a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q	r	s	t	u	a	b	c	d	a	b	c	d
Americas	Argentina	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−
	Brazil	+	+	+	+	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	+	−	−	−
	Peru and Chile	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	USA	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−
	Canada	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
	Uruguay	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
	Mexico	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
Australia	Australia	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
	New Zealand	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Africa	Egypt	−	+	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	Tunisia	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
	South Africa	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Asia	China	+	+	+	+	−	−	−	−	−	−	−	−	+	−	+	+	+	−	−	−	+	+	+	−	−	+	−	−	−
	India	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	+	+
	Bangladesh	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−
	Thailand	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
	Philippines	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	Japan	+	+	+	−	−	−	−	−	−	+	−	−	+	+	+	−	−	−	−	−	−	+	−	−	−	−	−	−	−
	Korea	+	+	+	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
	Mongolia	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
Europe	Austria	+	+	−	+	+	+	+	+	−	−	+	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
	Belgium	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
	Croatia	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

	Czech Republic	−	+	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	Denmark	−	+	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	Finland	−	−	−	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	France	+	+	−	+	+	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
	Germany	+	+	−	+	+	+	+	+	−	−	+	−	−	−	−	−	−	−	−	−	−	+	−	+	−	−	−	−
	Hungary	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	Ireland	+	+	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
	Italy	+	+	+	+	+	+	+	+	−	−	+	+	−	−	−	−	−	+	+	+	+	+	−	−	−	+	−	−	−
	Kosovo	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	Poland	−	+	−	+	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
	Portugal	+	+	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−
	Slovakia	−	−	−	+	+	+		+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
	Slovenia		+		+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	Spain	+	+	+	+	+	+	−	+	−	−	+	+	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−
	Sweden	+	+		+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	Switzerland		+	−	−	+	+	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	Turkey	+	+	−	+	−	+	−	−	+	+	−	+	−	−	−	−	−	+	−	−	−	+	+	−	−	−	−	−	−
	UK	+	+	−	+	+	+	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−

+ Present; − Absent

14.8.1 Genetic and Antigenic Diversity of BVDV-1

BVDV-1, which belongs to species *Pestivirus A*, is prevalent worldwide. Although initially divided into two subtypes, BVDV-1a (NADL-like) and BVDV-1b (Osloss-like), BVDV-1 isolates originating from different countries could be segregated into 11 subtypes subsequently (Vilcek et al. 2001). Further studies on genetic typing have revealed existence of at least 21 subtypes of BVDV-1 (Yesilbag et al. 2014, 2017; Giammarioli et al. 2015) and additional subtypes is likely in the future. Despite accurate distribution of BVDV subtypes in individual countries and continents is unknown, the published reports have revealed that BVDV-1b is the predominant subtype worldwide, followed by BVDV-1a and 1c. BVDV-1b is the predominant subtype in Europe, Asia and Americas, while BVDV-1c is predominantly prevalent in Australia and Mexico. Extensive genetic diversity of BVDV-1 has been reported from several countries in Europe and also in Turkey, China and Japan, but it is comparatively lesser in Americas, Africa and Australia as well as in India.

Although BVDV-1 isolates are antigenically closely related than BVDV-2 or HoBiPeV, significant antigenic differences between BVDV-1 subtypes have been found similar to that observed between BVDV-1 and BVDV-2 or HoBiPeV. Hence, significant antigenic differences should be taken into consideration while developing vaccines and designing effective control programs. Significant antigenic differences have been reported between BVDV-1a and BVDV-1b strains in Europe (Becher et al. 2003), between BVDV-1a, BVDV-1b and BVDV-1c strains in Chile and the USA (Pizarro-Lucero et al. 2006; Ridpath et al. 2010), between BVDV-1e and BVDV-1 k strains in Switzerland (Bachofen et al. 2008), and between BVDV-1n and BVDV-1o and BVDV-1a, BVDV-1b, BVDV-1c and BVDV-1j strains in Japan (Nagai et al. 2008).

14.8.2 Genetic and Antigenic Diversity of BVDV-2

Although distributed in all continents, BVDV-2 strains are genetically less diverse than BVDV-1 and occur less commonly than BVDV-1. Although BVDV-2 has been divided into four (2a–2d) subtypes, the strain belonging to 2d subtype is questionable due to a single report from Argentina. BVDV-2a is the most prevalent subtype in all continents, while BVDV-2b has been detected in Americas (Brazil, Argentina, Uruguay, USA), Europe (Portugal, Spain, Slovakia, Turkey) and Asia (India, China), and BVDV-2c has been detected only in Europe (Germany, Ireland) and North America (USA). BVDV-2 can occur also in sheep and goats. In sheep, BVDV-2a has been detected in the USA and Italy, while BVDV-2b has been reported from India (Mishra et al. 2008b) and Turkey (Yesilbag et al. 2008). However, only BVDV-2a subtype has so far been reported in goats (Mishra et al. 2007a) and significant antigenic variation between BVDV-2 subtypes has not yet been observed.

14.8.3 Genetic and Antigenic Diversity of HoBiPeV

Although less commonly found, clinical disease following natural HoBiPeV infections is similar to that caused by classical BVDV-1 and BVDV-2 infections. Till 2014, all the previously reported HoBiPeV strains, except the Thai and Bangladesh strains, were found to be closely related genetically. However, our previous work on the basis of sequence analysis of combined datasets of 5′-UTR and full N^{pro} gene showed that HoBiPeV strains can be classified into three subtypes with two highly divergent HoBiPeV lineages co-circulating in Indian cattle (Mishra et al. 2014). Further studies have shown that HoBiPeV strains can be classified into 4 subtypes (a–d). HoBiPeV-a has been detected in South America (Brazil) and Europe (Italy) and as contaminants of FBS, while HoBiPeV-b has been reported from Bangladesh and HoBiPeV-c and HoBiPeV-d have been detected in India (Mishra et al. 2014; Giammarioli et al. 2015). Marked antigenic differences exist between BVDV-1, BVDV-2 and HoBiPeV, while minor to moderate antigenic variation among HoBiPeV-a field isolates has been reported recently in Brazil.

14.9 Epidemiology of BVD

A large number of variables, such as clinical, pathological, virological, serological and production measures can be used to quantify the occurrence of BVDV infections. Long presence of antibodies (often lifelong) in acutely infected animals and presence of virus in PI animals throughout their life make prevalence studies more suitable for BVD. Sensitivity and specificity of diagnostic tests play a key role in determining true prevalence of BVDV infection. BVDV antibody prevalence studies are useful mostly in unvaccinated populations. Overall, PI animals play a major role in virus transmission than transiently infected cattle, since they shed virus in large amounts in all bodily fluids throughout their life, while virus shedding is limited to only for a few days or weeks in most other viral diseases of livestock. Vertical transmission and transmission through semen also play vital roles in epidemiology and BVDV is introduced into a susceptible herd mostly by introduction of PI animals or pregnant animals carrying a PI fetus.

14.9.1 Abroad

BVDV infections are widespread throughout the world except in a few countries in Europe, where it has been eradicated or in final stages of eradication. Cattle of all ages are susceptible to BVDV infection and are the primary hosts. Buffaloes and domestic non-bovid species including sheep, goats, new world camelids and swine have also been reported to carry and spread BVDV. Natural infection of BDV in cattle has been reported with clinical signs similar to BVD. Despite variation in prevalence rates among surveys, BVDV infection is endemic in many populations having 1–2% of the cattle being persistently infected (PI) and > 90% of the cattle

being antibody positive. Variations in BVDV prevalence rates among different countries or regions or introduction of virus into BVDV-naïve herds is often determined by cattle population density, cattle trade and pasturing practices.

BVDV prevalence is high in areas with high cattle population density and larger herds. BVDV-1 and BVDV-2 are distributed in all continents, while HoBiPeV has so far been reported in South America, Europe and Asia. Geographical patterns in distribution of BVDV-1 subtypes have been reported around the world. BVDV-1 subtypes, 1m, 1n, 1o, 1p, and 1q have been found only in some countries in Asia, while 1f, 1g, 1h, 1k, 1l, 1r, 1s and 1t have been found exclusively in Europe. Besides cattle, buffaloes, sheep and goats, a number of BVDV-1 subtypes have been identified in wild ruminants. BVDV-1a has been detected in Canadian bison; BVDV-1b in alpaca, pudu, Canadian bison and bongo; BVDV-1c in yak and deer; BVDV-1d in roe deer; BVDV-1f in mouse deer; and BVDV-1j in deer (Vilcek and Nettleton 2006; Mishra et al. 2008a).

BVDV-2, first detected in cattle of the USA and Canada in association with hemorrhagic disease with high mortalities, was found later in several other countries of South America, Europe and Asia and also in Australia (Vilček et al. 2005). BVDV-2a is the predominant BVDV-2 subtype circulating around the world. A very special case of fatal disease with high mortalities in cattle resulting from infection with BVDV-2c isolates consisting of 3 genomic variants (dup^+, dup_1^-, dup_2^-) has been reported in Germany (Jenckel et al. 2014). The recent association of HoBiPeV with severe respiratory and reproductive disease and mucosal disease in cattle and respiratory disease in small ruminants has raised concerns (Bauermann et al. 2013; Weber et al. 2015). Natural HoBiPeV infection in cattle has so far been reported sporadically in Brazil, Italy, Thailand, India and Bangladesh, but in some regions like in Northeastern Brazil, HoBiPeVs have been more frequently detected in cattle than BVDV-1 and BVDV-2. Natural infection has also been reported in buffalo in Brazil and in sheep and goats in China.

14.9.2 India

Serological evidence of BVDV infection in cattle in India was first reported in 1981 in Orissa State (Nayak et al. 1981) followed by a report from Gujarat State in 1989. A seroepidemiological study on sera collected from 17 states then demonstrated an overall apparent BVD seroprevalence rate of 17% in cattle in most parts of the country (Sudharsana et al. 1999). Subsequently, varying rates (up to 52%) of BVD seroprevalence in cattle and buffaloes in different parts of the country involving both commercial dairies and small holder units have been reported. Besides cattle and buffaloes, serological evidence of BVDV infection has been reported in sheep and goats (Mishra et al. 2009), in yaks (Mishra et al. 2008a), and also in mithun (Singh et al. 2017). A BVDV seroprevalence study involving sheep and goats from 13 states during 2004–2008 reported a true prevalence rate of 23.4% in sheep and 16.9% in goats and provided evidence of BVDV-1 infection predominantly and BVDV-2 occasionally (Mishra et al. 2009).

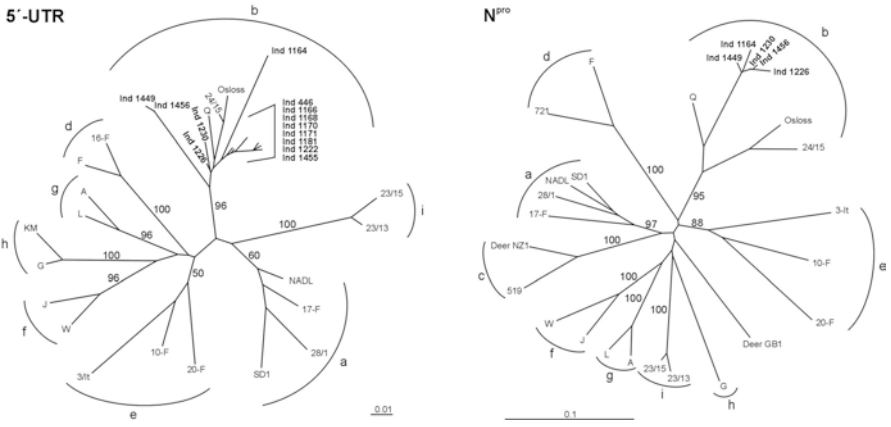

Fig. 14.2 Genetic typing of Indian cattle BVDV-1 isolates in 5′-UTR and N^{pro} regions. The unrooted tree was based on partial sequence analysis of the 5′-UTR (245 nt) and the N^{pro} (385 nt) and was prepared using the neighbor-joining method (Kimura 2-parameter method, transition/transversion 2.0). Sequences of BVDV isolates from India are labelled in bold and other sequences were taken from GenBank. The N^{pro} sequences of strains 519, 721, Deer NZ1, and Deer GB1 have the following GenBank Acc. numbers: AF144464, AF144463, U80903 and U80902. Numbers over branches indicate the percentage of 1000 bootstrap replicates. (Reprinted from our work Mishra et al. 2004)

Although earlier studies indicated serological evidence of BVDV infection, the conclusive evidence of BVDV in Indian cattle was provided by virus isolation and subsequent phylogenetic analysis of BVDV isolates in 2004 (Mishra et al. 2004). The phylogenetic analysis of 13 BVDV isolates originating from cattle in eastern, northern, and western India in 5′-UTR and N^{pro} genes revealed that they belong to BVDV-1b subtype and are closely related (Fig. 14.2). In the same year, BVDV-1 was also reported in lambs showing RVF-like symptoms (Yadav et al. 2004). Although systematic surveillance studies involving all the states are lacking, genetic typing of BVDV strains collected during later studies revealed that BVDV-1b is the predominant subtype circulating in cattle (Mishra et al. 2007b, 2014; Behera et al. 2011). Further studies have reported existence of BVDV-1b and BVDV-1c subtypes in buffaloes (Mishra et al. 2007b) and in sheep and goats (Mishra et al. 2012). Besides domestic ruminants, BVDV-1 was detected in yaks (*Bos grunniens*) in the Himalayan region for the first time and phylogenetic analysis revealed that they belonged to BVDV-1c subtype (Mishra et al. 2008a). It seems that BVDV has evolved well-developed strategies to become successful in replicating in different animal species.

BVDV-2a was detected in cattle from Jammu & Kashmir State in 2011 and recently in bull semen from Tamil Nadu (Behera et al. 2011; Mishra et al. 2018). BVDV-2a has earlier been detected in goats from Northern India, while BVDV-2b has been reported in sheep from Western India (Mishra et al. 2007a, 2008b). Molecular epidemiology studies on BVDV-2 have provided evidence of circulation of genetically divergent BVDV-2a strains in Southern India and in Northern India (Fig. 14.3).

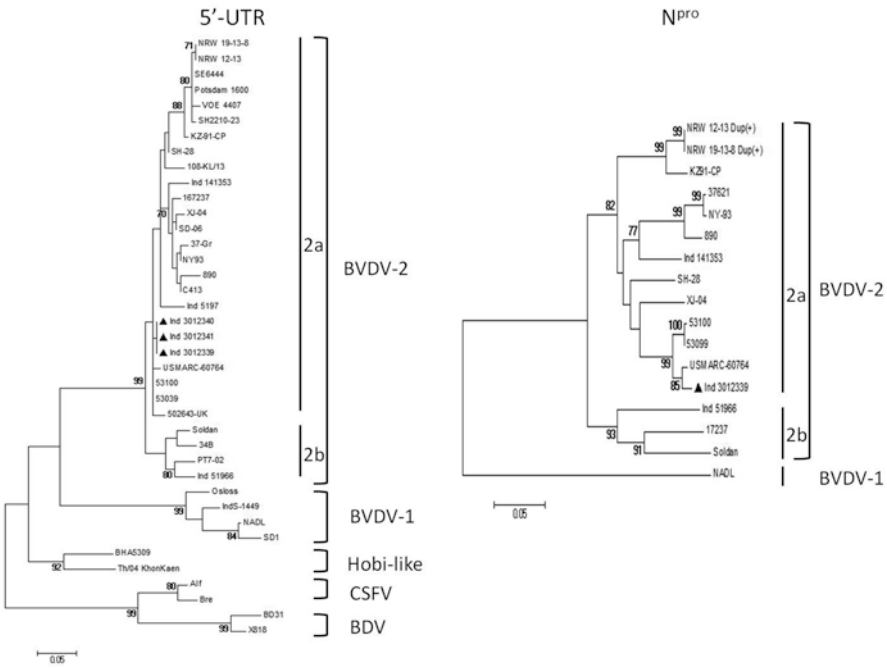

Fig. 14.3 Genetic typing and relationship of Indian BVDV-2 strains originating from cattle, sheep and goats in the 5′-UTR and N^{pro} regions. The phylogenetic tree was prepared based on 240 nt in the 5′-UTR and 474 nt in N^{pro} gene using neighbor-joining method in MEGA version 6.0. Numbers in nodes indicate the percentage of 1000 bootstrap replicates that support each group. BVDV strains (cattle) labelled as filled triangles originated from Southern India, while BVDV-2 strains Ind 141353 (cattle), Ind 5197 (goat) and Ind 51966 (sheep) originated from Northern India. (Reprinted from our work Mishra et al. 2018)

Natural infection with HoBiPeV, an emerging bovine pestivirus, was identified recently in cattle in the states of Maharashtra, Punjab and Chhattisgarh while conducting systematic surveillance in cattle from 21 dairy farms across India. Molecular characterization of HoBiPeV strains revealed co-circulation of two novel and divergent lineages of HoBiPeV (c, d) in India (Fig. 14.4), highlighting the independent evolution of at least 3–4 lineages of HoBiPeV strains globally (Mishra et al. 2014). These novel findings extended the knowledge on the epidemiology and genetic diversity of HoBiPeV strains globally which is important for management and control of BVD.

14.10 Risk Factors

Several risk factors have been associated with BVDV infection which may vary between different geographical regions and cattle rearing practices. The presence of PI animals in the vicinity of susceptible animals is considered as the highest proven risk of BVDV infection and spread. Purchase of animals without BVDV testing is a

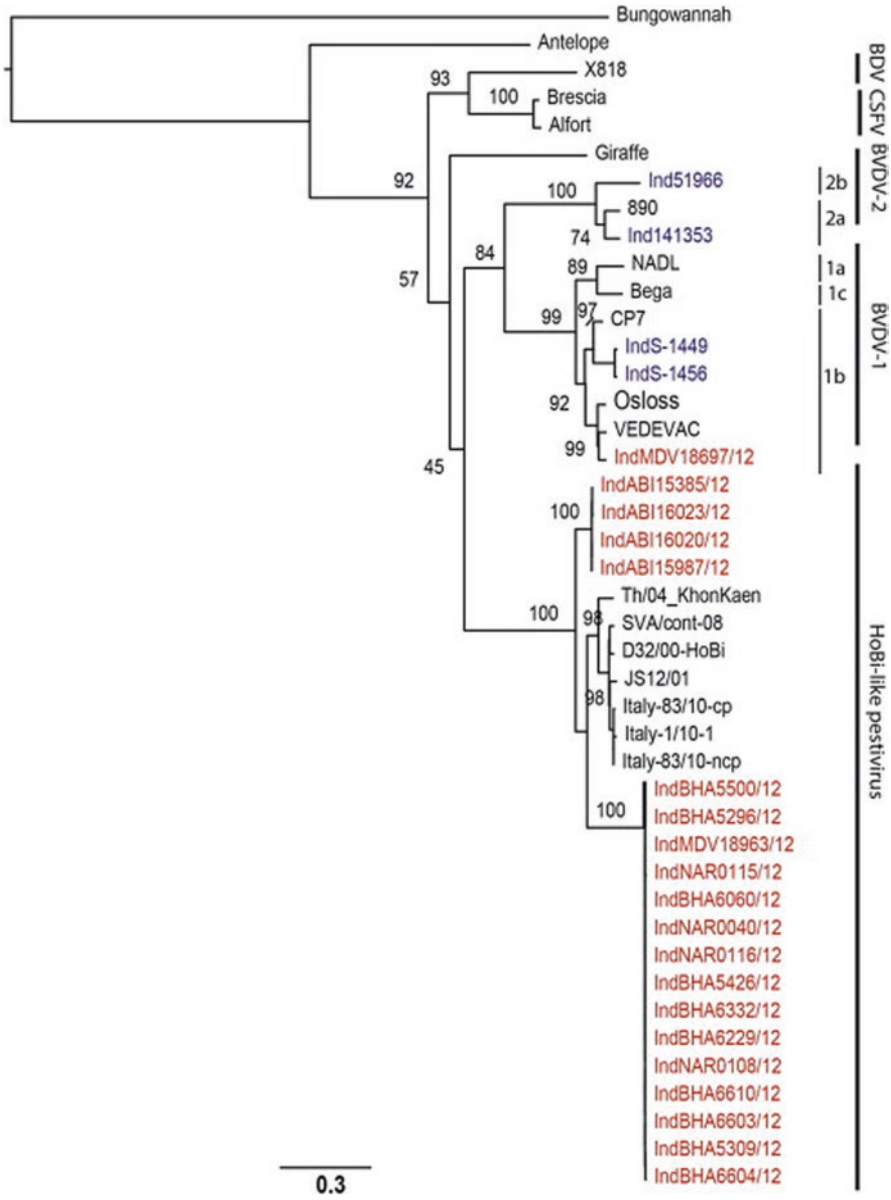

Fig. 14.4 Phylogenetic tree and genetic relationship of Hobi-like pestivirus (HoBiPeV) strains from Indian cattle with globally circulating HoBiPeV strains. The tree was based on the combined datasets of 5′-UTR (239 bp) and N^{pro} (504 bp) sequences and the maximum likelihood tree was generated using concatenated datasets of 5′-UTRand N^{pro} under the GTR + gamma substitution model in RAXML. Numbers indicate the percentage of 1000 bootstrap replicates that support each phylogenetic branch. The HoBiPeV isolates from India are labelled red, and previously reported Indian isolates of BVDV-1 and BVDV-2 are labelled blue. (Reprinted from our work Mishra et al. 2014)

high-risk factor in high prevalence regions than low prevalence regions. Some of the other risk factors include cattle on common pasture, sheep in pasture with cattle, over pasture fence contact, mixing of herds in pasture, wild animals in pasture, exchange of calves, large herd size, high cattle density, movement of animals pregnant with PI calves (Trojan animals), infection in contiguous farms, veterinarian reusing needles between farms, use of artificial insemination without testing semen for BVDV, use of contaminated live attenuated vaccine and livestock trade such as import of live cattle.

14.11 Host Range

BVDV has a wide host range and infects a variety of animals, both domesticated and wild. Among the domesticated ruminants, it infects cattle, buffalo, sheep, goats, and yaks, while among wild ruminants it infects buffalo, eland, Canadian bison, alpaca, pudu, bongo, deer, roe deer, mousedeer, reindeer, giraffe, European bison, chamois, pronghorn antelope and mithun. BVDV has been isolated in over 40 species of ruminants, and serological evidence indicates susceptibility of most free-ranging ruminants to BVDV infection (Vilcek and Nettleton 2006). Serological evidence of BVDV infection has also been reported in European rabbits. Although natural infection with BVDV occurs mainly in cattle, sheep and goats, it occurs also in pigs. Natural infection with BDV occurs mostly in sheep, but has also been reported in cattle and goats in many countries, whereas natural infection of CSFV and Bungowannah virus found in pigs has not been reported in cattle. However, natural or experimental BVDV persistent infection has been reported in mountain goats and domestic goats, domestic sheep, swine, alpaca, eland, mule deer, white-tailed deer and mouse deer and PI animals pose greatest risk of BVDV transmission.

14.12 Transmission

BVDV transmission occurs through several modes. BVDV spreads horizontally within a herd while vertical transmission occurs from cow to calf. PI animal results following infection of fetus with ncp BVDV in the first or second trimester (45–125 days) of pregnancy, before maturation of its immune system. The most common method of producing PI calves is through primary acute BVDV infections of pregnant cows. However, PI cows also invariably give birth to PI calves. Capability to cause fetal infections is exclusively a biotype-specific property of ncp BVDV. Horizontal transmission occurs not only by viremic transiently infected (TI) animals but also by PI animals that shed virus lifelong in all secretions, such as nasal and ocular discharges, milk/colostrum, semen, urine and feces (Van Campen and Frolich 2001). PI animals are the principal reservoirs of BVDV transmission, while TI animals transmit BVDV transiently and occasionally.

Semen from transiently infected bulls can transmit BVDV infection and virus can be detected up to 28 days in such bulls (Kirkland et al. 1994). In contrast, in both raw and extended semen of PI bulls, BVDV concentration remains high and

semen from PI bulls infects susceptible animals consistently. Persistent testicular infection (PTI) occurs following acute BVDV infection in bulls. BVDV persists in semen or testicular tissue of these non-viremic and seropositive bulls, and seronegative cows may be infected via artificial insemination. Environmental conditions favoring crowding and aerosol transmission enhance the chances of BVDV transmission from acutely infected calves having respiratory form of BVDV infection. Transient shedding of vaccine strains in animals vaccinated with modified live BVD vaccine has been reported with probable consequences of secondary transmission to pregnant animals in contact with vaccinated animals (Fulton et al. 2003).

BVDV can be transmitted indirectly from contaminated pens, rectal examination gloves, hypodermic needles, nose tongs, BVDV-contaminated live vaccines, and ambient air (Niskanen and Lindberg 2003). Sufficient evidence exists regarding the spread of BVDV from domestic ruminants to wild ruminants. But there is no conclusive evidence that BVDV spreads from wild ruminants to domestic ruminants. However, serologic data from camels and roe deer strongly suggest circulation of BVDV in these animals independent of cattle, sheep and goats.

The four main factors which affect BVDV transmission are infectiousness (virulence) of the virus strain, the number of adequate contacts per time period between infectious and susceptible animals, the prevalence of infectious animals in a herd and the presence of truly susceptible animals.

14.13 Immunopathobiology

14.13.1 Pathogenesis

BVDV replicates in epithelial cells and lymphoid tissues of the oropharynx following infection via oronasal route. The phagocytic cells carry BVDV and/or BVDV-infected cells to peripheral lymphoid tissues leading to viremia that occurs 2–4 days after exposure and spreads BVDV to internal organs. The pathogenesis of BVDV is a complex interaction between the agent, host and environmental factors, and hence clinical signs are highly variable (Baker 1995). The virulence of BVDV strains and biotype of BVDV (ncp and cp) are the major determinants from the agent's side, while immune status, immune competence and stage of pregnancy are the major determinants from host's side.

In cultured cells infected by cp BVDV strains, there is rounding up and detachment of cells and cells die due to apoptosis, whereas in cells infected by ncp BVDV, no microscopically detectable alterations are seen. But both cp and ncp BVDV isolates induce apoptosis of T and B cells in vivo. Moreover, ncp viruses are preponderant in nature and pathogenic to the host and establish persistent infection upon fetal infection leading to lifelong virus shedding by PI animals. The host interactions with BVDV are highly variable, ranging from lack of immune response to a purifying immune response and from lack of clinical signs to highly lethal infection. Hence, disease is not obligatory for BVDV replication in PI animals or for viral transmission. Reproductive failure or immunosuppression are major consequences

of BVDV infections because of its affinity for the fetus and for cells of lymphatic organs. Strong affinity of BVDV for lymphoreticular tissues causes necrosis in lymph nodes and spleen and destruction of Peyer's patches. The four major syndromes associated with BVDV pathogenesis are acute infection, transplacental infection, persistent infection and mucosal disease.

14.13.2 Acute Infection

Acute BVDV infections in cattle develop when seronegative and immunocompetent cattle are infected with BVDV and the disease may be subclinical, severe acute, or chronic. The majority of postnatal BVDV infections are inapparent and virulence of BVDV strains is the key determinant of outcome of acute infection. Incidences of field cases of acute BVDV infection associated with thrombocytopenia, severe clinical signs and mortality in all age groups have been reported with death of about 40,000 animals, due to high virulent strains of BVDV-2 (Perdrizet et al. 1987; Carman et al. 1998). However, subsequent studies showed that BVDV-1 is able to induce hemorrhagic disease and all the three bovine pestiviruses, BVDV-1, BVDV-2 and HoBiPeV, encompass strains of high, moderate, and low virulence (Ridpath et al. 2000; Decaro et al. 2012; Mishra et al. 2014). Moreover, virulence is not correlated with the biotype, since all the three bovine pestiviruses have both ncp and cp strains. The differences in replication have a great impact on the virulence of BVDV strains, since strains that produce highest degree of viremia result in the most severe clinical symptoms (Walz et al. 2001). Acute BVDV infection also causes immunosuppression by depletion of both B and T lymphocytes thereby leading to suppression of immune functions in the infected animal. BVDV-induced immunosuppression not only directly causes enteritis but also predisposes calves to development of bovine respiratory disease (BRD) and secondary bacterial infections and enhances severity of bovine rota viral enteritis. Chronic viral shedding may also occur following acute infection, which has been reported in some bulls, where BVDV was found in semen for up to 7 months.

14.13.3 Transplacental Infection

The major economic impact of BVDV is due to its ability to cause intrauterine and transplacental infection in cattle, which may result from acute infection during or immediately before pregnancy and contaminated semen through artificial insemination or natural service. The outcome of BVDV fetal infection depends on the stage of development of the fetal immune system at the time of infection. Fetal infections during the first two trimesters of gestation may have severe reproductive consequences and may result in persistent infections, fetal death and abortion, or congenital anomalies (Brownlie et al. 1998). Dual infection of the fetus with both BVDV-1 and BVDV-2 has also been reported. Although abortions due to BVDV occur mostly in the early stages of gestation (< 125 days), abortions in the late phase of gestation

do occur. Necrotizing inflammatory reaction with mononuclear cell infiltration in several tissues of fetus has been reported. The intrauterine infection of an immune-competent fetus in late gestation is similar to that of acute BVD. BVDV-induced congenital anomalies mostly occur during mid-gestation (80–150 days) and may involve the nervous system, eye, immune system, integumentary system, musculo-skeletal system, or respiratory system.

14.13.4 Persistent Infection

Pestiviruses including BVDV use novel strategies to persist and spread in the host population through persistent infection. PI animals develop following infection of fetus with ncp BVDV strains during the first trimester of gestation, before the development of lymphoid tissues and functional immune responses and the immunotolerance is specific to the persisting BVDV strain (McClurkin et al. 1984). Primary acute BVDV infection of pregnant cows is the predominant cause of producing PI calves, although PI cows most often give birth to PI calves, whereas Trojan cow, a non-PI cow carrying a PI fetus, is immune to BVDV and possesses significantly higher antibody titers during mid-late pregnancy. The ability to cause persistent infection is exclusively a biotype-specific feature of ncp BVDV. Immunotolerance develops through selective evasion of innate immunity in the fetus by inhibition of IFN-I synthesis following ncp BVDV infection in addition to complete avoidance of the adaptive immune system. However, immune tolerance is BVDV strain specific and immune response is induced in PI animals following infections with other BVDV strains (Fulton et al. 2003). BVDV remains widely distributed in organs and secretions of PI animals and these animals may live for several years without any immune response to the persisting BVDV strain. Besides cattle, persistent infection occurs also in buffaloes, sheep, goats, alpacas, mouse deer, mountain goats and white-tailed deer.

14.13.5 Mucosal Disease

Mucosal disease (MD) is a sporadic disease of cattle, in which mostly 6–24-month-old animals succumb, but it may arise in adult animals. MD is highly fatal and is associated with the presence of closely related ncp and cp biotypes of BVDV in these animals. The first hypothesis on the mechanism of MD was that it develops only in PI animals. The second hypothesis was that animals suffering from MD always harbor a cp virus along with the persisting ncp strain (Bolin et al. 1985; McClurkin et al. 1985). Then it was established that close antigenic relationship between ncp and cp strains from the same animal is obligatory for development of MD (Meyers et al. 1991). Mucosal disease arises in PI animals by complex mutations of the ncp biotype to a cp biotype, or by superinfection of PI animals with a cp BVDV. In acute MD, genome analyses showed cp strain-specific genomic alterations which are mainly due to recombination events resulting in insertion of cellular sequences or duplications and deletions of viral sequences. The genomic alterations

enhance NS3 protein production, which provides an apoptotic signal for the infected cells. Cp strains have been reported earlier for BVDV-1 and BVDV-2 and recently for HoBiPeV. Insertion of ubiquitin coding sequences upstream of the NS3 gene and insertion of host-cell origin Jiv sequences within NS2 upstream of the NS2/3 cleavage site are the most commonly observed genomic changes (Tautz et al. 1993; Becher and Tautz 2011).

In animals suffering MD, strikingly higher numbers of cp BVDV-infected cells have been found compared to ncp-infected cells in PIs before outbreak of MD. In chronic MD, the antigenic properties of the persisting ncp and the superinfecting exogenous cp viruses are more divergent, and such animals suffer clinically for a prolonged period. In some cases, MD can develop several weeks after superinfection and is known as late-onset MD, and cp viruses in them are recombinants between cp and ncp viruses, where structural genes are shared by ncp viruses and nonstructural genes are shared by cp viruses. Direct cell damage by cp BVDV is the major mechanism of disease in PI animals suffering from MD, while cp BVDV is mostly attenuated in acutely infected animals.

14.14 Clinical Signs

The manifestations of BVDV infections are complex and remain a challenge for the practitioners and researchers and BVD usually refers to acute infection in seronegative immunocompetent cattle. Most of the BVDV infections in adults are subclinical or mild. However, some strains produce severe disease with mortality. After 5–7 days of incubation period, there is fever, depression, inappetence, oculonasal discharge, and occasionally oral lesions such as erosions and shallow ulcerations (Baker 1987). In calves, respiratory and gastrointestinal symptoms occur, with occasional fatal enteritis. Growth is retarded and PI calves mostly die before weaning. BVDV produces venereal infections in bulls and semen from PI bulls is infective and failure of conception in cows occurs due to fertilization failure. Repeat breeding and more number of services per conception are common in BVDV-infected herds. Semen quality is reduced because of low motility and abnormal morphology of sperm cells. The prominent characteristics of mucosal disease are bloody diarrhea along with fever, anorexia, ataxia and general debility. Mortality is often 100% and within 15 days after onset of clinical signs (Baker 1987). During postmortem, extensive ulcerative lesions in the gastrointestinal tract, affecting especially the GALT in the mucosa, are observed.

14.15 Immunity and Immunosuppression

Immune responses to BVDV develop following vaccination, infection, exposure to cross-reactive pestiviruses, or by passively through colostrum. Antibody response to BVDV is detectable 2–3 weeks post-infection and may plateau after about 10–12 weeks and may persist for long. Passive antibodies protect neonatal calf from BVDV

infection but interfere with vaccination. Structural proteins, E2 and E^{rns} and nonstructural protein NS3 (P80) are immunodominant proteins and induce significant antibody responses following BVDV infection. The E2 glycoprotein elicits neutralizing antibodies and is the major determinant of protective immunity, whereas E^{rns} and NS3 elicit non-neutralizing antibodies (Donis et al. 1988). BVDV-1 E2 protein has one immunodominant epitope, while BVDV 2 has three and virus neutralizing test (VNT) is used to correlate protective immunity. Cattle vaccinated with inactivated vaccines develop a weak NS3 antibody response, while a strong NS3 antibody response is elicited following natural infection or vaccination with modified live vaccine.

BVDV causes general inhibition of cellular immune responses in cattle. Mild (10–20% decrease) or severe lymphopenia (50–60% decrease) is found depending upon the virulence of the BVDV strain and cytotoxic T-lymphocytes (CD8+) are affected more than helper T-lymphocytes (CD4+) cells (Brodersen and Kelling 1999). BVDV affects bovine monocytes, dendritic cells and macrophages and may alter function of TLRs, expression of cytokines and costimulatory molecules in bovine monocytes and macrophages resulting in an adverse effect on their ability to stimulate Th cells.

Interactions of BVDV and immune system are complicated and variable. The ncp BVDV elicits humoral immune response faster and traffics to more immune organs of mucosal immunity. Besides, BVDV antigen from ncp strains persists longer in immune tissues than the cp strains. BVDV cp strains elicit higher CMI response, while ncp strains avoid production of CMI response. Elimination of the adaptive immune response via infection before self-non-self-discrimination and inhibition of innate immune response are perfect strategies adopted by ncp BVDV for generation of PI animals. To inhibit innate immune response, ncp BVDV employs several strategies including preventing IFN-1 induction through N^{pro} and E^{rns} and strictly controls their RNA replication. BVDV E^{rns} and N^{pro} are crucial in establishment and maintenance of persistent infections and inhibit the innate immune response (Meyers et al. 2007).

14.16 Diagnosis

The control of BVD is highly dependent on confirmed laboratory diagnosis that defines exposure of an individual animal or population to BVDV. The clinician should have a clear intention about the diagnosis approach and communicate it to the laboratory. Laboratory diagnosis of BVD is either aimed at detecting BVDV, viral antigen, viral RNA, or antibodies against BVDV. Dramatic improvements have been made in laboratory methods for diagnosing BVDV infections during the last 20 years. In spite of recent advances in BVDV diagnosis, virus isolation and identification still remain the gold standard technique. Like other diseases, diagnosis of BVDV is an art that involves accumulation of data (laboratory test results) and reasoning from the data (interpretation).

BVDV-free animals upon testing are negative for antibody, antigen and virus, while acutely infected animals or immunocompetent fetuses are antibody positive and generally antigen or virus negative. PI animals in contrast are positive for

antigen or virus and negative for antibody. Since acutely infected animals become positive for BVDV antibodies within 2–3 weeks post-infection, testing for antibody 4–8 weeks after initial testing can distinguish between acute and persistent infection in animals with positive antigen ELISA or RT-PCR results. Confirmatory diagnosis of mucosal disease relies on confirmation of PI status followed by isolation of both cp and ncp BVDV from the affected animal. However, it can also be confirmed based on identification of PI and characteristic pathological lesions. It is of utmost importance that only well-validated diagnostic tests should be employed for providing confirmatory diagnosis of BVD.

14.16.1 Conventional Methods

14.16.1.1 Virus Isolation

Virus isolation (VI) is the gold standard test for BVDV diagnosis and is the OIE recommended test for certifying individual freedom of infection during international trade. VI relies on the growth of BVDV in specific cell lines, such as MDBK or BT (bovine turbinate) followed by detection using specific antibodies or molecular methods. The cells and serum used for VI should be BVDV free and BVDV antibody free. As most of the BVDV isolates are of ncp biotype, detection by immunostaining or immunofluorescence using E^{rns}- or NS3-specific monoclonal antibodies is recommended. In case of cp (cytopathic) strains, a characteristic cytopathic effect is observed. VI detects viremia in individual animals and is used to confirm the PI status of animals with positive ear notches, serum, or whole blood buffy coats. Microplate immuno-peroxidase assay in 96-well plates is commonly used for PI animal detection. However, these methods suffer certain limitations such as varying sensitivity and slower test results and colostral antibodies can mask BVDV in PI animals and interfere their detection.

In case a pestivirus is isolated during virus isolation, it has to be characterized antigenically further for identification of the agent, using a panel of monoclonal antibodies specific for BVDV-1, BVDV-2, or BDV in a microplate immunoperoxidase method (Paton et al. 1995). The interpretation of results needs caution due to serological cross-reactivity among pestiviruses.

14.16.1.2 Antigen ELISA

Direct detection of viral antigen in leukocytes, serum, or ear notch samples can be done by pestivirus antigen capture ELISA (PACE), and several commercial BVDV antigen ELISA kits are available which can be used in most of the veterinary diagnostic laboratories. PACE is usually recommended for PI screening, but it also detects some transiently infected animals, so a follow-up sample 4–6 weeks later is tested to confirm PI status. The test is based on a sandwich principle using monoclonal antibodies and usually detects BVDV NS2-3 or E^{rns} antigen in peripheral blood leukocytes, plasma, or serum. Skin biopsies or ear notches have become a

popular sample for PI testing by PACE, since PI animals are consistently detected by PACE tests regardless of antibody status and the site of the biopsy. Although the test is easy to perform and rapid, it suffers from low sensitivity and specificity. Besides, false positives are not unusual. E^{rns} mAb-based PACE is preferable since it can detect all the three species of bovine pestivirus, BVDV-1, BVDV-2 and HoBiPeV (Mishra et al. 2014). Moreover, antigen capture ELISAs based on the detection of the p80 (NS3) protein of BVDV have diagnostic gaps prior to the age of 90 days both for serum and ear notches, instability of the p80/NS3 protein and the stronger inhibitory effect of colostral antibodies.

14.16.1.3 Immunohistochemistry (IHC)

Immunohistochemistry using ear notch tissue samples detects PI animals with 100% sensitivity and hence is one of the popular methods of BVDV Ag detection. While IHC is considered robust, it has many disadvantages in that it is subjective and restricted to tissue samples and is labor intensive, requiring experienced staff and its unreliability for use on samples stored in formalin for >15 days.

14.16.1.4 BVDV-Specific Antibody Detection

Serological tests for BVDV can be used to determine previous exposure of animals to BVDV, colostral antibodies in calves and immune response in vaccinated animals and in confirmation of acute infection. However, antibody-negative animals should be further tested for BVDV or Ag to rule out PI status. High prevalence of antibodies is indicative of current infection at a herd or region level. Although several antibody detection methods such as AGID, dot ELISA, and microsphere-based immunoassay have been reported, the virus neutralization test (VNT) and Ab ELISA are most commonly used.

14.16.1.5 Virus Neutralization Test (VNT)

VNT is the gold standard test to detect anti-BVDV antibodies, although antibody ELISA can alternatively be used (OIE 2017). For demonstration of seroconversion, both acute and convalescent sera should simultaneously be tested. The test is based on determination of 50% neutralizing end point and both cytopathic and non-cytopathic strains of BVDV can be used. Most preferably local isolates of BVDV-1, BVDV-2 and HoBiPeV must be used as low levels of antibody to BVDV-1 may not be detectable by a VNT that uses only BVDV-2 and vice versa. A differential neutralization test against a BDV strain should also be carried out simultaneously for serological differential diagnosis of BVD as BDV can also infect cattle naturally and serological cross-reactivity occurs.

14.16.1.6 BVDV Antibody ELISA

BVD antibody ELISA is useful for screening large number of cattle herds for serological diagnosis of BVD in unvaccinated animals. Both mAb-based competition ELISA and indirect ELISA are available commercially and used for detection of

BVDV antibodies in serum, milk and bulk milk. Being rapid and cost effective, Ab ELISA is an efficient and economical alternative to VNT. But in BVDV-vaccinated animals, the usefulness of ELISA tests is fairly limited, while in unvaccinated populations and in eradication phase, it has more utility. Moreover, VNT detects a rise in Abs following vaccination or infection, while Ab ELISAs fail in this regard. Caution should be taken in result interpretation, since some commercial BVDV antibody ELISA kits have been reported to yield false-negative results when serum samples of calves harboring HoBiPeV antibodies were tested.

14.16.2 Modern Methods

Newer technologies are constantly being developed and evaluated for their use in BVDV diagnostic testing through genome detection and/or amplification. Besides, nucleotide sequencing and sequence analysis have dramatically improved the molecular epidemiology of BVDV in detecting new and divergent strains and in tracing the origin of outbreaks.

14.16.2.1 RT-PCR

In BVDV-infected cattle, viral RNA is detectable early and for a longer duration than virus isolation. RT-PCR employing pooled serum and milk samples has been found useful in identifying PI animals during BVDV surveillance. RT-PCR assays are able to detect acutely infected animals, PI animals and animals vaccinated with modified live vaccines, but follow-up testing is necessary to define the status of positive animals. Several RT-PCR protocols have been developed and evaluated and are being used for BVDV diagnosis. A range of samples, including blood, milk, follicular fluid, saliva, and tissue samples, can be tested successfully by RT-PCR (Dubovi 2013). The most widely used protocol utilizes primers 324/326, which is targeted at the highly conserved 5′-UTR and is pestivirus specific, but it fails to detect the highly divergent HoBiPeV strains. However, HoBiPeV-specific RT-PCR has recently been reported. Differentiation between BVDV-1, BVDV-2 and BDV is also possible by nested RT-PCR. However, extreme precautions should be taken during nucleic acid-based tests due to false-positive cases arising from cross-contamination, and a positive RT-PCR does not define the clinical status of an animal in a single animal test or in a pooled sample. Although several modifications of nucleic acid detection methods, such as RT-PCR ELISA, microarray, and LAMP tests, have been reported, real-time RT-PCR and RT-PCR are most commonly used for BVDV diagnosis.

14.16.2.2 Real-Time RT-PCR

As real-time RT-PCR assay provides simultaneous quantitation and genotyping of BVDV, it is used more commonly now not only during BVD outbreaks but also for routine BVDV diagnosis, due to its rapidity in obtaining the results. Several real-time PCR assays, in uniplex, duplex, or multiplex formats, are available

commercially for diagnosis and genetic typing of BVDV and differentiation from other pestiviruses using primers and probes targeted at 5′-UTR (Hoffmann et al. 2006; Baxi et al. 2006; Willoughby et al. 2006; Liu et al. 2008).

14.16.2.3 Sequencing and Next-Generation Sequencing

Genetic typing of BVDV provides useful information during BVD epidemiology and control. The more accurate genetic typing of BVDV strains is achieved from nucleotide sequencing or next-generation sequencing data followed by phylogenetic analysis. Although sequence analysis of 5′-UTR can be used for pestivirus species assignment, sequence analysis of complete N^{pro} and E2 genes or combined datasets can classify them more accurately into genotypes/subtypes (Becher et al. 2003; Vilcek et al. 2001; Mishra et al. 2014). Recently, analyses of whole genome sequencing data of BVDV strains are being more frequently used.

14.17 Prevention and Control

Vaccination against BVDV has been commonly used to prevent BVDV infection by enhancing immunity in cattle populations with an aim to prevent or reduce clinical disease and prevent the spread of infection within a herd by reducing BVDV viremia and preventing fetal infection and generation of PI calf. At present, both modified live vaccine (MLV) and killed vaccines are used to control BVD. Vaccination may reduce the incidence of acute and persistent infections but may not prevent all infections in individual animals and vaccination failure is likely due to failure in broad protection arising from existence of three bovine pestivirus species (BVDV-1, BVDV-2 and HoBiPeV) and multiple subtypes. Currently most of the commercially available BVD vaccines contain antigens of BVDV-1a, BVDV-1b, BVDV-2a, or bivalent vaccines of different combinations, but no vaccine is available against HoBiPeV. Vaccination is only successful when a minimum coverage of the population is achieved with maximum number of non-susceptible animals. Although vaccination has been effective in field conditions, when used as a lone measure, it has not resulted in the elimination of BVDV-induced clinical disease or a significant reduction in BVDV losses (Ridpath 2013).

Although initially it was thought that control of BVDV infection is not possible, a range of well-planned control strategies have been designed over the years, with successful implementation of BVDV control programs in many European countries. The common strategies for control programs are identification and removal of PI animals, movement control of infected animals, strict farm biosecurity and surveillance. BVD control programs without use of vaccination have been used successfully in Scandinavian countries, Austria and Switzerland, whereas control with vaccination has been implemented in Germany, Belgium, Ireland, and Scotland (Moennig and Becher 2018). While compulsory and systematic control programs have been found most successful than voluntary control programs, there is a lack of official guidelines on BVD control in most of the countries.

14.18 Vaccines

14.18.1 Inactivated Vaccines

Since protection against homologous strains is better than heterologous viral strains, multivalent vaccines containing both BVDV-1 and BVDV-2 are better than monovalent vaccines. Development and use of BVD vaccine based on the predominant subtypes of BVDV circulating in a country has been advocated as the most viable option. BVDV-inactivated vaccine elicits primarily a humoral response targeted mainly at E2 glycoprotein with minimal cell-mediated response. As inactivated vaccines contain viral antigen(s) incapable of replication, the risk of adverse effects in vaccinates and the fetus in pregnant animals is minimal. Hence, it is safe and can be administered at any stage of gestation. But protective immunity is shorter, and booster doses are required for its efficacy, and neonatal pancytopenia, associated with the use of inactivated vaccines with powerful adjuvants, has been reported in several European countries. Fetal protection varies from incomplete to satisfactory. Despite inactivated vaccines are predominantly used, several countries allow the use of inactivated BVD vaccine before breeding, while some countries implement use of inactivated vaccine first followed by modified live BVD vaccine (two-step vaccination). Use of inactivated BVDV vaccine first and then vaccination with a live attenuated vaccine after 4 weeks have shown long-lasting immunity in vaccinates and in prevention of fetal infection following BVDV-1 and BVDV-2 challenge (Moennig et al. 2005).

14.18.2 Live Attenuated Vaccines

Live attenuated or modified live vaccine (MLV) against BVD was initially developed in the early 1960s, and more MLVs were produced and used subsequently. Besides generating antibody responses against E2, MLV vaccines are better inducers of CD4+ and CD8+ T-cells immune responses and provide a solid fetal protection (Reber et al. 2006). Although quite efficacious, the MLV vaccines have safety concerns due to possibility of reversion of virulence of attenuated virus, its ability to cause in utero infections, ovarian lesions leading to infertility in cows and mucosal disease, risk of contamination with adventitious viruses, and immunosuppressive effects. Hence, the MLVs are not recommended in the first 6 months of unvaccinated pregnant animals especially with ncp BVDV which may cross the placenta and infect the developing fetus. Most of the modern MLVs are prepared using cp BVDV vaccine strains. Vaccination with MLVs is not advised in calves younger than 6 weeks. To improve the safety of MLVs, a mutant ncp BVDV virus strain was developed with the deletion of N^{pro} gene and inactivation of endoribonuclease activity of E^{rns} and found to elicit immune response without crossing the placenta of pregnant cattle (Platt et al. 2017).

14.18.3 Recombinant and Vectored Vaccines

The protective efficacy of BVDV E2 antigen has been shown using various delivery platforms like viral vectors and DNA immunizations or as a recombinant protein produced in various expression systems. However, these vaccines are not available commercially either due to prohibitive cost or low protective efficacy. An adenovirus vectored subunit vaccine against BVDV, consisting of recombinant adenoviruses expressing three novel mosaic polypeptide chimeras targeting N^{pro}, E2, and NS2-3 antigens has been reported recently and is promising (Lokhandwala et al. 2017). The prototype vaccine has been shown to induce higher BVDV-1-specific neutralizing antibody titers and lower clinical scores in calves following BVDV-2 challenge, compared to higher BVDV-2-specific neutralizing antibody titers found after MLV vaccination.

14.18.4 DIVA Vaccines

Although marker vaccines which allow differentiation of infected from vaccinated animals (DIVA) by serological tests have been successfully used in many other animal viral diseases, in the case of pestiviruses including BVDV, the benefit of a marker vaccine is questionable, because the PI animals, which are the main sources of infection, do not produce antibodies against the homologous strain. However, serological DIVA test may be useful in countries or regions where BVDV has been eradicated, and search for novel strategies should continue to find a solution.

14.19 Antivirals

There is no specific treatment for BVDV-infected animals currently, but several strategies have been tried in vitro and in vivo to identify the antivirals. Treatment of PI animal with DB772 (2-(2-benzimidazolyl)-5-[4-(2-imidazolino) phenyl] furan dihydrochloride) has shown decrease in the viral load of infected calves but it caused rapid selection of drug-resistant mutants (Newcomer et al. 2013). Similarly, iminosugar N-butyldeoxynojirimycin (NB-DNJ), an endoplasmic reticulum α-glucosidase inhibitor, has an antiviral effect against BVDV. Antiviral activity of bovine bovIFN-α and boIFN-τ against BVDV was tried but was unsuccessful, and boIFN-τ reduced the BVDV level in serum transiently when injected into PI cattle, but virus titer returned to the pre-administration level at the end of the treatment course. Essential oil of *Ocimum basilicum* (basil) and monoterpenes were reported to inhibit BVDV in an in vitro experiment.

14.20 Other Measures

14.20.1 Identification and Removal of PI Animals

PI animals act as BVDV reservoirs and permanently shed large amounts of infectious virus. Hence, identification and removal of PI animals are the hallmark of BVDV control. Virological screening using cost-effective BVDV antigen ELISAs and molecular tests have been found useful in identification and elimination of PI animals in successful control programs. All Scandinavian programs were successful following this method without vaccination, and the countries became mostly free from BVDV after a few years. Following success in Scandinavian countries, this approach was then implemented in Austria and Switzerland with promising outcomes.

14.20.2 Biosecurity

Strict farm biosecurity should be taken into account as a part of any BVD control program due to the vulnerability of susceptible herds to reinfection by PI animals. Purchase or trade with untested cattle is the predominant factor of BVDV introduction. Reports of high prevalence of BVDV-1 and BVDV-2 in sheep and BVDV transmission from sheep to cattle pose hindrances to the success of BVD control and eradication programs. Similarly wild ruminants also present a potential threat during grazing of cattle in certain geographical areas.

14.21 Conclusions

Molecular analyses during the last two decades have deciphered several astonishing features of BVDV and have added significantly on virus/host interplay, but many aspects of BVDV biology including the genetic basis of attenuation are still obscure and await further work at the molecular level. Similarly, problems associated with classification and nomenclature of pestiviruses including BVDV should be resolved soon and so also the subtype assignment criteria and consistency in phylogenetic analyses methods and target genes. Due to the recognition of severe acute BVDV infections earlier and with involvement of novel BVDV-2c strains recently, monitoring the role of newly emerging strains of BVDV on disease severity and on acute and persistent infections should be continued in the future, and acute BVDV infections with mucosal lesions should not be ignored. The genetic and antigenic heterogeneity of bovine pestiviruses, diverse host range, and clinical outcomes pose challenges for both laboratory diagnosis and clinical diagnosis. With regard to diagnosis, virus isolation should be carefully undertaken by eliminating cross-contamination from laboratory handling and identification of BVDV in unusual hosts should be reported with caution. Moreover, no uniform approach exists in selection of correct BVDV strains for use in serological studies, selection of antigen ELISA kits and the use of correct cells for virus isolation. Inconsistencies in

selection of primers and probes to detect existing and new BVDV strains and other pestiviruses and development of new tests for detection and differentiation of all the three species of bovine pestiviruses have to be resolved in the near future. As currently available BVD vaccines, consisting of BVDV-1 or BVDV-2 strains, provide only limited protection against HoBiPeV strains, future strategies should aim at development of efficient vaccines having ability of broad protection and complete protection from fetal infection. Since contact of cattle with other domestic and wild ruminants can favor BVDV transmission, and there is a risk of introduction of HoBiPeV, surveillance strategies may be reviewed to ensure optimal performance of laboratory diagnostic tests for identification of PI animals.

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Avian Reoviruses

15

Deepak Kumar, Kuldeep Dhama, R. K. Agarwal, Sonal, Praveen Singh, G. Ravikumar, Yashpal Singh Malik, and B. P. Mishra

Abstract

The viruses under *Orthoreovirus* genus of *Reoviridae* family are non-enveloped, segmented double-stranded RNA virus, possess icosahedral symmetry, and replicate in the cytoplasm. The avian reovirus (ARV) is ubiquitously distributed worldwide in poultry and in other wild birds and causes severe arthritis and tenosynovitis in the affected birds. The clinical manifestation in the affected birds is lameness, malabsorption-related enteric dysfunction, runting-stunting syndrome (RSS), respiratory infections, and immunosuppression. Infection with ARV can incur production losses that are estimated to be $23,000 per affected flock (28,000 birds/flock). Several methods for diagnosis of ARVs are reported, viz., virus isolation, immunofluorescent staining, and immunoperoxidase histochemistry offer straight detection of viral antigens in tendon tissues. Our laboratory has applied a real-time loop-mediated isothermal amplification technique to develop a rapid, sensitive, and specific method for virus detection and quantification. Additionally, we have standardized sigma B protein-based dot-ELISA

D. Kumar (✉) · Sonal · P. Singh · G. Ravikumar · B. P. Mishra
Division of Veterinary Biotechnology, ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Uttar Pradesh, India

K. Dhama
Avian Disease Section, Division of Pathology, ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Uttar Pradesh, India

R. K. Agarwal
Division of Livestock Product Technology, ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Bareilly, Uttar Pradesh, India

Y. S. Malik
ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Uttar Pradesh, India

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which can be used as a simple, reliable, and inexpensive alternative to commercial ELISA kits for serodiagnosis of ARV. A number of high-throughput sequencing studies deciphers the host pathogen interaction in ARV infection. Our laboratory has established the role of sigma B protein in ARV pathogenesis. Various live and inactivated vaccines are available for prevention of the disease. Currently, there has been a dramatic increase in the number of clinical cases of reoviruses in poultry and commercial vaccines are unable to provide adequate levels of protection against disease. Research focused on new-generation diagnostics, and vaccine may provide easy and effective substitute as vaccines and diagnostics candidates for these highly divergent viruses.

Keywords
Avian reovirus · Loop-mediated isothermal amplification (LAMP) · Diagnosis · Vaccine · Sigma B and sigma C

15.1 Prologue

The viruses under *Orthoreovirus* genus of *Reoviridae* family are non-enveloped and segmented and have an icosahedral capsid composed of an outer and inner protein shell and a double-stranded (ds) RNA virus, which replicates in the cytoplasm. On the basis of the ability of reoviruses to induce cell fusion, *Orthoreovirus* genus is further divided into two phenotypic groups, i.e., fusogenic and non-fusogenic. The fusogenic group of reovirus can cause multinucleated cells known as syncytia which is formed as result of fusion of several cells into one another, whereas this ability is absent in non-fusogenic group as it does not produce syncytia. The non-fusogenic group includes mammalian reoviruses, whereas other members of this genus such as avian orthoreoviruses (ARVs), baboon orthoreoviruses (BRVs), and reptilian orthoreoviruses (RRVs) are fusogenic (Chua et al. 2008). The genome consists of ten segments of double-stranded RNA (dsRNA) which are L-class (L1, L2, L3), M-class (M1, M2, M3), and S-class (S1, S2, S3, S4) (Varela and Benavente 1994), encoding at least ten distinct virus-specific primary proteins in the reovirion, eight of which (lA, lB, lC, μA, μB, σA, σB, and σC) are primary translation products encoded by mRNAs (Varela et al. 1996), whereas the other two, μBN and μBC, originate by post-translational cleavage of their precursor μB. The other four proteins (μNS, σNS, p17, and p10) are nonstructural, as they are not found in mature reovirions, but expressed in infected (Martinez-Costas et al. 1997; Varela and Benavente 1994). Studies have implicated *Orthoreovirus* in the expression of enteric disease in predisposed individuals (Bouziat et al. 2017). The virus can be detected in feces and may also be recovered from nasal or pharyngeal secretions, urine, cerebrospinal fluid, and blood. In animals such as baboons and reptiles and in avian population, fusogenic strains can cause more serious illness, viz., neurological illness and pneumonia. However, in birds, this virus may even cause death (Chua et al. 2011). Mammalian reovirus strain 3 (MRV3) was isolated from samples of live and

dead animals like cattle (Kurogi et al. 1976a, b), dog (Binn et al. 1977), and bats (Lelli et al. 2013) exhibiting the symptoms of respiratory and gastrointestinal tract disease. The main focus of this chapter is avian reovirus being the economically important pathogen among all the classes of reoviruses.

15.2 History

Reoviruses (respiratory enteric orphan viruses), members of the family *Reoviridae* (Attoui et al. 2000; Mertens 2004), are taxonomically classified into ten different genera (Chua et al. 2008). The name is actually mnemonic for respiratory (r) enteric (e) orphan (o) virus. The *Orthoreovirus* was named as an orphan virus because it was not known to be associated with any known disease (Chua et al. 2008). Mammalian reovirus was discovered in the early 1950s when it was isolated from the respiratory as well as gastrointestinal tracts of both sick and healthy individuals (White and Fenner 1994). Avian *Reovirus* was initially discovered as pathogenic agents that induced tenosynovitis in young chickens and were subsequently found to be ubiquitous among poultry flocks. They cause arthritis/tenosynovitis, respiratory diseases, enteric conditions, pericarditis, and infectious proventriculitis (Jones 2000; Robertson et al. 1984; van der Heide 2000). They have also been found associated with other disease conditions including ruptured gastrocnemius tendons, osteoporosis, pericarditis, myocarditis (Franca et al. 2010), and hydropericardium. *Reoviruses* have been recovered from a variety of other domestic and wild birds. These include turkey (Lozano et al. 1989; Simmons et al. 1972), goose (Hlinak et al. 1998; Palya et al. 2003), pheasant, pigeon (Vindevogel et al. 1982), quail and psittacine birds (Conzo et al. 2001), and several other wild bird species (Hlinak et al. 1998; Hollmen et al. 2002; Huhtamo et al. 2007; McFerran et al. 1976; Sakai et al. 2008; Sanchez-Cordon et al. 2002). *Reoviruses* have also been isolated from duck species, such as Pekin (Jones and Guneratne 1984), mallard, and Muscovy ducks (Gaudry et al. 1972; Kuntz-Simon et al. 2002; McFerran et al. 1976). *Reovirus* was firstly isolated in India in 1987 (Pradhan et al. 1987).

15.3 Incidence and Prevalence of *Orthoreovirus* Disease

Reovirus infections in poultry are global and have emerged in the poultry population of many countries. It is also known to cause immunosuppression and stress and reported to act synergistically with other infections like NDV, IBDV, IB, and CIA (Bhardwaj Nitin et al. 2004). Depending on the degree of severity, the affected birds may be unable to walk resulting in poor growth, poor production, and sometimes death. Emerging reoviruses can cause up to 10% mortality and 20–40% morbidity in broiler chickens, which may result in significant economic losses (Lu et al. 2015). ELISA-based surveillance showed 98.3% prevalence in Canada (Ayalew et al. 2017) and Iran (Bokaie et al. 2008); however, less prevalence is reported from Romania (85.5%) (Oana et al. 2014) and Nigeria (41%) (Ni and Kemp 1995).

Mammalian orthoreovirus was isolated from diarrheic pigs from North Korea (Kwon et al. 2012), the United States (Thimmasandra Narayanappa et al. 2015), northeastern China (Zhang et al. 2011), and Italy (Lelli et al. 2016).

15.4 Immunobiology

The disease caused by viruses of *Orthoreovirus* genus is very much dependent on the age and immune status of the host, virus pathotype, and route of exposure. Tenosynovitis/arthritis (Olson and Kerr 1966; Walker et al. 1972) and osteoporosis (van der Heide et al. 1981) were induced by *Orthoreovirus* at a younger stage in broiler poultry. These diseases cause acute lameness of birds affecting the tibiotarsal-tarsometatarsal joint (hock joint), the main load-bearing joint of birds. The affected joints are swollen with rupture of gastrocnemius muscle and, in severe cases, accompanied with hemorrhage causing green coloration of the skin at the joint. The mortality in birds is due to reduced feed conversion. Most importantly, S-class genome plays an important role in inflammation and apoptosis. Sigma B and sigma C proteins were found to be more conserved than other S-class proteins among *reoviruses* genes (Yin et al. 2013a, b). Sigma C is a component of the outer capsid layer of the virus and is shown to be the cell attachment protein. Sigma C protein expressed in *E. coli* has been used to detect ARV antibodies. Previous studies using mouse monoclonal antibodies revealed that three avian reovirus proteins (σB, σC, λB) are responsible for the induction of neutralizing antibodies (Meanger et al. 1995; Shapouri et al. 1996; Wickramasinghe et al. 1993). The sigma C outer capsid protein induces type-specific and to some degree group-specific neutralizing antibodies, and σB and λB proteins induce group-specific neutralizing antibodies.

Reovirus induces apoptosis in various cell lines including chicken embryonic fibroblast cells (Labrada et al. 2002; Shih et al. 2004). Apoptosis is initiated by death-receptor activation and subsequent action of the death receptor-associated initiator caspase 8 which translocates to the mitochondria. In the mitochondria, caspase 8 along with Bcl-2 leads to activation of intrinsic mitochondrial-associated apoptotic signaling pathways (Clarke and Tyler 2003). FAST protein serves as a membrane fusion protein as well as aids dissemination of infection as it stimulated apoptotic-induced disruption of syncytia (Salsman et al. 2005). Reoviral FAST protein-induced syncytial cells died in a manner characteristic of apoptosis. Reovirus FAST-induced syncytium formation triggers an apoptotic cell response and membrane leakage which was due to syncytium-triggered apoptotic response (Salsman et al. 2005). It has been reported that transient expression of a recombinant reovirus protein sigma C causes the activation of the apoptotic program (Shih et al. 2004), suggesting that the apoptosis is triggered in reovirus-infected cells by two different mechanisms before and after viral gene expression.

The gene expression profiles of vero cells in response to ARV strain S1133 infection and ARV-encoded pro-apoptotic protein σC overexpression were examined using microarray (Lin et al. 2011). Two naturally occurring ARV variant strain coinfections were identified, and complete genome was sequenced. Both viruses were

having the same M2 segment but were distantly evolved in nine other segments (Tang et al. 2016). A gene expression RNA-Seq study showed that ARV inoculation of chicken fibroblast DF-1 cells lines stimulates a prolonged antiviral response in host cells and interferes with cell growth and death pathways (Niu et al. 2017). Most recently, the significance of osteoarthritis pathway and the role of IL-17A was shown in ARV-induced arthritic changes (Praharaja et al. 2018). Cartilage degradation is mediated by osteoclasts, chondrocytes, and synovial fibroblasts when the inflamed synovium invades the adjacent cartilage. Cytokines such as IL-1, IL-6, IL-17, and TNF induce a switch in the synthesis pattern of chondrocytes from an anabolic state to a catabolic state. Figure 15.1 (unpublished data) shows the role of osteoclasts in ARV-induced arthritic changes.

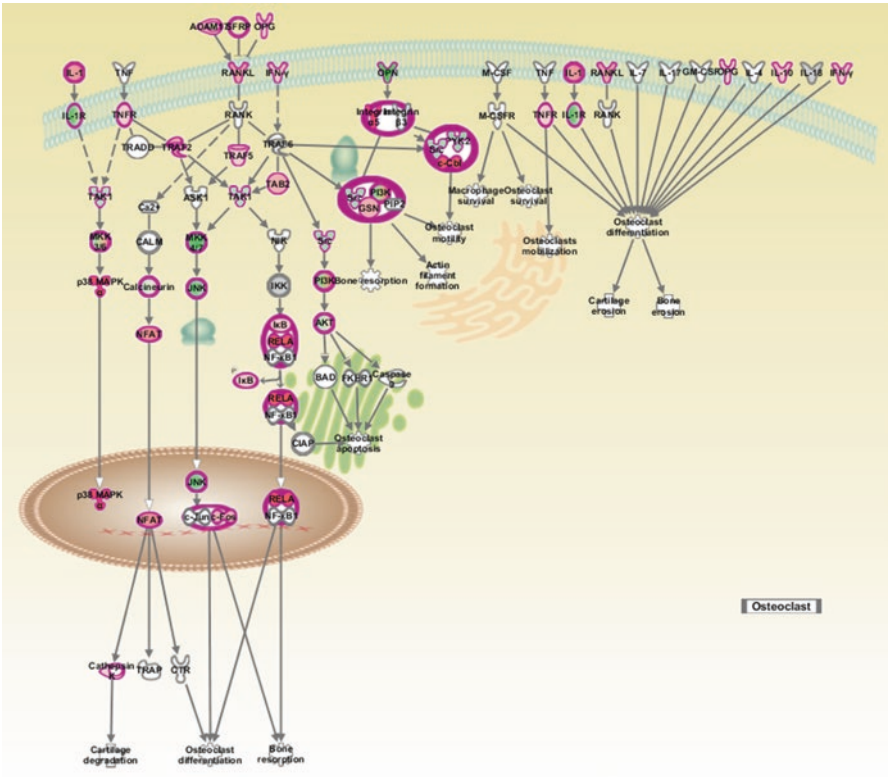

Fig. 15.1 Role of osteoclasts in ARV-induced viral arthritis
Delineated mechanism of the role of osteoclasts in ARV-induced viral arthritis. Pathway contains the differentially expressed genes related to ARV-infected cells showing their mode of action in arthritis activation. Red color indicates the upregulation and green color indicates the downregulation of a particular gene

15.5 Diagnostics

As reovirus infection is widespread worldwide, these viruses are rarely the only cause of a disease. In chickens, the major manifestation of disease is lameness. Isolation of reovirus from the joints may be considered as gold standard for diagnostic purpose, but isolation from the feces or gut tissue may be meaningless as Al-Mufarrej et al. found that after experimentally induced infection of hens with high titer of virus, no virus was detected in cloacal swabs, even though tissues of chicks hatched from eggs laid at that time were positive for virus (Al-Muffarej et al. 1996). Many laboratory methods have been developed for the detection of antibodies against reoviruses, including serum neutralization (Wickramasinghe et al. 1993), immunodiffusion (Meanger et al. 1995), immunoblot assay (Ide and Dewitt 1979), immunofluorescence (Ide, 1982), agar gel precipitation test (AGPT), indirect fluorescent antibody (IFA) assay, virus neutralization (VN) assay, agar gel immunodiffusion test (AGID) (Olson and Weiss 1972; Zhang et al. 2007), enzyme-linked immunosorbent assay (σB-σCELISA) (Yang et al. 2010), dot blot assay (Majumder et al. 2018), etc. Molecular-level diagnosis techniques include routine PCR (Smith et al. 1998), multiplex PCR (Caterina et al. 2004), reverse transcription loop-mediated isothermal amplification assay (RT-LAMP) (Xie et al. 2012), real-time probe-based loop-mediated isothermal amplification (RT-Cy5 qLAMP) (Kumar et al. 2017), etc. (Fig. 15.2).

15.6 Risk Factors

Previous studies suggested that the risk factors involved in *reoviruses* infection is age-linked. Infant or newborn individuals are at the highest risk of *reoviruses* infection due to their inability to mount an effective immune response against the pathogen (Jones and Guneratne 1984). Many strains of *reoviruses* have been identified in animals and birds around the world since its isolation (Fahey and Crawley 1954). *Orthoreoviruses* strains show a broad spectrum of pathogenicity, which can be challenging to reproduce experimentally (Clark et al. 1990; Rosenberger et al. 1989). In addition, approximately 80% of isolated *reoviruses* strains are nonpathogenic. Avian reovirus-associated diseases may be a result of coinfection with other infectious pathogens. A number of studies reported that infectious bursal disease virus, chicken anemia virus, and *Mycoplasma synoviae* have been found in the joint lesions of chickens with tenosynovitis (Bradbury and Garuti 1978; Jones and Guneratne 1984; McNeilly et al. 1995; Moradian et al. 1990).

15.7 Transmission

Reovirus is transmitted both vertically from parents and horizontally from pen mates. The vertical transmission occurs not only during acute phase of infection but also at intervals throughout life. The virus is excreted through intestinal as well as

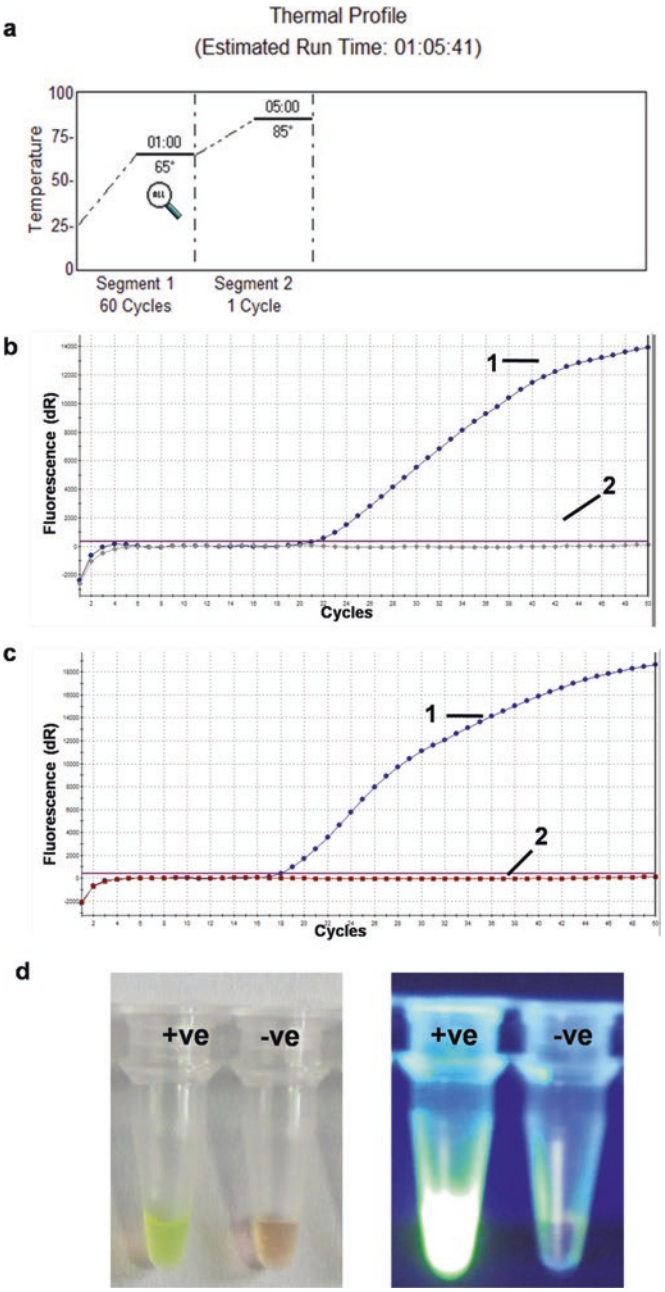

Fig. 15.2 Standardization of Cy5-RTqLAMP reaction for detection of ARV. (**a**) Thermal profile setup for running Cy5-RTqLAMP reaction. (**b**) Amplification plots with viral cDNA. (**c**) Amplification plots with the Sumo-S3 plasmid-gene construct. (**d**) Alternatively, end point detection of presence or absence of virus in the sample is possible in this assay by the addition of SYBR Green I dye under day and UV light

respiratory routes, which contaminate the environment and lead to virus intake orally. It may persist for long periods in the cecal tonsils and hock joint of birds infected at a very early age, which may act as a potential source of infection for pen mates. As a result of the short-lived persistence of the virus in trachea, it would appear that the spread is probably by the ingestion of fecal contaminated feed and water. Infection of chicken with pathogenic strains of reoviruses has been shown to result in a transient immunosuppression by affecting both humoral and cellular immune response (Al-Muffarej et al. 1996).

15.8 Prevention and Control

Vaccination is an important preventive measure against reovirus infections in birds. It involves a live S1133 vaccine followed by an inactivated vaccine containing strains 1733 and/or 2408. These strains are related to tenosynovitis and stunting syndrome pathologic conditions and belonging to the same subtype (Giambrone and Solano 1988; van der Heide et al. 1983). When a live vaccine is used, it should be administered before the onset of egg production. Attenuated reovirus is usually applied to the subcutaneous route. The commercial vaccines available in the market against viral arthritis/malabsorption syndrome are not effective against the newly encountered chicken viruses or against those causing arthritis in turkeys. In India, inactivated vaccines like Nobilis Reo inac from Intervet India Pvt. Ltd, live strain Nobilis® Reo *2177*, and Polyvax (NBGR) from Indovax India are available. Although chickens were vaccinated, *reoviruses* pathogens still persist in Tunisian poultry (Hellal Kort et al. 2013). Development of an edible vaccine against reovirus infection expressing σC protein in alfalfa cells is a new-generation vaccine candidate to control infection in the poultry industry (Huang et al. 2006). Sigma C protein is an important vaccine candidate as it represents the initial point of contact between the pathogen and the host. Lithium chloride is a potential antiviral agent against MRV3. Vero cells infected with mammalian orthoreovirus serotype 3 showed inhibition in early stage when treated with lithium chloride (LiCl) (Chen et al. 2016). The failure of conventional vaccines and the increased rate of diagnosis of the disease in the last 5 years are alarming, indicating that the emerging pathogenic *reoviruses* are becoming imminent threats to the livestock and poultry industry across the world. The effectiveness of a vaccine largely depends on the antigenic similarity between the vaccine and field strains (Ayalew et al. 2017).

Serotyping studies are recommended to further characterize the antigenic characteristics of these isolates and adapt the suitable vaccination program, which is time-consuming and difficult and requires technical expertise. New-generation diagnostics and vaccine can be an easy and effective substitute for the viruses having high antigenic diversity like ARV. Identification and selection of field isolates for use in development of new-generation diagnostics and autogenous vaccines can be difficult especially when multiple reoviruses are co-circulating among flocks. However, field data suggests that in some cases the custom diagnostics and vaccines

are providing adequate identification and protection against disease, but as new genetic variants emerge, new diagnostic assay and vaccines are needed.

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Conflict of Interest There is no conflict of interest.

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Avian Infectious Bronchitis Virus

16

Saravanan Ramakrishnan and Deepthi Kappala

Abstract

Avian infectious bronchitis (IB) is caused by avian infectious bronchitis virus (IBV) belonging to *Coronaviridae* family. The disease is prevalent in all countries with almost 100% incidence rate. Chicken and commercially reared pheasant are the natural host for IBV. Virus causes respiratory diseases, poor weight gain, feed efficiency in broiler, damage to oviduct, and abnormal egg production in mature hens resulting in economic losses. IBV also replicates in tracheal and renal epithelial cells leading to prominent tracheal and kidney lesions. Virus undergoes spontaneous mutation leading to continual emergence of new variants. The effectiveness of immunization program is diminished because of poor cross-protection among the serotypes. Identification of circulating serotypes is important in controlling IBV infection. Toll-like receptor 3 (TLR3) and TLR21 are involved in early recognition of virus resulting in induction of inflammatory cytokines. Both humoral and cellular immune responses are important in the control of infection. Humoral immunity plays an important role in recovery and clearance of viral infection. IBV-specific cytotoxic T lymphocytes induce lysis of IBV-infected cells. Effective diagnostic tools are required at field level to identify different IBV variants. Embryonated chicken eggs are effective model for virus isolation. Identification by other specific methods like virus neutralization (VN), hemagglutination inhibition (HI), enzyme linked immunosorbent assay (ELISA), immunohistochemistry, or nucleic acid analysis or by electron microscopy is also indispensable. VN test in tracheal organ culture is the best method for antigenic typing for surveillance purposes. Continuous epidemiological surveillance, strict biosecurity measures, and vaccine effective against various serotypes are necessary for controlling IB in chickens.

S. Ramakrishnan (✉) · D. Kappala
Avian Immunology Laboratory, Immunology Section, ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Uttar Pradesh, India

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16.1 Prologue

Avian infectious bronchitis (IB) is an acute, highly contagious upper respiratory tract disease affecting chicken of all ages with significant economic threat to the poultry industry. It is caused by infectious bronchitis virus (IBV), which belongs to the genus *Gammacoronavirus*, family *Coronaviridae*, in the order *Nidovirales*. The disease causes symptoms like watery eyes, mucus in the nares and trachea, sneezing, tracheal rales, and coughing. In layer birds, IB results in decreased egg production as well as quality, and some IBV variants cause interstitial nephritis. Infection with IBV causes ciliostasis in the trachea and predisposes to the secondary pathogens further complicating the disease. Morbidity is always 100%; however, mortality can vary from 0 to 82% depending upon the age of the birds, immune status, strain of the virus, and involvement of secondary pathogens. The virus possesses a single copy of positive-sense single-stranded RNA as its genome, which is approximately 27.6 kb in size. The genome undergoes genetic recombination and spontaneous mutation leading to the emergence of new variants having low level of cross-protection and complicates the control program by vaccination (Cavanagh and Gelb 2008; Jackwood et al. 2012). The IBV-like coronaviruses were also detected in pheasant, peafowl, turkey, teal, geese, pigeon, guinea fowl, partridge, penguins, and ducks (Dea and Tijssen 1989; Jonassen et al. 2005; Cavanagh 2005; Circella et al. 2007). The IBV is worldwide in distribution, and infection is acquired through inhalation or direct contact with infected birds or premises. Vertical transmission is not reported. Some of the IBV strains can also replicate in the kidneys, reproductive tract, and enteric surfaces resulting in the development of nephritis, misshapen eggs, and enteritis, respectively (Boltz et al. 2004). Induction of apoptosis in kidney cells is a major contributor of pathogenicity for virulent nephropathogenic IBV (Liu et al. 2017). The disease affects both egg and meat type of chickens. Respiratory disease is observed in the young growing chickens while reduced weight gain and feed efficiency in broiler chickens. IBV infection predisposes the broiler birds to secondary bacterial infection resulting in airsacculitis, pericarditis, and perihepatitis. In layers and breeder chickens, IBV replicates in the oviduct causing permanent damage or limited egg production. Pigment of the affected shell becomes paler and albumin is watery in consistency. The virus has not been reported to cause human infection.

The virus contains lipid envelope with a round to pleomorphic shape. The virus particles are approximately 120 nm in diameter with club-shaped spikes in their surface, which provide them a crown-like appearance (Jackwood and de Witt 2013). The genome encodes 4 structural proteins, 15 nonstructural proteins (nsp 2–16), and at least

5 lineage-specific accessory proteins (Dent et al. 2015). Structural proteins are spike (S), membrane (M), small membrane (E), and nucleoprotein (N). The spike protein is post-translationally cleaved into S1 and S2 subunits where the former is involved in virus entry to the host cell via sialic acid receptor and also harbors virus-neutralizing epitopes (Lai and Cavanagh 1997; Cavanagh 2007; Cavanagh et al. 1986; Koch et al. 1990; Niesters et al. 1987). S2 subunit anchors the spike protein, and the ectodomain of S2 protein also aids in the attachment of virus to the host cells (Promkuntod et al. 2013). M protein is the most abundant transmembrane protein that interacts with N and S glycoprotein for virion assembly (de Haan et al. 2000; Bande et al. 2015). E protein is scant and confined to the Golgi apparatus of the infected cell. This protein plays a key role in viral envelope formation, assembly, budding, ion channel activity, and apoptosis (Corse and Machamer 2003; Wilson et al. 2006). Ribonucleoprotein is formed by interaction of N protein to the genomic RNA of IBV and aids in transcription, replication, and translation of viral genome (Jayaram et al. 2005).

16.2 History

Schalk and Hawan in 1931 first reported a respiratory disease of chick that is characterized by gasping and listlessness in North Dakota, USA. Two years later, similar disease was reported by Bushnell and Brandly. Since the disease could be transmitted by Berkefeld filtered material, they recognized the causative agent as filterable virus and was confused with infectious laryngotracheitis virus (ILTV; Bushnell and Brandly 1933). The causative agent was established in 1936 by Beach and Schalm who proved that IBV was distinct from ILTV by cross-immunity studies. Although young chicks were found to be affected by IBV, later it was observed to be common in mature and laying hens. IBV was first cultivated in chick embryos by inoculating through chorioallantoic membrane (CAM). Unlike ILT, IBV did not show distinctive lesions on CAM and rather resulted in embryo death (Beaudette and Hudson 1937). Jungherr and coworkers first reported more than one serotypes of IBV. It has been shown that Massachusetts (Mass) isolate in 1941 and Connecticut (Conn) isolate in 1951 produced identical disease but did not cross-protect each other (Jungherr et al. 1956).

16.3 Transmission and Risk Factor

IBV is a highly infectious disease and birds show clinical signs within 36–48 h of infection. Infected chicks are the major source of virus contamination in the environment. Virus is transmitted through respiratory secretions and fecal droplet from infected chicken. Virus may also spread from one flock to another through inanimate objects including contaminated utensils, egg-packing materials, fertilizer, and farm visit. Vertical transmission of virus has not been reported. Trachea, kidney, and bursa of Fabricius show the presence of the virus as early as 24 h and through the seventh day following aerosol transmission (Cavanagh and Gelb 2008). The

presence of virus in the cecal tonsils up to 14 weeks and from the feces up to 20 weeks correlates with the viral transmission through fecal shedding (Alexander and Gough 1977). Birds recover from infection within 14 days with a rise in antibody titer. It has been reported that IBV re-excreted from virus-negative birds for several weeks following recovery from inoculation at 1 day of age. Isolation of virus from tracheal and cloacal swabs at the time of lay and 19 weeks of age was reported (Jones and Ambali 1987). Establishment of latent infection and erratic shedding of virus in feces and respiratory secretion for a prolonged period of time are possible in a very few cases (Ignjatovic and Sapats 2000). Kidney may be one of the sites of persistent infection (Raj and Jones 1997). Vaccine viruses persist up to 163 days or more in various internal organs (Gay 2000). Prolonged or intermittent shedding of virus is considered as a potential risk factor for transmission of virus from one flock to another through contaminated equipment or persons.

16.4 Incidence and Prevalence

Spontaneous mutation of S1 subunit of S gene resulted in the emergence of various serotypes (Abro et al. 2012). IBV strains can be classified by genotyping- and serotyping-based methods. Viral serotypes can be distinguished by cross-neutralization test and genotypes by reverse transcriptase polymerase chain reaction (RT-PCR) techniques, bioinformatics, and gene sequencing technology. S1 gene of IBV variants can be amplified and the sequences are analyzed by Basic Local Alignment Search Tool (BLAST; Posada and Crandall 2001). IBV strains like Mass, 4/91, D274, and QX-like are reported worldwide. Some of the IBV strains are more prevalent in particular geographical location. M41, Arkansas (Ark) and Conn are common in the United States, while 4/91 (793/B, CR88) and D274 are predominant strains in Europe. In recent times, the Chinese QX variants have caused outbreaks in Europe, Asia, the Middle East, and Africa (Bande et al. 2015).

Predominant strains of IBV isolated in China are QX-like strains (Ma et al. 2012). IBV QX strain-affected chickens showed proventriculus swelling which was first documented in 1996 (Yudong et al. 1998). The proportion of QX isolates has increased from 20% in 2000 to 60% in 2007. Recent isolates in China are LDT3 and 4/91 (Feng et al. 2014; Li et al. 2010; Ji et al. 2011). Attenuated live strains such as H120, LDT3, 4/91, and inactivated M41 are the commercially used vaccine strains in China. Previous studies reported that Mass type of strains is widely in circulation. Recombination of Mass type with other strain resulted in complete cross-protection of Mass strain by H120 strain. However, two recombinant strains (CK/CH/LDL/110931 and CK/CH/LHB/130573) are highly variable in S1 gene from Mass type and are not cross-neutralized by H120 strain (Chen et al. 2015; Han et al. 2011). Since 2009, the emergence of Taiwan group (TW-I and TW-II) of strains have been increased in China (Xu et al. 2016; Feng et al. 2014; Zou et al. 2010; Luo et al. 2012). Recombinants GD strain, created from QX and TW-I strains, induced renal lesions, respiratory symptoms, and 40% mortality (Xu et al. 2016; Yan et al.

2016). JP-I, JP-II, and JP-III are three genetic groups of IBV in Japan. Different variants of 4/91 strains are isolated (Inoue et al. 2008; Shimazaki et al. 2009). Two variants of 4/91 strain (JP/Wakayama/2003 and JP/Iwate/2005) are cross-neutralized by 4/91, JP-I and 4/91, JP-II strains, respectively. Ibaraki strain isolated in 2009 was distinctly related to other Japanese strain. Korean group-I and group-II are the two genetic groups of IBV that exist in Korea. Group-I is closely related to Mass type. There are three subgroups of group-II, namely, KM91-like, New Cluster 1 (NC1), and QX-like (Lim et al. 2011). Korean group-II isolates that emerged from 2005 to 2010 were nephropathogenic (Lim et al. 2011). Since 2009, predominant isolates circulating in Thailand are QX-like variants (Promkuntod et al. 2015). Indian IBV isolate PDRC/Pune/Ind/1/00 was found to have a unique S1 sequence compared to reference strains from the United States, Europe, Mexico, and Australia and found as nephropathogenic (Bayry et al. 2005). 4/91 type strain was first isolated in India by Sumi et al. in 2012. An Indian IBV strain (India/LKW/56/IVRI/08) showed 99% homology with a Thailand strain (THA280252), while another isolate (India/NMK/72/IVRI/10) showed similarity with the United Kingdom (4/91 pathogenic strain), Japan (JP/Wakayama/2/2004), and China (TA03) (Sumi et al. 2012). Indian strain that was isolated in 2015 by Patel and coworkers was similar to Mass type vaccine M41 strain. Six IBV genotypes, namely, 4/91, IS/1494/06, Mass, IS/885/00, Q1, and D274, were detected in the Middle East from 2009 to 2014 (Ganapathy et al. 2015). Analysis of complete genome sequence of a field strain of IBV from the northern part of India indicated the emergence of a genotype I variant of IBV (Jakhesara et al. 2018). China-like strains (CK/CH/Guangdong/Xindadi/0903 and CK/CH/LDL/97I) are also reported in the Middle East (Seger et al. 2016; Ababneh et al. 2012). LDL/97I-like strains isolated in the Middle East showed extensive tissue tropism such as trachea, kidneys, ovarian tissue, and cecal tonsils, whereas original LDL/97I strains were limited to the respiratory system and kidneys.

IBV isolates in Egypt are divided into the Egy/Var I, Egy/Var II, and Mass type groups (Zanaty et al. 2016). QX-like strains and Italy 02 type strains were first isolated in Zimbabwe (Toffan et al. 2011) and Morocco, respectively (Fellahi et al. 2015). Variants such as Conn, Mass, Florida, Clark 333, Ark, Holte, and Gray were identified in North America (Jungherr et al. 1956; Winterfield and Hitchner 1962; Brown et al. 1987; Butcher et al. 1989; Kinde et al. 1991). However, Mass, Conn, and Ark are the common serotypes that are reported. A nephropathogenic IBV strain (DMV/1639/11) was detected in Delmarva in 2011. When a vaccine containing a mixture of Mass, Conn, and Ark administered through intraocular route, virus shedding and renal lesions were decreased following DMV/1639/11 challenge (Gelb et al. 2013). Cal99 strain could affect only the respiratory tract, while Cal99 variants have a tendency to spread to the kidneys, gastrointestinal tract, and the bursa in addition to the respiratory system (Franca et al. 2011). Other IBV variants such as CAV, DE072, and MX97–8147 have also been reported (Jackwood et al. 2005). The D207, Mass, Conn, and Ark serotypes were detected in Brazil (Felippe et al. 2010).

16.5 Immunobiology

Innate Immunity Toll-like receptor 3 (TLR3) on innate immune cells is involved in the early recognition of the virus (Ariaans et al. 2008). TLR3 mRNA expression was more following IBV-M41 strain infection (Wang et al. 2006), and upregulation of TLR3 and TLR7 mRNA was detected in the trachea and lungs when chicks were intratracheally infected with Conn strain (Kameka et al. 2014). Nephropathogenic IBV infection significantly increases chicken myeloma differentiation antigen 5 (MDA5) expression in the kidneys, which indicate the role of chicken MDA5 against IBV infection (Cong et al. 2013). Chicken mannose-binding lectin (MBL), a member of C-type collectin family, has antiviral activity against IBV. It binds to S1 protein of IBV and blocks the attachment of viral particles to the surface of the susceptible cells in the chicken trachea (Zhang et al. 2017). In addition, MBL also plays a major role in shaping innate as well as adaptive immune responses against IBV (Hamzic et al. 2016; Juul-Madsen et al. 2011; Kjaerup et al. 2014b). Further, a high level of MBL contributes to the clearance of IBV from the trachea (Juul-Madsen et al. 2011; Kjaerup et al. 2014a).

During viral infection, cytokines are involved in the protection of adjacent cells as well as facilitate the activation and migration of T lymphocytes to the infection site (Guo et al. 2008). IBV infection induces interferon in the trachea, lung, kidney, liver, and spleen (Otsuki et al. 1987). Replication of IBV strains both in vitro and in vivo was inhibited in the presence of IFN-α. Both IFN-α and IFN-β transcripts are elevated in the trachea after IBV infection. Among type I interferon, IFN-β has a dominant role in the innate immune response against IBV infection (Yang et al. 2018). IFN-γ contributes to the tracheal lesions, and IFN-λ plays a major role in the host resistance against IBV especially in the trachea (Yang et al. 2018). IBV can act as a polyclonal stimulator of IFN-γ production in the chicken leukocytes, which is IBV-specific as other chicken viruses were unable to do the same (Ariaans et al. 2009). Proinflammatory cytokines like IL-6 and IL-1β were upregulated at 3 days post-IBV-M41 infection. These cytokines have association with increased viral load and tracheal lesions as well as defects in eggshell components in the laying hens (Okino et al. 2014; Nii et al. 2014).

Macrophages and heterophils are important innate immune cells during the initial phase of infection. Heterophils are the first phagocytic cells recruited at the infection site. Upon phagocytosis, heterophils degranulate cytotoxic molecules like cathepsin and bactericidal permeability increasing protein and thus try to neutralize phagocytosed pathogen. Heterophils are responsible for destruction of IBV-infected cells by phagocytosis and oxidative lysosomal enzyme release during the initial phase of infection (Guo et al. 2008). Further, depletion of heterophils causes more severe nasal exudation in comparison with control when infected with IBV (Raj et al. 1997). Activation of macrophages is based mainly on the action of IFN-γ (Caron 2010). Macrophages were found to increase in number in the tracheal and bronchial lumen of chickens at 24 and 96 h post-IBV-M41 infection (Fulton et al. 1993). Similarly, chickens infected with Conn strain significantly increased macrophages in the lungs

and trachea at 24 h post-infection (hpi), suggesting the role of respiratory macrophages in limiting replication of virus within respiratory tissues (Kameka et al. 2014). Recruitment of macrophages and production of IL-1β play a major role in the host responses against IBV infection (Amarasinghe et al. 2018).

Adaptive Immunity Humoral and cellular immune responses are important in the control of infection. Local antibody level but not serum antibodies are involved in the protection of respiratory tract epithelium (Raggi and Lee 1965). Hyperplasia of goblet cells and alveolar mucous glands resulting in seromucous nasal discharge and catarrhal exudates in the trachea are believed to be the first component of innate immunity (Nakamura et al. 1991). Serum anti-IBV IgG can be detected at 4 days post-infection (dpi) and peaking at about 21 dpi. In an in vitro study, activation of memory B cells by IBV results in the secretion of antibodies at 21 dpi (Pei and Collisson 2005). IgG antibody titer was highest at 7 dpi in the lachrymal fluid and possibly remaining up to 23 dpi. Similarly, in oviduct washes, antibodies were detected at 7 and 23 dpi (Raj and Jones 1996).

IBV-infected chickens showed anti-IBV IgA antibodies in the lamina propria, trachea, and between the epithelial cells of trachea (Joiner et al. 2007; Nakamura et al. 1991). Anti-IBV IgA was detected in the lachrymal fluid at 10 days post-live attenuated Ark DPI-type vaccination. However, there was no further increase in IgA level upon challenge, suggesting the significant role of neutralizing antibodies in reducing the potency of IBV infection at the time of challenge (Joiner et al. 2007). In our lab, administration of resiquimod (R-848), a TLR-7 agonist with inactivated or live IBV vaccine increased the secretory IgA, which was mediated through the enhanced expression TGF-β4 in the chicken (Matoo et al. 2018).

Humoral immunity plays an important role in recovery and clearance of viral infection. Bursectomized chicks showed increase in tissue viral load as well as severe and long-lasting illness (Cook et al. 1991). As the titer of humoral antibodies increased, re-isolation of virus from kidneys and genital tract decreased (Macdonald et al. 1981). This increase in antibody titer also protected against drop in egg production and viral spread from trachea to other susceptible organs (Box et al. 1988; Raj and Jones 1997). Conversely, it has also been shown that chickens were more susceptible to IBV with high tear antibody titer, whereas low tear antibody titer protected the birds from IBV. This suggested that not only antibody but other immune defense mechanisms are also important in clearance of infection in tear (Gelb et al. 1998).

Nephropathogenic Gray strain-infected chickens showed increased IBV-specific cytotoxic T lymphocytes (CTLs) at 3 dpi with peak level at 10 dpi (Seo and Collisson 1997). A study assessing the immunophenotypes of mononuclear cells in the tracheal mucosa revealed the presence of CTLs at 3 or 4 dpi suggesting the role of CTLs in viral clearance during the early phase of infection (Kotani et al. 2000). CD8+ T cells play an important role in controlling IBV infection. This IBV-specific CTL activity was dependent on S and N proteins of IBV, and it can induce lysis of IBV-infected cells (Collisson et al. 2000). While CD8+ T cells play an important role in early immune response, CD4+ T and B cells are involved in long-term control of IB.

Maternally Derived Antibodies IgG antibodies were transferred from vaccinated hens to the respiratory mucus of newly hatched chicks (Hawkes et al. 1983). One-day-old chicks with high level of maternal antibodies showed more than 95% protection against IBV-Mass strain challenge. However, this was not evident at 7 days of its age. Protection was associated with local respiratory antibodies but not with serum antibodies (Mondal and Naqi 2001). Day-old chicks when vaccinated with live IBV-Mass strain showed no IBV-specific antibody response. This may be due to binding and neutralization of vaccine virus by maternal antibodies (Mondal and Naqi 2001). Prime boost strategies involve vaccination with live strain followed by inactivated vaccine to protect hens throughout their laying period as well as to transfer high level of antibodies to their offspring (Chhabra et al. 2015).

16.6 Diagnosis

Virus Isolation Since trachea is the primary site of IBV infection, tracheal swab and tracheal tissues are the preferred sampling material within 1 week of infection. Cloacal swabs or cecal tonsils are the sample of choice during postmortem examination if time is elapsed more than 1 week from the start of infection. This may be due to the initial growth of virus in the upper respiratory tract and eventual spread to the non-respiratory organs. This results in the early clearance of virus from trachea than from the intestinal tissues (Alexander and Gough 1977; Jones and Ambali 1987; Lucio and Fabricant 1990). Based on clinical history, sampling of other organs like the lung, kidney, and oviduct should also be considered. Sentinel chickens should be placed in the problematic flock where direct sampling method is difficult. After 1 week of contact exposure, sentinels are removed for direct sampling method (Gelb et al. 1989).

Isolation in Embryonated Eggs Embryonated chicken eggs infected with IBV shows characteristic signs including dwarfism, curling, hemorrhages, and death (Fig. 16.1). Field samples were inoculated through intra-allantoic route of 9- to

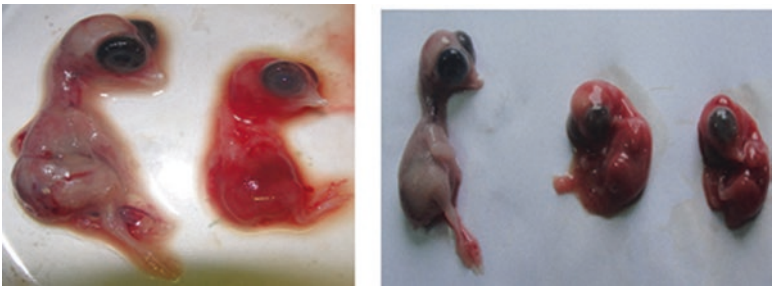

Fig 16.1 Comparison of 12-day-old normal (left) and dwarfed and IBV-infected embryos (right) of the same age

10-day-old embryonated eggs and incubated for 4 days. Allantoic fluid were collected and again passaged to analyze the morphology of the embryo. IBV titer reaches its maximum about 1 to 2 days post-inoculation. Although chicken embryonated eggs are effective model for virus isolation, it has the drawback of need for three successive passages for manifestation of characteristic lesions. In addition, reduced virus yield may be observed when IBV is inactivated due to improper preservation (Villarreal 2010).

Cultivation in Tracheal Rings Tracheal rings were collected from 19- to 20-day-old SPF embryo and placed in a tube containing culture media and antibiotic. This was incubated for 48 h, and only the rings with more than 50% ciliary motility are used. After removing the culture media, 0.1 ml of sample was added and incubated for 1 h for viral adsorption. One ml of culture media was once again added and incubated. Tracheal rings were evaluated for ciliary motility at 24, 48, 72, and 96 h post-inoculation. Ciliary motility decreases as IBV replicates in the tracheal cells (Epiphanio et al. 2002). This technique also has disadvantage of not being sensitive for IBV field samples and may result in false-negative results. Thus, IBV should be confirmed by other specific methods like virus neutralization (VN), hemagglutination inhibition (HI), ELISA, immunohistochemistry, or nucleic acid analysis or by electron microscopy.

Serology Different serotypes of IBV show high amino acid similarity within N, M, and S2 protein, and hence these regions are called group-specific antigens. The antibodies against group-specific antigen of one serotype can neutralize different serotypes, while antibodies against S1 protein of the virus are type-specific and are not cross-protective between different serotypes (Cavanagh and Gelb 2008). The serological tests like, ELISA, immunofluorescence, and immunodiffusion test cannot differentiate different serotypes because antibodies bind to both group- and type-specific antigens, while VN and HI are serotype-specific tests. Therefore, VN, HI, and ELISA are routinely used serological methods (de Wit 2000). Precipitating antibodies in the group-specific agar gel precipitation test (AGPT) test are short lived which may lead to under-detection. Positive AGPT is indicative of recent infection. Birds infected with the same serotype as vaccine virus lead to poor production of antibodies as detected by AGPT. Thus, AGPT is not recommended for detection of IBV antibodies and rather for detection of IBV antigen (de Wit et al. 1997, 1998). IBV ELISAs are group-specific methods and detect IBV antibodies within 1 week of infection. Paired serum samples are required with first sample collected at the beginning of infection and second sample at 4 weeks later. The first sample should be collected without any delay for detection of seroconversion. Hence, ELISA analyzes post-vaccination and infection responses by IgG detection as an indicator of humoral immunity (Cavanagh and Gelb 2008). VN and HI are serotype-specific techniques for detection of antibodies induced by S1 protein. VN can be carried out in embryonated egg, cell culture, and tracheal ring culture to

detect embryo alterations, cytopathic effect, and ciliostasis, respectively. This assay requires neutralizing antibodies that are highly specific and do not cross-react with other serotypes. IBV requires treatment with type C phospholipase enzyme to expose hemagglutinin. HI test usually detects antibodies between 1 and 2 weeks after infection (de Wit 2000).

Antibody-Based Methods Immunofluorescence and immunoperoxidase assays are used to detect IBV antigen from tracheal mucosa or other tissues using IBV-specific polyclonal sera or monoclonal antibodies (Handberg et al. 1999). The presence of virus in the tracheal organ culture can be detected by immunofluorescence without fixation of the culture using low-power microscopy.

Nucleic Acid-Based Methods IBV can be detected directly from the clinical sample by real-time RT-PCR or quantitative RT-PCR (Callison et al. 2006). This technique is cost- effective, as a number of samples can be examined in a short period of time, and also gives an indication of the level of viral nucleic acid in the sample. Although conventional RT-PCR identifies IBV nucleic acid, it requires passage in embryonated eggs before positive result is obtained. Swabs containing only a small amount of viral RNA can be detected by nested RT-PCR. However, this method is highly prone to cross-contamination resulting in a false-positive test. Positive RT-PCR indicates only the presence of viral nucleic acid but not IBV type. IBV types can be identified by genotyping methods by analyzing the amplicons from the S1 gene. IBV types can be differentiated based on their unique electrophoresis banding pattern using restriction fragment length polymorphism (RFLP) (Marquardt et al. 1981; Nakamura et al. 1994).

Recent Advances An intelligent electronic device was developed by Banakar and coworkers for fast diagnosis of Newcastle disease, IB, and avian influenza based on chicken's sound signals (Banakar et al. 2016). Laamiri and coworkers developed a one-step multiplex real-time RT-PCR for simultaneous detection of four respiratory avian viruses including IBV (Laamiri et al. 2018).

16.7 Prevention and Control

Vaccination and strict biosecurity measures are important for the prevention and control of IBV. Both live attenuated and killed vaccines are used to control IBV in commercial poultry farm. Since serotypes of IBV do not cross-protect each other, multivalent vaccine containing two or more antigenic types would be beneficial in providing broad protection (Cavanagh 2007; Dhama et al. 2014). In most countries,

low virulent IBV vaccines are administered in day-old chicks with subsequent booster immunization with virulent vaccines in drinking water. Unlike highly virulent vaccine, low virulent IBV do not cause respiratory reaction. However, immunity produced by low virulent IBV is not always enough to protect the respiratory tract (Kataria et al. 2005). Prior to immunization with oil-emulsion-inactivated vaccines, breeders and commercial egg layers are initially primed with live attenuated IBV vaccines in order to maintain a good level of local protection of the respiratory tract. Oil-emulsion-inactivated vaccines are given at 17 weeks of age (prior to egg laying of breeder or layer) and never during laying (Jackwood et al. 2009; Liu et al. 2009).

Embryonated chicken eggs are serially passaged with IBV strains to prepare live attenuated vaccines. Prolonged or lifelong immune response can be achieved through vaccination under optimal conditions (Cook et al. 2012; Dhama et al. 2014). Mass serotype, such as H120, is the most commonly used strain in live vaccines. Initially, birds are either vaccinated individually by eye drop instillation, intranasal and intratracheal routes, or mass vaccination by coarse spray or drinking water. Such mass vaccination procedures are cost-effective and induce both local and systemic immune responses. Ma5 is a single-component vaccine that can be included in first vaccination programs with IB 4/91 vaccines and inactivated vaccines. This type of vaccination program provides broad protection against different IBV serotypes. Selection of vaccine must be based on the prevalent strains in the local area. Administration of different vaccine strains together confers cross-protection against various field isolates in the SPF chicken (Li et al. 2008; Marandino et al. 2015). Combination of Mass and Conn or Mass and JMK provides higher degree of cross-protection to some heterologous strains. IB 4/91 serotype or IB 274 vaccine virus gives specific protection against IBV. These provide broad protection when combined with Ma5 and IB multi-vaccines (Ma et al. 2012). Inactivated vaccines induce higher level of antibody response.

However, live vaccine plays a significant role in protecting commercial layers because of better induction of T cell response and local antibody (IgA) production. In North America, Mass, Conn, and Ark serotypes are included in both live attenuated and inactivated vaccines. California and Georgia 98 vaccine strains are being used in the United States. Holland variants such as D-274 and D-1466 along with the IB H120-based vaccines are used in most parts of Europe. Live, freeze-dried vaccine serotypes (Ma5 and 4/91) provide long-lasting protection. In Korea, newly evolving IBV recombinants can be controlled by K2 vaccine strain (Lim et al. 2011). Mass vaccine strain is the only live attenuated vaccine approved in Brazil (Brandao 2010). Vaccination program involves initial administration of Mass-type vaccine followed by a variant shown to be efficacious against Italy 02 and QX (Jones 2010). The only vaccine strain used in India is Mass strain 41 (M41). DNA, subunit, and vectored vaccines using S1 glycoprotein gene and reverse genetics vaccines have been identified (Dhama et al. 2008; Lin et al. 2012). Protection against two or more serotypes can be achieved through introduction of antigen from two or more viruses in recombinant or vector-based vaccines. These new-generation

vaccines can be administered safely in ovo, and efficacy is needed to be tested before introduction for commercial purposes (Matthijs et al. 2003; Brandao 2010; Ullah et al. 2013). Recombinant IBV vaccine using fowl adenovirus vector backbone expressing the S1 glycoprotein gene provides different levels of protection against homologous challenge (Johnson et al. 2003; Toro et al. 2014).

The recent emergence demands the inclusion of QX-like IBV strains in the vaccination program against IB. Yan and coworkers attenuated the QX-like IBV strains by continuous passage in chicken embryos for 130 generations, and it was found to be safe and effective for inducing protection against QX-like IBV strains (Yan et al. 2018). The accessory proteins 3a, 3b, 5a, and 5b contribute individually to the pathogenicity of IBV, and deleting any one of these genes results in attenuation of the virus and can be a potential vaccine candidate (Laconi et al. 2018). Another research team also reported the deletion of accessory genes 3ab and/or 5ab in IBV resulting in attenuation with the ability to induce protection in chickens (van Beurden et al. 2018). Recently, inactivated IBV vaccine encapsulated in chitosan nanoparticles induced an early and stronger IgA and IgG anti-IBV antibodies, when it was vaccinated alone or in association with a live attenuated vaccine (Lopes et al. 2018).

Some of the alternative control measures have been tried and found to be partially successful for the control of IBV. The chicken TLR21 is a functional homologue of mammalian TLR9, recognizes CpG motif, and results in the induction of nuclear factor-kappa B (NF-κB) and its related cytokines (Brownlie et al. 2009). Prophylactic administration of CpG to 18-day-old embryos decreased the viral load (Dar et al. 2009). Astragalus polysaccharides isolated from a traditional Chinese medicinal herb, *Astragalus mongholicus*, inhibit IBV infection, in vitro, in a dose-dependent manner. The lower viral replication was associated with reduced mRNA levels of the proinflammatory cytokines IL-1β, IL-6, IL-8, and TNF-α (Zhang et al. 2018).

16.8 Future Perspectives

Avian infectious bronchitis is one of the most difficult diseases to be controlled in the chicken. Continued emergence of different variants of IBV complicates the control program. Both live attenuated and killed vaccines are used in the field; however, IB outbreaks even in the vaccinated flocks are constantly reported from many parts of the world. Epidemiological surveillance and improved knowledge on the circulating field variants of IBV are necessary for implementing a better control program. Research works are still warranted for improving our understanding on immune response against IBV particularly cellular immunity and a strategic vaccine having capacity to control diverse variants of the virus.

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Newcastle Disease Virus

17

Sohini Dey, Dinesh Chandra Pathak, Ashis Debnath, Narayan Ramamurthy, Rahul, Ajai Lawrence D'Silva, and Madhan Mohan Chellappa

Abstract

Newcastle disease virus (NDV), also known as avian paramyxovirus 1, causes a devastating disease globally in over 250 species of birds known as Newcastle disease. All the viruses belong to a single serotype but categorized into eighteen genotypes based on sequence analysis of the fusion gene. The virus is continuously evolving leading to generation of new genotypes. The clinical manifestation of the disease varies depending on the pathotype of the virus. Very virulent viruses cause severe mortality in susceptible birds whereas less virulent ones cause mild or inapparent symptoms. Diagnosis of the disease is carried out by conventional and molecular tests. The virus can be controlled by live as well as killed vaccines prepared out of less or moderately virulent viruses with considerable level of protection. Recently, genotype-matched vaccines are prepared by reverse genetics for disease control. The virus has a potential to be used as a vector for delivery of foreign immunogenic genes of poultry and other livestock as live-vectored vaccines. Considerable improvements have been made in using the virus as a potential anti-cancer therapeutic for ameliorating cancers of animals and humans. The present chapter delves the various epidemiological dynamics of the virus, diagnosis strategies, and control measures.

Keywords

Newcastle disease virus · Avian paramyxovirus 1 · NDV genotypes · Pathotypes · Pathology · Diagnosis · Vaccines

S. Dey · D. C. Pathak · A. Debnath · N. Ramamurthy · Rahul · A. L. D'Silva · M. M. Chellappa (✉)
Recombinant DNA Laboratory, Division of Veterinary Biotechnology, ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Uttar Pradesh, India

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17.1 Prologue

Newcastle disease (ND) is one of the most important poultry viral diseases and causes severe economic impact on the poultry industry. Due to its global distribution and the fact that it is a great economic threat, ND is regarded as one of the most devastating diseases in the poultry industry. The disease causes a serious impact due to the involvement of a diversity of domestic and wild bird populations in its epizootiology and its global distribution. In the past decade, the disease was endemic in 57% of the countries' rearing poultry while further 23% suffered one or several introductions of the virus (OIE 2008). The aim of this chapter is to give the readers with updated information on the different aspects of the disease and the virus and the recent methodologies to diagnose and control it.

17.2 History

Newcastle disease started as a highly pathogenic disease of chickens in 1926 (Doyle 1927) with the first outbreak to have occurred on a farm near Newcastle upon Tyne in England. Simultaneously, the disease had also emerged on the Island of Java, Indonesia (Kraneveld 1926) and in Ranikhet in India (Edwards 1928). This highly virulent disease of poultry appeared within a short time in England, Java, Philippines, India, Sri Lanka, Korea, and Japan and the disease is sufficiently different from other highly virulent diseases to be recorded as distinct and recognized as the same disease (Rodier 1928; Ochi and Hashimoto 1929; Crawford 1930).

17.3 Epidemiology

17.3.1 Hosts

NDV has a wide range of hosts as more than 250 bird species were found to be susceptible by natural or experimental infections. To this end, it is to note that chickens are highly susceptible to disease; varied levels of susceptibility occur in turkeys, pheasants, partridges, quail and guinea fowl, parrots, cockatiels, with wild birds and waterfowl harboring the virus subclinically. The disease is also demonstrated in young cormorants, ostriches, and pigeons. Raptors are usually resistant to ND, except for reports of acute disease in bearded vulture, white-tailed sea eagle, and wild osprey. NDV also affects gulls, owls and pelicans, passerine birds (order Passeriformes), crows, ravens, and penguins.

Besides, NDV has also been shown to experimentally infect calves, swine, sheep, mice, guinea pigs, rabbits, ferrets, hamsters, mink, and non-human primates such as monkeys (Reagan and Lillie 1947; DiNapoli et al. 2007; Subbiah et al. 2008; Zhao et al. 2017). Further, both avirulent and virulent strains of NDV can infect and cause clinical signs in humans (Chang 1981). The virus usually causes only mild, transient conjunctivitis or flu-like symptoms. However, natural human infection with NDV is extremely rare with no report of human to human transmission.

17.3.2 Transmission

The primary route of transmission appears to be direct contact. The secretions of infected birds primarily via ingestion (fecal/oral route) and inhalation contribute to its spread with feed, water, implements, premises, human clothing, footwear, sacks, egg trays, and soiled eggs exacerbating the spread. Vertical transmission of highly virulent isolates is uncommon. Vectors do not play a role in transmission of the virus.

17.3.3 Viral Pathotypes

Newcastle disease varies widely in its severity spanning from per acute disease with almost 100% mortality to subclinical disease with no lesions. Nowadays, pathotypes are classified based on pathogenicity from least to most pathogenic as lentogenic, mesogenic, and velogenic. The velogenic viruses have been further divided into viscerotropic or neurotropic according to their ability to cause primarily visceral or nervous signs (Alexander 2003). Certain laboratory tests using numerical criteria are done in embryos or chickens using standard pathogenicity parameters to pathotype the virus. These include MDT (mean death time), IVPI (intravenous pathogenicity index), and ICPI (intracerebral pathogenicity index) analysis.

The MDT is the numerical measure in hours the time to death, after inoculation of 9–11-day-old embryonated eggs. Velogenic viruses kill the embryos within 60 h, mesogenic ones between 60 and 90 h, and embryos survival beyond 90 h are classified as lentogens (OIE 2008). The IVPI test involves intravenous inoculation of NDV into 6-week-old chickens and scoring the subsequent illness as 0 = normal, 1 = sick, 2 = paralyzed or nervous signs, and 3 = death. The computed IVPI scores range from 0 to 3 with velogenic NDV having IVPI scores between 2 and 3, mesogenic between 0.0 and 0.5, and lentogens having 0. The IVPI test is not in widespread use. The most definitive and sensitive test to measure the virulence of the virus is based on the ICPI test which is based on inoculation of virus intracerebrally into ten 1-day-old chicks. The chicks are then observed every day for 8 days and scored as 0 = normal, 1 = sick, and 2 = dead. The mean score per bird, per observation over the experiment period, is analyzed. The scores range from 0 to 2 and any virus strain with an ICPI >0.7 is considered virulent or “notifiable” to the OIE. The test can be problematic with viral strains isolated from the bird species other than chicken which could be overcome by passaging the virus in chickens or embryonated chicken eggs prior to performing the test.

17.3.4 Occurrence

Velogenic NDV is endemic in Mexico and Central and South America, widely spread in Asia, the Middle East, and Africa, and in double-crested wild cormorants in the United States and Canada. Lentogenic strains of NDV are worldwide in their distribution while widespread mesogenic pathotypes with a special adaptation to

pigeons (i.e., pigeon paramyxovirus) do not appear to infect other poultry readily. The countries of Oceania are relatively free from ND, probably as a result of effective quarantine and geographical distribution. The disease is currently under control in the United States, Canada, and some western European countries.

17.4 The Virus

Newcastle disease virus (NDV) is classified as a member of the order *Mononegavirales*, family *Paramyxoviridae*, subfamily *Paramyxovirinae,* genus *Avulavirus*. NDV is the only member of the *Paramyxovirinae* whose host is a bird and not a mammal. The viruses in the genus *Avulavirus* are classified into nine serotypes, avian paramyxovirus (APMV) 1–9 based on hemagglutination inhibition and neuraminidase inhibition assays (Alexander 1998), and NDV belongs to APMV-1.

17.5 Viral Pathogenesis

NDV initially attaches its virion through HN glycoprotein to sialic acid-containing cell receptors to initiate the infection. This process stimulates the F protein-mediated fusion of the viral envelope with the host cell plasma membrane in a pH-independent mechanism (Lamb and Parks 2007). The RNA genome is encapsidated with NP and associated with the polymerase complex composed of the P and L proteins to form the viral nucleocapsid or ribonucleoprotein complex. The viral nucleocapsid is released into the cytoplasm subsequent to its dissociation from the M protein. The polymerase complex transcribes the viral genomic RNA to produce the mRNAs that are required for the synthesis of the viral proteins (Curran 1996). After sufficient quantities of viral proteins have been accumulated, the virus switches from the transcription to replication mode. Here, the polymerase complex synthesizes the full length plus strand antigenomic RNA, which serves as the template for synthesis of minus strand genomic RNA. New viral nucleocapsids are then formed by association of NP with genomic RNA and with the polymerase complex. The M protein then directs the assembly of all viral components on the plasma membrane from where the virions are budded off. The virus detaches itself from the host through the neuraminidase activity of HN protein. Finally, the sialic acid residues from progeny virus particles are removed to prevent self-aggregation (Takimoto and Portner 2004).

17.6 Newcastle Disease Virus Classification

Varying degrees of antigenic and genetic diversity are seen among different genotypes of NDV across the globe (Aldous et al. 2003; Alexander et al. 1997; Kim et al. 2007a). NDV is classified into class I and class II viruses. Class I is further divided into nine genotypes and class II into eighteen based on the sequences isolated over time

(Ballagi-Pordány et al. 1996; Czegledi et al. 2006; Kim et al. 2007b). NDV has evolved over the period of time since its first incidence and is known to have at least three genome lengths comprising of 15,186, 15,192, or 15,198 nucleotides (Czegledi et al. 2006).

Class I viruses are generally avirulent with an exception of one known virulent virus from Ireland. These viruses have the longest of the genomes at 15,198 nucleotides (Czegledi et al. 2006) and have been recovered from waterfowl (Family *Anatidae*) and shorebirds (Alexander et al. 1992; Kim et al. 2007a). There are at least nine (1–9) genotypes among the class I viruses and are distributed across the world in wild birds and are being isolated in live bird market samples (Kim et al. 2007a, b).

Class II viruses are classified into eighteen (I–XVIII) genotypes till date. Genotypes I, II, III, IV, and IX contain 15,186 nucleotides and are referred to as "early." Viruses belonging to genotypes V, VI, VII, and X contains 15,192 nucleotides and are termed as "late" (Czegledi et al. 2006; Kang et al. 2016). Genotype II includes lentogenic viruses that are used as vaccine viruses worldwide, such as LaSota, B1, and VG/GA. It also includes the neurotropic virulent chicken/U.S. (TX) GB/1948 (TXGB) isolate and the mesogenic vaccine strain R2B used commonly in some Asian countries (Dey et al. 2014). The study of NDV genotyping and phylogeny analysis is an evolving field with new scientific literature being added to the existing ones. A brief account of distribution of viruses belonging to different genotypes is presented below∗:

Genotype	Predominant distribution/circulation	References
III	Japan (before 1960), Taiwan (1969), Zimbabwe (1990)	Yu et al. (2001)
IV	Europe (1970); no report since 1989	Czegledi et al. (2006)
V	South and Central America (1970), Europe (1970), Florida (1971, 1973), California (1971, 2002)	Ballagi-Pordány et al. (1996), Wise et al. (2004), and Perozo et al. (2008)
VI	Asia (1960–1985)	Mase et al. (2002); Courtney et al. (2013)
VI (a–g)	Mexico (1947), Dominican Republic (1986–2008)	
VII		Aldous et al. (2003), Bogoyavlenskiy et al. (2009), Wang et al. (2006), and Snoeck et al. (2009)
VII a	Far East to Europe and Asia	
VII b	Far East to South Africa	
VII (c–e)	China, Kazakhstan, South Africa	
VII (f–h)	South Africa	
VIII	South Africa, South East Asia	Abolnik et al. (2004)
IX	China (1948)	Wang et al. (2006)
X	Taiwan (1969, 1981)	Tsai et al. (2004)
XIII	Russia, Iran, Pakistan, India	Diel et al. (2012), Jakhesara et al. (2016), and Bhuvaneswari et al. (2014)
XIV	West Africa, Central Africa	de Almeida et al. (2013)
XV	China (1997–2004)	
XVI	Mexico (1947); Dominican Republic (1986–2008)	Courtney et al. (2013)

Genotype	Predominant distribution/circulation	References
XVII	West Africa (2006–2011)	Cattoli et al. (2010) and de Almeida et al. (2013)
XVIII		Cattoli et al. (2010) and de Almeida et al. (2013)
XVIIIa	Ivory Coast, Mali, Mauritania (2006–2010)	
XVIIIb	Ivory Coast, Mali, Nigeria, Togo	

∗For a detailed review of the genotypes of NDV, readers are advised to refer to the article by Dimitrov et al. (2016)

New viruses continue to evolve over a period of time leading to assignment of new genotypes. A set of criteria are followed to counteract any ambiguities in classification of viruses (Diel et al. 2012).

17.7 Newcastle Disease Virus in India

The Indian subcontinent is endemic to ND since its emergence in 1926. The endemicity of the disease is attributed to the porous border it shares with many of the neighboring South Asian countries where backyard poultry rearing is in vogue and also of the strategic location of the country in the route of the several migratory birds which act as reservoir for the virus. Recently, based on the whole genome sequencing of the 12 field isolates and the 5 vaccine strains used in the country, it has been observed that genotypes II, IV, VI, VII, XIII, and XVIII are circulating in India (Dey et al. 2014; Jakhesara et al. 2016). Based on the complete genome sequence the field NDV strain isolated from the 2012 outbreaks farm in Nagpur, India, the velogenic virus is placed in the phylogenetic clade as genotype VII in class II (Gogoi et al. 2015). Based on the nucleotide sequence analysis of fusion (F) and hemagglutinin protein genes (HN) in 2014–2015, NDV isolated from different chicken flocks showed a close similarity with genotype XIII strains of NDV and the amino acid sequence of F protein confirmed about the virulent cleavage site ^{112}R-R-Q-K-R-F^{117} (Nath et al. 2016). During the outbreak of 2012 in northwest India, NDV isolated from wild peacock was phylogenetically placed as genotype II, class II of NDV strains (Khulape et al. 2014). Based on the percentage of divergence of amino acid sequence present at the F protein cleavage site, three field isolates were characterized as velogenic and two isolates were lentogenic in nature in Indian subcontinent (Nanthakumar et al. 2000a). In Tamil Nadu state of India, two NDV strains isolated from a chicken and a pigeon were characterized by complete genome sequence analysis and pathotyping. Based on the sequence analysis of the matrix gene, a pigeon isolate was grouped into APMV-1 and sequences analysis of the fusion and hemagglutinin genes and complete genome sequence grouped these viruses into genotype IV. Most of the NDV isolates obtained from southern states of India belong to genotype II. However, some of the strains from Tamil Nadu and

majority from Uttar Pradesh belong to genotype groups VI and VII with three isolates recovered from Tamil Nadu being grouped with genotype IV viruses (Tirumurugaan et al. 2011).

17.8 Clinical Signs and Pathologic Findings with NDV Infection

Majority of the clinical signs presented below relate to the observations made in chickens as this is the most commonly affected species of bird. Although the severity of clinical signs varies with the pathotype of the virus, some host related factors such as age, route of infection, immune status, and concomitant environmental stress also play a role in manifestation of disease symptoms.

Respiratory and nervous signs, or both, occur in the most widespread form of the disease. Signs appear almost simultaneously throughout the flock 2–15 days after exposure. Young chickens are more susceptible and show signs sooner than older ones. Respiratory signs include gasping and coughing and nervous signs include drooped wing, dragging legs, twisting of head and neck, circling, depression, inappetence, and complete paralysis but usually follow the respiratory signs (Kommers et al. 2003a, b). The presence of multifocal hemorrhages seen through the serosal surface of the intestines, multifocal areas of necrosis and/or ulceration of the gut-associated lymphoid tissue, and disseminated foci of necrosis in the spleen are highly suggestive of viscerotropic velogenic NDV infection (Alexander 2001; Brown et al. 1999). Clonic spasms are seen in moribund birds. Laying flocks may have partial or complete cessation of production and not recover. Eggs from infected flocks may be abnormal in color, shape, or surface and have watery albumen.

Viscerotropic signs which predominate in the peracute disease include watery and greenish diarrhea and swelling of the tissues around the eyes and in the neck. The primary clinical signs of neurotropic velogenic NDV infection are neurologic and consist of head twitch, tremors, opisthotonus, and paralysis (Terregino and Capua 2009). Gross lesions are minimal with mesogenic strains with severe morbidity seen in birds due to concurrent viral and secondary bacterial infections (Nakamura et al. 1994). Histologically, non-suppurative encephalitis (i.e., perivascular cuffing and gliosis) similar to those caused by the neurotropic velogenic NDV strains are seen. Some birds have myocarditis and splenic and pancreatic necrosis especially within 5–10 dpi. Lentogenic viruses rarely cause disease in adult chickens. Some lentogenic isolates caused non-suppurative tracheitis in association with *E. coli* in field outbreaks (Hooper et al. 1999). Mortality depends on virulence of the strain, environmental conditions, and condition of the flock.

17.9 Diagnosis of Newcastle Disease

Diagnosis of the disease at the first instance is by looking for the typical clinical signs of the disease, namely, mild respiratory symptoms in the form of coughing, rales, gasping, and sneezing (lentogenic strains), acute respiratory disease and neurologic signs in some species (mesogenic strains), greenish or white watery diarrhea, respiratory distress, neurological symptoms, and paralysis (velogenic strains).

Laboratory diagnosis is usually carried out for the identification of the causative agent that includes isolation of the agent in embryonated chicken eggs or in cell culture systems, serology, and using a plethora of molecular methodologies.

For virus isolation, 9–11-day-old SPF embryonated chicken eggs are injected with 0.1 ml of suspected samples into the allantoic cavity. The eggs are incubated at 37 °C and observed twice daily. Dead embryos and eggs after 5–7 days of incubation are chilled at 4 °C and allantoic fluid harvested to check for the presence of the virus by hemagglutination (HA) test. Related avian paramyxoviruses and avian influenza viruses which also cause HA can be overruled by a hemagglutination inhibition test by using specific sera (OIE 2008).

Direct detection of NDV in tissues can be achieved through immunohistochemistry, *in situ* hybridization, and immunoperoxidase assay (Russell and Alexander 1983; Lockaby et al. 1993; Brown et al. 1999). Serological tests such as virus neutralization and HI assays are also used for detection of NDV. These tests are simple and inexpensive but are time-consuming and less sensitive, and there is difficulty in reproducing the test among different laboratories (Beard and Wilkes 1985).

The indirect enzyme-linked immunosorbent assays (ELISAs) are being used and are being correlated to the HI tests (Brown et al. 1999; Cvelic-Cabrilo et al. 1992; Cadman et al. 1997; Schelling et al. 1999). Several formats of ELISAs are in vogue either by using complete virus as coating antigens (Miers et al. 1983; Wilson et al. 1984; Jestin et al. 1989) or by using expressed NDV proteins. Recombinant nucleoprotein expressed in *E. coli* or baculovirus (Errington et al. 1995; Makkay et al. 1999), recombinant phosphoprotein expressed in *E. coli* (Das and Kumar, 2015), and recombinant hemagglutinin protein expressed in *E. coli* (Mohan et al. 2006b) have all been used as coating antigens in quantifying serum antibodies against NDV infection. A monoclonal antibody (mAb)-blocking ELISA in which mAbs recognizing well-conserved serotype-specific epitopes were used was also developed thus making it more sensitive and specific than indirect ELISA and HI (Czifra et al. 1996).

17.9.1 Molecular Diagnosis of the Disease

Molecular tools have aided greatly the pathotyping of NDV. The initial report used specific primers to amplify F-gene sequences of various strains in infected chicken allantoic fluids in a reverse transcription PCR (Jestin and Jestin 1991). Restriction enzyme analysis of the RT-PCR products of the F0 cleavage site and parts of the M

gene led to the identification and differentiation of NDV isolates (Ballagi-Pordány et al. 1996; Wehmann et al. 1997; Kou et al. 1999; Gohm et al. 2000). The same methodology was able to differentiate lentogenic and mesogenic viruses (Nanthakumar et al. 2000b) and also cell-culture-adapted viruses (Mohan et al. 2006a, 2007). Sequencing the RT-PCR products with several sets of different primers for different strains of NDV was also carried out (Collins et al. 1993).

Real-time PCR has also been employed for the detection of the virus (Nidzworski et al. 2011; Gopinath et al. 2011). The advent of real-time PCR using fluorogenic hydrolysis (TaqMan) probes provided highly sensitive and rapid testing procedures. Multiplex RT-PCR was developed to differentiate type I and type II NDV (Liu et al. 2011). Several real-time PCR assays have been developed around the world to detect viruses circulating in their locations (Miller et al. 2010). An M-gene-based real-time PCR assay was developed for the detection of NDV isolates (Kim et al. 2007a). Since there are variations in M gene sequences, an L-gene-based real-time assay in combination with M-gene assay is used to detect a broad range of isolates (Miller et al. 2010). Some of the other assays used for NDV detection include use of light upon extension fluorogenic primers (Antal et al. 2007); loop-mediated isothermal amplification assay (Pham et al. 2005), and real-time PCR assays using TaqMan minor groove binder probes (Farkas et al. 2007). The limit of detection for these assays was established between 10^1 and 10^3 EID_{50}/0.1 ml.

Phage capturing dot blot assay, oligoarrays, and bioactive amplification with probing and diagnosis by quantum dots and magnetic beads are some of the recent diagnostic protocols which are being put into use for diagnosing the disease (Lee et al. 2006; Ma et al. 2010).

17.10 Vaccines Against Newcastle Disease

A robust vaccination program for NDV considers the following: the vaccine type to be used, the disease and immune status of the birds to be vaccinated, the level of maternally derived antibodies in young chickens, and the protection level required to combat infection with field virus under local conditions. Likewise, three important criteria need to be satisfied to combat NDV with usage of vaccines: (i) decrease or eliminate clinical disease; (ii) decrease the amount of virulent virus shed; and (iii) increase the infectious dose of the challenge virus. The success of any ND vaccination program also may depend on a minimum of 85% of the flock receiving a proper dose and responding to vaccination to achieve herd immunity.

Traditional NDV vaccines have been live attenuated prepared from NDV isolates that were termed as “early” having been isolated in the 1940s and 1960s. The conventional commercial live virus vaccines are categorized into two groups: lentogenic vaccines, such as Hitchner-B1, LaSota, V4, NDW, I2, and F, and mesogenic vaccines, such as Roakin, Mukteswar, and Komarov. These vaccines belong to the class II genotype II viruses. Usually a dose of 10^6 EID_{50} or higher is sufficient to give humoral immune response that protect the birds from NDV infection. One important aspect of NDV vaccination is the ability of the vaccine to

prevent the shed of the virulent virus. Antigenically matched vaccines wherein the entire backbone of the virus is kept same as that of the circulating virulent strain with a change in the fusion protein cleavage site to a lentogenic one or genotype matched vaccines can be handy. A thermostable NDV for the tropical countries would have an added advantage over the existing live attenuated ones as maintenance of cold chain in vaccination process is a major drawback in these countries. Recent literatures suggest that hemagglutinin-neuraminidase, fusion, and phosphoprotein genes contribute to the thermostability of the virus (Zhao et al. 2018; Liu et al. 2019). Inactivated vaccines for NDV have a disadvantage over the live vaccines as they require a withdrawal period before the birds can be used for consumption.

17.10.1 New-Generation Newcastle Disease Virus Vaccines

Gene immunization provides an easy and flexible way to modulate immune responses. The glycoproteins F and HN are immunogenic in nature and multiple immunizations using them enhanced immunity in chickens (Loke et al. 2005). Delivery agents like ISCOMS and virosomes were also studied against NDV (Homhuan et al. 2004). Enhancement of mucosal immunity to NDV vaccine was achieved by intranasal delivery in conjunction with CPG ODNs (Zhang et al. 2008). A combined *in ovo* vaccine against AI and NDV has been reported by Steel et al. 2008. Chitosan when used along with live vaccine improves antigen-specific cell-mediated immunity (Rauw et al. 2010).

Biotechnological vaccines include immunization with the HN or F gene expressed in recombinant fowl pox virus (Boursnell et al. 1990, Taylor et al. 1990), vaccinia virus (Meulemans et al. 1988), or turkey herpes virus (Morgan et al. 1993). A commercially available fowl pox-vectored NDV vaccine, Vectormune FP-ND, is available in the market in several countries. Edible vaccines provide an alternate vaccine delivery platform for mucosal immunity (Berinstein et al. 2005). HN protein expressed from plant cell lines has been registered by Dow Agrosciences but not marketed. Calcium phosphate-coupled NDV virus enhanced humoral as well as cellular immunity (Koppad et al. 2010). Antiviral peptides against NDV and infectious bronchitis virus were reported to be effective by Wang et al. 2011. Recently, nanoparticle-delivered ND vaccines has been attempted. Two chitosan derivatives, O-2′-hydroxypropyltrimethyl ammonium chloride chitosan and N-2 hydroxypropyl trimethyl ammonium chloride chitosan, have been utilized to make nanoparticles as a mucosal delivery vehicle for live attenuated ND vaccines (Dai et al. 2015; Zhao et al. 2016b). Similarly, Silver @SiO2 and double hydroxide @ SiO2 nanoparticles have been developed for intranasal delivery of DNA ND vaccines (Zhao et al. 2016a). A virus-like particle vaccine against NDV involving nucleoprotein, matrix, fusion, and hemagglutinin genes has also been reported (McGinnes et al. 2010).

17.10.2 Newcastle Disease Virus-Vectored Vaccines

Recent works suggest that NDV could be used as a viral vector to deliver other immunogenic genes of important viral diseases of poultry and livestock. This was made possible with the inception of reverse genetic system. Due to the modular nature of the genome of NDV, engineering additional genes from several different pathogens or tumor-specific antigens to design contemporary vaccines for animals and humans could be achieved. The initial rescue system for NDV was based on LaSota strain reported by two groups simultaneously (Peeters et al. 1999; Romer-Oberdorfer et al. 1999). The biological properties of the rescued virus were similar to the wild virus with a slight reduction in virulence and growth characteristics. A number of strains of NDV were subsequently rescued prominent ones being Hitchner B1 (Nakaya et al. 2001), F (Dey et al. 2017), Clone-30 (Romer-Oberdorfer et al. 1999), Beaudette C (Krishnamurthy et al. 2000), and R2B (Chellappa et al. 2017; Yadav et al. 2018).

The recombinant viral-vectored vaccines hold many promises for the future as the foreign antigens are expressed naturally in the context of an infected cell, thereby inducing cellular, as well as humoral, immune responses. Certain characteristics of NDV suggest that recombinant NDV expressing a foreign viral protein would be very good vaccine candidate. NDV grows to very high titers in many cell lines and eggs, and it elicits strong humoral and cellular immune responses in vivo. NDV naturally infects via respiratory and alimentary tract mucosal surfaces, so it is especially useful to deliver protective antigens of respiratory disease pathogens. Recombination with NDV is very rare as the viral life cycle occurs in the cytoplasm of the host cell. NDV can also be engineered as a surrogate virus in which the viral envelope can be completely replaced with other viral envelope proteins or by chimeric envelope proteins (Collins et al. 1999). In the field of poultry vaccinology, NDV has been used as a vector to deliver some of the immunogenic genes of other poultry viruses including highly pathogenic avian influenza viruses A/H5 (Ge et al. 2007) and A/H7 (Park et al. 2006), VP2 of infectious bursal disease virus (Huang et al. 2004; Dey et al. 2017), S2 gene of infectious bronchitis virus (Toro et al. 2014), spike genes S1 and S2 of M-41 strain of infectious bronchitis virus (Shirvani et al. 2018), gB, gC, and gD of infectious laryngotracheitis virus (Basavarajappa et al. 2014; Zhao et al. 2014), and glycoprotein of avian metapneumovirus subgroup C in turkeys (Hu et al. 2011). A surrogate virus has a great potential for gene therapy or treatment of cancer or to prevent diseases for which no effective vaccines are available. Certain NDV strains can selectively kill human tumor cells (Reichard et al. 1992), and there is an increasing interest in the use of NDV for cancer therapy (Nelson 1999).

17.11 Conclusions

Newcastle disease has been an age-old disease since its discovery eight decades ago. There had been several innovative discoveries regarding the diagnosis and vaccines for this disease. The brutal force with which the velogenic virus causes the disease in organized farms as well as village poultry is phenomenal. As poultry meat becomes a cheaper source of quality protein for the world population, the effect it has on the poultry-rearing countries in the event of an outbreak is a matter of great concern. With the advent of recent molecular tools and the discovery of the virus rescue by reverse genetics, this virus has provided the scientists and researchers worldwide with an insight into its pathogenesis, the viral determinants of virulence, and its genome organization. As the virus has become an attractive tool to deliver foreign genes and its ability to multiply in human cells, many more exciting discoveries are bound to happen in the near future.

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Avian Paramyxoviruses

18

Sachin Kumar

Abstract

Avian paramyxoviruses (APMVs) are well-known infectious agents of the avian species. These viruses belong to the family *Paramyxoviridae* under genus *Avulavirus*. APMV outbreaks produce serious economic impact worldwide by implementing significant trade restrictions and escalating costs of production from culling and quarantines for the infected regions. As APMVs affect domestic, wild and migratory bird species, their surveillance is under-reported. Thus it results in a lack of control measures and fuel evolution of its new serotypes. Scientists all over the world have reported till date 18 serotypes of APMV. The following chapter provides a glimpse of isolation, genome integrity and pathogenicity of the confirmed strains. The precise interpretation of the molecular nature of the emerging APMV strains could help researchers to develop suitable means for their management. APMVs are excellent vectors for vaccine use and are oncolytic in nature. Therefore, a good understanding of this group of viruses could benefit humankind.

Keywords

Avian paramyxoviruses · APMV · *Avulavirus* · Isolation · Genome · Pathology · Diagnosis · Management

S. Kumar (✉)
Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati, Assam, India
e-mail: sachinku@iitg.ac.in

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18.1 Prologue

Avian paramyxoviruses (APMVs) are ubiquitous pathogens present in a wide variety of bird species around the globe. The viruses infecting avian species have economic significance due to associated high mortality and morbidity rates. There are 17 different serotypes of APMV reported to date. These viruses are taxonomically classified in the family *Paramyxoviridae* under genus *Avulavirus.* Newcastle disease virus (APMV-1) is a well-characterized member. Complete genome sequence of all 17 APMVs is known. In recent years, APMVs have attracted molecular virologists and vaccine biologists for its advantage as a viral vector for different pathogens. Recombinant APMV-based vaccine is a pertinent choice for virologists. Absence of DNA phase during its replication, minimum recombination frequency and modular nature of transcription are some properties that favour recombinant NDV construction as a live attenuated vaccine. Although information regarding the APMV serotypes is not vast, availability of advanced molecular biology tools and high-throughput sequencing facility has led to increased research in this area.

18.2 Avian Paramyxovirus-2

The first isolation of avian paramyxovirus-2 (APMV-2) strain from poultry was reported in Yucaipa, USA (Bankowski et al. 1960). APMV-2 naturally causes infection in turkeys, while chickens are the accidental host. The virus causes mild respiratory disease, drop in egg production and infertility in turkeys (Bankowski et al. 1981; Lipkind et al. 1979). The passerine and psittacine species are active carriers of the APMV-2 and act as reservoir host (Lipkind et al. 1979; Goodman and Hanson 1988; Lang et al. 1975; Mbugua et al. 1985; Weisman et al. 1984). A recent report of the APMV-2 outbreak from Brazil in penguins suggested the high risk of its spread to non-natural hosts (Fornells et al. 2012). APMV-2 strain Yucaipa is the prototype strain with a genome length of 14,904 nucleotides (nts). APMV-2 strain Yucaipa genome has six overlapping genes in order 3′-N-P-M-F-HN-L-5′ (Fig. 18.1). Based on sequence and antigenic analyses, APMV-2 strains are classified into two genetic subgroups (Fig. 18.2) under a single serotype considering

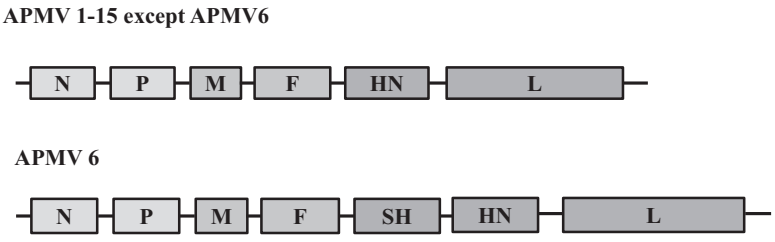

Fig. 18.1 Schematic representation of the genome of avian paramyxoviruses

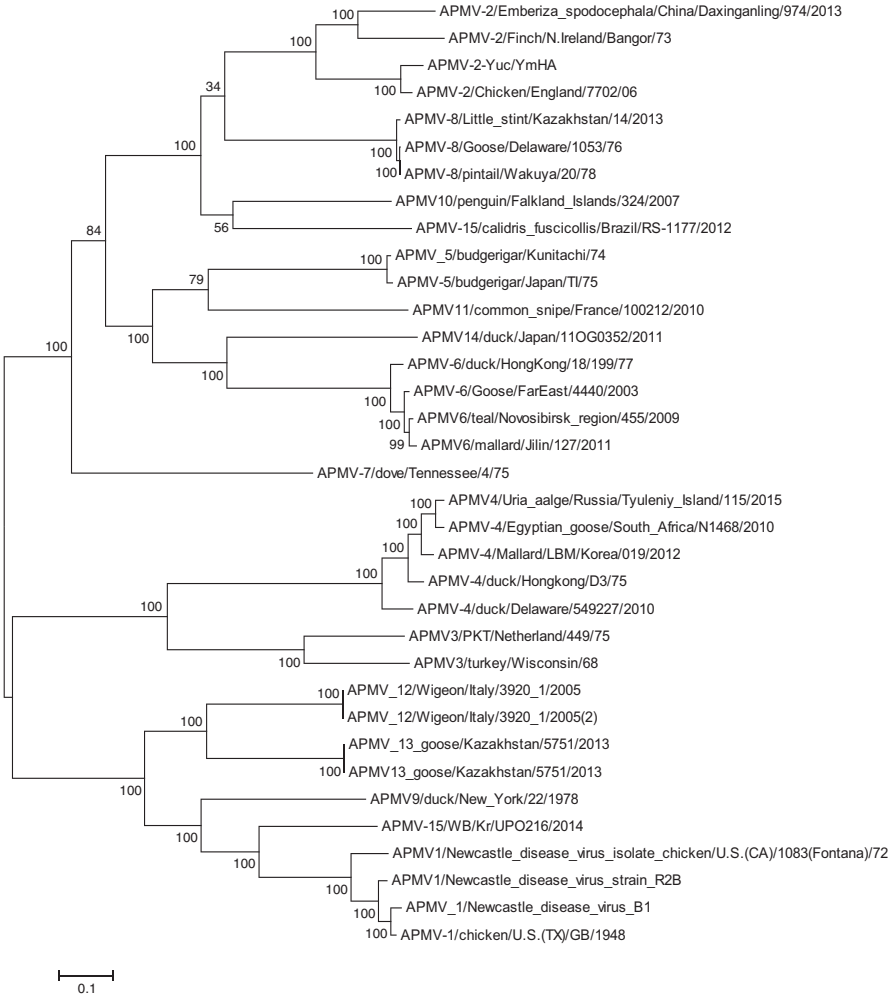

Fig. 18.2 Phylogenetic analysis of different avian paramyxoviruses

sequence and antigenic differences (Subbiah et al. 2010). Antibodies against APMV-2 were seen in both poultry and wild birds (Maldonado et al. 1994).

18.3 Avian Paramyxovirus-3

In 1968 the first isolate of APMV-3 was reported from turkeys in Ontario in 1967 and Wisconsin in 1968 (Tumova et al. 1979). Like APMV-1 and APMV-2, APMV-3 also has a wide range of hosts. APMV-3 strain parakeet/Netherlands/449/75 is the prototype strain and has 16,272 base pairs with an extra-long trailer (Kumar et al. 2008). Later complete genome of APMV-3 strain turkey/Wisconsin/68 showed a genome

length of 16,182 nts, which is 90 less than strain parakeet/Netherlands/449/75 (Kumar et al. 2010a). Similar to other APMVs, APMV-3 genome has 3′-N-P-M-F-HN-L-5′ genes. APMV-3 has been established to cause encephalitis leading to high mortality in birds and respiratory disorders in turkeys (Tumova et al. 1979; Kumar et al. 2010b). APMV-3 was hypothesized to be a better vector to express foreign proteins against other avian pathogens. Moreover, the gene junction between phosphoprotein and matrix protein was recognized as optimum location for the high-level gene expression by reverse genetics approach (Yoshida and Samal 2017). The reverse genetics of APMV-3 was used to decipher the importance of fusion protein against the virulent strain of Newcastle disease virus in chickens (Kumar et al. 2011).

18.4 Avian Paramyxovirus-4

The APMV-4 was first isolated from the duck in 1975 (Shortridge et al. 1980). APMV-4 does not have a broad host range as compared to previous APMVs. It is isolated from ducks, geese and poultry (Stanislawek et al. 2002). The Duck/Hong Kong/D3/75 strain is the representative strain of APMV-4 and has 15,054 nts with a short trailer of 17 nts (Nayak et al. 2008). Recently, the complete genome sequence of an APMV-4 strain isolated from a domestic mallard duck was reported from South Korea (Tseren-Ochir et al. 2017a). Dispersal and transmission of APMV-4 among wild birds and domestic poultry have been reported (Yin et al. 2017).

18.5 Avian Paramyxovirus-5

The APMV-5 was first isolated from budgerigars (*Melopsittacus undulatus*) at Kunitachi, Tokyo, Japan, in 1974 (Nerome et al. 1978). APMV-5 is asymptomatic in chickens, but the disease shows symptoms like depression, dyspnoea, diarrhoea and torticollis and causes high mortality in budgerigars. APMV-5/budgerigar/Kunitachi/74 is the prototype strain of this group and has a genome length of 17,262 nts (Samuel et al. 2010). Although the virus has not been well-characterized, it differs from other APMV serotypes majorly in the failure to propagate in the allantoic cavity of embryonated chicken eggs and the lack of haemagglutination with chicken red blood cells (Nerome et al. 1978). Surprisingly, the APMV-5 was found avirulent to chickens even though it has polybasic amino acids in its fusion protein cleavage site. APMV-5 genome is shown to be more closely related to APMV-6 than to other APMVs in phylogenetic analyses.

18.6 Avian Paramyxovirus-6

The first isolate of APMV-6 was from healthy ducks in Hong Kong in the year 1977. APMV-6 strain duck/Hong Kong/18/199/77 is the prototype strain with 16,236 nts and encodes an additional protein, small hydrophobic (SH) along with regular six

proteins encoded by other APMVs. The genome of APMV-6 has a 55-nt leader sequence and a 54-nt trailer sequence (Chang et al. 2001). APMV-6 has more sequence relatedness to APMV-1 (NDV) and APMV-2, with an identity ranging from 22 to 44%. Notably, the APMV-6 differs from other APMVs by encoding an extra SH gene. The APMV-6 genome consists of 3′-N-P-M-F-SH-HN-L-5′ (Fig. 18.1). Through phylogenetic analysis, APMV-6, APMV-1, APMV-2 and APMV-4 all cluster into a single lineage, distinct from other paramyxoviruses. APMV-6 has been reported as a vaccine vector for highly pathogenic avian influenza virus in chickens (Tsunekuni et al. 2017). The virus causes severe disease and egg drop in turkeys (Rosseel et al. 2011).

18.7 Avian Paramyxovirus-7

The first isolate of APMV-7 was from a hunter-killed dove in Tennessee in 1975 and further from ostriches (Woolcock et al. 1996). The APMV-7 prototype strain Tennessee is 15,480 nts long with six genes in the order of 3′-N-P-M-F-HN-L-5′ with 3′-55-nts leader and 5′-127-nts trailer sequences. The fusion (F) protein had a monobasic cleavage site ^{101}-TLPSSRF-^{107} and is capable of in vitro replication without exogenous protease. The APMV-7 was shown to have a restricted host range. Sequence alignment and phylogenetic analysis of the predicted amino acid sequence of APMV-7 proteins showed more relatedness to APMV-2, APMV-6 and APMV-8. The APMV-7 was suggested to be avirulent for chickens based on pathogenicity index test in embryonated chicken eggs. APMV-7 strains represent promising attenuated candidates for vector development (Khattar et al. 2013).

18.8 Avian Paramyxovirus-8

The first isolate of APMV-8 was from feral Canadian goose in 1976 from the USA and pintails in Japan (Yamane et al. 1982). The APMV-8 strain goose/Delaware/1053/76 is the representative strain and is 15,342 nts long (Paldurai et al. 2009). The virus has been recently reported from wild birds in Mongolia, and Kazakhstan (Fereidouni et al. 2017; Tseren-Ochir et al. 2017b). APMV-8 immunization failed to protect against Newcastle disease¸ suggesting it to be serologically distinct from APMV-1 (Grund et al. 2014).

18.9 Avian Paramyxovirus-9

The APMV-9 was first isolated from ducks in New York (Sandhu and Hinshaw 1981). The APMV-9/domestic duck/New York/22/78 is the prototype strain and is 15,438 nts long. The genome of APMV-9 consists of six non-overlapping genes arranged in the order 3′-N-P/V/W-M-F-HN-L-5′ (Samuel et al. 2009). The APMV-9, F protein cleavage site is different from the standard cleavage site for the

universal cellular protease furin. Similar to APMV-1, APMV-9 consists of different lineages (Dundon et al. 2010). Based on the nucleotide sequence and phylogenetic analysis of the predicted amino acid sequences of APMV-9 proteins, it shows maximum sequence relatedness to APMV-1 than to other APMVs (Samuel et al. 2009). The pathogenicity index test in embryonated chicken eggs suggests APMV-9 as avirulent for chickens.

18.10 Avian Paramyxovirus-10

The APMV-10 was first isolated from penguins in the Falkland Islands and Brazil (Fornells et al. 2012; Miller et al. 2010). The strain Penguin/Falkland Islands/539/2007 is 15,456 nts long (Miller et al. 2010). Phylogenetic evaluation of APMV-10 coding regions reveals it to be similar to APMV-2 and APMV-8. The calculated evolutionary distance between APMV-10 and APMV-2 is 0.843, while the distance between APMV-10 and APMV-8 is 0.892. APMV-10 was also found as an efficient vector for expressing avian influenza proteins (Tsunekuni et al. 2014).

18.11 Avian Paramyxovirus-11

The first isolate of APMV-11 was reported from the common snipe in France (Briand et al. 2012). The genome of APMV-11 is 17,412 nts, the largest among APMVs. APMV-11 shows highest genomic nucleotide identity (48.9%) with APMV-2. Sequence data of phosphoprotein gene from APMV-11 suggests similar editing sites as reported for mumps virus or simian virus 5.

18.12 Avian Paramyxovirus-12

The APMV-12 was isolated during surveillance of wild birds in November 2005 in the Rovigo province of Northern Italy (Terregino et al. 2013). The prototype strain APMV/pigeon/Italy/3920-1/2005 is 15132 nts long. The cross-HI tests suggested a low-level relationship of APMV-12 to APMV-1. A similar pattern was observed in the phylogenetic analysis, where APMV-12 was found quite distinct from APMV-1. APMV-12 shows 52.1% genome identity with APMV-1 and 50.1% identity with APMV-9. However, other APMVs exhibited lower identity of 42%.

18.13 Avian Paramyxovirus-13

The first report of APMV-13 came in 2015 with only sequence analyses of F gene of its strain Shimane 67. In 2000, the APMV-13 was reported from the faecal samples of geese in Shimane prefecture. Further, two more novel APMV-13 strains were reported in white-fronted goose from Kazakhstan and Ukraine in 2013 and

2011, respectively (Karamendin et al. 2016; Goraichuk et al. 2016). The complete genome length of the APMV-13 isolates Kazakhstan and Ukraine were 15,996 and 16,146 nts, respectively. The genome length of an isolate from Ukraine was 150 nts longer than that of isolates from Kazakhstan. However, both the isolates showed a nucleotide sequence identity of 97%. The F protein cleavage site of APMV-13 isolate Shimane 67 showed QVRENR/LVG, which is similar to lentogenic strains of APMV-1 (Yamamoto et al. 2015). Similarly, F gene sequence analyses of Kazakhstan and Ukraine showed a maximum identity of 96% and 98% with isolate Shimane 67, respectively. The intracerebral pathogenicity index (ICPI) test of APMV-13 strain Ukraine suggested it to be a lentogenic pathotype.

18.14 Avian Paramyxovirus-14

APMV-14 was isolated from duck faeces during surveillance of avian influenza virus in migratory wild birds in Hokkaido Prefecture, Japan, in 2011. The complete 15,444 nts of APMV-14 showed 46.3–56.1% identity with other APMVs (Thampaisarn et al. 2016). The genetic characterization of the APMV-14 suggested the putative RNA editing site similar to other APMVs. Presence of K at the F protein cleavage site is a unique feature (R-E-G-*K*↓L) identified in APMV-14. The relevance of unique K at the F protein cleavage site has not been explored (Thampaisarn et al. 2016). Cell culture infectivity of APMV-14 suggested the virus to be restricted to only avian cells.

18.15 Avian Paramyxovirus-15

APMV-15 was reported nearly at the same time from South Korea and Brazil (Lee et al. 2017; Thomazelli et al. 2017). The APMV-15 strain UPO216 was isolated in January 2014, from faecal droplets of wild birds from UPO wetland, South Korea. The virion of UPO216 indicated a pleomorphic shape with 80–300-nm diameter and a complete genomic length of 15,180 nts (Lee et al. 2017). The genetic characterization of APMV-15 suggested maximum nucleotide sequence identity (64.0%) with APMV-1. However, the serology based on cross-HI test suggested its cross-reactivity with APMV-1 ($R = 0.088$) and APMV-9 ($R = 0.125$); still, UPO216 was found to have distinct antigenicity from other APMV serotypes. In addition, a specific cleavage site in its F protein (LVQAR↓L) indicated it to be unique among previously confirmed APMV serotypes. The APMV-15 strain RS-1177 was initially isolated from migratory bird cloacal swab in April 2012 from South Brazil (Thomazelli et al. 2017). Further, the genome analyses of APMV-15 strain RS-1177 revealed a 14,952-nts-long genome with a maximum nucleotide sequence identity (60.1%) with APMV-10. The absence of serological cross-reactivity and significant genetic phylogenetic distance of APMV-15 suggested it be considered as a new serotype. The pathogenicity index of APMV-15 gave an MDT index of >120 h and an ICPI value of 0.00, indicating its low virulent nature.

18.16 Avian Paramyxovirus-16

APMV-16 is not designated at present to be a separate serotype. However, as suggested, one of the two viruses UPO216 or RS-1177 reported in the group APMV-15 should be re-annotated as APMV-16 (Jeong et al. 2017). However, more serological and genetic analysis will give us more insight into the biology of AMPV-16.

18.17 Avian Paramyxovirus-17

In late 2017, a novel serologically and genetically distinct avian paramyxovirus, Cheonsu1510, was characterized and suggested to be a new APMV serotype 17. The virus was initially isolated in 2015, from a faecal sample coming out of a migratory birds nesting area in the western region of South Korea. Full genome sequence of APMV-17 revealed 15,408 nts, comprising six genes (3′-N-P-M-F-HN-L-5′) similar to other APMVs (Jeong et al. 2017). Although maximum sequence identity of the virus was revealed with APMV-9 (63.0%) followed with APMV-15(Kr) (55.8%), APMV-1 (55.7%) and APMV-12 (51.9%), a unique F protein cleavage site (D-R-E-G-R↓L) resembling a lentogenic strain of APMV was identified (Jeong et al. 2017). Moreover, phylogenetic analyses based on the F and HN gene placed Cheonsu1510 in a separate phylogenetic group. A putative RNA editing site (3′-UUUUUCCC-5′) was also predicted in the P gene suggesting the possible presence of V and W proteins. Although several genome features of APMV-17 are similar to other APMVs, the cross-haemagglutination-inhibition (HI) test implied serologically weak cross-reactivity with APMV-1 and APMV-9. The cross-neutralization of other APMVs with the APMV-15 antibody could give us a better insight about its cross-reactivity.

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Conflict of Interest There is no conflict of interest.

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Orthomyxoviruses

19

Elsayed M. Abdelwhab and Ahmed S. Abdel-Moneim

Abstract

The viruses under the family *Orthomyxoviridae* are responsible for a variety of important respiratory diseases in humans and different animal species. The seven genera under the family are the influenza viruses A, B, C and D, *Quaranjavirus*, *Thogotovirus* and *Isavirus*. Viruses are highly evolving, and the genetic reassortment among viruses is seen only within the same genus and never been reported in between viruses from different genera. Influenza A viruses (IAVs) infect humans and different animals including birds, pigs, equines, dogs, cats, whales and seals. To date, there are 18 different haemagglutinins (H1 to H18) and 11 different neuraminidases (N1 to N9) for influenza A viruses. Influenza B viruses (IBVs) are exclusively human pathogens, while influenza C virus (IVC) affects humans and pigs. Serological evidence of ICV was recently detected in camels. Influenza D virus (IDV) was reported in pigs with influenza-like symptoms. IAVs cause recurrent epidemics of varying severity in humans and different animal species due to antigenic drift, gradual accumulation of point mutations, during replication under immune pressure induced by vaccines or prior infections. Several animal species act as important mixing vessel hosts. This chapter provides information on various orthomyxoviruses emphasizing upon virus properties, strains/types, genome, host, ecology, pathobiology, diagnosis and control.

E. M. Abdelwhab
Institute of Molecular Virology and Cell Biology, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Greifswald-Insel Riems, Germany

A. S. Abdel-Moneim (✉)
Microbiology Department, Virology Division, College of Medicine, Taif University, Al-Taif, Saudi Arabia

Virology Department, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef, Egypt
e-mail: asa@bsu.edu.eg; asa@tu.edu.sa

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19.1 Preamble

Members of the family *Orthomyxoviridae* are responsible for a variety of important respiratory diseases in humans and different animal species. The family of *Orthomyxoviridae* possesses a negative-sense, single-stranded RNA segmented genome. It possesses seven different genera: the influenza viruses A, B, C and D, *Quaranjavirus*, *Thogotovirus* and *Isavirus*. Viruses of the same genus can undergo genetic reassortment; however, reassortment has never been reported in between viruses from different genera. Influenza A viruses (IAVs) infect humans and different animals including birds, pigs, equines, dogs, cats, whales and seals. To date, there are 18 different haemagglutinins (H1 to H18) and 11 different neuraminidases (N1 to N9) for influenza A viruses. Influenza B viruses (IBVs) are exclusively human pathogens, while influenza C virus (IVC) affects humans and pigs. Serological evidence of ICV was recently detected in camels (Salem et al. 2017). Influenza D virus (IDV) was reported in pigs with influenza-like symptoms. The virus and/or its serological evidence was detected also in cattle, sheep, goats and dromedary camels (Su et al. 2017). The genus *Thogotovirus* contains viruses of ticks in two different species, Dhori virus and Thogoto virus. The genus *Isavirus* contains infectious salmon anaemia virus as a prototype. The genus *Quaranjavirus* included two new species, Quaranfil virus (QRFV) and Johnston Atoll virus (JAV), and a tentative member, Lake Chad virus (LKCV). Although recently recognized as orthomyxoviruses, they were detected a long time ago: QRFV in Egypt, in 1953; JAV in the North Pacific, in 1964; and LKCV in Nigeria, in 1969 (Clifford et al. 1968). QRFV was isolated from ticks (*Argas arboreus*), children with febrile disease and seabirds. It also causes a lethal respiratory disease and meningoencephalitis experimentally in mice (Baskerville and Lloyd 1976).

Johnston Atoll virus (JAV) was isolated from ticks (*Ornithodoros capensis*) collected in 1964 from a Noddy Tern (*Anous stolidus*) nest, Johnston Atoll in the central Pacific (Clifford et al. 1968). No human disease has been associated with JAV, but experimentally, it is lethal to newborn and weanling mice and to 1- to 2-day-old chicks. LKCV is also lethal to newborn mice and is shown to be antigenically related to QRFV. Wellfleet Bay virus (WFBV) was the responsible pathogen for causing cyclic mortality events since 1998, in common eiders (*Somateria mollissima*) in the United States (Allison et al. 2015). In 2010, a Cygnet River virus (CyRV) induced a fatal disease in captive Muscovy ducks (*Cairina moschata*) in South Australia. WFBV is closely related to CyRV suggesting that they may be geographic variants of the same virus (Allison et al. 2015).

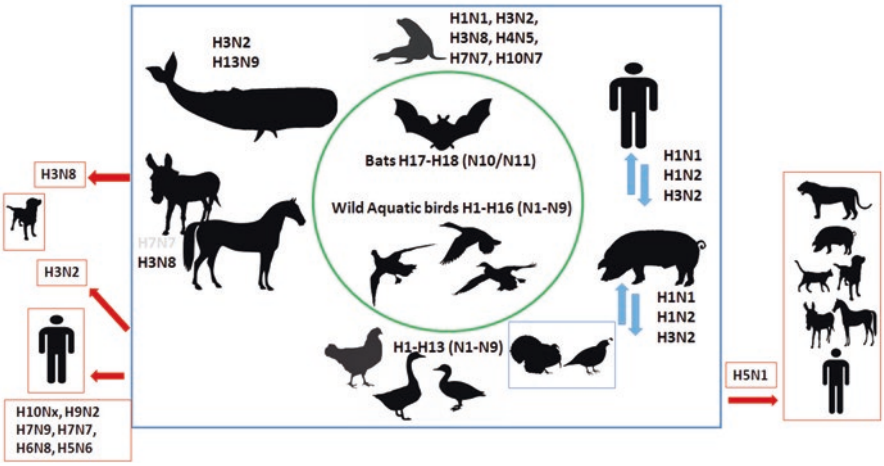

Fig. 19.1 Influenza A virus subtypes in final and reservoir hosts. Bats are reservoir hosts of H17 and H18 (N11 and N12), while wild aquatic birds are reservoir host for all other influenza subtypes. Some influenza A viruses cross species barrier and infect other species (red boxed)

IAVs cause recurrent epidemics of varying severity in humans and different animal species (Fig. 19.1) due to antigenic drift, gradual accumulation of point mutations, during replication under immune pressure induced by vaccines or prior infections. Moreover, reassortment, swapping of gene segments of two different IAVs, during replication enables continuous evolution of IAVs in nature causing devastating panzootic in different animal species and occasionally human pandemics. Domestic pigs, turkeys and quails are important hosts that could act as a mixing vessel. The influenza A viruses circulate in reservoir hosts, mainly wild aquatic birds: especially ducks, gulls and shorebirds that spread IAVs across continents with subsequent transmission to the respective final host including human and different animal species (Maclachlan et al. 2017). More recently, bats are reservoir for H17N10 and H18N11 (Maclachlan et al. 2017). In the wild bird reservoirs, IAVs mainly replicate in the intestinal epithelium resulting in an efficient faecal excretion of the virus (Maclachlan et al. 2017; Suarez and Sims 2013).

19.2 History

In September 1872, a panzootic of equine influenza was reported in Canada which was associated with a fatal epidemic in poultry in the United States between 15 November and 15 December 1872 and followed by major influenza epidemics in 1873 and 1874 (Morens and Taubenberger 2010). Major epizootics of equine influenza recurring in the United States was also recorded in 1880–1881, 1900–1901 and 1915–1916 (Morens and Taubenberger 2010), with no record of associated avian influenza outbreaks. In 1901, a filterable agent was isolated from chickens suffering

from fowl plague that was later on classified as an influenza virus of H7 subtype (Suarez and Sims 2013).

Swine IAV was first isolated from pigs in 1930, 15 years after the greatest 1918 Spanish human flu pandemic. These early viruses have been proven to be H1N1, and it has been circulated in swine for about 80 years without great antigenic changes (Maclachlan et al. 2017). Influenza vaccines are used in humans since the 1940s and in horses 20 years later (Daly et al. 2011). In poultry, blanket vaccination strategy against HPAIV was applied in the 1990s during the Mexican H5N2 outbreaks.

19.3 Virus Properties

19.3.1 Morphology

Virions are spherical or pleomorphic with helical symmetry with a size range of 80–120 nm in diameter. Filamentous forms of the virions were also detected.

19.3.2 Viral Genome

Orthomyxoviruses possess 6–8 negative-sense, single-stranded RNA segments. Influenza virus types A and B and *Isavirus* contain eight RNA segments; influenza C and influenza D, seven RNA segments; and *Quaranjavirus* and *Thogotovirus*, six RNA segments. The segment length ranges from 736 to 2396 nt, and the total genome size ranges from 10.0 to 14.6 kb. Distinguishing features and conservative terminal sequence are presented in Tables 19.1 and 19.2. The RNA segment contains complementary sequences with promoter activity.

19.3.3 Proteins

Viral proteins of orthomyxoviruses include (i) replication proteins (RNA-dependent RNA polymerase: RNdRp) including PA, PB1 and PB2; (ii) internal core protein (nucleoprotein [NP]), which is associated with each RNA segment forming ribonucleoprotein (RNP); (iii) surface type I membrane glycoproteins (haemagglutinin [HA, HE {HEF} or GP]) that are involved in attachment, fusion and neutralization; and (iv) a non-glycosylated matrix protein (M1 or M). M2 and BM2 function as proton-selective ion channels in mammalian cells, acidifying the virion interior that is important for uncoating and fusion and equilibrating the intraluminal pH of the trans-Golgi apparatus

with that of the cytoplasm. The ion channel activity of only the former is inhibited by anti-influenza A drugs, amantadine and rimantadine: (v) orthomyxoviruses may code for two non-structural proteins (NS1, NS2 [NEP]). Virion enzymes (variously represented and reported among genera) include a transcriptase (PB1 in

Table 19.1 The genomic segment organization of different genera of *Orthomyxoviridae*

	Influenza A	Influenza B	*Isavirus*	Influenza C	Influenza D	*Quaranjavirus*	*Thogotovirus*
Seg. no.	8	8	8	7	7	6	6
Seg. 1	PB2	PB2	P[a]	PB2	PB2	PB2	PB2
Seg. 2	PB1	PB1	P[a]	PB1	PB1	PB1	PB1
Seg. 3	PA	PA	NP	PA	P3	PA	PA
Seg. 4	HA	HA	P[a]	HE[b]	HE	Unknown	GP[d]
Seg. 5	NP	NP	F	NP	NP	GP	NP
Seg. 6	NA	NA	HE	M1/CM2	P42	Unknown	M and ML
Seg. 7	M1 and M2	M1 and M2	NS[c]	NS1/NEP [NS2]	NS1/NEP [NS2]	–	Unknown
Seg. 8	NS1 and NEP (NS2)	NS1 and NEP (NS2)	M1 and RNA binding protein	–	–	–	–

[a]The segments 1, 2 and 4 encode the P proteins based on limited homologies to other RdRp.
[b]Receptor binding and fusion activities. It also functions as the receptor-destroying enzyme, 9-0-acetylneuraminyl esterase.
[c]Interferon antagonistic function (34.2 kDa) and a protein of unknown function (17.6 kDa).
[d]It is unrelated to any influenza virus protein but shows amino acid sequence similarity with the glycoprotein gp64 of baculoviruses.

Table 19.2 Terminal sequences of different genera of *Orthomyxoviridae*

Genus	Terminal sequence
Influenza A	5′-AGUAGAAACAAGG and 3′-UCG(U/C)UUUCGUCC
Influenza B	5′-AGUAG(A/U)AACAA and 3′-UCGUCUUCGC’
Influenza C	5′-AGCAG(U/G)AGCAAG and 3′-UCGUCUUCGUC
Influenza D	NA[a]
Isavirus	5′-AGUAAAAA(A/U) and 3′-UCG(U/A)UUCUA
Quaranjavirus	5′-AGCAAUCACAA and 3′-UCGUUAGUGU(A/U)(A/G)
Thogotovirus	5′-AGAGA(U/A)AUCAA(G/A)GC and 3′-UCGUUUUUGU(C/U)CG (segments 1–5) or 3′-UCACCUUUGUCCG (segment 6)

[a]NA [not identified]: To date, no available sequences for the gene termini of influenza D viruses.

influenza viruses A, B and C and thogotoviruses), an endonuclease (PA in influenza viruses A, B, C) and a receptor-destroying enzyme (neuraminidase [NA] for influenza A and influenza B viruses or 9-0-acetylneuraminyl esterase in the case of the influenza C virus HE [HEF] protein).

19.3.3.1 PB2

In addition to its role in viral RNA transcription/replication, PB2 plays a role in host range restriction. Amino acid substitution at amino acid residue number 627 from glutamic acid (found in avian isolates) to lysine (found in human influenza viruses) confers efficient replication in mice and humans. However, viruses without Glu 627 Lys mutation were detected in both severe and fatal human cases (Shaw et al. 2002).

19.3.3.2 PB1

PB1 is required for the initiation and elongation of the viral RNA. A second open reading frame PB1-F2 polypeptide is present in some influenza A viruses (Chen et al. 2001).

19.3.3.3 PA

PA is an important component for the polymerase complex and is needed for viral RNA replication by acting as an elongation factor or through facilitating the binding of PB1 to viral RNA and in the transcription process. It possesses a serine protease that supports efficient viral growth but not in cell culture (Fodor et al. 2003).

19.3.3.4 Haemagglutinin

HA is responsible for the attachment and penetration of viruses into cells. HA (HA0) is cleaved by cellular proteases into HA1 and HA2 subunits. The signal sequence at the N-terminal part of the protein is removed. HA cleavage is essential to expose the N-terminal part of the HA2 (hydrophobic terminus), which is responsible for fusion of the viral envelope and the endosomal membrane. The protease cleavability of the HA is affected by the number of basic amino acids at the cleavage site and the presence of the carbohydrate. The highly pathogenic (HP) avian influenza viruses (AIVs), members of influenza A viruses, have multibasic cleavage site motifs, whereas low pathogenic (LP) AIVs possess monobasic cleavage site motifs

(basic amino acids are arginine “R”, lysine “K” and rarely histidine “H”). The presence of carbohydrate adjacent to the cleavage site may sterically hinder the access of proteases to the cleavage site. However, the insertion of two basic amino acids in the cleavage site restores the HA cleavability probably due to the formation of a loop structure which is accessible to the cellular proteases. The amino acid downstream of the cleavage site (the amino terminal residue of the HA2) also affects HA cleavage (Horimoto and Kawaoka 1995).

19.3.3.5 Receptor Binding

Influenza A and B viruses bind to α2,6-sialyllactose, N-acetylneuraminic acid, α2,6-galactose-(NeuAca2,6Galb1,4Glc) and α2,3-sialyllactose-(NeuAca2,3Galb1,4Glc). The majority of avian and equine influenza A viruses bind the NeuAca2,3Gal, whereas human and swine influenza viruses bind the NeuAca2,6Gal. Swine tracheal epithelium harbours both types of sialyloligosaccharides: hence, pigs are potential mixing vessel for both influenza viruses. Human viruses infect preferentially nonciliated cells with SAα2,6Gal sialyloligosaccharides, while avian viruses infect ciliated cells with SAα2,3Gal sialyloligosaccharides. Duck intestinal epithelium and equine tracheal epithelium possess SAca2,3Gal but not SAa2,6Gal. α2,3-N-Glycolyl sialic acid (NeuGca2,3Gal) is prevalent in the equine tracheal and duck epithelium supporting the replication of influenza A with specificity to this type of receptor but not allowing replication of viruses that bind to N-acetyl sialic acid (Matrosovich et al. 2004; Ito et al. 1998).

The amino acids of HA at position 226 and 228 (H3 numbering) are determinants for receptor-binding specificity. Substitution of Gln to Leu 226 and Gly to Ser 228 changes the receptor-binding specificity from avian to human receptor binding. Amino acid substitution at amino acid residue numbers 136, 190, 195 and 225 (H3 numbering) also alters the binding affinity in a variable degree (Martin et al. 1998). Human influenza viruses grown in mammalian cell culture contain the same HA amino acid sequences of those initially isolated from humans; however, receptor-binding variant mutants can be selected during propagation in chicken eggs (Mochalova et al. 2003).

19.3.3.6 NP

The viral RNA wraps the NP protein and the RNA-binding region of the NP of influenza A virus is located between amino acid residues 91 to 188. NP is important for viral RNA synthesis and some critical mutations in NP lead to defects in RNA replication. NP is presumed to be an important determinant of host range restriction (Maclachlan et al. 2017).

19.3.3.7 NA

The NA protein of influenza A viruses is the second major glycoproteins on the virus surface. The NA is a receptor-destroying enzyme that is essential for both virus entry and release from infected cells. The balance between the HA and NA functions is critical for influenza virus replication. The NA plays a role in host range restriction, and the NA substrate corresponds to the preferential receptor

recognition by the HA molecule and is determined by the NA amino acid at position 275 (Kobasa et al. 1999). Currently, there are eleven types of NA among the influenza A viruses. With the exception of N10–N11, the nine NA subtypes are separated into two main groups based on the structure and the phylogenetic analysis. Group 1 NA included N1, N4, N5 and N8, while group 2 included N2–N3, N6–N7 and N9. NA is an attractive target for anti-influenza drugs due to its role in virus release from infected cells. In humans, oseltamivir and zanamivir are active against both group 1 and group 2 NA as well as influenza B NA. Meanwhile, laninamivir is another long-acting NA inhibitor including oseltamivir-resistant viruses in adults. Peramivir is approved in Japan for use in patients above 1 month of age (Gaymard et al. 2016).

19.3.3.8 M1

M1 is a type-specific antigen, determines the virus morphology of influenza viruses and is a determinant of virus budding and assembly. It is also required for nuclear export of viral RNP complexes. M1 acts as a molecular switch that inhibits RNP transcription activity and initiates the final step of virus assembly (Rossman and Lamb 2011).

19.3.3.9 M2

M2 functions as a pH-activated ion channel that permits protons to enter the virion during uncoating and that modulates the pH of intracellular compartments, an essential function for the prevention of acid-induced conformational changes of intracellularly cleaved HAs in the trans-Golgi network. The activity of the M2 ion channel is targeted by amantadine and rimantadine hydrochloride. Drug-resistant mutations include mutations in amino acid residue numbers 27, 30, 31 and 34. The M2 ectodomain may play a role in virion incorporation (Rossman and Lamb 2011).

19.3.3.10 Other M Gene Products of Type B and C Viruses

BM2

BM2 is encoded by the M gene of type B virus (Horvath et al. 1990). It possesses equivalent function of the type A M2 protein ion channel activity. It also prevents HA from being subjected to low-pH-induced conformation during transport to the cell surface (Horvath et al. 1990).

NS1

NS1 inhibits of interferon (IFN) response to ensure efficient viral replication. It also induces apoptosis in infected cells with other viral proteins such as NA. NS1 inhibit mRNA splicing and the nuclear export of cellular mRNA, to increase the viral mRNA synthesis (Marc 2014).

NS2(NEP)

NS2 is referred to as nuclear export protein (NEP). It contains a nuclear export signal (NES) and interacts with the cellular nuclear export factor that mediates export of proteins containing NESs. It also connects the cellular export machinery with viral RNPs through M1 (Akarsu et al. 2003).

HEF

The HEF protein of influenza C virus is post-translationally cleaved into two HEF1 and HEF2 subunits. The head of the HEF contains the receptor binding site. Two regions located under the receptor-binding site in HEF1 form the esterase domain. The HEF facilitates the binding of influenza C virus to the cell receptor, an oligosaccharide with a terminal 9-O-acetyl-N-acetylneuraminic acid. Unlike the NA, HEF does not catalyse the cleavage of the linkage between sialic acid and the adjacent sugar residue, but cleave of the 9-O-acetyl group of 9-O-acetyl-N-acetylneuraminic acid. It also possesses fusion activity. Influenza C virus uses the acetylesterase activity for cell entry (Strobl and Vlasak 1993).

19.4 Avian Influenza

19.4.1 Introduction

Avian influenza is a highly infectious virus disease of poultry with potential zoonotic importance. It also affects pet, zoo and wild birds. In poultry, the LP AIVs induce a mild or subclinical infection including diarrhoea and drops in egg production in layers. The HP AIVs induce severe respiratory disorders, diarrhoea and up to 100% mortalities in domestic birds. HPAI is usually associated with H5 and H7 influenza A virus subtypes, although LPAI of the same subtypes was recorded in birds (Suarez and Sims 2013). Avian influenza viruses pose potential zoonotic importance, and many human cases were infected with different avian influenza subtypes: H10N8, H10N7, H9N2, H7N7, H7N9, H6N8, H5N6 and H5N1, among others. The latter subtype induces very high case fatality with a potential of being a pandemic strain with all catastrophic consequences.

19.4.2 History

HPAI was recognized in 1878 in Italy and was confused with fowl cholera (Suarez and Sims 2013). Although it is a filterable agent, it was detected in 1901 by Centanni and Savonuzzi; however, avian influenza was identified as an influenza virus in 1955. By the mid-twentieth century, HPAI had been diagnosed in most parts of the world. H7N1 and H7N7 subtypes were found to be the causative agents of 1901–1950s' HPAI outbreaks. In 1959 in Scotland and in 1961 in South Africa, H5N1 and H5N3 induced outbreaks in chickens, respectively (Suarez and Sims 2013).

LPAI was first reported in Germany (Dinter strain), in chickens in 1949, that was later identified as A/chicken/Germany/49 (H10N7) in 1960. LPAI viruses from domestic ducks with respiratory distress were isolated between 1953 and 1963 in Canada, Czechoslovakia, England and Ukraine. The LPAI was associated with mild respiratory disease and drops in egg production in turkeys in Canada and the United States. LPAI H5 subtype was isolated in Canada and the United States in 1966 and

1968, respectively. In 1971, a LPAI H7N3 subtype was isolated from turkeys with mild respiratory distress and diarrhoea (Suarez and Sims 2013).

During 1972, AIVs were isolated from migratory birds. Although AIVs isolated from wild aquatic birds have low pathogenicity to poultry, a few HPAIVs have been isolated from wild birds: H5N1, H5N3, H5N8 and single isolations of H7N1 (A/finch/Germany/72), H7N7 (A/gull/Germany/79) and H7N3 (A/Peregrine Falcon/UAE/2384/98) (Suarez and Sims 2013).

19.4.3 Virus Strains and Genotypes

All IAVs can infect birds except H17N10 and H18N11 that have been isolated from bats. AIVs that infect birds have two main genetic lineages: Asian lineage and North American lineage. Such lineages are further classified into sublineages and clades within each main subtype. AIVs are also classified according to their virulence in birds and their molecular characteristics into highly pathogenic avian influenza (HPAI) and low pathogenicity avian influenza (LPAI). Classical disease in poultry is usually represented by infections with AIV subtypes H5, H7 and H9 associated with N1–N9. In contrast to H5 and H7 that contain both HPAI and LPAI, all H9 viruses identified worldwide in wild birds and poultry are LPAI viruses.

In areas where LPAI is endemic or when AIV vaccination is used in the control policy, genetic variants emerge due to genetic drift which could be due to immunological pressure from vaccine or endemic infections by the circulating field strains. Antigenic shift due to reassortment could also occur and reassortment was reported for the Hong Kong H5N1.

Amino acid substitutions in different genes of H5N1 and H7N1 were associated with airborne transmission in ferrets (Sutton et al. 2014).

19.4.4 Ecology

Healthy wild aquatic birds, mainly in the orders Anseriformes and Charadriiformes, were confirmed as asymptomatic reservoirs of AIVs. In wild waterfowl, AIVs are maintained by passage in susceptible birds throughout the year, especially in juvenile birds prior to fall migration. Such migratory birds infect susceptible resident waterfowl. Wild birds may play an essential role in initial introduction of AIVs in terrestrial poultry (Olsen et al. 2006).

There are five man-made ecosystems including (i) village, backyard and hobby flocks, (ii) range-raised poultry, (iii) intensive commercial poultry, (iv) live poultry markets (LPM) and (v) bird collection and trading systems. In both developed and developing countries, rural and village poultry as well as LPM possess a high rate of infection (Suarez and Sims 2013).

Since 2003, H5N1 HPAI that began in Southeast Asia has become endemic in many countries. During 2010–2016, H5 subtype (H5N1, H5N2, H5N8, H5N6, H5N9, H5N5, H5N3) was the major circulating subtype, followed by H7 subtype (H7N9,

H7N7, H7N3, H7N2, H7N1, H7N6) and then H9 subtype (H9N2, H9N1). Other subtypes include H3N8, H3N2, H10N?, H1N2, H4N6, H10N7, H1N1 and H11N9. Interestingly, considerable percentages of wild bird isolates were due to HPAI.

19.4.5 Geographical Distribution

Avian influenza is a common disease that is widespread worldwide.

19.4.6 Host

AIV was isolated from more than 100 species of birds representing 13 different orders; however, the actual number could be much greater. AIV affects different domestic birds. AIVs cross species barrier and infect mink, seals and whales causing epidemics of respiratory distress. H5N1 HPAI virus causes sporadic infection in tigers, leopards, cats, Owston's palm civets, a stone martin and pigs as well as equines. In addition, it causes a highly fatal disease in humans. Experimentally, H5N1 can infect pigs, ferrets, rats, rabbits, guinea pigs, mice, cats, mink and nonhuman primates (Suarez and Sims 2013; Yee et al. 2009).

19.4.7 Transmission

Sources of AIV infection in commercial poultry flocks include infected domestic and confined birds, migratory waterfowl and other wild birds, as well as domestic pigs or pet birds. AIV transmission occurs by horizontal route of transmission by both direct and indirect contact through aerosol droplets or exposure to virus-contaminated fomites, people (e.g. contaminated shoes and clothing) and equipment shared in production, or live-bird marketing. Eggshell surface and the internal contents of the eggs are potential source of HPAI virus. Transmission could also occur by airborne dissemination in short distances. Swine are potential source of transmission of swine influenza viruses (H1 and H3) in turkeys. AIV is excreted for up to 36 days in chickens, 22 days in turkeys, 17 days in ducks and 56 days in pheasants (Suarez and Sims 2013).

19.4.8 Clinical Signs

Most infections by LPAI viruses in wild birds produce no clinical signs. In broiler-type chickens and turkeys, LPAI leads to mild to severe respiratory signs, while layers and breeders exhibit decreased egg production (Suarez and Sims 2013).

In wild birds and domestic ducks, most HPAI viruses produce mild clinical signs. In the last decade, HPAI H5 subtype resulted in sudden death without apparent clinical signs (e.g. neurological signs, depression, anorexia).

In turkeys, chickens and other poultry, HPAI induces severe highly fatal disease up to 100% mortality. Mortalities may appear in the absence of any clinical signs or gross lesion in the peracute form of the disease. The acute form of the disease causes cyanosis and oedema of the head, comb, wattle or snood in turkeys and reddish-blue discoloration of the shanks and feet. Diarrhoea may also be a common sign (Suarez and Sims 2013). Torticollis and opisthotonos as well as other nervous manifestations may appear in ducks and geese and birds recovering from the peracute form of the disease (Suarez and Sims 2013).

19.4.9 Post-mortem Lesions

19.4.9.1 Low Pathogenic Avian Influenza in Birds

Low pathogenic avian influenza virus causes congestion and catarrhal to serofibrinous inflammation of the trachea and sinuses. The tracheal mucosae are oedematous with congestion. Haemorrhagic ovary and the presence of yolk in the abdominal cavity may be observed in the reproductive tract of laying hens. Airsacculitis, peritonitis, visceral urate deposition and nephritis may be found in some birds.

19.4.9.2 Highly Pathogenic Avian Influenza in Birds

Birds that die from the peracute form of the disease may have no lesions. In the acute form in chicken and turkeys, oedema and cyanosis of the head, wattle and comb are common symptoms. Petechiae on the viscera, epicardium and sometimes the muscles especially the pectoral muscles, necrotic lesions and congestions of the pancreas, spleen and heart as well as hepatosplenomegaly with parenchymal mottling were observed. Haemorrhagic enteritis and haemorrhages in the mucosa of the proventriculus, ventriculus and Peyer's patches and/or atrophied thymus and bursa can be seen in some birds.

In ostriches, there are oedema of head and neck, severe haemorrhagic enteritis, airsacculitis, hepatosplenomegaly, peritonitis, renal congestion and enlarged and firm pancreas (Suarez and Sims 2013).

19.4.10 Diagnosis

19.4.10.1 Clinical Diagnosis

High mortality rate; cyanosis and oedema of the head, comb, wattle or snood in turkey; and reddish-blue discoloration of the shanks and feet are highly suggestive of HPAI.

19.4.10.2 Samples

Tracheal, oropharyngeal or cloacal swabs from live or dead birds placed in a sterile virus transport medium containing high levels of antibiotics can be used for virus isolation or detection. Specimens from the lungs, liver and spleen, as well as

secretions and/or excretions from respiratory and intestinal tracts, are also useful (Suarez and Sims 2013).

19.4.10.3 Laboratory Tests

The direct detection of influenza A nucleoprotein in avian specimens and allantoic fluid of inoculated ECE using antigen capture immunoassays is a sensitive method for rapid detection of AIV infection but less efficient than virus isolation (Suarez and Sims 2013). Detection of the M, HA and NA genes using real-time RT-PCR is commonly used for the diagnosis of field cases. For virus isolation, the sample is inoculated in the allantoic cavity of 9–11 chicken embryos. Several egg passages may be required to isolate LPAIVs, while HPAIVs kill the embryos within 48 h. The virus detection of the chilled allantoic fluid is indicated by the presence of haemagglutinating activity using chicken erythrocytes. Newcastle disease virus (NDV) should be first excluded using specific antibodies against NDV in haemagglutination inhibition assay. If negative, then AIV detection should be screened using (monoclonal) antibodies against the type-specific nucleoprotein or matrix protein viral antigens by rapid chromatographic strips or other commercial antigen capture immunoassays (Suarez and Sims 2013).

19.4.10.4 Serology

ELISA assays have been developed to detect antibodies to AIVs.

19.4.11 Control

Control of AIV depends on the subtype, the public health importance, the country's economic status, and the epidemiologic nature of the disease (e.g. HPAI or LPAI, endemic or sporadic infections). A proper control programme for HPAI should include education and awareness, biosecurity, regular influenza surveillance and depopulation of infected poultry. In optimum conditions, HPAI outbreaks can be eradicated within 6 months to a year by traditional stamping-out programmes. In suboptimum conditions, especially in areas with high poultry production at the village or rural level, management of the disease to a low infection rate has been a realistic option. In some countries, live poultry markets, rural poultry, mixed poultry population and mixed animals including pig and poultry raising pose a serious risk for the introduction of influenza to commercial poultry. In HPAI, control procedures are accomplished by depopulation and disposal of dead birds, eggs and manure by composting, incineration or hygienic burial (Suarez and Sims 2013).

19.4.12 Vaccination

Vaccines are used in endemic areas to provide protection from LPAI and HPAI viruses. Inactivated whole AIV vaccines or reverse genetic-generated vaccine

strains, followed by chemical inactivation and oil emulsification, are commonly used. Moreover, chickens can be immunized successfully by the in ovo administration of inactivated oil emulsion vaccine. Different chimeric vaccines including fowl pox-AI haemagglutinin (H5) recombinant vaccine (rFP-AI-H5), NDV-AI-H5 and avian influenza–Marek's disease vaccine are commercially available for use in birds. Vaccination does not guarantee that the flocks are free from influenza and vaccinated birds must be monitored for the presence of AI virus until slaughtered (Suarez and Sims 2013).

19.5 Swine Influenza Virus

19.5.1 Introduction

Swine influenza virus (SIV) is a highly contagious mild swine viral disease caused by influenza A virus, mainly H1N1, H1N2 and H3N2 subtypes. Although the disease shows 100% morbidity, infected pigs might not show signs of disease manifestation and infection. SIV infections are manifested as acute respiratory disease characterized by fever, inactivity, decreased food intake, respiratory distress, coughing, sneezing, conjunctivitis and nasal discharge. The disease is zoonotic and can be transmitted to humans who come in contact to infected pigs (Vincent et al. 2014).

19.5.2 History

Swine influenza (SI) was first reported as an epizootic of respiratory disease in pigs in the Midwestern United States in 1918. It coincided with the incidence of human major influenza pandemic: the Spanish flu. The first SIV was isolated from pigs in 1930, 3 years before the isolation of swine H1N1 in humans.

19.5.3 Virus Strains and Genotypes

H1 and H3 subtypes mainly affect pigs, in addition to sporadic infections with other subtypes including: H2, H4, H5 and H9. Two distinct variants of the H1N1 swine influenza virus exist: the avian variant in Europe and the other variant in United States, similar to the original virus strain. Other strains that infect swine include human H3N2 strains in China, Europe and North America and H1N2 (triple reassortants) (Fig. 19.2). In China, three types of H3N2 were reported in swine: human-like H3N2 virus, double reassortants and triple reassortants. Infection of pigs with avian H5N1 and H9N2 types were also recorded in China (Vincent et al. 2014).

19.5.4 Ecology

There is a strong evidence that the 1930 swine strain was antigenically related to the 1918 pandemic influenza strain (reviewed by Stuart-Harris et al. (1985)). The American swine influenza A subtype H1N1 viruses spread to Europe in 1976 and have been replaced by swine H1N1 of avian origin in 1979 (Pensaert et al. 1981).

Pigs constitute a mixing vessel in which different influenza viruses can reassort. This is related to the fact that pigs are susceptible to infection by swine, avian and human influenza viruses and possess both avian-type and human-type sialic acid receptors, with subsequent possibility of emergence of pandemic influenza virus strains. In Europe, avian–swine influenza reassortants were first detected in 1979 and human–avian H3N2 viruses emerged between 1983 and 1985 and then repeatedly detected in pigs (Webster et al. 1992). Human H3N2 influenza infected the North American swine herds around 1995 and a double-reassortant swine H1N2 arisen that possessed PB1 from human H3N2. The double reassortants further acquired PA and PB2 avian internal protein genes. The triple reassortant H1N2 spread widely in the pig population. Pandemic *pdm09*H1N1 acquired the HA and NA genes from Eurasian avian-like swine H1N1 and other genes from the triple reassortant swine H1N2. It contains HA, NP and NS from classical swine H1N1, PB2 and PA from avian source, and PB1 from a human seasonal H3N2 (Fig. 19.2) (Garten et al. 2009; Vincent et al. 2014).

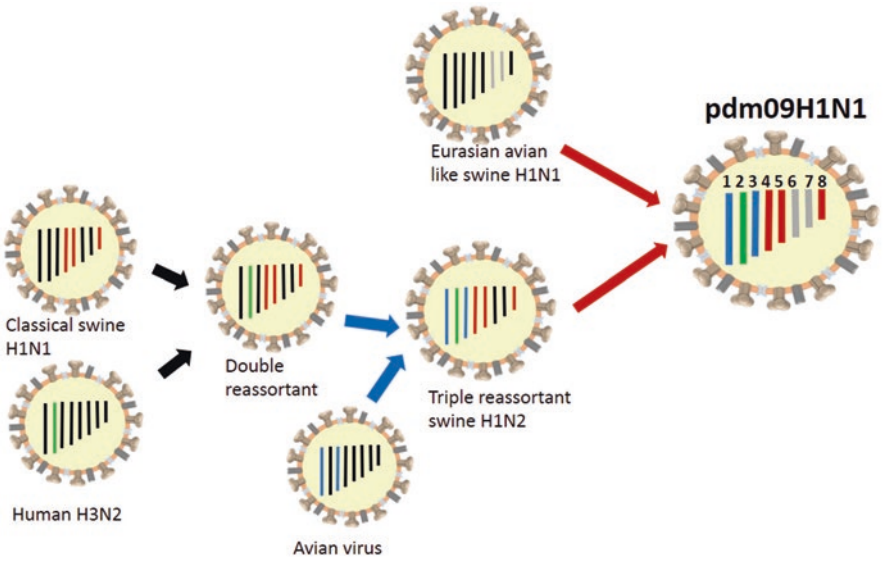

Fig. 19.2 Emergence of *pdm09*H1N1 due to the reassortment of influenza viruses

19.5.5 Geographical Distribution

Swine influenza is the most common disease in Europe, parts of Asia, North and South America and some parts of Africa.

19.5.6 Host

Swine influenza strains are enzootic in pig populations in many parts of the world and have also infected turkeys in the United States (Vincent et al. 2014; Suarez and Sims 2013).

19.5.7 Transmission

SIVs spread among pigs by direct and indirect contact to infected animals, contaminated equipment and utensils from infected pig herd.

19.5.8 Clinical Signs

The disease appears abruptly after an incubation period of 1–3 days. Most animals show subclinical infection. Clinically infected pigs exhibit fever, inappetence, huddling, weight loss, coughing, sneezing and nasal discharge; however, severe bronchopneumonia was also recorded in some animals (Janke 2014).

19.5.9 Post-mortem Lesions

Lungs of infected pigs showed a purple-red, multifocal to coalescing consolidation. The lesion usually appears in the cranio-ventral portions of the lung (Janke 2014).

19.5.10 Diagnosis

19.5.10.1 Clinical Diagnosis

Swine influenza is characterized by sudden onset of respiratory disease that may be misdiagnosed with other diseases including *Mycoplasma hyopneumoniae* and *Actinobacillus pleuropneumoniae* (Janke 2014).

19.5.10.2 Samples

Nasal swabs or oral fluids can be collected from living animals. Post-mortem samples include nasal turbinates, tonsil, trachea or lung.

19.5.10.3 Laboratory Tests

SIVs can be isolated in the allantoic sac of embryonated chicken eggs. MDCK cells are the most commonly used cell line for isolation, propagation and titration of SIV, a trypsin-containing overlay. Although they have not been explored for primary isolation, newborn swine kidney (NSK), newborn pig trachea (NPTr), porcine intestinal epithelial cell line (SD-PJEC) and bone marrow support SIV replication (Janke 2014; Khatri and Saif 2011).

For routine diagnosis, the real-time RT-PCR test has replaced virus isolation; however, virus isolation is still used to increase the initial virus titer in the original sample for genetic analyses. Identification of the virus genotype is conducted by real-time RT-PCR tests for both HA and NA genes. The results are confirmed by sequence analysis of both genes, or with specific monoclonal antibodies. The virus can be detected in tissue samples by immunofluorescence or by immunohistochemistry.

19.5.10.4 Serology

Among the serologic tests available, indirect fluorescent antibody test (IFAT), serum neutralization (SN), haemagglutination inhibition (HI) and ELISA are currently being used for swine. The most commonly used are the HI and ELISA (Janke 2014).

19.5.11 Control

Swine influenza is controlled by strict biosecurity measures and vaccination. All-in–all-out policy is used in many commercial swine producers. Biosecurity may be sufficient to minimize or exclude influenza virus infection.

19.5.12 Vaccination

Inactivated influenza vaccines are used to protect the pregnant sow and her suckling piglets or during the grow/finish phase of production. They are also used to reduce the zoonotic transmission of the disease to humans. Inactivated SIV vaccine includes two or more representatives of H1 and H3 which provide efficient homologous but only partial protection against heterologous SIV (Rahn et al. 2015).

19.6 Equine Influenza Virus

19.6.1 Introduction

Equine influenza virus (EIV) is a highly contagious rarely fatal respiratory disease of equines. EIV is associated with two subtypes of influenza A viruses: H3N8 and H7N7. H3N8 virus is currently associated with equine influenza worldwide. H7N7

virus was a potential cause of EIV with H3N8 viruses in horses for many years; the former virus has not been detected in equines for more than 25 years (Webster et al. 1992). Avian IAV subtype H5N1 was isolated from a respiratory disease in donkeys in Egypt (Abdel-Moneim et al. 2010). H3N8 infection of naïve horse population may be severe, or fatal, especially in the young or debilitated animals. EIV links to sialic acid receptor with α2,3-galactose linkage similar to AIVs.

19.6.2 History

An influenza-like epizootic in horses was reported in 1872 in the United States (Webster et al. 1992). This outbreak was speculated to be an evidence of cross-species barrier of avian influenza. The first serological evidence of EIV was reported in 1955 in Sweden, while the first isolation of the EIV from horses occurred in Czechoslovakia in 1956 that was subsequently demonstrated to be a H7N7 (A/equine/Prague/1/56). EIV subtype H3N8 (A/equine/Miami/I/63) was first reported in Florida in animals recently imported from Argentina. This virus is widespread globally and reached Europe in 1965. The first EIV oil-adjuvant vaccine was developed around 1965.

Both H7N7 (A/equi-1) and H3N8 (A/equi-2) were the only detected subtypes reported in equines. Currently, only H3N8 is detected among equines, while H7N7 viruses were not detected since the late 1970s (Webster 1993); however, the antibody to H7N7 has been detected in unvaccinated horses suggesting that the virus may still be circulating.

19.6.3 Virus Strains and Genotypes

EIVs, like other influenza A viruses, are subjected to antigenic drift, although in a rate lower than avian and human influenza viruses. In the late 1980s, European and American lineages evolved (Daly et al. 1996). Two clades were further emerged from the American lineage. Clade 1 (Florida clade I) EIV strains are widespread in American continents and also in Africa, Asia and Australia as well as Europe (Cullinane and Newton 2013). On the other hand, clade II EIV strains (Florida clade II) are responsible for EI infections in Europe and Asia. Currently, EIV vaccine contains one strain from each clade (Cullinane and Newton 2013).

19.6.4 Ecology

EIV strains are probably evolved from AIV, but constitute independent lineages with little evidence of reassortment between EIV and AIV.

H7N7 viruses between 1973 and 1977 were reassortants carrying H3N8 internal-protein-encoding genes except the M gene (Murcia et al. 2011). EIV H3N8 subtype arose probably from AIV in the early 1950s (Murcia et al. 2011). The H3N8 strain

possessed the PB2 and matrix proteins from North American avian strains (Gorman et al. 1990b; Ito et al. 1991), while PB1, PA, HA and NP from other influenza A viruses (Gorman et al. 1990a; Kawaoka et al. 1989; Okazaki et al. 1989). Equine NS was found to be subtype specific (Nakajima et al. 1990), as the NS segments of the H3N8 viruses were close to each other but not to H7N7 viruses.

19.6.5 Geographical Distribution

H3N8 EIV is not a seasonal disease and it is enzootic in Europe, North and South America and Asia. It is also present in India and Africa. Australia and Hong Kong, as well as New Zealand, Japan and South Africa, are now thought to be EIV-free (Cullinane and Newton 2013).

19.6.6 Host

EIV causes a respiratory disease of horses. EIV H3N8 subtype cross species to canine in 2004 causing an outbreak of canine influenza virus (CIV) in the United States (Crawford et al. 2005). Subsequently, CIV evolved as a distinct cluster of the H3N8 EIV. However, there is no current evidence of transmission of equine influenza virus from dogs to horses. Cats also showed clinical disease signs after experimental infection of EIV H3N8 subtype (Su et al. 2014). EIVs have also been isolated from swine. H3N8 EIV strains are not considered to be human pathogens (Cullinane et al. 2010).

19.6.7 Transmission

The virus is highly contagious and transmitted directly by the respiratory route through direct contact and indirectly thorough personnel, fomites and vehicles. International traveling of horses for breeding or race purposes plays an important role in the introduction of EIV strains into different countries (Cullinane and Newton 2013).

19.6.8 Clinical Signs

The clinical signs include fever (peak 42 °C), a serous to mucopurulent nasal discharge, dry cough, depression, anorexia, limb oedema and enlarged mandibular lymph nodes. Broncho-interstitial pneumonia was recorded in young foals with no maternal immunity against EIV with subsequent hypoxia and acidosis. Although not a common sign, enteritis was frequently reported in 1989 avian H3N8 epidemic in China. Anaemia, leukopaenia and lymphopaenia were recorded (Daly et al. 2011). In vaccinated exposed populations, the spread of disease is not rapid and the

clinical signs are less severe and may be limited to suboptimal performance in well-vaccinated horses, and many horses may be subclinically infected (Daly et al. 2011; Elton and Bryant 2011).

19.6.9 Post-mortem Lesions

Equine influenza infections rarely result in fatal consequences. Post-mortem finding may include bronchiolitis, peribronchiolitis and subacute interstitial pneumonia (Elton and Bryant 2011).

19.6.10 Diagnosis

19.6.10.1 Clinical Diagnosis

Rapid spread of clinical signs especially cough is suggestive of EIV in unvaccinated horses; however, in vaccinated populations clinical signs are not inconclusive (Elton and Bryant 2011).

19.6.10.2 Samples

Nasopharyngeal swabs should be collected from acute cases. Virus shedding may persist for up 7–10 days in non-vaccinated horses but 1–2 days in immune horses (Elton and Bryant 2011).

19.6.10.3 Laboratory Tests

Antigen capture ELISAs and real-time RT-PCR are used for the diagnosis of EIV. EIV-specific ELISA and human influenza ELISA kits have been used in the diagnosis of equine influenza. These kits detect the nucleoprotein which is highly conserved among influenza viruses. However, RT-PCR was found to be more sensitive than AC-ELISA. Although virus isolation is less sensitive than both ELISA and RT-PCR, it is necessary for virus characterization and strain surveillance. EIV is isolated in ECE and less frequently in MDCK (Cullinane and Newton 2013; Elton and Bryant 2011).

19.6.10.4 Serology

HI is used for testing the seroconversion against EIV. Pretreatment of sera with receptor-destroying enzymes (RDE), periodate and trypsin–periodate is used to remove nonspecific inhibitors (Subbarao et al. 1992). Virus antigen is treated with Tween-80/ether (John and Fulginiti 1966) to increase the HI sensitivity. The single radial haemolysis (SRH) assay is more reproducible than the HI test (Mumford 2000). It was estimated that SRH titers of 120–154 mm2 is needed for complete protection from homologous virus challenge and 200 mm2 for heterologous protection (Newton et al. 2006). An EIV nucleoprotein ELISA was used to differentiate horses vaccinated with a canary pox recombinant vaccine from horses that had been exposed to virus by natural infection (Garner et al. 2011).

19.6.11 Control

International movement of horses is considered one of the key factors responsible for the spread of EIV. Australia and New Zealand are free from equine influenza due to routine vaccination of imported horses and adequate quarantine periods to prevent the introduction of EIV. Some countries adopt vaccination policy to indigenous equine populations to reduce the infection of EIV (Daly et al. 2011). In countries where equine influenza virus is endemic, the economic losses due to influenza can be minimized by vaccination of highly mobile horses. In EIV-enzootic countries, surveillance is limited, vaccination is seldom required, and importation policies regarding EIV are less rigorous. The OIE recommends vaccination 21–90 days prior to shipment. Horses should be screened for EIV by RT-PCR in the quarantine. All-in–all-out quarantine should be implemented.

19.6.12 Vaccination

EIV vaccines are inactivated vaccines. Other licensed vaccines include ISCOM, MLV and recombinant canarypox. For protective antibody titer, revaccination every 4 and 6 months in young horses (Newton et al. 2000). Biannual boosters are recommended for racehorses aged 2 years and older. In older horses that received multiple vaccinations, annual booster could be satisfactory. Mismatching between the vaccine and field strains leads to subclinical infection in vaccinated horses. Equine influenza vaccines are reviewed and updated annually (Cullinane et al. 2010). Since 2010, it is recommended that international vaccines should contain a clade 1 and clade 2 virus of the Florida sublineage especially for racehorses that are frequently transported from place to place.

19.7 Infectious Salmon Anaemia Virus (ISAV)

19.7.1 Introduction

Infectious salmon anaemia virus (ISAV) is the causative agent of infectious salmon anaemia (ISA), one of the most important diseases of farmed Atlantic salmon. Virulent strains may be transmitted back to the wild population. Although the disease affects farmed Atlantic salmon held in or exposed to seawater, evidence of infection in the freshwater stage has been reported. The disease is contagious and manifested by severe anaemia and multi-organ haemorrhages with an initial low mortality rate (less than 1%); however, cumulative mortalities may reach 90%. The disease leads to severe economic losses that in certain areas result in $4.8–5.5 million annual losses (New Brunswick) and millions of fish culled to control the disease (Kibenge et al. 2004).

19.7.2 History

The disease was first described in Norway in 1984, and it was initially named haemorrhagic kidney syndrome. ISAV was then detected in Canada, Scotland and Chile in the 1990s. The Chilean virus was assumed to be introduced during the initial importation of salmon to America from Europe (Cottet et al. 2011; Kibenge et al. 2004).

19.7.3 Virus Properties and Classification

ISAV possesses the haemagglutinin esterase (HE) and fusion protein (F). HE is responsible for receptor-binding and receptor-destroying activities. It agglutinates the RBCs of several fish species. The virus replicates in endothelial cells and leukocytes. Nucleotide sequences of all eight ISAV genome segments have been described. The genome encodes at least 10 proteins (Table 19.1). Segments 1, 2 and 4 encode the viral polymerases: PB1, PB2 and PA, respectively. Segment 3 encodes the nucleoprotein (NP), 68 kDa. Segments 5 and 6 encode fusion (F) protein (50 kDa) and HE (the 42 kDa) responsible for receptor-binding and receptor-destroying activities. Segment 7 encodes a non-structural protein (non-spliced mRNA) that interferes with the interferon type 1 response and another not yet characterized protein (spliced mRNA). Segment 8 encodes a 22-kDa matrix protein and an RNA-binding structural protein (26 kDa) with interferon antagonistic activities (Cottet et al. 2011; Kibenge et al. 2004).

19.7.4 Virus Strains and Genotypes

There are two major lineages of ISAV – genotype I (the European genotype) and genotype II (the North American genotype) – based on the sequence variation of segments 2, 6 and 8. Various clades occur within these genotypes. The European isolates are divided into three subgroups: G1–G3 based on the sequence variation of the extracellular region of haemagglutinin. Within these two major groups, viruses with deletions in the high polymorphic region (HPR) of the haemagglutinin esterase (HE) appear to be more virulent and can be isolated in cell culture. The HPR is characterized by the presence of gaps instead of single nucleotide mutations. The viruses that cause disease outbreaks in farmed fish have deletions in HRP in comparison to the putative ancestral variant with a longer HPR (HPR0). Those viruses are classified to more than 28 different HPRs (e.g., HPR1, HPR2, HPR3). HPR and F protein are responsible for the viral virulence. ISAV is subjected to reassortment, and a four-reassortment ISAV related to the European clade was reported in outbreaks in Chile (Cottet et al. 2011; Kibenge et al. 2004).

19.7.5 Geographical Distribution

ISAV genotype I (the European genotype) is detected in Norway, Scotland, Faroe Islands and Chile, while genotype II (the North American genotype) is detected in Canada and the United States. Both Norway and Chile constitute 33 and 31% of the total salmon production farms worldwide, respectively (Cottet et al. 2011; Kibenge et al. 2004).

19.7.6 Host

ISAV induces a disease with variable mortalities in rainbow trout. ISAV also experimentally infects brown trout and herring (*Clupea harengus*). It was isolated from diseased farmed Pacific coho salmon in Chile in 1999 (Kibenge et al. 2004).

19.7.7 Reservoirs

Several species of salmonids (brown trout, sea trout, rainbow trout, steelhead trout, chum salmon, Chinook salmon, coho salmon and Arctic char) can carry virulent ISA viruses asymptomatically. Conversion of these non-/low pathogenic strains to pathogenic or virulent strains was detected after small genetic changes (Kibenge et al. 2004).

19.7.8 Transmission

The virus is excreted from infected fish into the water from skin, mucus, faeces and urine. The virus enters the fish through the gills and skin lesions; however, transmission by coprophagy is also proposed. ISAV spreads by water-borne transmission. ISAV may be transmitted vertically. The sea louse (*Lepeophtheirus salmonis*) is a possible vector for ISAV. ISAV is detected by RT-PCR in water samples 1.5 km away from infected areas (Kibenge et al. 2004). The virus is stable at pH 5.7–9.0. The virus replicates in the salmon cell lines SHK-1, TO and ASK, with a replication optimum of 10–15 °C. Some strains also replicate in the CHSE-214 cell line. ISAV possesses a unique gene organization.

19.7.9 Clinical Signs

The clinical signs appear after 2–4-week incubation period. Prior to an outbreak, mortality slightly increased over a period of 1–3 weeks. Signs include lethargy, anaemia, pale gills, leukopaenia, ascites, exophthalmia, dark skin and increased mortality. Haemorrhages in the anterior chamber of the eye and jaundice on the ventral portion of the body with yellowing of the base of the fins and on the abdomen were also observed (Thorud and Djupvik 1988).

19.7.10 Post-mortem Lesions

Yellow- or blood-tinged fluid in the peritoneal and pericardial cavities were detected, as well as petechiae on the eye, internal organs, visceral fat and skeletal muscles, together with enlarged and congested spleen and liver. The liver may be dark brown or black or covered with a thin layer of fibrin in some cases. The kidney may be swollen and dark. The gastrointestinal tract may also be congested. Hydropericardium and severe myocarditis are observed in some outbreaks (Godoy et al. 2008).

19.7.11 Diagnosis

19.7.11.1 Clinical Diagnosis

ISA is suspected in farmed Atlantic salmon with signs of anaemia and increased mortality. Haematocrit less than 10% is indicative of the disease. ISAV is confirmed in case of the presence of typical clinical signs.

19.7.11.2 Samples

Heart and mid-kidney are collected for virus isolation and/or RT-PCR. The detection of ISAV from gills or gill mucus by RT-PCR should be avoided to exclude the possibility of presence of the virus as a contaminant rather than a primary infection.

19.7.11.3 Laboratory Tests

Outbreaks of infectious salmon anaemia can be diagnosed by virus isolation, detection of antigens and RT-PCR. Avirulent virus strains can usually be detected only by RT-PCR. ISAV can be isolated in Atlantic salmon head kidney (SHK-1) or Atlantic salmon head kidney leukocyte (ASK), Chinook salmon embryo (CHSE-214), salmonid cell culture (TO) or epithelioma papulosum cyprinid (EPC) (Kibenge et al. 2004). Virus identification can be confirmed by RT-PCR, immunofluorescence and haemadsorption. Immunochromatographic strips can also be used in some countries.

19.7.11.4 Serology

ELISA can be used as a supplemental test to other assays.

19.7.12 Control

ISA is among the notifiable diseases. A single year-class fish should be stocked together. Boats and equipment as well as nets should be cleaned and disinfected regularly. Divers should disinfect their gear before and after diving and between cages. Youngest fish cages should be dived first and then cages with the older fish cages. Proper hygienic decontamination of waste water and dead fish should be

observed. Sea lice should be controlled, and stress should be minimized. ISAV can be inactivated by sodium hypochlorite, chloramine-T, chlorine dioxide, iodophors, sodium hydroxide, formic acid, formaldehyde and potassium peroxymonosulfate (Torgersen 1998).

19.7.13 Vaccination

Commercial inactivated ISA vaccine although prohibited in the European Union is available in some countries including Canada. The vaccines do not provide complete virus clearance and the fish may become carriers (Kibenge et al. 2004).

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Part III

Concepts in Virology

Epidemiological Perspective in Managing Viral Diseases in Animals

20

Mahendra Pal Yadav, Raj Kumar Singh, and Yashpal Singh Malik

Abstract

Since the first report of a viral disease associated with plants, the fascinating field of virology has evolved and aided mankind altogether. Viral infections are known for inflicting colossal economic losses worldwide in food/work/companion animals. During the last few decades, emergence of a number of new viral diseases in animals, humans and plants has been visualized. Animal disease surveillance and monitoring is essential for the sustainability of healthy livestock production systems internationally. Preparedness for combating the emerging, re-emerging, exotic and transboundary diseases requires comprehensive monitoring and precision detection systems that are pliable under the field situations. With collective and concerted scientific interventions, a few of the animal viral diseases have been stamped out globally or regionally. Rinderpest, popularly called cattle plague, was eradicated from India in 2006 and globally in 2011. Notably, India achieved the disease-free status by OIE in 2014 for African horse sickness (peste equine), a deadly viral disease of equines. Likewise, equine infectious anaemia (EIA) and equine influenza (EI) have been controlled to a greater extent in India by adopting surveillance and monitoring along with zoo sanitary measures. Overall, there is a need for developing the 'One World, One Health' concept using multidisciplinary, regional and international networking to control major

M. P. Yadav
ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Uttar Pradesh, India

Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut, India

R. K. Singh
ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Uttar Pradesh, India

Y. S. Malik (✉)
ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Uttar Pradesh, India

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economically important emerging/re-emerging infectious diseases of humans and animals. This chapter describes various strategies for combating viral diseases of livestock.

Keywords
Virology · History · Livestock diseases · Diagnosis · Epidemiology · Disease eradication · Animal health programme · Control · Vaccines

20.1 Prologue

Ever since the identification of the causative agent of tobacco mosaic disease in plants, as a filterable agent (later named as 'virus') by D. Ivanovsky, a Russian scientist in 1892, marking the beginning of the science of 'virology', the fascinating field of virology has progressed and benefitted mankind globally. Subsequent to the isolation of the first plant virus, namely, tobacco mosaic virus (TMV), the first animal virus – foot-and-mouth disease (FMD) virus – was reported in 1897 by Loeffler and Frosch (1898). The virus aetiology of yellow fever in humans was established in 1900 by Walter Reed (Reed et al. 1901). In India, animal virology started around 1900 with research work on rinderpest (cattle plague) at the Imperial Bacteriological Laboratory, Mukteswar (now Indian Veterinary Research Institute), Izatnagar/Mukteswar. One of the most important achievements for Indian animal virologists was the development of vaccines that proved efficient and safe. These were used for rinderpest and African horse sickness (AHS) eradication programmes (Yadav 2011; Yadav et al. 2016) and control of important viral, bacterial and parasitic diseases of livestock and poultry.

The creditable animal disease surveillance is critical for the sustainability of healthy livestock production systems of any country globally, as the threat of infectious diseases in the climate change scenario is large, diverse and dynamic which adversely affect the socio-economic conditions and welfare of livestock farmers/keepers (Malik et al. 2018). Preparedness for combating the emerging, re-emerging, exotic and transboundary diseases requires sound monitoring and precision detection systems that are cost-effective, flexible and adaptable under prevailing field conditions. There is also an international obligation for OIE (World Organization for Animal Health) reportable diseases of major significance in the trade of animals and animal products globally by all member countries of the World Trade Organization (WTO).

20.2 Epidemiological Perspective

Animal viral diseases inflict heavy economic losses globally in livestock, poultry and humans. The term 'epidemiology' originated in the last part of the nineteenth century from the Greek word 'epidēmia', meaning 'the knowledge of the prevalence of disease'. Different definitions have been coined for 'epidemiology'. The simplest

definition describes epidemiology as 'the scientific study of the spread and control of disease in populations'. In other definitions, it has been described as the 'branch of medicine which deals with the incidence, distribution, and possible control of diseases and other factors relating to health' or 'the study and analysis of the distribution and determinants of health and disease conditions in defined populations'. Epidemiology has been classified as descriptive epidemiology, analytical epidemiology, spatial epidemiology, landscape epidemiology, temporal epidemiology, local and global epidemiology, molecular epidemiology and applied epidemiology. The data collected on the epidemiology of important livestock diseases is of immense use in formulating appropriate disease control and management strategies.

The technological advances made in proteogenomics and immunomics have showed the way for understanding the genetic basis of host–pathogen interactions influencing the host immune response. New proteomic approaches including T-cell and B-cell epitope mapping have given a boost to the pace to discover antigen–antibody relationships, thus giving a push to the development of newer diagnostics and vaccines for infectious diseases. Innovations in genomic technologies have paved the way for unravelling interactions between the microorganisms and cells of the innate immune system. The advent of molecular techniques in recent decades has made visible impact on the study of the epidemiology and resultant boost to the understanding of disease dynamics, aetiology, diagnosis and charting of suitable control measures at faster speed. A large number of molecular techniques have been developed and used to address epidemiological concerns. Different techniques are now available for different aspects of investigations (Joshi et al. 2013). At the core of 'molecular epidemiology' is the need for high accuracy and specificity in typing the disease-causing agents; to monitor the spread of pathogens in populations and different species and regions; to trace back the original source of the causal agent; to study variations in the antigenicity, pathogenicity and immunogenicity; to differentiate between enzootic and panzootic infections; to discern the mode of transmission of the causative viral agent from host to host; and to sense the existence of strain variants in the vulnerable population and/or individual, besides addressing other epidemiological parameters and issues (Chakraborty et al. 2014; Singh et al. 2017). Availability of newer innovative molecular technologies in recent years has revolutionized the study of patho-immunobiology and understanding the disease dynamics, leading to better diagnostics and vaccines. Molecular subtyping, being more discriminating, is considered to be better than most of the phenotypic subtyping methods as it is least influenced by the organism's responses to environmental factors.

The interactions between the host, pathogen and environment are known to influence the epidemiology of the disease. Numerous factors are known to influence the epidemiology of disease, such as sex; age; nutrition; immune status of the host; climatic conditions (heat, cold, humidity, wind velocity, ambient temperatures); role of vectors; survival of virus in nature; duration and extent of excretion of virus from infected host; reservoirs/carriers of the infectious agent; susceptibility of the pathogen to common disinfectants; reactivation of latent viral infection under immune suppression; vaccination campaigns; presence of naturally occurring attenuated strain of virus in the population; spillover of the infectious agent/virus from their

natural niches due to encroachments; developmental projects for laying railway tracts, roads, etc., leading to mixing of human and domestic animal populations with wildlife; ecological changes on account of deforestation and making dams, canals, etc.; antigenic multiplicity/stability of the virus; intimate contact of humans and livestock with wildlife/wildlife products; social/religious customs; and extent of the availability of funding for capacity building.

20.3 Diagnosis of Viral Diseases of Livestock

Viral infections have been identified as an important cause for inflicting huge losses worldwide in food/work/companion animals, including sheep, goats, cattle, buffaloes, equines, camel, yaks, mithun, canines, pigs, poultry and fish. In the last 40 years, the world has witnessed the emergence of a number of new viral diseases in animals, humans and plants in various parts of the world with more severe consequences due to ecological, demographic and climatic changes. While about 60% of the infections are zoonotic, 75% of new viral diseases reported during the last three decades are zoonoses, i.e. transmissible between animals and humans. Interplay of host, pathogen and environment-related factors in the epidemiology of diseases justifies to cover viral and other infections under the 'One World, One Health' umbrella. Timely detection and accurate diagnosis of the viral aetiologies allows better selection and adoption of appropriate and timely management practices including prophylactic vaccination of the susceptible population or therapeutic vaccination in the affected livestock population. Therefore, development/standardization of diagnostic techniques which are reliable, time-efficient, cost-effective, sensitive, specific and feasible under field conditions is of utmost importance for prevention, control, eradication, monitoring and forecasting of infectious and contagious diseases (Dhama et al. 2014). Diagnosis of viral infections has greatly advanced in recent decades with the use of state-of-the-art technologies, using modern biotechnology, nanotechnology and molecular biology.

For the diagnosis of animal viral diseases, both conventional and molecular tools as well as new-generation diagnostic techniques are employed. Apart from observing clinical signs, postmortem lesions and histopathology, isolation and identification of the viral agent employing *in vitro* cell culture techniques and embryonated chicken eggs, *in vivo* isolation in the host animals and demonstration of the virus particles by electron microscopy or viral proteins/nucleic acid in tissue sections or infected cells using immunofluorescence/immunoperoxidase technique are also utilized. Other conventional tests include haemagglutination, haemagglutination inhibition (HI), haemadsorption, haemadsorption inhibition (HADI), agar gel immunodiffusion, counterimmunoelectrophoresis, enzyme immunoassays, latex agglutination test (LAT), etc. These conventional disease diagnostic techniques are time consuming and laborious, and some of these even require *in vivo* systems. Moreover, it is difficult to differentiate antigenic variants and virulent strains from classical strains by conventional methods. Therefore, it has been emphasized time and again to develop newer diagnostics with improved sensitivity and specificity which can also

differentiate newly evolved pathogen types from classical or vaccine strains (Dhama et al. 2014).

Advances in molecular biology and recent knowledge of virus pathogenesis have paved the way for the development of highly sensitive and specific nucleic acid-based detection systems for many viral diseases. By using advanced state-of-the-art modern tools, the detection of animal pathogens has become more reliable and rapid. Molecular tools and techniques are commonly used nowadays for detection, differentiation, characterization, monitoring, pathogenicity study, and analysis of epidemiological status to assess the genomic relationship or variations and tracing the probable origin of viral pathogens. The nucleic acid- and antigen detection-based molecular techniques are gaining preference over the conventional diagnostic tests based on demonstration of specific antibody in the serum or antigen(s) in the tissues of the host species. The polymerase chain reaction (PCR) and its variations (RT-PCR, real-time RT-PCR with TaqMan, multiplex real-time PCR, nested RT-PCR, q-PCR, RAPD-PCR, REP-PCR), RFLP, RISA, SNP, SNR, VNTR, AFLP, polymerase spiral reaction (PSR), PCR-ELISA (enzyme-linked immunosorbent assay), loop-mediated isothermal amplification (LAMP), surface plasmon resonance (SPR), sensor-based microarray DNA chips, immuno-biosensors, next-generation sequencing (NGS), mass spectrometry, genomic hybridization, nucleic acid probes, sequence-based typing, single nucleotide polymorphism, nucleotide sequencing, phylogenetic analysis and whole genome sequencing are used with precision. Techniques like real-time reverse transcription PCR (RRT-PCR) have made it possible for real-time detection and allowing confirmation of virus within a couple of minutes.

Further, advances in biomedical instrumentation techniques and nanobiotechnology have led to the development of microarray, biochips and biosensor platforms that have revolutionized the modern-day diagnostics, and fully automated small micro devices have become a reality for providing instant 'point-of-care' (PoC) diagnosis (Rout et al. 2018). Apart from being very sensitive, specific and quick, these can also be used even if the pathogen has lost infectivity. With these techniques it is possible to differentiate closely related organisms directly from clinical samples. These technologies can be used to pinpoint the origin/evolution of the pathogen, making them very powerful tools for studying epidemiology. The present era also demands highly sensitive, specific, rapid, cost-effective, labour-friendly and off-the-shelf, pen-side diagnostic assays for diagnosing metabolic disorders and infectious diseases. With a plethora of decisive advantages, the nanodiagnostics are proving to be a promising substitute to in-use diagnostic techniques. The prospective applications of nanodiagnostics are manifold. To name a few, these have an edge in the area of detecting infectious agents, tumours, intracellular and tissue imaging, immunohistochemistry, multiplexed diagnostics and fluoroimmunoassays. The increasing use of quantum dots, decorated gold nanospheres and nanoshells, nanobarcodes and nanobiosensors in the field of diagnostics is quickly taking over diagnostic techniques of the past as these provide accurate, faster and sensitive monitoring and surveillance tools and intensify network approaches assisting greatly in formulating effective disease prevention and control strategies. The

microfluidic technology offers as a cost-effective substitute for disease diagnosis in the field.

The nanodiagnostics have a promising future to shift the paradigm from organized laboratories and skilled personnel to point-of-care testing and lab-on-chip technologies, which are user-friendly and can provide instant diagnosis right at the doorstep of livestock owners. One of the prerequisites for developing any farmer-friendly diagnostic assay is to use the reagents that do not require any cold chain facilities. In this direction, colloidal nanogold particles fabricated with either antigens or antibodies have been used for developing field diagnostic assays for animal diseases. The immuno-comb assay has been developed for rapidly detecting PPR virus-specific antibodies in serum samples. Similarly, colloidal gold particles fabricated with antibodies have been used to detect the PPR virus in a lateral flow assay. Both these diagnostics can be used at the doorstep of farmers without requiring the use of any sophisticated instrumentation. Recently, peptide, nucleic acid and colloidal gold nanoparticle-based visual diagnostic assay has been developed for Newcastle disease virus.

To keep pace with the recent advancements in the diagnostic arena, researchers are working on biosensor-based diagnostic platforms. In this direction, research work on the development of surface plasmon resonance (SPR) optical sensor-based label-free diagnostic assays has been initiated, and biosensor assays for detecting PPR virus and specific antibodies in clinical samples have been optimized recently (Rout et al. 2018). The technique is advantageous because it can quantitatively detect the target in real time within less than 10 min and also can automatically analyse a number of samples in high-throughput manner to provide rapid and confirmatory diagnosis of this disease. Efforts need to be directed towards developing this type of label-free biosensor assays for other viral diseases of livestock also.

Among the antigen/antibody detection-based tests, ELISA and its modifications, namely, DIVA C-ELISA, IC-ELISA, sandwich ELISA, strip ELISA, dot ELISA, immune stick ELISA, liquid-phase blocking ELISA and pen-side diagnostic kits, have advanced the identification and management of viral infections globally. The metagenomic approach has opened a unique method for the detection of hitherto unknown/unexpected infectious viruses, variants of existing viruses and other pathogens. Recently, several new novel viruses, namely, bocaviruses, torque teno viruses, astroviruses, rotaviruses and kobuviruses, have been identified in porcine disease syndromes. Nowadays, in veterinary diagnostic virology, the metagenomic approach of detecting viral pathogens is becoming a useful cultivation-independent tool.

For accurate and rapid diagnosis, capacity building of diagnostic laboratories adopting GLP, trained human resource, biosafety and containment facility according to the category of the virus being handled in the laboratory are prerequisite for checking spillover of the infectious agents from the laboratory to the environment or posing risk to the laboratory staff, when dealing with zoonotic agents. Development of indigenous diagnostic reagents, kits, vaccines and DIVA test-compliant vaccines is required for cost-effectiveness and better immune response and interpretation of vaccinal immunity and efficacy of mass vaccination campaigns in hand (Rout et al. 2014). Harmonization of the diagnostic tests, reagents and SOPs

between the laboratories in networking mode is a must exercise to be followed for comparative results. Development of thermo-resistant vaccines will have added advantage in tropical countries having problems in maintaining cold chain in rural areas. Thus, as far as possible, innocuous reagents and reverse genetics should be used for the development of diagnostic tests and/or vaccines.

20.4 Animal Disease Monitoring and Surveillance Database

In India, the ICAR-National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI), Bengaluru, is responsible for livestock disease informatics, seromonitoring of important livestock diseases, forecasting and forewarning, as well as to assess the economic losses due to animal diseases. An innovative epidemiological software, the National Animal Disease Referral Expert System (NADRES), was developed at the institute as a web-based dynamic and interactive disease relational database supported by geographic information system (www.nadres.res.in.). To suit the needs of veterinary epidemiology in India, EpiInfo© software of CDC Atlanta, USA, has been considered. An access-based software for 'National Livestock Serum Repository' (India.admasEpitrak – an exclusive epidemiology offline software) has also been created at this institute.

The epidemiology of important livestock diseases is studied by the institute and used in formulating appropriate disease control strategies. Furthermore, they have also established a few spreadsheet modules to assess the economic impact of certain abortive diseases. Some of the other significant achievements include development of a forecasting module for predicting possible occurrence of nationally important viral and bacterial diseases of livestock 60 days preceding the likely outbreak (Anonymous 2011, 2013), identification of eco-pathozones for the economically important livestock diseases, development of bluetongue disease map in the endemic states of Karnataka and Tamil Nadu, and systematic seroprevalence studies on infectious bovine rhinotracheitis (IBR) and *peste des petits* ruminants (PPR) in animals.

The livestock sector in India, known for its magnificent animal wealth, has been recognized as a potential solution for addressing the national nutritional insecurity and an ideal platform for addressing the unemployment problem in women and youth. Livestock rearing is central to the livelihoods and nutritional security of millions of small and marginal farmers and landless agricultural labours across the country. India is blessed with rich livestock resources with diverse species, breeds and strains and impressive production performance. It has the world's largest bovine dairy herd of around 300 million comprising of cows and buffaloes and stands first in milk production globally since about two decades (1998 onwards). Among the many areas of concern that limit the realization of the full potential of the livestock sector, the rising and unescapable outbreaks of viral diseases among animals are posing considerable challenges to livestock health and production. Although exact estimates due to various viral diseases to livestock industry are not available due to inadequate reporting of the disease outbreaks, the viral diseases are most important as they cause heavy economic losses through morbidity, mortality and other direct

and indirect costs on treatment, hygiene, disinfection and sanitary measures; loss in production, reproduction and working capacity of animal; and replacement costs of stock. Unlike bacterial, fungal, parasitic and mycoplasma diseases, non-availability of cost-effective antiviral drug therapy, rapid spread, etc., make the task of their control more difficult.

Over the past few decades, many of the bacterial and viral diseases have negatively impacted the socio-economically deprived people, sustaining mainly on livestock. Agricultural activities including ploughing and tilling of the fields and agricultural product transportation suffer adversely due to the assault of viral diseases. With the collective and concerted scientific interventions and enabling policy support, a few of the animal viral diseases have been stamped out globally or regionally. Rinderpest, popularly called cattle plague, an ancient viral disease of bovines, caprine and swine was eradicated from India in 2006 and globally in 2011 (Yadav 2011). India has been given disease-free status by OIE in 2014 for African horse sickness (peste equine), a deadly viral disease of equines. Equine infectious anaemia (EIA) and equine influenza (EI) have been controlled to a greater extent in India by adopting surveillance and monitoring along with zoo sanitary measures (Singh et al. 2018).

20.5 Success Story of Eradication/Control of Animal Viral Diseases

20.5.1 Rinderpest

Rinderpest was once a serious threat to the livestock industry in several regions of the world, especially in Asia, Africa, Europe and the Americas. The infection and death rates in newly exposed naïve population were as high as 95–100% leading to colossal economic losses. A death rate of around 200,000 animals per annum was recorded among the affected bovine population of 400,000 during the first half of the 1950s in India. Throughout the history of mankind, the social, economic and ecological consequences due to rinderpest had been more serious and severe. In India, the presence of rinderpest was confirmed by the Cattle Plague Commission (Hallen et al. 1871). This disease has been conquered successfully by following mass vaccination along with zoo sanitary measures. The FAO declared the global eradication of rinderpest on 28 June 2011, marking it as the first ever viral disease of animals eradicated globally about three decades after the eradication of small pox, a viral disease of humans in 1980 (Yadav et al. 2016). In India, dividing the country into four zones based on the epidemiological picture of the disease and adopting strategic and focused vaccinations at interstate and international borders and migration routes of bovines and caprine for creating immune belts, coupled with rigorous clinical and serosurveillance, were of great help in achieving freedom from the infection. With the successful eradication of rinderpest, the livestock sector across the globe became safer and consequently the livings of livestock farmers improved.

Eradication of rinderpest helped in assuring inclusive growth as it mainly benefitted landless, marginal and smallholder livestock keepers, besides providing much

needed animal protein, food and nutrition security and livelihood security. The milk production in India enhanced 2.99 times from the year 1955 to 1995 and further stepped to 4.796 times by 2006. Similarly, the bovine meat production increased by 17.99 times between 1959 and 1995. The income from bovine milk and meat increased to 102.06 and 193.96 times, respectively, between 1950–1951 and 2005–2006. In value terms these benefits amounted to 15563.56 million US dollars in respect of milk and 435,011 million US dollars for bovine meat from the year 1950 to 1996. It is estimated that India gained additional food production valuing 289 billion US dollars from 1965 to 1998 due to reduction in rinderpest incidence. This is one of the greatest contributions of veterinary scientists to crop production and dairy development programmes in India after Independence (Uppal 2011).

The success of rinderpest control and eradication proved a rewarding experience and landmark for the veterinary services in India, providing confidence and capacity building to undertake a control programme of livestock diseases at the national level. The freedom of the country from rinderpest not only enabled the growth of the dairy industry in India but has also boosted the export of meat and other dairy products in recent decade. Today India tops not only in milk production in the world but also the largest exporter of buffalo meat. Cost–benefit analyses indicated that every dollar spent on rinderpest control programme gained about 20 dollars to the Indian dairy industry through more milk, meat and draft power for better agricultural productivity (Uppal 2011).

20.5.2 African Horse Sickness

African horse sickness (AHS) is a devastating, highly infectious, non-contagious, insect (biting midge)-transmitted viral disease of equines. After the detection of the first animal virus (foot-and-mouth disease virus), AHS virus was the second animal virus discovered by John McFadyean at the Royal Vet College London in blood samples from Africa. This virus affects all species of *Equidae* family including horses, mules, donkeys and zebras. In susceptible horse population, the consequence of AHS can be dreadful, resulting in up to 95% mortality. At present as there is no treatment available against AHS virus, vaccination is the only weapon available against this dreaded disease. On 27 May 2014, India touched a major landmark by getting official disease-free status from African horse sickness (peste equine). As per Resolution No. 19 (82nd General Session), India was declared as member country recognized free from African horse sickness according to the provisions of Chap. 12.1 of the Terrestrial Code by the World Organization for Animal Health (OIE) (82 GS/FR – PARIS, May 2014).

20.5.3 Animal Viral Disease Control in India

Trade of livestock and livestock products, within and between countries, has resulted in enhanced risk of spread of diseases to livestock, poultry and human beings. The

occurrence of highly contagious viral diseases of livestock and poultry, namely, foot-and-mouth disease (FMD), *peste des petits* ruminants (PPR), classical swine fever (CSF), bluetongue, equine infectious anaemia (EIA), equine influenza (EI), highly pathogenic avian influenza (HPAI) A virus, infectious bursal disease (IBD) and zoonotic diseases such as West Nile fever, Rift Valley fever, SARS-coronavirus, Nipah virus infection, Hendra virus, swine influenza virus A (H1N1) and Crimean–Congo haemorrhagic fever (CCHF), has compelled to formulate policies and regulatory procedures to prevent the entry of transboundary and exotic diseases on the one hand and interstate spread of livestock diseases through uncontrolled movements of animals for work, migration, grazing, etc., on the other hand, for checking the dissemination of endemic diseases within the country.

For the planning and execution of control programmes against viral disease, it is necessary to fully understand the disease, particularly the interaction between the host and the pathogen as well as between the pathogen and the host vis-à-vis the environment. This knowledge should include the duration of incubation period; pathogenesis; route of entry of virus in the host species; extent and duration of excretion of the virus from the host; reservoir and carrier hosts; duration and mechanism of interepidemic survival of the virus; survival of the virus in nature including its susceptibility to high and low temperature, freezing and thawing, acid and alkaline pH and disinfectants; survival in body fluids; and antigenic variations, such as types, subtypes, clades and genotypes, particularly in RNA viruses. Appropriate knowledge of these parameters will be very useful in planning and implementing cost-effective disease control programmes. For example, in case of equine influenza, the knowledge that the virus is excreted for not more than 10 days from the infected animal can be used with advantage to check the spread of infection by restricting animal movements from infected to healthy premises and vice versa for 2 weeks. Similarly, all direct and indirect contacts between the sick and healthy animals and farms need to be avoided, besides symptomatic palliative treatment and complete rest to the sick animal to avoid secondary bacterial complications. In case the fever lasts for more than 3 days, antibiotic therapy should be considered to combat secondary bacterial infections. A three-day rest is recommended for each day of fever the animal had run in equine influenza. Influenza virus is highly susceptible to freezing and thawing and common disinfectants, such as Dettol, Savlon, 70% alcohol, phenyl, $KMNO_4$, ultraviolet light and sunlight, but fairly resistant to sodium carbonate (Na_2CO_3) and sodium hydroxide (Yadav et al. 1993). Thus, suitable cost-effective disinfectants may be used for decontamination of the stables and adjoining premises, ropes of animals, hands, shoes and clothing of animal attendants. The outbreaks of influenza, which are more common during winters, fade away in summer due to the susceptibility of the virus to high ambient temperatures.

Equine infectious anaemia (EIA), caused by a retrovirus of Lenti group, is transmitted by blood-sucking insects, including mosquitoes and flies. The virus remains lifelong in latent form in animals which survive from the disease. The disease was reported in India for the first time in 1987 (Uppal and Yadav 1989). It can relapse due to immunosuppression on account of stress conditions due to pregnancy, hard

work, cortisone therapy or other factors. Such carrier animals are thus potential source of infection for spreading the disease throughout their life. In the absence of a suitable vaccine, the only solution to limit the infection is by destruction of seropositive animals. However, in the absence of proper legislation, the owners did not agree to put down their EIA-positive animals immediately and have to be convinced about the accuracy and validity of the test by repeat tests of the animals on fresh serum samples in the same laboratory or at other laboratories in the same country or abroad. The authors, while confronted with this problem in India, made use of an old observation of a researcher wherein it was shown that an insect after feeding on an EIA-affected equine does not go beyond 200 meters and prefers to come back to the same host for the next blood meal, by isolating the seropositive animal in Coggins test (agar gel precipitation test) more than 200 meters away from other equines before they were put down. This method was employed on hundreds of EIA-seropositive animals and always found effective.

20.5.3.1 Foot-and-Mouth Disease Control Programme

After successful eradication of rinderpest, foot-and-mouth disease (FMD) of cloven-footed animals is another OIE-listed important viral disease inflicting heavy economic losses and adversely affecting the trade of livestock and livestock products from India to other countries. Direct losses due to FMD in India have been estimated to the extent of INR 2,30,000 million per annum. Accordingly, to combat FMD, the Government of India (GoI) initiated the FMD Control Program (FMD-CP) in 2003–2004 during the 10th Five Year Development Plan in 56 select districts of seven states having bearing on milk production. The FMD-CP envisaged vaccination of cattle and buffalo population using indigenously produced killed adjuvanted trivalent (O, A and Asia1) vaccine under nationally coordinated and monitored mass vaccination programme following OIE progressive pathway. Encouraged with its success in reducing the incidence of the disease, the programme was expanded to further 221 districts in 14 states in the 12th Plan. Now, since 2016 the entire country has been covered under FMD-CP with the ultimate objective to eradicate the disease by 2040. FAO/OIE have targeted to control FMD by 2035. This will be followed by stopping vaccination and conducting surveillance for freedom from clinical disease followed by freedom from infection. These activities are expected to be over by 2040. The control programme involving six monthly rounds of vaccinations has shown encouraging results as reflected in reduction in the number of outbreaks and incidence of the disease. The number of outbreaks declined from 1911 in 2003–2004 to 149 in 2017–2018 reflecting about 92% drop in FMD incidences. The years 2016–2017 and 2017–2018 were mainly dominated by serotype ‘O’ FMD virus as there were 296, 03 and nil outbreaks due to serotypes ‘O’, ‘Asia1’ and ‘A’, respectively (Anonymous 2017–18).

20.5.3.2 PPR Control Programme

Subsequent to the detection of PPR in the southern peninsula in India in the late 1980s, the disease became widespread and endemic by 1995–1996. The disease is estimated to cause global losses between 1.45 billion and 2.1 billion US dollars per

year. The disease causes economic losses in India to the tune of INR 11070 million per year (Tripathi et al. 2018). After developing diagnostic facilities and a safe and potent vaccine indigenously by the year 2001–2002, a national control programme on PPR (NCP-PPR) was started in 2010 in five states (Kerala, Tamil Nadu, Karnataka, Andhra Pradesh, Maharashtra) and five union territories (UTs). During February 2014, the programme was extended to all the states and UTs. The aim of the programme is to undertake intense immunization of sheep and goats and their three subsequent generations. With judicial use of vaccine and diagnostics, the NCP-PPR has shown encouraging results in Karnataka, Andhra Pradesh and Chhattisgarh with 75% reduction in the disease incidence (Tripathi et al. 2018). India is expected to attain infection-free status of the disease within the time frame of 2030 set by FAO and OIE for eradication of PPR in small ruminants from the globe.

20.6 Zoonotic Viral Diseases

Viral zoonotic diseases of animal origin also pose threat to human welfare and livelihood through morbidity, mortality, reduced nutrition and working capacity. The explosive growth in human population, increasing urbanization, high density of livestock and poultry populations in modern livestock farms, environmental degradation, deforestation, contact with wildlife and climatic changes are some of the factors responsible for emergence of zoonotic diseases in recent decades (Dhama et al. 2018). The chances of a spillover of a pathogen from domestic or wildlife species are more in countries where the public health infrastructure is suboptimal and the interaction between humans and animals is more intimate. The hotspots for infectious disease emergence are generally places where wildlife, livestock and human interactions are more frequent. This is best exemplified by the emergence of Nipah virus in Malaysia and SARS in Guangdong Province of China. As per a report (Mapping of Poverty and Likely Zoonoses Hotspots, 2012) from the International Livestock Research Institute (ILRI), Ethiopia, Nigeria, and Tanzania (Africa) and India (Asia) exhibit the highest disease burden of zoonoses, with parallel illness and death. The zoonotic diseases cost US $ 6.7 billion a year worldwide.

There are more than 1000 known animal pathogens, of which about 40% pathogens of domesticated livestock species and 70% of domestic carnivores have zoonotic potential. Though at present only 11–18% of zoonotic pathogens from domestic livestock and carnivores are viruses, a significantly high number (55–59%) of emerging zoonotic pathogens are viruses. Approximately, 90 novel human pathogens were discovered during the last 30 years, averaging 3 per year, 66% of which were viruses, and more than 80% of these are RNA viruses. The propensity for emergence of new variants of RNA viruses is very high because of their small and segmented genome, rapid rate of multiplication and polymerase enzymes that lack proofreading capability. As a result, these viruses are more prone to exchange

genetic material from related viruses when coinfecting the host by recombination or reassortment events. Pathogens that can infect multiple species and those that find a closely related host species in close proximity can jump species under suitable conditions and may cause the emergence of a new disease which may lead to epidemics/pandemics. Bovine spongiform encephalopathy (BSE) and scrapie, caused by prions, are examples of species jumping. Feeding of cows on scrapie-infected sheep offal and meat meals in the UK resulted in the development of BSE or mad cow disease. Subsequently, consumption of beef from BSE-affected cows in people developed another version of this disease, namely, Creutzfeldt–Jakob disease.

20.6.1 Combating Zoonotic and Non-zoonotic Viral Diseases

In the present scenario of constantly increasing worldwide population, urbanization; globalization; industrialization; mutable lifestyles and food habits; tourism; intensified animal farming; ecological and biodiversity changes; emerging antimicrobial/drug resistance, coupled with lack of effective, safer and cost-effective treatment regimens and vaccines; immunosuppression due to multiple factors; and global warming are resulting in higher susceptibility of the host to the pathogen(s) and vaccine failures. This emphasizes the development of the 'One World, One Health' concept using multidisciplinary, regional and international networking to counter economically important, emerging/re-emerging infectious diseases of humans, livestock and poultry. The important infectious diseases/pathogens to be tackled include FMD, PPR, coronavirus, rotavirus, parvovirus, infectious bovine rhinotracheitis (IBR), bluetongue, toroviruses, infectious bursal disease (IBD), avian influenza, Newcastle disease (ND), Marek's disease (MD), avian infectious bronchitis (IB), chicken infectious anaemia (CIA) and hydropericardium syndrome. Additionally, there is a need to counter several pathogens of zoonotic significance including rabies virus, Rift Valley fever, West Nile virus, avian influenza A (bird flu), swine influenza A virus (H1N1), viral encephalitis, Crimean–Congo haemorrhagic fever (CCHF), Hendra virus, Nipah virus, Ebola and Zika viruses.

There is also a need to monitor wildlife, migratory birds and important vectors and reservoirs of various infectious agents having a role in the maintenance/survival and spread of the pathogens. Recent threats of epidemic/pandemic nature of severe acute respiratory syndrome (SARS), highly pathogenic avian influenza (HPAI) A virus (H5N1) and swine influenza A virus (H1N1) warrant strengthening of global health issues through capacity building for enabling comprehensive preventive and control measures. Besides this, preparedness and prompt response are a must for successful control and management of the devastating diseases posing grave threats to humans as well as to food and companion animals and posing immense socioeconomic burdens. For the efficient management and control of viral zoonoses, well-planned strategic approaches and interventions through collaborative working of medical and veterinary professionals are needed.

20.7 Networking of Animal Health Programmes

The advent of geographical information system (GIS) has proved of great help for efficient disease detection, reporting and recording among populations, cluster analysis, spread of infections and their modelling, assessment of outbreak magnitude and planning of efficient and effective control strategies. The GIS is of immense help for mapping the location of herds/flocks and other related parameters. Use of GIS has greatly facilitated the knowledge of epidemiologists, diagnosticians, clinicians and researchers. Multidisciplinary efforts are required to realize the 'One World, One Health' concept at global, national and local levels in the overall interest of our society. A number of crucial issues at the global level, such as rapid population growth, emerging antibiotic resistance, climate change and global warming, international trade and travel, food safety, migration of people from rural to urban areas, biosecurity and biosafety concerns, ecotourism and disease surveillance and monitoring, have underlined the importance of networking of these programmes for faster solutions. Increasing risk for the emergence/re-emergence of deadly/debilitating zoonotic diseases including rabies, highly pathogenic avian influenza virus, swine influenza A (H1N1) virus, Nipah virus infection, tuberculosis and brucellosis has led to advancement of novel diagnostic techniques and vaccines and contributed immensely to human and animal health impacting global health as a whole. FAO of the United Nations has established linkages with various funding institutions at regional levels in various parts of world through GIS. It ultimately led to engagement of manpower as well as investment for strengthening of veterinary services along with the whole spectrum of disciplines leading to the protection and promotion of animal health.

Efficiency of control programme for viral diseases will depend on several factors, such as rapid and accurate diagnosis of the disease, incidence and/or prevalence, molecular epidemiology of the disease to understand antigenic variation at protein or gene level, choice of the diagnostic test and stage of the disease, correct interpretation of the test results and decision regarding undertaking control and preventive measures including vaccination, disinfection, stamping out, type of vaccine (live modified, killed, adjuvanted), adjuvant used in the vaccine and duration of immunity, route of vaccination, extent of humoral and CMI response induced, frequency of booster vaccination, affordability and availability of vaccine and cold chain availability under field conditions. For ease of vaccination and reducing the stress to the animals and the vaccinator in controlling the animals, in future combined vaccines, multiple vaccines – peptide, split, thermostable, and recombinant vaccines – will be more in demand. Similarly, intradermal and pressure vaccination may become a common practice. Vaccination in advance stage of pregnancy should not be practiced to avoid immune tolerance in the newborn. Merits/demerits of in ovo vaccination in poultry also deserve due consideration, as the newborn chicks get antibodies from the mother hen through the yolk.

20.8 Journey Through Animal Viral Vaccine Development

Infectious diseases are one of the most hazardous enemies mankind has faced as these are capable of destroying all his economic strengths by affecting their pets, domesticated animals and themselves. For profitable animal husbandry practices, prevention and control of diseases should be considered on cost–benefit ratio basis. The colossal losses are incurred during disease outbreaks like FMD, IBR, PPR, bluetongue, sheep pox, goat pox in livestock and IBD, MD, IB, EDS, CIA and avian influenza in poultry. Vaccines and judicial vaccination procedures are only ways to prevent and control such diseases in a cost-effective manner. The concept of vaccination was first popularized by Edward Jenner in 1798 as a method to fight against the deadly human small pox disease. However, prior to it, Chinese people were practicing a form of vaccination called 'ovination' where virulent sheep pox virus was inoculated in sheep to generate immunity against further infection with sheep pox virus. Similarly, in FMD, in the absence of a vaccine, 'apthization' (deliberately rubbing the infected fluid or lesions of diseased animals into the healthy animals of the farm/herd) was practiced with the objective of reducing the time, labour and cost towards the management of the outbreak in one go at the farm. The ILT virus, a herpes virus which causes respiratory disease in poultry, was recommended to be inoculated in the cloacal region to impart immunity in want of vaccine availability. However, now with the availability of dependable vaccines against most of the infectious diseases it is not advisable to use such methods as it has the risk of spreading the virulent virus to susceptible animals. After the successful initial approaches by Jenner using cow pox virus as vaccine against small pox in human, the concept of killed vaccine was made into use by Louis Pasteur in 1885 to prevent post-bite rabies disease in man. The mid-1940s designated as 'the era of cell culture' made a revolution in vaccine research to develop more attenuated live organisms as vaccine. Vaccine research has moved from the application of whole organism approach (inactivated or live attenuated) to synthetic antigenic peptide-based or gene-based vaccine approaches for man and animals against a number of infectious diseases.

Vaccine discovery, though initially made by chance due to keen observation or hit and trial method, later became a deliberate attempt to combat infectious diseases. In initial times the whole organisms were applied in scarified wounds to give the host an opportunity to mount an immune response against an infectious agent. But later more specific and safer killed or attenuated live organism-based vaccines became important in vaccine research even though the vehicles added to make the vaccines more immunogenic were causing serious health problems in the individuals given the vaccines. Availability of desired vaccines against the lethal, wasting and debilitating diseases has backed immensely the attainment of successful public health programmes. As of now, scientific challenges still exist to develop safer, effective and reliable vaccines that boost protection against the pathogens of major significance. With the recent advancement in the field of immunology and vaccinology, modern biotechnological innovative approaches are becoming available to counter such diseases.

To ameliorate these problems, recombinant DNA technology-based vaccine research gained strength and advanced the idea of gene delivery as vaccines using shotgun method, replication-defective live virus vector vaccine, single-copy virus vaccine and split, peptide and edible vaccines have been researched. Thus, vaccinology has traversed through three successive phases/eras. The first one was with initial stages using scabs or infective material of related diseases in animals to prevent reinfection. The second era started with the advent of cell culture or chicken embryo-based techniques and introduction of inactivated and attenuated viruses as vaccine, whereas the inclusion of rDNA technology in vaccine production made them to advance to the third era. The innovative technologies have transformed the concept of vaccine development and will go a long way in the generation of safer and more effective vaccines. Scientific advancements and their application will certainly provide better-quality health-based products. Based on cost-effectiveness, vaccination still remains a high priority for the prevention and control of animal diseases. These advancements in vaccine development will meaningfully lessen the prevalence of diseases in man and animals. An ideal vaccine is expected to have minimum threshold of the antigenic (immunogen) mass devoid of extraneous antigens and capable of inducing solid and long-lasting immunity.

20.9 Handling of New Viral Disease Outbreaks

When an exotic viral disease struck a country for the first time, it may initially affect one animal, few animals or a large number of animals. The strategy to be adopted for containing the outbreak will depend on the nature of the virus, its spread, risk assessment and country legislation on disease control and prevention. Thus, there is a need to develop strategic plans for the prevention and control of exotic and transboundary animal diseases on case-to-case basis. Examples of such viral diseases from Indian perspective include African swine fever; transmissible gastroenteritis (TGE) in pigs; swine vesicular disease; Rift Valley fever; West Nile fever; Eastern equine encephalomyelitis (EEE); Western equine encephalomyelitis (WEE); Venezuelan equine encephalomyelitis (VEE); AHS; FMD virus types ‘C’, ‘SAT I’, ‘SAT II’ and ‘SAT III’; Nipah virus; Hendra virus; SARS-coronavirus; and prion diseases – bovine spongiform encephalopathy (BSE) and scrapie.

20.10 Biosecurity Measures to Combat Viral Infections

Institution of appropriate timely biosecurity methods is important for preservation and improvement of animal health in order to minimize the risks from infectious diseases. Lapses in biosecurity in the management of livestock and poultry are often responsible for higher incidence of infectious and zoonotic diseases of animals. This is more so in case of viral diseases of animals and poultry. Close contacts between animals, wildlife and humans will facilitate the spread of viral and other infectious diseases. The emergence of new viral diseases/infections, such as Rift

Valley fever, West Nile fever, SARS-coronavirus, Hendra virus, avian influenza A H5N1, Nipah virus, Zika virus and swine influenza A (H1N1) virus, from time to time is a glaring example of zoonotic disease threats adversely affecting animal health and public health, national economies and food and nutrition security globally. Due to breach in the biosecurity, deadly viral diseases like FMD had re-surfaced in countries which were having disease-free status for decades. Disease incursions through imported livestock and poultry, fish and their products in the past in India have been responsible for the introduction of a number of diseases like PPR, bluetongue, IBR, IBD, CIA and classical swine fever (CSF) in India. AHS was responsible in the death of around 3,00,000 equines between 1959 and 1961 in Asia including India and other countries (Kumar 1976). Subsequent to its first occurrence in 1996 in China, the highly pathogenic avian influenza (HPAI) A virus H5N1 has affected more than 60 countries in Asia, Europe, Africa and North America. The virus affected wild birds as well as domestic poultry. Sporadic cases of transmission to humans in close contact of infected birds with sizeable mortality raised pandemic concern of 'bird flu'. Since the first reporting of H5N1 virus from India and Bangladesh in 2006 and 2007, respectively, both these countries are experiencing outbreaks almost every year. The zoonotic disease outbreaks due to animal pathogens underline their double-sword action on animal human health, livelihoods of livestock farmers and food and nutritional security and safety.

20.11 Misuse of Viral Agents and Biosecurity

In the present-day advancements in biotechnology, genetic engineering, gene editing, new-generation sequencing and artificial intelligence (AI), there is a common concern about the risk from likely misuse of deadly pathogens of animal or human origin. Massive destruction is possible with the misuse of some of the highly virulent viruses/bacteria/biotoxins as bioterrorism agents upsetting animal and human health and food security and safety. The biowarfare agents can spread widely through animal to animal which may take heavy economic toll due to morbidity, mortality and loss in production. During wartime, particularly involving army operations in difficult hilly or desert terrains, requiring the use of equines or other animal species for transport purpose, natural outbreak of infectious disease or its deliberate introduction by the enemy may cripple the fighting units.

Biosecurity is essential for avoiding the disease entrance across the borders or their spread within defined zones and keep the natural resources (water, soil, feed, food) safe for use. Contemporary farming stresses for a more complete tactic for managing diseases that integrates biosecurity and lay more emphasis on prevention and protection from animal diseases. Introduction of high-producing exotic stock on livestock farms often has inbuilt risk of introducing new viral and other diseases. Biosecurity is a must to safeguard animal health and reduce the risk of new pathogen entry or spread. Biosecurity measures combine the modules of 'external biosecurity' where force is on averting entrance of transboundary animal diseases (TADs) and 'internal biosecurity' where there is focus on stopping disease spread within the country, covering at the zonal and farm level.

To halt the intrusion of new pathogens through animal trade, the OIE has framed certain standards and guidelines. These guidelines are followed for animals (livestock, poultry, fish, aquatic animals and wildlife) and animal products, including meat and meat products, milk and milk products, egg and egg products, fish and fish products. The standards developed by the OIE from 1995 onwards have been formally recognized by the agreement on the application of sanitary and phytosanitary measures (SPS agreement) of the WTO.

20.12 Epidemiological Concerns to Shape Research Agenda

While dealing with the control and management of viral diseases/infections in animal populations, the greatest obstacle is posed by the emergence of antigenic and/or genetic variants of the existing virus types resulting into new types, subtypes, genotypes, clades and lineages, etc. which may not be covered by the existing vaccines in vogue in providing adequate protection leading to vaccine failures. This necessitates redesigning and updating of the vaccines to include the new antigenic variants. Compared to DNA viruses, RNA viruses are more prone to such changes due to their inherent biology, such as segmented genome and possibility of exchange of genes from related virus types resulting into recombination and reassortment. These changes in influenza viruses are described as antigenic drift (gradual minor changes) and antigenic shift (major antigenic alterations). Thus, the need for continuous surveillance and monitoring of virus types in the population and updating of the vaccines becomes a costly and time-consuming proposition. It would be appropriate here to mention the phenomenon of 'antigenic sin' in case of influenza viruses where the infection of a new type of virus may give rise/boost up a detectable HI antibody response against a previous heterologous type/subtype infection. Such findings may confuse in arriving at correct diagnosis based on antibody detection alone.

20.13 Origin of New Diseases and New Viruses

Another phenomenon faced by the virologists is the encounter/spread of new infections, diseases from their natural niches (mostly forests, mountains) to new territories and new hosts due to climate change or other conditions leading to close contact of humans and animals with the pathogen(s) under changed conditions. A few examples in this regard include the spread of fox rabies in Europe to human inhabitations due to forcing out of foxes from forest areas as a result of their higher population density. Similarly, in India where jackals serve as important wildlife reservoir of rabies virus, the disease spreads to urban cycle at the beginning of the monsoon when the jackals are forced to come out of their dens (due to filling with rainwater) in the foothills of the Himalayas forcing them to move to nearby human habitations/villages, thus coming into contact with stray dogs. Fights between dogs and jackals lead to the transmission of the rabies virus from jackals (sylvatic cycle) to urban cycle. In the case of bluetongue disease of sheep and goats, there is now proven

evidence that the rising temperatures due to climate change have facilitated the spread of the *Culicoides* vector of the bluetongue virus further northwards into Europe. This had resulted into outbreaks of the disease into new areas/countries.

There is a possibility of exposure of the host to hitherto unknown new viruses from space along with meteorites or 'permafrost' as a result of the melting of snow on polar surfaces due to rising temperatures under climate change scenarios. This will facilitate the release of viruses and other microbes trapped in ice since ages into water streams. Consumption of raw bush meat in African countries is suspected to be the source of new virus pathogens in human.

New viral diseases may arise due to the broadening of the host range of the virus influenced by the genetic makeup of the virus, mutation rates, transmission mechanism such as direct contact transmission or indirect transmission involving vectors (insects, rodents, monkeys, etc.) and reservoir host(s) and the possibility of the new host to acquire receptors of the virus as a result of the coinfection of mammalian or avian hosts with RNA viruses having segmented genome. The best example is provided by influenza viruses when a prevailing human influenza virus and an avian influenza virus coinfect pig, an intermediate host, which has receptors for surface HA antigens of both human and avian viruses. Thus, reassortment of the genetic material of two viruses in the intermediate host could give rise to a new strain that can infect humans leading to pandemics. There have been instances when coinfection of avian virus and influenza viruses of horses resulted in the development of new virulent strains due to genetic shift as a result of genetic reassortment or recombination. The identification of the conditions involving the host, pathogen and the environment, combinations and sequences of events that are likely to change the pattern of infections under a particular set of circumstances, is crucial in understanding and combating viral diseases.

Several factors contribute to the emergence of new viruses, including zoonotic viruses. Some of these include continued increase in global human and livestock populations, thus providing more chances of direct contact between the hosts and the development of modern fast movement and transport by air making it possible to circumnavigate the globe in less than the incubation period of most infectious agents/viruses.

When confronted with a new viral disease, knowledge at the system level, including evolutionary biology, ecosystems, epidemiology and population dynamics of the hosts, vectors and parasites, would be helpful for working out strategies for the prevention of infection/disease. Information on the history, symptoms, lesions and fate of the affected host populations will be of help in the development of diagnostic and differential diagnostic tests (Asokan et al. 2003).

20.14 Latent Viruses

Some viruses, including herpes viruses, undergo two phases inside the host, which include the active stage characterized by the multiplication of virus and expression of disease symptoms. In the second phase, i.e. the 'latent phase', the nucleic acid of

the virus gets integrated with the host cell genome. It is believed that this phase, characterized by the absence of virus multiplication, does no harm to the host. However, whether integration of the virus genetic material/genes with the host genome influences the expression of host genes is a subject for further research. After primary infection, human beta herpesvirus (HHV-6) may establish lifelong latency by integrating with the cellular DNA. The latent virus can be activated under certain conditions, such as immunodeficiency/immunosuppression. Recent studies suggest that HHV-6 is suspected to play a role in the pathogenesis of several diseases of the central nervous system. MicroRNAs produced during active infection and reactivation can prove as biomarkers for this virus. The latent viruses pose challenges in the diagnosis in the dormant phase, control of the disease or establishing disease-free herds.

Immunosuppression due to cortisone therapy, stress of work, pregnancy, higher production and toxicity due to heavy metals, aflatoxin and other mycotoxins is known to make animals more susceptible to diseases. These conditions also reduce the immune response to vaccination. As parasitic infection/infestation leads to debility of the host and intracellular protozoon parasites cause immunosuppression of the host, it is advisable to deworm animals about 2–3 weeks before administering the vaccine. After giving PPR vaccine to sheep and goats, the animals should not be transported or moved to long distances for 3–4 weeks to avoid stress.

20.15 Naturally Occurring Mild Virus Strains as Vaccines

There are several examples of naturally occurring mild strains of viruses in the host species or other species which have been used as vaccine candidates. Herpes virus of turkeys (HVT) has been successfully used as vaccine against Marek's disease (a cancerous disease) by administering the vaccine in day-old chicks before they get a chance of infection with the virulent Marek's disease virus (MDV) from the environment. A naturally occurring mild strain of Ranikhet disease virus isolated from pigs in India had been successfully used as vaccine for providing protection against this disease. Similarly, avirulent infectious laryngotracheitis (ILT) virus isolated from apparently healthy chickens in India probably provides natural immunity to birds against the virulent strain of the virus.

20.16 Interrupting the Contact of Pathogen with the Host

While confronted with the control of viral disease in poultry, all-out (depopulation)–all-in (repopulation) policy is preferred. It includes destruction of all affected and in contact birds followed by disinfection of the premises and repopulation after keeping the sheds empty for an adequate time to ensure complete freedom from infection under consideration. Depopulation of the susceptible animal species at the international borders up to sufficient distance can be adopted when confronted with a new fatal infectious disease in a neighbouring country, in case no vaccine is

available to protect the animals. Separation of healthy and infected animals due to natural boundaries, rivers and mountain passes, quarantine or restriction of animal movements can be used with advantage to restrict the spread of the disease. During the equine influenza outbreak in India due to A/Equi-2 virus in 1987 (Uppal and Yadav 1987), setting of isolation camps for sick equines for 2 weeks with provision of potable water and feeding arrangements in the state of Madhya Pradesh in Central India was able to stop the further spread of the disease to the southern states.

In mass vaccination programmes undertaken at national level following OIE pathway, zoning of large countries on the basis of the epidemiological picture of the disease after initial vaccination rounds along with strategic and focused vaccinations at international and interstate borders and animal movement routes to create immune zones and belts of sufficient depth had been a success with saving on vaccine and time taken for getting negative status of the infection. This strategy was followed in India during the rinderpest eradication campaign.

20.17 Physicochemical Characteristics of the Virus

Information on the excretion of the virus from body fluids, exhaled air, feathers, etc., is of vital importance in disease management. For example, knowledge that BHV1 virus is excreted intermittently in the semen of bulls can be helpful in using the semen of elite bulls after testing of the semen of the ejaculates found negative by PCR test for freedom from virus. Information that Zika and Ebola viruses may be excreted in the semen for about 6 months is of vital importance in planning appropriate strategies for control of the disease. Similarly, information on the survival of the virus in nature at ambient temperatures, urine, blood, soil, air, water (tap water, canal water), sewerage, vector (if applicable) and common disinfectants can be used with advantage in disease control strategies.

20.18 Vaccination in the Face of Outbreak

The objective of vaccination usually had been prophylactic, i.e. to impart specific acquired immunity in the host against a potent pathogen in advance, in case the host animal or human is exposed to that particular pathogen in the near future. Under field conditions, a question is usually asked: if the host is already showing the symptoms of the disease, in that case, should vaccination be undertaken or not? The simple answer is ‘no’ as the antigen(s) present in the vaccine may make the host more susceptible to the disease as a result of the neutralization of existing humoral antibodies/immunity and diversion of the host immune system to respond to non-immunogenic proteins in the vaccine will weaken the immune response against the vaccine. However, if the disease occurrence (outbreak) is detected in a closed (animal farm) or defined herd and we are able to detect and diagnose in the beginning, vaccination can be attempted with certain conditions. The first author has experienced handling an outbreak in a dairy farm of the institute, having about a hundred

cows. On day 1, one cow was reported to be sick with high fever. Examination of the herd revealed that only one cow was having fever as well as lesions of FMD. Only one cow had fever but no lesions in the foot or mouth. Examination of the material collected from the foot-and-mouth lesions of the diseased cow confirmed an FMD virus antigen. The remaining cows in the herd were apparently normal and had no fever or FMD lesions. Two sick cows were separated at the farm. The remaining cows were administered inactivated aluminium hydroxide gel FMD vaccine. The sheds, dung, urine, fodder and feed waste were thoroughly decontaminated with 4% sodium carbonate (Na2 CO3) disinfectant before disposal for a week. The interior of the animal sheds including mangers, floor, walls and roof were also disinfected similarly. The milk was properly boiled before feeding to the suckling calves or discarded. This exercise proved effective as no further case of the disease was detected at the infected farm or in other dairy units or experimental animals maintained at the campus. The success may be attributed to the availability of expertise, diagnostic facilities and vaccine, taking prompt decision and action in dealing with the outbreak. Another situation is with rabies subsequent to the bite of a rabid animal; in that case, vaccination is recommended irrespective of the fact whether the animal had been earlier vaccinated against rabies or not. This type of vaccination practiced in rabies is termed as ‘therapeutic’ vaccination.

20.19 Vaccination of Pregnant Animals

In classical textbooks, vaccination in the last quarter of pregnancy is not recommended. One of the reasons is the possibility of abortion while using live vaccines. The possibility of the foetus/newborn developing immune tolerance against the vaccine virus was another consideration. Another view held is that vaccination of pregnant animals in late pregnancy diverts the protein synthesis towards immunoglobulins against vaccine antigens rather than synthesizing the proteins required for the development of the foetus. Thus, it is detrimental for the health of the foetus as well as the newborn. However, there is a need to study the immunobiology of vaccination of animals in the last quarter of pregnancy and its possible effect on the foetus/newborn in response to killed as well as live vaccines.

20.20 Killed Versus Live Attenuated Vaccines

Both inactivated and live modified vaccines have merits and demerits. While live vaccines induce long-lasting immunity, are easy to produce at a large scale and require freeze-drying and cold chain during storage and transport, there is remote possibility of the attenuated virus to revert back to become virulent; the killed vaccines on the other hand have weak immunogenicity and require frequent booster doses, costly adjuvants and more space for storage and transport. When the incidence of the disease is high and infection is endemic, live vaccines should be

preferred. However, killed vaccines may be preferred when the incidence has come down to become negligible after stamping out policy or using live vaccine initially. In case safe live vaccine is not available against a particular virus, the use of killed vaccine is the only alternative left.

When a particular virus affects multiple species, it should be ensured that the attenuated vaccine candidate strain is non-pathogenic to all the susceptible species. In case of FMD virus which affects a number of ruminants and other species, it has been observed that after passage of the virus in cell culture while it became attenuated for one species, it remained pathogenic or became more virulent to other susceptible species. As a result, no safe live vaccine could be developed against FMD.

20.21 Vaccinated Animals as Carriers of Viral Pathogens

A common concept is that vaccinated animals pose no risk of spreading the disease to susceptible animals. However, it may not be always so, particularly while using inactivated vaccines and the route of infection is respiratory tract, because inactivated vaccines elicit a poor secretary IgA response which is not adequate to neutralize the virus in the respiratory tract. As a result, the virulent virus if contracted may be harboured in the respiratory tract of the host. Equine influenza and FMD viruses can be cited as examples. The FMD and influenza viruses may persist in the respiratory tract of vaccinated animals due to partial immunity. The FMD virus has been detected in the oesopharyngeal fluid for several months. Similarly, the presence of rabies virus in a few dogs as long as up to 2 years without showing any symptom necessitated the need to revise the policy of recommending vaccination even after the bite of a person by an apparently normal dog.

20.22 Spillover of Viruses from Laboratory

To avoid spillover of viruses from research laboratories, strict biosafety and biocontainment procedures need to be followed and adhered to. Viruses can be carried on the hairs of laboratory workers associated with virus research. Hence, as a biosafety precaution, a researcher should not visit an animal farm/unit after working in the laboratory. Similarly, after visiting the experimental or farm animals, one should not visit the laboratory without taking a complete bath with soap and plenty of water. If strict hygienic and biosafety measures are not taken, there are strong chances of transmission of viral infections through farm workers or visitors to animals in rural or urban areas and vice versa.

Application of peptide antibodies and RNAi to inhibit virus multiplication in the host, use of non-coding small RNAs, use of nanotechnology for efficient delivery of drugs and vaccines and developing point-of-care diagnostics and CRISPR-cas gene editing technology are some of the future innovative technologies to be used with advantage in the management and control of viral diseases.

20.23 Strategies, Best Practices and Way Forward

Efficient management of animal viral diseases is a must for accomplishing livestock health protection and promotion to enhance production, productivity and profitability by adopting the suggested action points, strategies, best practices and policy imperatives as follows:

20.23.1 For Diseases with Eradicated Status

So far only one viral disease of animals, namely, rinderpest, has been eradicated from India as well as globally. Besides this, African horse sickness (AHS) has also been eradicated from India. Thus, India needs to be prepared for tackling both of these diseases in an emergency situation, preferably using non-infectious diagnostics and vaccines, if these diseases re-emerge. There must be availability of modernized laboratories for quick detection of the etiological agent with skilled manpower. There is need to keep biological material of value, such as vaccine strains, virulent viruses for challenge and antisera under strict biocontainments in BSL III and BSL IV facilities with periodic checkup by FAO/OIE experts/national consultants, or destroyed if so decided. There is also a need to have technical competence and preparedness to develop appropriate vaccine using reverse genetic engineering.

20.23.2 Capacity Building/Policy Options

- Veterinary vaccine institutes/biological units in the country should be strengthened and equipped to produce the required diagnostics and combined/polyvalent, thermo-resistant and easy-to-administer vaccines against prevalent major viral and other infectious diseases of livestock.
- Creation of disease-free area/zones with emphasis on export-oriented production.
- Cooperation of village panchayat institutions should be sought for 80–100% vaccination through incentives to the vaccinators and farmers for getting their animals vaccinated.

20.23.3 For Other Eradicable Diseases

- After global eradication of rinderpest, we may succeed in eradicating PPR, a serious disease of small ruminants, caused by a morbillivirus similar to rinderpest virus by 2030 following the OIE pathway and timeline. Similarly, with concerted efforts, adequate vaccine production and funding support, it should be possible to eradicate FMD from India by 2040 adopting the OIE Progressive Control Pathway (PCOP) with significant economic and trade dividends. Presently, India is in the Stage 3 of FAO's COP for the control of FMD (Anonymous 2017–18).

- Strengthening of the disease diagnostic laboratories at the national, regional and state level with state-of-the-art facilities having desired biosafety and containment facilities of BSL-2/BSL-3 status depending on the type of bioagent being handled is warranted on priority. Other viral diseases, namely, CSF, sheep pox, goat pox, fowl pox and rabies, may also be included in national eradication programmes as potent vaccines are available against these.
- As India has long porous land borders with the neighbouring countries all around, there is always threat of transboundary diseases. Ideally disease control/eradication programmes should be taken in network mode involving all the neighbouring SAARC/ASEAN countries. A venture fund should be created by the participating countries for routine programmes to meet emergency situations in the face of disease outbreaks.
- Farmers need to be updated on useful livestock technologies, best practices and innovations in animal health, nutrition and management. This will require strengthening of extension services for livestock, poultry and fishery in PPP mode using modern ICT tools.
- A comprehensive livestock health policy should be developed at the national and state level.
- Capacity building for developing cold chain; testing the quality of vaccines, medicines, mineral mixture and residues in livestock feed; and production of disease-free semen, chicks, and fingerlings.
- The success story of rinderpest eradication and FMD-CP needs to be replicated for PPR, classical swine fever, HS, brucellosis and other dreadful diseases of livestock and poultry.
- A state-level **zoonoses coordination committee** with subcommittees at the district level should be constituted under the umbrella of the Department of Animal Husbandry GoI to bring about close association among veterinarians, medical professionals, wildlife experts and other related departments focusing on the 'One World, One Health' concept for efficient handling of the newly emerging and re-emerging deadly viral and other infectious diseases having zoonotic significance.

20.24 Epilogue

Animal disease surveillance is critical for the welfare and sustainability of healthy livestock productivity systems of any country, as the threat of infectious diseases is large as well as diverse and dynamic. Preparedness and combating the prevailing emerging, re-emerging and transboundary diseases require sound monitoring and precision detection systems that are flexible and adaptable under field conditions. There is also an international obligation for OIE reportable diseases of high importance from the point of view of trading in animals and animal products harbouring potent infectious agents/pathogens by all member countries of WTO. It is the right time to apply developed diagnostics and molecular detection tools at the field stage to ensure fast detection and confirmation of pathogens capable of causing diseases

in humans and animals. This must be accompanied by national-level disease surveillance, monitoring and networking to enable an early warning system for infectious diseases based on forecasting (Saminathan et al. 2016). Due priority is also required for application of new vaccines, developing vaccine delivery systems and adopting prudent vaccination programmes and immunomodulatory and effective therapeutic modalities, which would help in devising timely prevention and control strategies against viral and other infectious diseases. Besides these, good management and standard biosecurity measures/practices and appropriate hygienic and zoo-sanitary and quarantine measures should be observed. Moreover, on-the-spot control and checking the spread of pathogens and adequate trade restrictions as envisaged under the SPS agreement of WTO also need to be followed. A holistic vision is required for timely implementation of these concepts and strategies along with strengthening of various multidimensional research and development programmes backed with appropriate funding resources. These measures will greatly help minimize disease incidences and outbreaks and lessen economic burdens due to infectious animal diseases and boost livestock and poultry health, reproduction and production to strengthen sustainable growth of livestock and poultry industry. Reduction in pandemic threats and public health concerns eventually lead to improvement in the socioeconomic status and welfare of the society at large.

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Virus-Mediated Cancers in Animals

21

Catherine Paul and Rajeev Kaul

Abstract

Ever since the discovery of the first tumour-associated virus in hen by Peyton Rous in 1911, cancer-causing viruses have been extensively investigated over the last century. Subsequently, tumour-associated viruses were discovered in several other animal species. Some of these are responsible for severe economic losses to farmers. Exposure to at least one animal virus has lately been reported to be related with cancer in humans. The ability of some of tumour viruses to cross species barrier and infect wild birds or animals to establish a natural reservoir of circulating virus, which can further evolve, may pose a major challenge to veterinary as well as human medicine. Animal tumour viruses have also been used as model system in their natural host as well as in laboratory animals to study the molecular basis of cellular tumorigenesis. This review attempts to summarize the important historical advances and some of the recent developments in the field of tumour viruses of animals with a focus on inter-species transmission.

Keywords

Viral cancer · Pathogenesis · Animals · Inter-species transmission

21.1 Prologue

The hunt for tumour-associated viruses was initiated as a desire to identify the aetiology for cancer and apply prophylactic principles that have been long associated with microbiology in order to prevent the development of cancer. Although this search for viruses began with the aim of preventing human cancers, the problem is

C. Paul · R. Kaul (✉)
Department of Microbiology, University of Delhi, South Campus, New Delhi, India
e-mail: rkaul@south.du.ac.in

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no less complex in animals. The first ever demonstration of a viral aetiology of cancer was done by Peyton Rous in 1911 who found that a tumour can be induced upon injecting a cell-free filtrate prepared from a chicken sarcoma into healthy chickens (Rous 1911). Subsequently, several other viruses associated with cancer in animals were discovered including Shope papillomavirus, mouse mammary tumour virus, murine polyomavirus, and simian vacuolating virus 40 (SV40). It was then generally believed that tumour formation in animal occurs through an entirely different process than tumorigenesis in humans, and viruses were considered to be agents of aetiological significance in causation of cancers only in animals. It was only after the discovery of the Epstein-Barr virus (EBV), the first human tumour-associated virus, that viruses were identified as a causative agent for cancers in humans thereby leading to initiation of search for other human tumour-associated viruses (Epstein et al. 1964). After the discovery of EBV as a cause of Burkett's lymphoma, other groups of viruses causing cancers in humans were subsequently discovered which include human papilloma virus, hepatitis C virus, hepatitis B virus, human T-cell lymphotropic virus type I, Kaposi sarcoma-associated herpes virus, and Merkel cell polyomavirus.

Approximately 12% of cancer in humans are caused by oncoviruses; however, tumour-associated viruses are also widespread among domestic animals and lead to great economical loss in animal husbandry. Animals with a large body size have a greater number of cells and live longer and are expected to develop more tumours than small short-lived animals during their lifetime. However, mice have been shown to be more susceptible to cancer than large animals or even humans. A recent study has shown that larger-bodied species have significantly lower levels of endogenous retrovirus (ERV) activity, whereas genomes of smaller animals have much higher numbers of ERVs suggesting that these endogenous retroviruses may account for high rates of cancers in these animals (Katzourakis et al. 2014). Other than these, the viruses belonging to several other families are known to be associated with cancers in animals of veterinary importance, some of which are described ahead.

21.2 Types of Oncoviruses

Even though oncovirus infections are common in humans as well as animal hosts, the cancers do not develop immediately after infection. Instead, it can take up to several years post viral infection for virus-mediated cellular transformation and oncogenesis. Oncoviruses may either be directly or indirectly associated with cancers. Direct carcinogenic oncoviruses can directly contribute to neoplastic cellular transformation as they possess viral oncogenes. On the other hand, indirect carcinogenic viruses can cause chronic inflammation leading to oncogenic transformation. Another classification for oncoviruses can be on the basis of their genetic material as either DNA oncoviruses or RNA oncoviruses.

21.3 Virus-Mediated Oncogenesis

In virus-associated cancers, the replication of virus is either absent or significantly reduced as active virus replication can prevent tumourigenesis due to lysis of the host cell. Thus the viral genome exists as naked nucleic acid either in episomal form or cellular-genome integrated form. DNA viruses can directly integrate their genomes into the genome of the host, while RNA viruses first generate a DNA copy of their genome by undergoing reverse transcription which can then integrate into the host genome. All oncogenic viruses modulate a few common signalling pathways including p53 and RB tumour suppressor pathways to promote tumourigenesis (Levine 2009). Other major pathways that are targeted include NF-κB, JAK/STAT, TNF receptor-associated factors (TRAFs), TERT, PI3K-AKT-mTOR, β-catenin, interferon signalling pathways, and MHC-1. DNA viruses in particular can also affect the host DNA damage response pathway (DDR). DDR pathways are involved in detection and repair of damaged DNA in cells via PIKK family of serine/threonine kinases, including ATM and ATR and DNA-PK. Thus, DDR can cause a delay in cell cycle progression until the DNA is completely repaired or no foreign viral DNA is detected. Some viral proteins can activate functions of the DDR such as repair factor recruitment that prove beneficial for the viral replication or cellular transformation and it can inactivate activities such as apoptotic pathways that are detrimental to viral DNA survival. Among RNA viruses, tumour is induced rapidly by the viral oncogene carrying acute transforming oncogenic retroviruses in comparison to other retroviruses that cause slow tumour progression. Acute transforming retroviruses have been extensively investigated in the past. The cellular proto-oncogenes are responsible for cell growth, whereas viral oncogenes constitutively stimulate cell multiplication by mechanisms similar to those used by cellular proto-oncogenes. Other retroviruses transcriptionally activate the cellular proto-oncogenes and thus induce tumours. This is usually as a result of modulation of cellular proto-oncogenes by retroviral long terminal repeats.

21.4 Animal Tumour Viruses

The first report of cancers mediated by an infectious agent in livestock animals described pulmonary adenocarcinoma in sheep. The causative agent was later identified as jaagsiekte sheep retrovirus. Viruses as causative agents of cancers have been identified in several animal species of veterinary importance. Oncogenic viruses of veterinary importance belong to four major virus families which have been summarized in Table 21.1. The timeline of important discoveries in the field of tumour virology has been summarized in Fig. 21.1.

Table 21.1 List of oncogenic viruses of veterinary importance

Virus family/ subfamily	Virus genus	Virus	Genome type	Genome size	Host which causes cancer
Retroviridae	α-Retrovirus	Avian leukosis virus	ssRNA	7.5 kb	Poultry
		Rous sarcoma virus		9.3 kb	Poultry
		Avian sarcoma virus		3.7 kb	Poultry
	β-Retrovirus	Jaagsiekte sheep retrovirus	ssRNA	7.4 kb	Sheep
				8 kb	Mouse
		Mouse mammary tumour virus			
	γ-Retrovirus	Feline leukaemia virus	ssRNA	7.6 kb	Cat
		Murine leukaemia virus		8.3 kb	Mouse
		Viper retrovirus			Reptile
	δ-Retrovirus	Bovine leukaemia virus	ssRNA	8.7 kb	Cattle
	ε-Retrovirus	Walleye dermal sarcoma virus	ssRNA	13 kb	Fish
	Lentivirus	Feline immunodeficiency virus	ssRNA	9.5 kb	Cat
Papillomaviridae	Papillomavirus	Bovine papillomaviruses 1–4	dsDNA	7.9 kbp	Cattle
		Canine oral papillomavirus		7.8 kbp	Dog
Herpesviridae	Mardivirus	Gallid herpes virus-2	dsDNA	177 kbp	Poultry
Adenoviridae	Mastadenovirus	Canine adenovirus-1	dsDNA	30.5 kbp	Hamster

Modified from Truyen and Lochelt (2006)

21.5 Retroviruses

Retroviruses are the aetiologic agents of most of the naturally occurring lymphomas, leukaemias, sarcomas, and other haematopoietic neoplasms of many animal species including bovine, sheep, chickens, mice, cats, and gibbon apes. The viruses belonging to this group are also responsible for causing non-neoplastic diseases in sheep, horse, and humans (Olsen et al. 1986). A variety of animals are thought to be infected by numerous retroviruses, some of which interfere with the immune system and cause substantial disease due to immunosuppressive effects, while some of them cause cancer. Most animals have normal genetic elements in their genome that

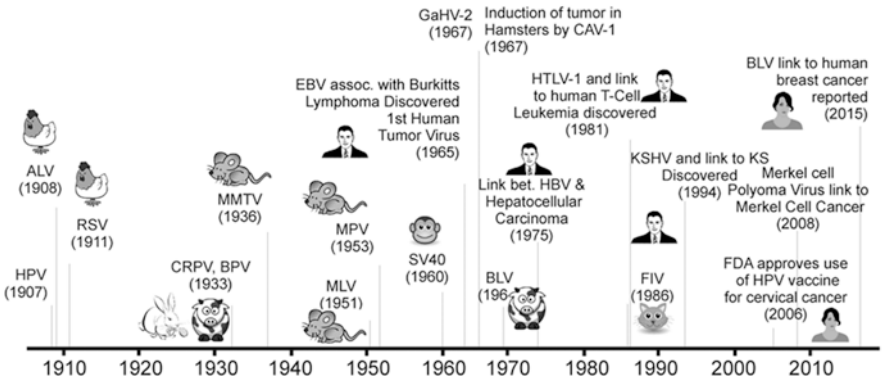

Fig. 21.1 Timeline of important discoveries in field of tumour virology. The years mentioned against virus names refer to year of their discovery. *HPV* Human Papilloma Virus, *ALV* Avian Leucosis Virus, *RSV* Rous Sarcoma Virus, *CRPV* Cottontail Rabbit Papilloma Virus, *BPV* Bovine Papilloma Virus, *MMTV* Mouse Mammary Tumour Virus, *MLV* Murine Leukaemia Virus, *SV40* Simian Virus 40, *EBV* Epstein Barr Virus, *GaHV-2* Gallid Herpesvirus 2, *HTLV-1* Huma T- cell Lymphotropic Virus, *HBV* Hepatitis B Virus, *KSHV* Kaposi's Sarcoma Associated Virus, *FIV* Feline Immunodeficiency Virus, *BLV* Bovine Leukemia Virus, *HPV* Human Papilloma Virus

are termed as endogenous retroviruses which probably evolved from transposable elements. In addition, the RNA containing horizontally transmitted exogenous retroviruses are also known to cause infections in animals, which can be either replication-competent or replication-incompetent viruses. Few genes of replication-incompetent retroviruses got deleted during evolution and they acquired some of the cellular oncogenes; therefore, they require gene products of helper viruses for their replication. Retroviral-mediated cancers in animals like avian, cattle, and fish have been extensively studied and the mechanism of pathogenesis described.

21.5.1 Avian Retroviruses

Infection of avian alpharetrovirus to the cell is mediated by surface (SU) envelope protein of virus which interacts with cellular receptors. Based on the nature of the viral receptor, alpharetroviruses have been grouped into ten subgroups (A–J). Rous sarcoma virus which belongs to subgroup A of alpharetroviruses infects domestic chicken and has also been shown to infect mice cells in culture indicating that it can infect and transform mammalian cells in certain conditions even in the absence of a receptor (Svoboda 1986). RSV was discovered when Peyton Rous found that sarcoma in the domestic fowl was transmissible to uninfected chickens by transplantation of tissue and later by cell-free tumour extract (Rous 1910). Successful isolation of the virus was achieved from the first in vivo passage of sarcoma tissue (Rous 1911). Eventually later, this virus was known as Rous sarcoma virus (RSV) which for the first time demonstrated virus as a cause of cancer. The *src* oncogene identified in RSV has led to identification of several other oncogenes in retroviruses. It

has been suggested that the cell-to-cell contact plays a role in the first stages of virus transmission in addition to other factors. RSV has also been shown to be successfully transmitted to pigeons by using RSV-infected tissue of chicken (Duran-Reynals 1947) and to neonatal rats inoculated with minced chicken RSV tissue (Svet-Moldavsky 1958). However, the attempts to isolate RSV or detect its genome in these tumours remained unsuccessful and role of RSV in tumourigenesis in heterologous species could not be proved. It was observed that the RSV genome that gets integrated in non-permissive rodent cells are mostly not fully expressed, but cell association with permissive fibroblast can lead to production of the virus thus triggering cell fusion between rodent and chicken cells.

Other than RSV, the most common avian retroviruses that are associated with tumours in poultry birds are avian leucosis virus (ALV) and reticuloendotheliosis virus (REV). Among these, ALV is responsible for significant economic losses to poultry farmers. Pheasants and quails are resistant to ALV; however, both species could be experimentally infected with this virus in a recent study indicating the ability of ALV to cross species barrier (Shen et al. 2016). Avian leukosis virus subgroup J can also be transmitted to New World quails with a possibility to establish a natural reservoir of circulating virus which can further evolve (Plachy et al. 2017). Viruses of the avian leukosis/sarcoma group (ALSV) have been shown to infect human cells in culture (Kawai et al. 1989). However so far, there is no evidence that avian oncogenic viruses have any role in cancers in humans or whether they can infect and replicate in human host (Schat and Erb 2014).

The reticuloendotheliosis viruses (REVs) are a cluster of several cognate amphotropic retroviruses isolated from birds. A study published in 2013 analysed the phylogenetic and historical evidence and proposed a model which suggested that these viruses emanated as mammalian retroviruses. In the late 1930s during the laboratory experiments on an assumed malaria parasite, the virus was inadvertently infected into avian hosts (Niewiadomska and Gifford 2013). Consequently they were incorporated into the genomes of fowlpox virus (FWPV) and gallid herpesvirus type 2 (GHV-2) resulting in generation of viruses with recombinant DNA, which are now circulating in wild birds and poultry (Niewiadomska and Gifford 2013). This study for the first time showed that the unpremeditated consequences of experimental procedures can result in virus evolution leading to broadening of host range of previously existing viruses. It is therefore important to improve investigation of viral genetic diversity.

21.5.2 Feline Retroviruses

Domestic cats are frequently infected by retroviruses belonging to three genera: Lentivirinae, c-retrovirus, and Spumavirinae. Feline immunodeficiency virus (FIV), which is a lentivirus, and feline leukaemia virus (FeLV), which is a c-retrovirus, are exogenously transmitted retroviruses that cause pathology in cats. Based on the epidemiologic studies using FeLV-positive leukaemia cluster households, the initial evidence of the relationship between a virus and the disease was established.

Naturally cats are thought to become infected with the FeLV mainly by contact with salivary and nasal secretions of cats that are actively shedding FeLV. Within weeks after exposure to FeLV, cats exhibit one of two major host-virus relationships: a progressive and persistent infection or a self-limiting regressive infection. Development of anaemia, acquired immune deficiency syndrome (AIDS), and eventually neoplastic disease can also be observed in cats (Olsen et al. 1986). FeLV infects up to 10% of cats in different populations. When infected naturally, the virus is cleared from majority of cats after a period of transient viremia. Vaccines are available against this virus which includes subunit vaccines or inactivated whole-virus preparation-based vaccines. Felines that are not able to eliminate the virus from the system fall prey to the disease and die within 3–4 years. Among the previously described three FeLV subgroups, FeLV subgroup A virus is the most significant subgroup. Subgroup B evolved from recombination within *env* gene of subgroup A virus, whereas subgroup C evolved due to mutation in the same gene. The p25 protein is released in high amounts in the bloodstream of the persistently infected animals and thus serves as a diagnostic antigen. Subsequent to infection, the production of replication-incompetent 'feline sarcoma viruses' as a result of recombination between FeLV and cellular genes is associated with oncogenicity of FeLV. Many of the cellular oncogenes including c-myc, c-sis, and c-fes have been detected in these replication-incompetent viruses (Truyen and Lochelt 2006).

Several studies in the late 1960s had shown that some variants of FeLV can replicate to high titres in human cells in cell culture system (Jarrett et al. 1969). This led to concerns regarding the possibility of its zoonotic spread. However, no proof of any serological responses against this virus in exposed individual has been detected (Butera et al. 2000). A recent study has shown that the cancer-derived cell lines of human origin commonly show a fully permissive infection, but cells of haematopoietic origin are normally less permissive. It has been suggested that various cellular barriers protect primary human blood cells which could be critical in defence against zoonotic infection with FeLV (Terry et al. 2017). However, cross-species infection of FeLV to wild feline species has been reported (Krengel et al. 2015).

Feline immunodeficiency virus (FIV) is an analogue of human immunodeficiency virus (HIV) in cats. An augmented incidence of neoplasia has been reported in cats which are infected with this virus. FIV infection most commonly results in lymphoma of B-cell origin whose mechanism of development is not clearly understood but has been suggested to arise *via* indirect mechanisms (Magden et al. 2011). A study had shown natural cross-species transmission of puma lentivirus A (PLVA), which is a subtype of FIV, between bobcats and mountain lions (Lee et al. 2014). Subsequent study by the same group showed that FIV transmission from reservoir host (bobcats) into a closely related but different species (mountain lions) in the same geographical region can occur frequently (Lee et al. 2017). The study showed lentiviruses have the capacity to evolve and adapt to new environments allowing them to even overcome host restriction mechanisms over time and under certain ecological circumstances (Lee et al. 2017).

Feline foamy virus, which is a spumavirus, is considered non-pathogenic although it is transmissible (Jarrett 1999). A study which tested for the presence of

antibodies to the feline foamy virus in cats showed no significant association with clinical disease (Romen et al. 2006). Several studies have revealed that the foamy viruses of non-human primates are transmitted to humans where they establish persistent infection, whereas bovine foamy virus and feline foamy virus do so infrequently (Tachibana et al. 1997). In contrast, retroviral infections of dogs are not well-characterized; however, retrovirus-like particles have been occasionally isolated from immunosuppressed dogs (Modiano et al. 1995).

21.5.3 Bovine Retroviruses

Bovine leukaemia virus (BLV) belongs to genus *Deltaretrovirus* of the *Retroviridae* family. This virus is used as a model for studying the pathogenesis of human T-cell leukaemia virus type 1 (HTLV1). BLV infects cattle all over the world and causes enzootic bovine leucosis (EBL) that results in economic losses in meat industry (Wang et al. 2018). Although the occurrence of leukosis was described in the late 1800s in Europe, the virus was first identified much later in 1969 and transmissibility confirmed in 1972. BLV infects lymphocytes and produces a lifelong infection. It is believed that cell-free virus appears only during acute infection before the production of neutralizing antibodies and is rarely detected in vivo or it possibly appears in advanced stages with the development of clinical disease. Thus, most BLV infections arise from transfer of BLV-infected lymphocytes between cattle, whereas a small proportion of infections occur in utero. In terms of infective media, only blood, colostrum, and milk would ordinarily be expected to contain significant numbers of lymphocytes compared to semen, ova, saliva, nasal secretions, urine, and faeces, unless an exudative process were present. Many different exposures, some iatrogenic, may potentially transmit BLV. Most of the BLV infections are asymptomatic; however, persistent lymphocytosis is seen in less than half of infected animals (Moratorio et al. 2010). Malignant monoclonal B-cell lymphosarcoma (LS) develops in a very small percentage (<5%) of infected cattle. Other symptoms of BLV infection in cattle involve reduced milk yield, general weakness, and complications associated with digestive and nervous systems (Polat et al. 2017).

BLV infection in the United States is widespread even though the virus has been eradicated in 22 countries worldwide. The pathogenesis of the virus, however, is not well understood. EBL/BLV genome encodes the Tax protein which functions as a regulatory protein and has been investigated for its role in virus pathogenesis (McGirr and Buehring 2005; Rice et al. 1987). The Tax protein activates the promoter within the LTR sequence and promotes transcription of virus-coded genes. Based on the studies in some neoplastic B cells, it has been suggested that the X region of the virus and expression of the tax gene alone is sufficient to transform infected cells. Persistent lymphocytosis is caused not because of delayed apoptosis of infected B cells, but rather it is caused due to increased cell proliferation. However, the exact mechanism by which Tax protein may play a role in transformation and tumourigenesis is not well understood.

Exposure to BLV has recently been reported in the United States to be associated with breast cancer in humans (Buehring et al. 2015). It is not known how humans become infected with BLV. However, transmission from cattle through raw milk and inadequately cooked beef and from infected humans is considered to be potential routes. A subsequent study performed on samples from Australia showed BLV DNA in breast tissue of 80% of women with breast cancer versus 41% of women with no history of breast cancer, indicating significant association (Buehring et al. 2017). It must also be mentioned that there have also been a couple of studies performed in China and Belgium which did not show any association between breast cancer and BLV (Gillet and Willems 2016; Zhang et al. 2016). These suggest that further investigations are required to explore the association between BLV and breast cancer.

21.5.4 Fish Retroviruses

Members of several virus families have been implicated in the aetiology of tumours in fish. Walleye dermal sarcoma virus (WDSV) is a retrovirus found in the freshwater fish *Stizostedion vitreum* and has been reported to cause tumourous disease in fishes (LaPierre et al. 1999). The development of tumours is observed during winter. However in most cases, tumour regression is observed during the spring and summer seasons. It is believed that fishes that experience tumour regression develop an antiviral immunity as the tumours do not reappear in these fishes. Natural infection of WDSV occurs during spring season when spawning of walleyes takes place where they gather in big numbers in streams. Infection of walleyes takes place via topical, oral, and intramuscular route experimentally.

The genome of WDSV is complex with three coding regions (ORF A–C) that encode for proteins. Downstream to the 5-LTR is ORF C which encodes for a protein that can contribute to tumour regression by inducing apoptosis (Nudson et al. 2003). Downstream of the 3-LTR are ORFs A and B that are gene duplicates and encode for rv-cyclin which is a cyclin homolog (LaPierre et al. 1998). The rv-cyclin protein causes cell proliferation by interacting with cellular cyclin-dependent kinases (CDKs) and repressing the expression of some cellular genes (Holzschu et al. 2003). The mechanism of action of WDSV and Kaposi sarcoma herpesvirus (KSHV) has been shown to be similar as WDSV cyclin also interacts with CDK8 similar to KSHV in Kaposi sarcoma of humans (Rovnak and Quackenbush 2002). Transmission of WDSV to humans or other animal species has not been reported.

21.5.5 Ovine Retrovirus

Enzootic nasal tumour virus (ENTV-1) associated with enzootic nasal adenocarcinoma (ENA) is an ovine betaretrovirus that causes tumour in mucosal nasal glands of goats and sheep. The disease has been reported to occur naturally almost worldwide. Recently a study reported the generation of ENTV-1 molecular clone that can

be used to produce mature virus particles (Walsh et al. 2016). ENTV-1 has been previously suggested as a causative agent of ENA (Walsh et al. 2013); however, the virus could not be successfully cultured. Experiments are being performed using the virus produced from the molecular clone of ENTV-1 to test whether ENTV-1 is necessary and sufficient for induction of ENV. Transmission of ENTV-1 to humans or any other animal species has not been reported.

21.6 Papillomaviruses

Papillomaviridae family includes bovine papillomaviruses (BPV), consisting of numerous small DNA tumour viruses which infect mucosal epithelial tissues in several species of animals resulting either in benign growths or occasionally causing cancers. So far 13 BPV genotypes have been identified out of which type 1, 2, 5, and 13 have been reported to infect other species. Papillomavirus-mediated cellular transformations were initially investigated using BPV-1 as a model organism (Campo 1992). BPV-1 has since been extensively investigated to understand the molecular basis of virus-mediated pathogenesis due to its ability to infect and transform murine cells. Papillomaviruses can also infect both canines and felines. In canines these viruses have been suggested to cause papillomas of the oral cavity and pigmented plaques on the skin; whereas in felines they cause sarcoids, papillomas of the oral cavity, and bowenoid in situ carcinomas of the skin. It has been further suggested that papillomaviruses can also cause squamous cell carcinomas and basal cell tumours in felines.

Papillomaviruses were reported to be associated with cancers for the first time in 1935 based on experiments performed in rabbits. The association of these viruses as a causative agent of human cervical cancers was reported almost half a century later in 1981 (zur Hausen et al. 1981). Papillomaviruses have been reported to infect many species of mammals and some species of birds. Papillomavirus genomes consist of circular dsDNA that codes for several early and late genes. The L1 ORF sequences of papillomaviruses are used for its classification. Papillomaviruses with more than 60% L1 ORF similarity generally belong to the same genus and mostly infect closely related host species. The virus can spread by both direct and indirect contact as they do not get inactivated in the environment. The infection of basal cells occurs when the virus comes into contact with the mucocutaneous epithelium due to micro-abrasions on the skin surface. The infection results in the generation of circular episomal viral genomic DNA copies that are maintained inside the cells and replicate along with host cell genomic DNA. Papillomaviruses disrupt the process of terminal differentiation of infected cells resulting in abnormal cell growth and tumourigenesis. Bovine deltapapillomaviruses are a unique group of papillomaviruses that infect the mesenchymal cells, although replication of the virus is not permitted within these cells (Jelinek and Tachezy 2005).

The papillomaviruses that have shown evidence of transmission to other species by crossing species barriers are bovine papillomavirus 1 (BPV-1) and BPV-2. These are responsible for causing benign warts in bovine host and cause sarcoids in equids (Nasir and Campo 2008). An analysis of the transcriptional promoter region of BPV-1 that was associated with causation of sarcoids in equine hosts has suggested the possibility of multiple cross-species transmission events into horses (Trewby et al. 2014).

21.7 Herpesviruses

Marek's disease virus or gallid herpesvirus 2 (GHV-2) belongs to the *Mardivirus* genus of the *Alphaherpesvirinae* subfamily and is the only member of the group that can cause Marek's disease. The other members of this subfamily include gallid herpesvirus 3 and herpesvirus of turkeys (HVT). A study on multiple GVH-2 strains with varying oncogenicity showed the role of 7700-bp sequence within the internal long repeat of their genome in modulation of GHV-2 oncogenicity. The activated CD+ T cells are latently infected where the virus remains hidden for long periods. GHV-2 has been previously suspected to be associated with multiple sclerosis (MS) in humans due to the fact that the serum of human suffering from MS was found to react with GHV-2 antigen (McHatters and Scham 1995). A study also showed detection of GHV-2 DNA in human sera (Laurent et al. 2001). Whether these findings are of any concern to human health or if GHV-2 plays any role in the pathogenesis in humans has never been shown.

Herpesvirus saimiri (HVS) and herpesvirus ateles (HVA) isolated from Old and New World monkeys, gorillas, and chimpanzees are oncogenic only in heterologous hosts. Herpesvirus saimiri, unlike other oncogenic herpesviruses, is lymphotropic for T lymphocytes. H. saimiri could be isolated from squirrel monkey which was naturally infected with this virus but it did not cause any disease in squirrel monkey. However, it was found to be leukaemogenic in owl monkeys, several marmoset species, and spider monkeys. Herpesvirus ateles has been detected in spider monkeys in which it causes no recognizable disease in the natural host. Other closely related host species such as marmosets and owl monkeys are highly susceptible to infection, with resultant neoplastic disease. Infection of susceptible animals can result in outcome that ranges from lymphoid hyperplasia to lymphomas. Like H. saimiri, H. ateles is also a T-cell lymphotropic virus.

21.8 Adenoviruses

Adenoviruses are one of the most extensively investigated DNA tumour viruses. The gene products of this virus have been shown to transform mammalian cells in vitro, even though the virus has not been found to be associated with cancer in its natural host. Adenoviruses have long been used as tools with which to probe critical cellular processes, including oncogenic transformation. These viruses have been

studied as a model system to understand the molecular mechanisms of tumourigenesis and the associated biological events.

Adenoviruses are non-enveloped, icosahedral viruses, with a diameter of 70–90 nm (Wigand et al. 1982). The genome of the virus consists of a linear dsDNA of 20–30 Kbp size. This virus family consists of two genera that include *Mastadenovirus*, which infects mammals, and *Aviadenovirus* that infects birds. Adenoviruses of veterinary importance are the avian adenoviruses (AAV) which comprise of three distinct subgroups. A common group antigen is shared among group 1 AAV and are commonly called the 'conventional' AAV.

Although group 1 AAV has been previously isolated from faecal matter and tissues of birds that showed symptoms of gastrointestinal diseases, their role in the pathogenesis of the disease has not been understood or proven. Group 1 AAV have been associated with several conditions in birds that include inflammation of several organs such as the ventricles in the brain, the proventriculus part of the digestive system, and the intestine and malabsorption syndrome.

Canine adenovirus type 1 (CAV-1) causes infectious canine hepatitis (ICH) in dogs which is characterized by symptoms of abdominal pain, fever, loss of appetite, vomiting, and diarrhoea. Simian adenoviruses were isolated from macaques, baboons, chimpanzees, and squirrel monkeys. Under experimental conditions, some of the virus strains are oncogenic in newborn hamsters but play no role in neoplastic disease in any of the host species (Olsen et al. 1986).

21.9 Conclusions

The discovery of infectious causes of cancers initially in animals and later in humans has pointed to a direct role of viruses in tumourigenesis. Animals and humans have lived along each other for a long period in human history. Several reports in the last few years have suggested a link between exposure to bovine leukaemia virus and development of breast cancer in humans (Baltzell et al. 2018; Buehring et al. 2017; Pavlenko 1973). While the causative relationship between BLV and breast cancer has not been established, its potential role in initiating the malignant process has been pointed. The possibility of existence of other tumour viruses of animals that could be of zoonotic importance cannot be ruled out. Extensive studies of tumours in animals of veterinary importance are a must to screen for novel infectious viral causes and their association if any with tumourigenesis in other species of animals and humans.

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Antivirals: Past, Present and Future

22

Mohammed Bule, Fazlullah Khan, and Kamal Niaz

Abstract

The uses of antiviral agents are increasing in the new era along with the development of vaccines for the effective control of viral diseases. The main aims of antiviral agents are to minimize harm to the host system and eradicate deadly viral diseases. However, the replications of viruses in host system represent a massive therapeutic challenge than bacteria and fungi. Antiviral drugs not just penetrate to disrupt the virus' cellular divisions but also have a negative impact on normal physiological pathways in the host. Due to these issues, antiviral agents have a narrow therapeutic index than antibacterial drugs. Nephrotoxicity is the main adverse reaction of antiviral drugs in human and animals. In this chapter, we summarize the antiviral agents' past, present and future perspectives with the main focus on the brief history of antiviral in animals, miscellaneous drugs, natural products, herbal and repurposing drugs.

Keywords

Virus · Therapy · Bacteria · DNA · RNA

M. Bule
Department of Pharmacy, College of Medicine and Health Sciences, Ambo University, Ambo, Ethiopia

F. Khan
Department of Toxicology and Pharmacology, The Institute of Pharmaceutical Sciences, Tehran University of Medical Sciences, Tehran, Iran

K. Niaz (✉)
Department of Pharmacology and Toxicology, Faculty of Bio-Sciences, Cholistan University of Veterinary and Animal Sciences (CUVAS), Bahawalpur, Pakistan

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22.1 Prologue

Vaccine development has long been the first and foremost approach in the control of viral diseases (Saminathan et al. 2016). However, with the development of the first human antiviral drug, idoxuridine, and its later approval in 1963, a new era of antiviral treatment development began (De Clercq and Li 2016). Because of viral replication cycle, which involves usage of host cell biochemical machinery, antiviral drugs can also affect the function of the host's pathways resulting in the great risk of toxicity. Therefore, the major concern in antiviral drug development is the identification of specific targets with increased selectivity and reduced side effects, which limit the therapeutic use of antiviral drugs in comparison to antibacterial agents (Dal Pozzo and Thiry 2014). In the early 1900s, state-of-the-art review articles on antiviral chemotherapy in veterinary medicine listed several drawbacks of low antiviral drugs use in veterinary medicine. Those included usage restricted to a single virus and specific animal species, problems with high spectrum activity and low cytotoxicity, high costs of development of new chemical compounds and absence of rapid diagnostic techniques allowing prompt use of a specific antiviral agent in the course of an acute infection (Rollinson 1992a, b). Most of the antiviral drugs used in animal medicine have been originally developed against human viral infections and their clinical use in veterinary medicine is not widespread and common. Nevertheless, several licensed human antiviral agents are being used with cascade principle for treatment of animal diseases (e.g. acyclovir, idoxuridine and trifluridine against feline herpesvirus-1 ocular infection in cats) (Thiry et al. 2009). Currently, the only licensed antiviral drug in veterinary medicine is feline interferon-omega (IFN-ω), whose mechanism of action involves a combination of antiviral and immunostimulatory activity (De Clercq and Li 2016; Bracklein et al. 2006). Most antiviral agents interfere with the synthesis or regulation of viral nucleic acids (Fig. 22.1) and act by nucleoside analogues that block elongation of newly synthetized DNA or RNA chain. Other antiviral agents used in veterinary medicine act as neuraminidase (oseltamivir) or amino acid (L-lysine) inhibitors, while novel treatment options such as small inhibitory RNAs are also under investigation (Dal Pozzo and Thiry 2014; Sykes 2013). In recent years, the use of antiviral agents in veterinary medicine has become more favourable with growing interest in its research. This is partially due to successful outcomes of antiviral therapy in some human diseases and partially due to advances in internal veterinary medicine with the development of novel and sophisticated diagnostic and treatment protocols. In addition to that, current measures for control of viral infections such as vaccination or removal of infected animals from breeding stock by culling have many limitations. Therefore, antiviral agents represent a promising alternative for the treatment of viral diseases in veterinary medicine (Dal Pozzo and Thiry 2014).

One of the most common approaches in antiviral drug discovery is rational drug design, which is based on the understanding of the structures and functions of target molecules. It comprises three steps of drug design: (1) identification of the receptor or enzyme relevant for the disease that the drug is being developed for, (2) discovery of the structure and function of the receptor or enzyme of interest and (3) use of the

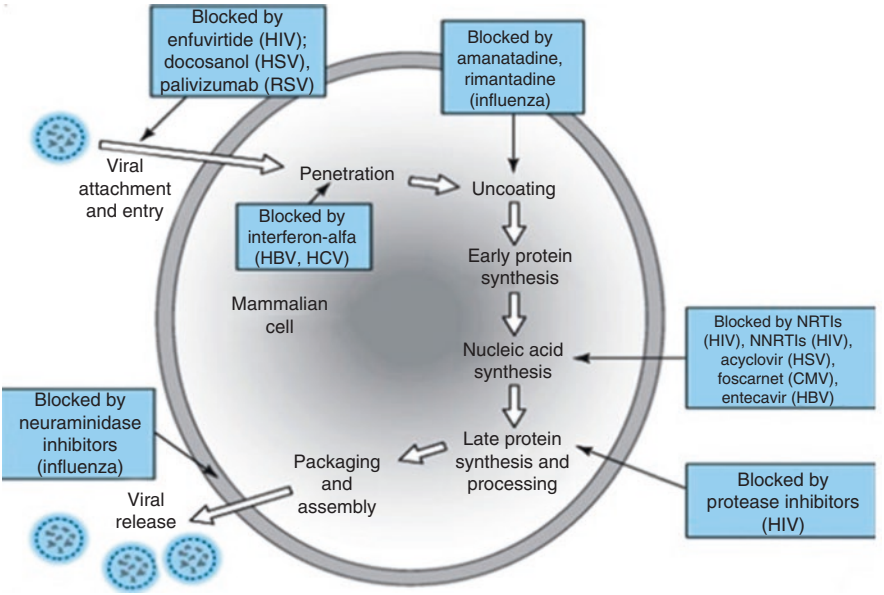


Fig. 22.1 Possible general mechanism of action of antivirals

information from step 2 in order to design a drug molecule that would interact with the receptor or enzyme in a therapeutically beneficial way. The best known example of this approach is azidothymidine (AZT), used in the treatment of human immunodeficiency virus (HIV), which acts by inhibiting HIV reverse transcription. Interestingly, it was originally developed to target reverse transcription of avian retroviruses that may cause cancer and was later successfully applied to HIV as well (Olivero 2018). The other widely used approach is high-throughput screening (HTS) methods, which enable validation of a number of biological modulators against a chosen set of defined targets. They yield rich data sets over a short span of time by combining expertise in liquid handling and robotic automation, multiplatform plate reading and high-content imaging. The number of thus emerged "active hits" is normally around 2% of the total number of potential biological modulators screened. Steps in HTS pipeline can be summarized as follows: (1) sample preparation, (2) sample handling and (3) readouts and data acquisition. The most common HTS methods are targeted/selected screens, diversity and high-content screens and RNAi screens (Szymański et al. 2012). Targeted or selected screening is based on identification of compounds that can selectively inhibit or bind to a specific protein of interest. If the crystal structure of the protein of interest is known, it is usually done by in silico three-dimensional (3-D) modelling, while if the ligand for the protein of interest is known, software can search libraries for the other compounds with similar characteristics and binding properties. Examples of such approach include identification of compounds against HIV, filoviruses, poxviruses, arenaviruses, etc. (Marriott et al. 1999). Another commonly used screening method is

diversity screening, based on identification of compounds that inhibit viral replication or pathogenesis at any level. This approach involves a much broader target base, instead of focusing the screening against one specific protein of interest. It has been applied in the identification of candidate small-molecule inhibitors against dengue virus, yellow fever virus and New World arenaviruses (Valler and Green 2000). High-content screening (HCS) is a subclass of diversity screening method developed upon automation of cellular imaging and analysis techniques. It allows imaging multiple cells at the same time and measurement of multiple parameters, such as shape, texture, staining localization and intensity, total number of cells, size of the nucleus and percentage of virus-positive cells (Brodin and Christophe 2011). Another type of screening for antiviral drug discovery is RNAi screens. siRNAs incorporate into the RNA-induced silencing complex and bind to the target mRNA, thereby inducing degradation of the mRNA and thus preventing its translation into a protein. shRNA, on the other hand, silences protein by forming a "short hairpin" loop through folding back upon itself. Genome-wide RNAi screens have been used to study the pathogenesis of HIV, influenza virus, West Nile virus, Ebola virus, etc. (Hirsch 2010). In addition to the above-mentioned methods, recent advances in genomics, bioinformatics and associated technologies offer new opportunities in antiviral drug discovery. Computational methods enabled construction of databases that contain information related to biological function, chemical structure, biologic activity and many other properties of potential antiviral compounds that can all contribute to identification of new lead bioactive species (Prichard 2007).

22.2 Vidarabine

Vidarabine (9-d-arabinofuranosyl adenine) was the first antiviral agent licensed for systemic treatment of herpes viral infections in humans (Fenner et al. 2014). It is an adenosine analogue that is converted by cellular enzymes to its active intracellular derivate, vidarabine triphosphate. Obtained triphosphate form further acts as competitive inhibitor of both viral and host DNA polymerase, where viral enzymes are much more susceptible to the drug than that of the host cell (Sykes 2013; Schaechter 2010). However, independence on viral thymidine kinase-mediated phosphorylation results in greater host cell toxicity (Sykes 2013). It is used as a topical treatment for feline herpes keratitis, albeit in vitro studies have shown it to be less potent against feline herpesviruses than trifluridine and idoxuridine (Nasisse 1990). In vitro activity has also been demonstrated against feline end equine rhinopneumonitis (Ayisi et al. 1980). Five to six times daily administration as a 3% ophthalmic ointment was reported to be well tolerated by cats and effective in the treatment of feline keratoconjunctivitis sicca. It has also been reported to be effective against idoxuridine-resistant strains (Sykes 2013; Stiles 1995).

22.3 Acyclovir

Acyclovir (acycloguanosine) is an acyclic analogue of the purine nucleoside deoxyguanosine that has been widely used to treat herpesvirus family infections (Sykes and Papich 2013; Perazella and Shirali 2014). The activation involves phosphorylation of the drug firstly by virus-encoded thymidine kinase enzymes into monophosphate form, followed by further phosphorylation to triphosphates by host cell enzymes (Sykes and Papich 2013). Acyclovir triphosphate is a better substrate to viral than host DNA polymerase, resulting in its concentration in infected cells. Due to the lack of 3′-hydroxyl group, the drug inhibits viral DNA polymerase enzyme, as upon its incorporation further DNA chain elongation is disabled (Sykes and Papich 2013; Salvaggio and Gnann 2017). Hence, as a therapeutic, it is mostly used to treat DNA virus infections, in particular herpes simplex virus types 1 and 2 and varicella-zoster virus (Salvaggio and Gnann 2017). In animal medicine, it has been primarily used against feline herpesvirus-1 (FHV-1) infections, but not as efficiently as against the same human virus in vitro, which is related to its low oral bioavailability in cats (Maggs and Clarke 2004; Gaskell et al. 2007; Nasisse et al. 1989). The acyclovir prodrug valacyclovir shows better pharmacokinetic properties in terms of its enhanced bioavailability, resulting in faster absorption after oral administration upon which it gets rapidly metabolized to acyclovir. Acyclovir and entecavir had the ability to block nucleic acid synthesis (Fig. 22.1). However, administration of valacyclovir as well as subsequent increased plasma acyclovir concentrations has been associated with adverse effects, such as nephrotoxicity, myelosuppression and renal and hepatic necrosis, and yet was not effective against FHV-1 infection (Nasisse et al. 1989). These findings suggest systemic administration of neither acyclovir nor valacyclovir is recommended for treatment of herpesvirus infections in cats. Ganciclovir, another purine nucleoside analogue that resembles acyclovir and is widely used to treat cytomegalovirus infections in human medicine, has been shown to be more effective against FHV-1 in vitro, but unfortunately there is lack of data on its efficacy and safety in animals (Sykes 2013). On the other hand, topical acyclovir treatment was shown to be effective against FHV-1 conjunctivitis and keratitis when applied at least 5 times a day and did not produce toxic effects (Williams et al. 2005). Apart from cats, the existing studies provide data on acyclovir treatment in horses, where intravenous administration resulted in 9,6-hour half-life, contrary to very low oral absorption (< 3%) (Williams et al. 2005; Riviere and Papich 2013). It has therefore been suggested that IV treatment could be administered twice daily for equine herpesvirus-1 (EHV-1) (Riviere and Papich 2013). In birds, oral treatment with 120 mg/kg of acyclovir every 12 h has been shown as the minimum dose necessary to maintain concentrations that exhibit antiviral effect in pheasants (Rush et al. 2001). Studies in dogs report oral absorption of acyclovir of 80–90%, but it becomes saturated at high doses (de Miranda et al. 1981).

22.4 Penciclovir

Penciclovir [9-(4-hydroxy-3-hydroxymethylbut-1-yl)] is guanosine analogue that resembles acyclovir in structure, mechanism of action and antiviral activity spectrum. Comparing to acyclovir, penciclovir-triphosphate accumulates in virus-infected cells in much higher concentrations and for longer half-life (10–20 times longer than acyclovir) (Salvaggio and Gnann 2017; Gill and Wood 1996). However, it is less potent than acyclovir triphosphate as it exhibits lower affinity for viral DNA polymerase enzyme, which would allow lower and less frequent dosage in clinical use. In vitro studies have proven its efficacy against FFHV-1 virus and hepatitis B virus (Dannaoui et al. 1997; Shaw et al. 1994; Korba and Boyd 1996). Because of penciclovir's poor oral bioavailability (<5%), famciclovir was developed as the oral formulation (Salvaggio and Gnann 2017). The use of penciclovir will be further described along with famciclovir.

22.5 Famciclovir

Famciclovir (the diacetyl ester of 6-deoxy-penciclovir) is the oral prodrug of acyclovir with the improved bioavailability which, following oral administration, gets rapidly converted to its active metabolite penciclovir by di-deacetylation and oxidation. However, the pharmacokinetics of penciclovir and famciclovir in cats appears to be nonlinear (saturable) and absorption variable compared to other species. This is supported by the observation that administration of the same doses of famciclovir to cats and other species resulted in much lower plasma concentrations and longer time required to reach peak plasma concentrations in cats (Thomasy et al. 2007). Thus, limited famciclovir metabolism stems from deficiency of hepatic aldehyde oxidase enzyme in cats, which converts famciclovir to its active form (Dick et al. 2005). Even though famciclovir has to be administered in high oral doses to develop adequate plasma concentrations in cats, it seems to be well tolerated and successful in the treatment of FHV-1-associated conjunctivitis (Thomasy et al. 2007; Malik et al. 2009; Thomasy et al. 2012). Due to saturable metabolism, oral administration of both 40 and 90 mg famciclovir/kg to cats resulted in equivalent serum and tear penciclovir concentrations, implying that 40 mg/kg is equally effective against FHV-1 as the higher dose (Thomasy et al. 2012). In rats and dogs, famciclovir absorption and metabolism appear to be similar to those previously reported in people, despite the observed slower conversion of famciclovir to penciclovir in both species (Filer et al. 1994).

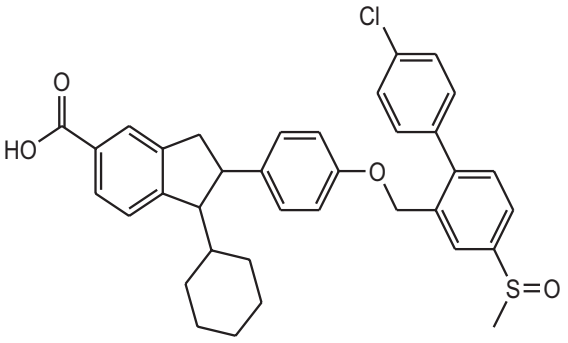
22.6 Ribavirin

As first described by Witkowski et al. in 1972, ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is a triazole nucleoside analogue that inhibits replication of both DNA and RNA viruses by interfering with viral mRNA synthesis (Witkowski

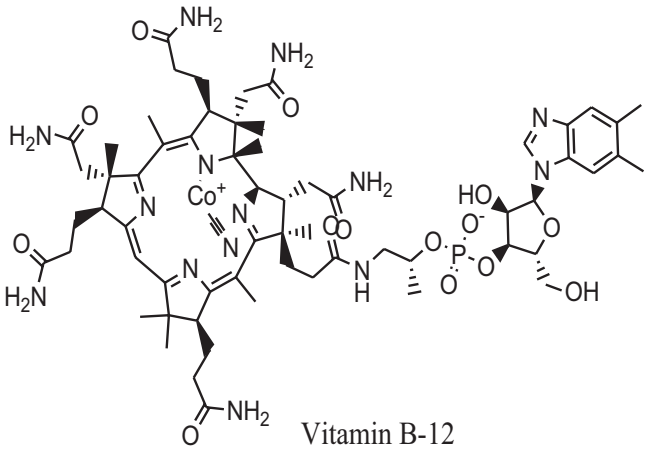
et al. 1973). It is active against a wide range of viruses including adenoviruses, arenaviruses, bunyaviruses, herpesviruses, orthomyxoviruses, paramyxoviruses, picornaviruses, poxviruses, retroviruses, rhabdoviruses and rotaviruses, but the strongest effect exhibits against influenza viruses and, in combination with interferon, against hepatitis C virus (Dolin 1985; Gustafson 1986; Te et al. 2007). It has several possible mechanisms of action. Firstly, as ribavirin monophosphate, generated by adenosine kinase-mediated phosphorylation, it can indirectly inhibit the synthesis of guanine nucleotides. Further, the phosphorylated triphosphate form competitively inhibits binding of ATP and GTP to RNA polymerase (Riviere and Papich 2013). Orally administered ribavirin has been shown to worsen the condition of cats experimentally infected with calicivirus. Toxic effects mainly resulted from drug-induced thrombocytopenia and include depression of red and white blood cells, increased alanine aminotransferase activity, icterus and body weight loss. However, observed clinical symptoms withdrew within one week after treatment discontinuation (Riviere and Papich 2013; Povey 1978). Interestingly, these side effects were not observed in dogs treated for 2 weeks with 60 mg/kg of the drug (Canonico 1985). In kittens experimentally infected with feline infectious peritonitis virus (FIPV), treatment with neither free nor liposomal ribavirin improved survival rate and, similarly to animals infected with calicivirus, resulted in intrinsic toxicity (Riviere and Papich 2013; Weiss et al. 1993). Activity of ribavirin has also been demonstrated against bovine viral diarrhoea virus, bovine herpes virus-1 and parrot bornavirus 4 in cell culture models (Glotov et al. 2004; Musser et al. 2015).

22.7 Benzimidazoles

The antiviral activity of benzimidazole nucleosides was first reported by Tamm, Folker and co-workers in 1954. They designed 5,6-dichloro-1-(β-D-ribofuranosyl) benzimidazole (DRB), which had various biological activities including antiviral activity against RNA and DNA viruses. The antiviral activity of DRB is via inhibiting cellular RNA polymerase II thus inhibiting viral and cellular RNA synthesis (Migawa et al. 1998; Porcari et al. 1998; Chen et al. 2000; Townsend et al. 1995). In pharmaceutical chemistry, heterocyclic compounds particularly the benzene-fused are of great importance. In the class of benzene-fused compounds, benzimidazole and its derivatives are known for their wide variety of biological activities. Biologically active compounds such as vitamin B12, albendazole, mebendazole and thiabendazole contain a benzimidazole nucleus in their structure (Fig. 22.2) (Shaharyar et al. 2016). Structure–activity relationship (SAR) studies show that due to a change in the group on the basic structure, benzimidazoles display a wide array of biological activities including analgesic, antibacterial, antifungal, anticancer and antiviral (Alaqeel 2017). Moreover, a range of structurally varied nonnucleoside inhibitors (NNI) of the HCV polymerases sharing the benzimidazole pharmacophore has been reported. Among these classes of compounds, JTK-003, which is an orally active benzimidazole derivative, is in its Phase I and II clinical trial stage in Japan (Tan et al. 2002; Tomei et al. 2003). Currently, a number of benzimidazole



JTK-003



Vitamin B-12

Fig. 22.2 The chemical structure of bioactive benzimidazole derivatives JTK-003 and vitamin B12

derivatives are available in the market such as omeprazole and rabeprazole used for gastric ulcers, telmisartan and candesartan for hypertension, astemizole and mizolastine for allergic rhinitis, and albendazole, oxibendazole and mebendazole for parasitosis (Wang et al. 2015). Benzimidazoles readily interact with the biopolymers of the living system due to the fact that they are bioisosteres of cellular nucleotides (Starčević et al. 2007).

22.8 Arildone

Arildone is an antiviral drug of the 4-[6-(2-chloro-4-methoxy)phenoxyl]hexyl-3,5-heptanedione class, which is active against both DNA and RNA viruses (Kuhrt et al. 1979). It is primarily suggested to be used as a broad-spectrum antiviral agent

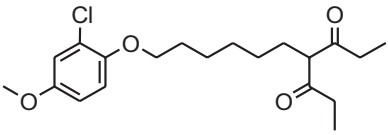


Fig. 22.3 The chemical structure of the antiviral agent arildone

because it is relatively a less toxic drug and inhibits viral replication at lower concentration (Kim et al. 1980). SAR studies demonstrated that omission of the lipophilic substituents of arildone diminished the antiviral activity (McSharry et al. 1979). Arildone (Fig. 22.3) inhibits replication of enterovirus, particularly poliovirus, via interaction with the viral capsid and hence blocking viral uncoating (Nikolaeva-Glomb and Galabov 2004). Further in vitro studies provided evidence that a direct interaction of arildone with the poliovirus capsid stabilizes the virion against heat and alkaline treatment, resulting in loss of the VP4 capsid polypeptide and blocked release of viral RNA (Fox et al. 1986). The uncoating inhibition action of arildone at lower dose can block replication of herpes virus at an earlier stage than the polymerase. Arildone is administered as a solution in dimethyl sulphoxide (DMSO) due to its poor solubility in water, and the solvent properties of DMSO may have augmented its antiviral action (Hutchinson 1985). In an animal model experiment, the ability of arildone to block the virion uncoating property to prevent paralysis and death was studied in mice intracerebrally infected with a higher dose of poliovirus type-2 (strain MEF). Moreover, IP administration of arildone suspended in gum tragacanth successfully protected the animals from paralysis and death in a dose-dependent fashion (minimal inhibitory dose = 32 mg/kg, 2X/day) (Mckinlay et al. 1982). Arildone is the first of the capsid inhibitors that demonstrated in vitro inhibition of poliovirus replication and prevented paralysis and death in poliovirus-infected mice. Such compounds with better bioactivity showed potency orally in the mouse model, even when administered days after intracerebral infection (McKinlay et al. 2014).

22.9 Phosphonoacetic Acid

Compounds consisting the carbon-phosphorous bond are rare in nature and were considered non-existent till recently. It was in 1924 that phosphonoacetic acid (PAA) was first synthesized and its antiviral activity was discovered almost 50 years later in 1973 (Shipkowitz et al. 1973). The discovery of PAA (Fig. 22.4) as an antiviral drug gave rise to intense research on its biological activities, which demonstrated PAA and its derivatives' ability to inhibit the replication of a number of viruses such as immunodeficiency, hepatitis and herpes viruses. In animal studies it was shown that PAA is active against herpes keratitis in rabbits and herpes dermatitis in mice. PAA and its derivatives being analogues of antimetabolites of pyrophosphates have their action against herpes viruses, especially in Epstein–Barr virus, CMV and HSV, through inhibiting DNA polymerase, which is important in herpes virus replication (Alimbarova et al. 2015; Overby et al. 1974). In addition, it was

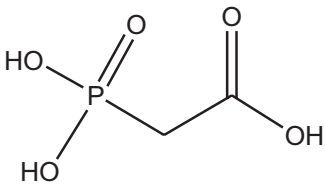


Fig. 22.4 Structure of phosphonoacetic acid

depicted in a study that polymerase activity was inhibited in lysed cultures of infected cells by PAA without affecting the enzyme in normal cells. Polymerases from both normal and infected cells were highly purified and investigated to verify their differential sensitivity towards PAA (Mao et al. 1975).

22.10 Rifamycins

Amycolatopsis rifamycinica is the first soil bacteria that provided the rifamycins in 1957. For a while, it was considered the only bacterial source of rifamycins till their discovery in *Salinispora* group. Although there are several rifamycins isolated from bacteria, the most widely used derivative of rifampicin (rifampin) is a semisynthetic rifamycin. Rifamycins are preferable as they can cross mammalian tissue and cell membrane easily (Bhattacharjee 2016). As a result, rifamycin-SV and its derivatives are deemed first line in the treatment of intracellular pathogens and demonstrated inhibitory action in various biological systems. Among the antibacterial agents of these derivatives, some act by inhibiting the bacterial DNA-dependent RNA polymerase. Furthermore, rifampin inhibits poxvirus replication in vitro via a mechanism other than inhibiting DNA-dependent RNA polymerase. In vitro screening for selective inhibition of RNA-dependent DNA polymerase (reverse transcriptase) on a number of derivatives revealed that certain derivatives prevented focus formation by RNA tumour viruses (Szabo et al. 1976). Rifamycin derivatives were also found to act against type II DNA topoisomerases. Besides, phylogenetic studies showed that viral type II DNA topoisomerase and their bacterial counterparts have similarities indicating that the antibacterial topoisomerase inhibitors can act against African swine fever (ASFV) replication. In fact, fluoroquinolones, a class of synthetic antibacterial drugs, were shown to inhibit the ASFV replication by interacting with type II topoisomerase (Zakaryan and Revilla 2016). Rifampicin, rifapentine and rifabutin (Fig. 22.5) are semisynthetic and water-soluble derivatives of 3-formylrifamycin SV, used in therapies against different Gram-positive and Gram-negative bacterial strains including methicillin-resistant *Staphylococcus aureus* (MRSA), mycobacteria (*Mycobacterium bovis* or *Mycobacterium tuberculosis*) and leprosy, legionella. They are also able to prevent viral infections (e.g. influenza) (Czerwonka et al. 2016).

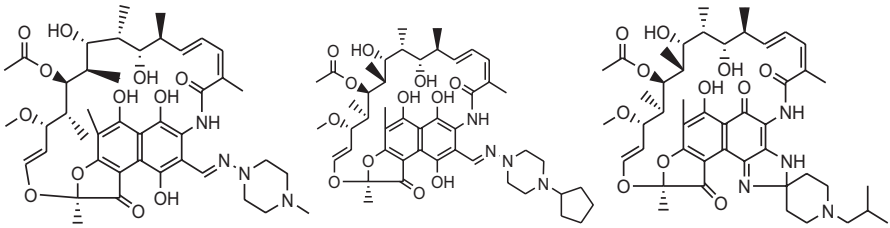


Fig. 22.5 Rifamycin-SV derivatives having antiviral activity

22.11 Other Antibiotics

The antiviral potential of antibacterial drugs has been studied on various drugs. Minocycline is among the well investigated for its actions against a number of ailments. It is a synthetic second-generation tetracycline derivative with immunomodulatory and anti-inflammatory action and widely used for the treatment of acne, rheumatoid arthritis and UTIs. Potential antiviral action of minocycline against human immunodeficiency virus, Japanese encephalitis virus and West Nile virus has been reported. It was also found promising in reducing *dengue virus* infection, with a prompt action against all the four serotypes of the virus. Minocycline generally diminished viral RNA synthesis, intracellular viral protein synthesis and thus infectious virus production. It was also found to decrease ERK1/2 phosphorylation, which is associated with intensifying pathogenesis and organ damage in *dengue virus* infection (Leela et al. 2016). Furthermore, the quinolones have showed an antiviral activity towards HIV and hepatitis C virus (HCV) in addition to their antibacterial and anticancer activity. Particularly the antimalarial drugs chloroquine and amodiaquine displayed activity against viruses like dengue virus, West Nile virus and Ebola virus by interfering with viral entry and replication (Savoia 2016). On the other hand, the compound teicoplanin isolated from an *Actinobacteria* member, *Actinoplanes teichomyceticus*, is a fermentation product that exerts bactericidal action through inhibiting bacterial cell wall biosynthesis. This semisynthetic glycopeptide teicoplanin showed a significant inhibitory activity against Ebola envelope pseudotyped viruses in Vero cells when used in the clinic. Besides, teicoplanin and other glycopeptide antibiotics, including dalbavancin, oritavancin and telavancin, but not vancomycin, had inhibitory action against the entry of Ebola virus, SARS-CoV and MERS-CoV transcription and replication-competent virus-like particles. With regard to teicoplanin's antiviral activity, various studies have reported about its action against HIV, hepatitis C virus, flaviviruses, coronaviruses, respiratory syncytial virus and influenza virus (Colson and Raoult 2016).

22.12 Several Natural Products

The world has benefited from the phenomenal discovery of penicillin by Alexander Fleming in 1928 and its development in the 1940s by Chain, Florey, Heatley and Abraham at Oxford. Similarly, the 1940s invention of important streptomycete products by Waksman, Woodruff, Schatz and Lechevalier at Rutgers University has resulted in the selective action of antibiotics against pathogenic bacteria and fungi. Ever since the invention of penicillin, microbes have played a very significant role in the discovery of newer natural product-based drugs. Currently, over 23,000 active compounds of microbial origin including antimicrobials, antivirals and cytotoxic and immunosuppressive compounds, of which 42% are made by fungi and 32% by filamentous bacteria, the actinomycetes, are available (Demain 2014). Cyclosporin 72, which is a fungus-derived potent immunosuppressant that acts through inhibition of cyclophilin, is found to have antiviral activity. Nevertheless, its immunosuppressive and calcineurin-related side effects have made it impossible for use as an antiviral agent. Therefore, continued search for structurally related cyclosporin analogues with minimal immunosuppressive activity and strong cyclophilin inhibitory action resulted in its derivative NIM 811 73. On the contrary, NIM 811 73 had 1700 times less immunosuppressive activities than cyclosporin 72 with a lesser toxicity profile and has demonstrated to possess anti-HIV and HCV activity. NIM 811 73 has passed evaluation in Phase I trial for the treatment of HCV (Butler 2008). Even though majority of natural products have been produced from terrestrial environments, marine organisms have also contributed quite a large number of bioactive compounds. Between 2000 and 2003, about 129 bioactive compounds have been isolated from marine microbes only. Various compounds with anticancer, antibacterial, antiviral, immunomodulatory and protease-inhibition activities have been isolated from marine cyanobacteria. Marketed marine products include cytarabine (Cytosar) for non-Hodgkin's lymphoma, the antiviral vidarabine (Vira-A), ziconotide (Prialt) and trabectedin (Yondelis) (Demain 2014).

22.13 Herbal Antiviral

The history of herbal drug use is widespread in both developed and developing countries, and they are still utilized because of several reasons such as fewer side effects, relatively less expensive, patient tolerance and acceptance due to long history of use (Vermani and Garg 2002). Veregen (polyphenon E ointment), which is a green tea leaf extract and a mixture of catechins, was the first herbal remedy to obtain FDA approval in 2006 for treating genital warts. Additionally, a perennial herb *Glycyrrhiza glabra* has been in use for over 20 years in Japan for treatment of hepatitis. Its dried and processed root *licorice* has a unique odour and sweet taste. Various studies have investigated the pharmacological activity of licorice against viral hepatitis. A randomized controlled trial conducted on *Glycyrrhiza glabra* derived compound glycyrrhizin and its derivatives demonstrated diminished hepatocellular damage in chronic hepatitis B and C (Fiore et al. 2008). The herb

Caesalpinia pulcherrima Swartz (Leguminosae) is a common medicinal plant in Taiwan. Its flower contains a number of metabolites like lupeol, lupeol acetate, myricetin, quercetin and rutin. Quercetin has been reported to have activity against bacteria, fungi and viruses [human immunodeficiency virus (HIV), poliovirus, herpes simplex virus (HSV)], indicating that it can be a potential antibiotic. Furthermore, rutin has also been stated to inhibit replication of parasites, bacteria, fungi and viruses (rotavirus and HSV) (Chiang et al. 2003). On the other hand, in China and Taiwan, *Ocimum basilicum* is widely used traditionally against a number of infections. A number of compounds have been reportedly found from *Ocimum basilicum* including monoterpenoids (carvone, cineole, fenchone, geraniol, linalool, myrcene and thujone), sesquiterpenoids (caryophyllene and farnesol), triterpenoid (ursolic acid) and flavonoid (apigenin). In particular, ursolic acid was shown to have inhibitory activity against herpes simplex virus (HSV)-1 and human immunodeficiency virus (HIV), as well as tumour growth (Chiang et al. 2005).

22.14 Repurposing of Drugs

Drug repurposing (or drug repositioning) is the method of assigning a new medical indication for an existing drug. The repositioned drug might be currently on the market for other medication, withdrawn due to adverse effects or proved to be less efficacious. As a matter of fact, most of the drug repositioning emerged as a result of beneficial side effects (by serendipity); however, current efforts to attain repurposing are accomplished in a more systematic way (Naveja et al. 2016). Nowadays, the problem of antimicrobial drug resistances poses a growing threat to global public health and demands newer or repositioned drugs. With regard to utilizing already FDA-approved drugs for another indication, the entities can be used for treating the new indication without any further structural modification of the compound at hand (though dosing and formulation could be modified) (Savoia 2016; Klug et al. 2016). The case of Ebola virus outbreak in West Africa that reached to a scale not ever seen in history was the greatest public health emergency. Antibody-based therapy was proved effective in a macaque model and had been used to treat few patients; however, the supply of the drug was quite limited. Therefore, drug repurposing was the best option to come up with an old drug with new indication to speed up the discovery and development of anti-Ebola virus drugs for the treatment of patients with Ebola virus infection. As a result, initial drug repurposing screen subsequently provided 53 approved drugs with Ebola virus-like particle entry-blocking activity including the macrolide antibiotics azithromycin and clarithromycin, which block bacterial protein synthesis (Kouznetsova et al. 2014). Finally, six antibiotics which inhibit Ebola virus infection (azithromycin, erythromycin, spiramycin, dirithromycin, maduramicin, clarithromycin) were selected for anti-Ebola activity out of 3828 FDA-approved drugs (Veljkovic et al. 2015). There was no herbal therapy for Zika virus infection; however recently, two antiviral agents have been approved by FDA for Zika virus infection (Cheng et al. 2016). It is important to design or develop a therapeutic approach to overcome Zika virus infection with a special focus on drugs

targeting the virus helicase protein, nucleosides, inhibitors of NS3 protein, small molecules, methyltransferase inhibitor and repurposed drugs. Repurposed drugs such as chloroquine, azithromycin and niclosamide are used for the treatment of Zika virus infection (Munjal et al. 2017). New studies revealed that Alzheimer's drugs may moderate Zika virus-mediated neuronal damage. So, Alzheimer's disease drugs which overstimulate N-methyl-d-aspartate receptors (NMDARs) lead to damage neuronal death interlinked with Zika virus infection. Therefore, blocking of NMDAR channels with memantine and/or other antagonists helps to lessen the neuronal damage associated with Zika virus infection, which act as a pre-approved drug from the Food and Drug Authority (FDA) which need more clinical trials (Sirohi and Kuhn 2017).

Modification of specific or non-specific immune responses is a promising intervention for ongoing viral infections. The most suitable for immunotherapy are chronic viral infections (Hegde et al. 2009). The first example is monoclonal antibodies, which are specific for one to one antigen or one epitope. In fibroblasts and neuroblastoma cells, monoclonal antibodies specific for nucleoprotein and non-structural protein of the nucleocapsid have been shown to inhibit rabies virus, in a dose-dependent way, by impairing transcription of the genome or neutralizing newly translated proteins (Lafon and Lafage 1987). In addition, another study found that monospecific antibody against rabies virus nucleoprotein recognizes lyssavirus-specific antigen (Inoue et al. 2003). Monospecific antibodies have shown to be effective against non-capsid proteins of poliovirus (Pasamontes et al. 1986) and various livestock diseases as rotaviral diarrhoea, bluetongue, classical swine fever, Hendra and Nipah viral infections (Deb et al. 2013). Neutralization by antibody can be mediated by different mechanisms such as destabilization of the virion structure, aggregation of virions, inhibition of virion attachment to target cells, inhibition of the virion lipid membrane fusion with the membrane of the host cell, inhibition of the entry of the genome of non-enveloped viruses into the cell cytoplasm and inhibition of a function of the virion core through a signal transduced by an antibody (Reading and Dimmock 2007). Another therapeutic strategy for infectious viral diseases are recombinant antibodies, which, unlike monoclonal antibodies, do not need hybridomas and animals in the production process, but only synthetic antibody coding genes, and are delivered in high reproducibility, specificity and scalability (Echko and Dozier 2010). Examples include avian antibody against VP2 of infectious bursal disease virus protecting against viral infection in chicken (Zhang et al. 2017), porcine circovirus type 2 (Yang et al. 2014), the E2 protein of classical swine fever virus (Chen et al. 2018) and capsid protein of bovine immunodeficiency virus (Bhatia et al. 2010).

Antiviral drugs have been tested for various viral diseases of animals. Antiviral therapy has been developed against a number of RNA viral infections in livestock. First among them, against foot-and-mouth disease (FMD), involves vaccine that contains an inactivated whole-virus antigen. However, since vaccinated animals cannot be differentiated from the infected ones, the vaccine is not useful in eliminating FMD outbreaks from previously disease-free countries. Hence, interferons (IFNs) have emerged as another treatment agent, including IFN-α, IFN-β and

IFN-γ, which are used both individually and synergistically. IFN-γ has been described to have several targets that possess antiviral properties, such as indoleamine 2,3-dioxygenase and inducible nitric oxide synthase (Moraes et al. 2007). One of the most widespread diseases in domestic livestock is caused by another RNA viral infection, bluetongue virus (BTV). An aminothiophenecarboxylic acid derivative named compound 003 (C003) and its derivative compound 052 (C052) have been identified as virostatic molecules against BTV. They exert their effect by inhibiting BTV-induced apoptosis via inhibition of caspase-3/caspase-7 activation and inhibition of host autophagy activation (Gu et al. 2012). Furthermore, feline herpes virus type 1 (FHV-1) is a common cause of various diseases in cats, such as ocular surface disease, respiratory disease, dermatitis and potentially intraocular disease. A number of antiviral agents have been described against the virus, but the most effective antiviral therapies are the ones that target viral proteins involved in DNA synthesis, many of which have been used against closely related human herpes simplex virus type 1. For example, nucleoside and nucleotide analogues have been reported for topical administration, such as vidarabine that affects DNA polymerase and subsequently disrupts DNA synthesis, trifluridine which acts as a fluorinated nucleoside analogue of thymidine and cidofovir which is a cytosine analogue acting on two host-mediated phosphorylation steps (Thomasy and Maggs 2016). Purine analogues and their oral prodrugs have also been described as well as other antiviral drugs, such as foscarnet that inhibits pyrophosphate binding site on viral DNA polymerases, while numerous novel compounds have been investigated against FHV-1 including siRNAs which target the FHV-1 glycoprotein D (gD) alone or jointly with DNA polymerase genes (Wilkes and Kania 2010). To conclude with, equine herpesvirus type 1 (EHV-1) infection causes outbreak of respiratory and various neurological diseases in horses, against which acyclovir and valacyclovir are the most common drugs, but also IFN targeting IFNGR complex as a key mediator of virus-specific cellular immunity (Poelaert et al. 2018).

Feline immunodeficiency virus (FIV) is a complex lentivirus causing immunodeficiency disease in cats, manifested as the body's inability to develop normal immune response. As a retrovirus, it inserts copies of its genetic material into the DNA of a host cell, where it can replicate. The most commonly used antiretroviral drugs are reverse transcriptase inhibitors (RTIs), in particular the ones acting as nucleoside analogues, which are similar in structure to intrinsic nucleosides and can therefore block enzymatic activity by binding to the active centre of the enzyme (Hartmann et al. 2015). The first among them, zidovudine (AZT), has been reported to improve the immunologic and clinical status of FIV-infected cats, increase quality of life as well as prolong life expectancy. It has been shown to increase the CD4/CD8 ratio in naturally FIV-infected cats and acts by inhibiting RT but also cellular polymerases, which can lead to bone marrow suppression (Hartmann 1998). Another drug acting as RTI is stavudine that has been shown to be active against FIV in vitro, however with many resistant strains arisen. Similarly, didanosine and lamivudine have shown potency against FIV in in vitro conditions (Schwartz et al. 2014). Additionally, a combination of zidovudine and lamivudine has been investigated, resulting in synergistic anti-FIV effects in cell cultures. Also, a high-dose

zidovudine/lamivudine combination was shown to protect from infection when treatment was initiated before virus inoculation (Arai et al. 2002). Moreover, all of the above-mentioned antiviral agents are also effective against HIV infection.

Interferons (IFNs) are a multigene family of inducible cytokines that possess antiviral activity. The IFN system comprises the cells synthesizing IFN in response to an external stimulus, such as viral infection, and cells that respond to IFN by establishing the antiviral state. They represent an early host defence, the one that occurs prior to the immune response onset. IFNs are classified as IFN-α and IFN-β, which are produced by the cell in response to virus infection, and IFN-γ, synthetized upon antigen or mitogen stimulation (Samuel 2001). Their activity has been reported against a number of feline viruses, including feline calicivirus (FCV), where they act by stimulating downstream genes such as 2′-5′oligoadenylate-dependent ribonuclease L (RNase L), which degrades single-stranded viral RNAs. Also, feline IRF-1, shown to be reduced upon FCV infection, has been reported to positively regulate IFN signalling by triggering the production of endogenous IFN and the expression of downstream targeted genes (Liu et al. 2018). As mentioned before, another feline virus reported for IFN therapeutic solutions is FIV. The best known among them, recombinant RFeIFN-ω, is the first interferon compound that has been licensed for use in veterinary medicine, shown to significantly increase levels of acute phase proteins (APPs) (Doménech et al. 2011). RFeIFN-ω has also been reported for anti-inflammatory properties, exerted via interleukin-6 (IL-6) (Leal et al. 2015). Recombinant human interferon alpha-2b (rHuIFN-alpha2b) and recombinant feline interferon omega (RFeIFN-omega) have also exhibited an antiviral effect against feline herpesvirus (FHV)-1 in in vitro settings, as evidenced by significant reduction in plaque size (Siebeck et al. 2006). Interferons also showed therapeutic potential against feline leukaemia virus (FeLV), with recombinant feline interferon RFeIFN-omega, resulting in improvement of clinical signs and survival of infected cats (de Mari et al. 2004).

Idoxuridine and trifluridine are structurally similar thymidine analogues that inhibit synthesis of DNA. They have been applied in the treatment of feline herpesvirus-1 (FHV-1), significantly reducing the number of viral plaques in vitro (Nasisse et al. 1989). Idoxuridine has also exhibited therapeutic potential against equine herpesvirus type 2 (EHV-2) by alleviating the ocular symptoms caused by the infection (Collinson et al. 1994). It has also been reported that idoxuridine in the concentration of 0.1% and 0.3% trifluridine can limit the viral replication but do not kill the virus (Plummer et al. 2014).

Antiviral drugs and vaccines are the most powerful tools to combat viral diseases. However, they mostly selectively target only a single virus, known as a “one drug–one bug” principle. On the contrary, broad-spectrum antivirals (BSAs) cover multiple viruses and genotypes, therefore reducing the likelihood of resistance development. They can, hence, reduce the complexity of the treatment, ensuring management of new or drug-resistant viral strains, first-line treatment or prophylaxis of acute infections, as well as co-infections (Zhu et al. 2015). Against some viruses, such as hepatitis C, a direct-acting antiviral agents (DAAs) have been developed in the past few years. They act on NS3/4A protease inhibitors, NS5A

inhibitors or NS5B inhibitors and ensure efficient, tolerable, safe and interferon-free oral therapies (Das and Pandya 2018). Furthermore, further development of antiviral agents will not only focus on viral factors as the potential targets for inhibition but also on the host factors as well, such as cellular receptors, adhesion molecules, cyclophilins and microRNAs. Therefore, an effort will be put on the combination of viral and host inhibitors, eventually leading to interferon-free therapies for consistent clearing of infection (Bryan-Marrugo et al. 2015).

Perspectives for use of antiviral drugs in livestock animals are envisaged as the mass treatment for the control of the disease (on a large scale), whereas treatment in companion animals favours an individual approach. The main prerequisite for successful veterinary antiviral chemotherapy is a better understanding of the viral infection pathogenesis as well as development of sophisticated means for drug delivery. These will mainly focus on targeted approaches that aim specific molecular targets with a narrow niche, allowing for better specificity and less side effects of antiviral agents. Advances in the field of molecular biology, in particular computational approaches, would contribute to development of a new generation of antiviral therapy, which would be of importance in the control of various kinds of animal diseases.

22.15 Conclusions

The use of animal models for viruses of human and veterinary importance is still abundantly used to develop therapeutic agents. However, the current interest of these various viruses leads to multiple drug resistance due to the use of higher-dosage therapies. The approaches are different for companion animals as a single method is preferred, while for large-scale livestock, mass treatment therapy is used; that is why antiviral drugs and other natural as well as herbal products are characterized through a novel and optimistic approach. Still it is worthy to notice that these therapies lead to multiple drug resistance which should be overcome in the future.

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Bioinformatics Applications in Advancing Animal Virus Research

23

Ablesh Gautam, Ashish Tiwari, and Yashpal Singh Malik

Abstract

Viruses serve as infectious agents for all living entities. There have been various research groups that focus on understanding the viruses in terms of their host-viral relationships, pathogenesis and immune evasion. However, with the current advances in the field of science, now the research field has widened up at the 'omics' level. Apparently, generation of viral sequence data has been increasing. There are numerous bioinformatics tools available that not only aid in analysing such sequence data but also aid in deducing useful information that can be exploited in developing preventive and therapeutic measures. This chapter elaborates on bioinformatics tools that are specifically designed for animal viruses as well as other generic tools that can be exploited to study animal viruses. The chapter further provides information on the tools that can be used to study viral epidemiology, phylogenetic analysis, structural modelling of proteins, epitope recognition and open reading frame (ORF) recognition and tools that enable to analyse host-viral interactions, gene prediction in the viral genome, etc. Various databases that organize information on animal and human viruses have also been described. The chapter will converse on overview of the current advances, online and downloadable tools and databases in the field of bioinformatics that will enable the researchers to study animal viruses at gene level.

A. Gautam (✉)
Central Research Institute, Kasauli, Himachal Pradesh, India

A. Tiwari
University of Kentucky, Louisville, KY, USA

Y. S. Malik
ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Uttar Pradesh, India

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Preamble
Viruses are notorious to infect all forms of life ranging from bacteria to chordates. In humans, viruses are known to cause infectious diseases such as influenza, hepatitis, AIDS, diarrhoea, encephalitis, dengue fever and, more recently, severe acute respiratory syndrome (SARS), Ebola (Singh et al. 2017a), Zika (Singh et al. 2017b), etc. Despite the vaccines and treatments for such diseases, morbidity and mortality both occur as a result of the viral infections. Viral disease of animals not only affects the production but also is a threat to humans (Saminathan et al. 2016). A rapid growth in the availability of sequencing methods and a vast amount of viral sequence data have been generated during recent times. Thus, it is imperative to decipher this data using more advanced tools such as bioinformatics resources. A large number of bioinformatics tools that can aid in the analysis of viral genomes and develop preventive and therapeutic strategies have been developed for human as well as animal viruses. This chapter will introduce virologists to some of the common as well virus-specific bioinformatics tools that the researches can use to analyse viral sequence data to elucidate the viral dynamics, evolution and preventive therapeutics.

23.1 Applications of Bioinformatics in Virology

Analysis of viral sequence involves use of certain tools that are employable on any novel sequence, for example, gene identification, ORF identification, functional annotation and phylogeny. However, due to small genome size, viruses have complex methods to maximize the coding potential of genomes and evolution. Many viruses utilize overlapping reading frames or translational frameshifts to code for multiple proteins from limited genome sequences. Also, higher rates of mutations and recombination between related viruses pose a challenge in accurate phylogenetic and evolutionary analysis of viruses using general-purpose softwares. Lately, enormous growth in the volume and diversity of viral sequences in the databases has been seen. Now, it has become imperative to organize data of these viral sequences in virus family-specific resources tailored for accurate analysis of a specific virus.

23.1.1 Phylogeny and Molecular Epidemiology

One of the most common applications of bioinformatics in virology was to use phylogenetic analysis of the viral isolates to aid in the epidemiological analysis of viral outbreaks. General-purpose phylogeny programs such as PHYLIP (Felsenstein 1989) have been used extensively for the phylogeny and molecular epidemiology of viruses. A comprehensive list of these packages and web servers is maintained by Joe Felenstein at http://evolution.genetics.washington.edu/phylip/software.html.

23.1.2 ORF/Gene Discovery

An open reading frame (ORF) is the part of genome that translates into a protein. Finding ORF is one of the key steps in viral genome analysis. It forms the basis for further analysis such as homologous search, predicting proteins, functional analysis and viral vaccine and antiviral target discovery. If an ORF translates a surface protein that is unique to that virus, it may elicit immune responses and could potentially be a vaccine candidate. ORF Finder by NCBI is a ORF prediction program (Rombel et al. 2002). The program outputs a range of each ORFs along with its protein translation in six possible reading frames from the input DNA sequence. It can be used to search newly sequenced DNA for potential protein encoding sequences and to verify predicted proteins using SMART BLAST or BLASTP (Altschul et al. 1990). However, the web version of the program is limited to a query sequence length of 50 kb only. A standalone system has no limitation on length but is available only for the Linux 64 operating system. NEG8, a 167-codon novel ORF in segment 8 of influenza virus, was visualized using ORF Finder (Clifford et al. 2009). Using the ORF Finder in association with the basic local alignment search tool BLAST, 154 ORFs were found in the Hz-1 virus genome (Cheng et al. 2002). Due to small genome size, viruses employ multiple strategies to maximize the coding potential including frameshifts and alternative codon usage. Thus, virus-specific programs have been developed to overcome these challenges. GeneMark (http://opal.biology.gatech.edu/GeneMark/genemarks.cgi) provides gene prediction tools for viruses (Besemer and Borodovsky 2005). Viral genome organizer (VGO) – a Java-based web tool – offers identification of gene and ORF identification in viral sequences (Upton et al. 2000).

23.1.3 Epitope Recognition

Identification of immune epitopes is important in designing new vaccine candidates and in diagnostics. An epitope is the part of an antigen that is recognized by the receptors of immune system components such as antibodies, B cells or T cells.

Epitopes have been generally classified as either linear or conformational epitopes. T cells recognize linear epitopes, short continuous strings of amino acids derived from protein antigen, presented with MHC class I molecules. B cells and antibodies, on the other hand, recognize conformational epitopes which are formed by interactions of amino acids with multiple discontinuous segments forming a three-dimensional antigen (Barlow et al. 1986). Owing to the simple linear structure of T cell epitopes, their interaction with receptors can be modelled with high accuracy (DeLisi and Berzofsky 1985). A large number of prediction databases and servers thus are available for linear epitope prediction. MHCPEP (Brusic et al. 1998), SYFPEITHI (Rammensee et al. 1999), FIMM (Schonbach et al. 2005), MHCBN (Bhasin et al. 2003) and EPIMHC (Reche et al. 2005) are some of the commonly used T cell epitope prediction programs. Immune epitope database and analysis resource (https://www.iedb.org) (Vita et al. 2015) offers the most comprehensive set of tools for epitope analysis for epitope prediction covering HLA-A and HLA-B for humans as well as chimpanzee, macaque, gorilla, cow, pig and mouse and is one of the few databases that cover such a variety of organisms. Since 2011, IEDB uses NetMHCpan as prediction method. NetMHC server uses the artificial neural network method to predict binding of peptides to different alleles from human as well as 41 animals including cattle and pig (38 from core). The database also contains curated data for many viruses including influenza and herpesviruses. B cell receptors and epitope interactions are more complex in nature than the linear epitopes for T cells; thus, accuracy of B cell epitopes is relatively low. Furthermore, most of the current databases are centred on linear rather than conformational epitopes. Bcipep is a tool developed for predicting the linear epitope of B cells (Saha et al. 2005). Epitome is a database of structure-inferred antigenic residues in proteins (Schlessinger et al. 2006). Epitome is especially useful in the prediction of antibody-antigen complex interaction. The database is available at http://www.rostlab.org/services/epitome/. AntiJen is an intricate database with entries on both T cell and B cell epitopes. It emphasizes on integration of kinetic, thermodynamic, functional and cellular data within the context of immunology and vaccinology (Toseland et al. 2005) (Fig. 23.1a).

23.1.4 Structural Modelling

Three-dimensional prediction of viral proteins can be used to predict the correlation between actual protein structure and antigenic sites, folding surfaces and functional motifs. Such structural modelling tools may be implicated to identify and design novel candidates for antiviral inhibitors and vaccine targets. Secondary structures may be predicted using the tool PredictProtein (http://www.predictprotein.org/) (Rost et al. 2004). Using this online tool, along with secondary structures, solvent accessibility and possible transmembrane helices can be predicted. Further, it also provides expected accuracy of prediction methods. SWISS-MODEL (http://

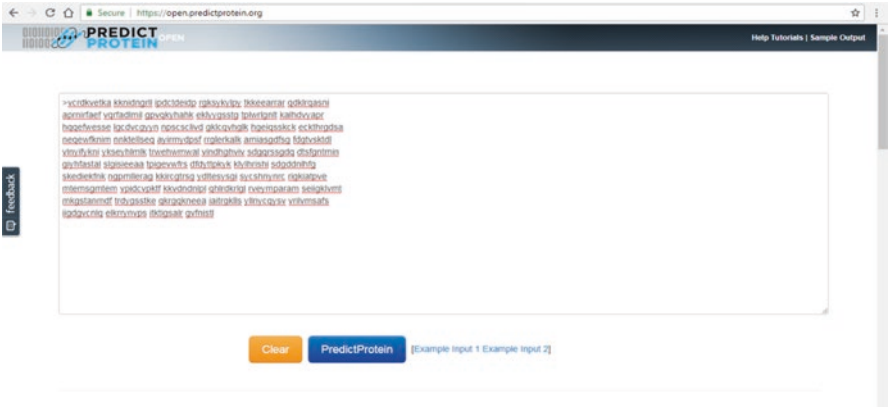

Fig. 23.1a The online tool PredictProtein predicts various secondary structures in a given viral protein. The amino acid sequences of viral protein are required to be fed in Fasta format

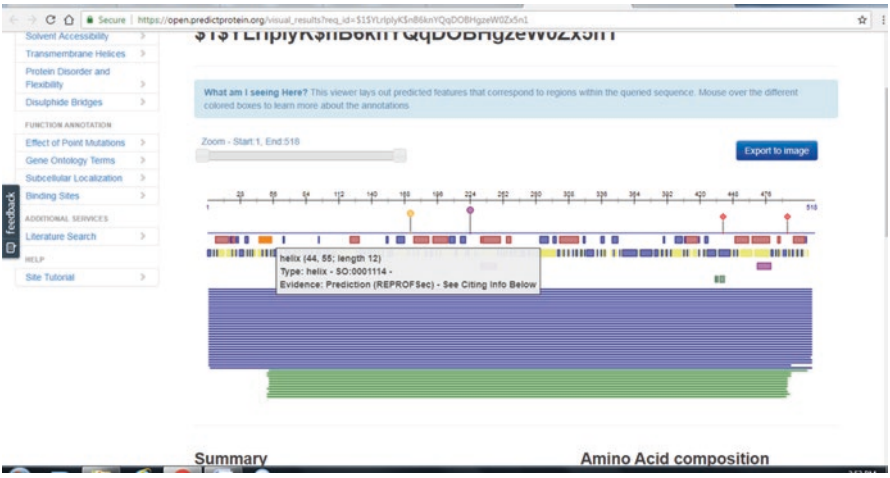

Fig. 23.1b Prediction of various secondary structures (Helical) in a given viral protein using the online tool Predictprotein

swissmodel.expasy.org/) is a popular tool for the prediction of a 3-D structure of a protein. 3-D structure prediction programs usually employ homology searching using similar and known protein structures as templates. One of the most commonly used database for such templates is Protein Data Bank (PDB) (Reddy et al. 2001). Output from the SWISS-MODEL program includes the template selected, alignment between the query sequence and the template, and the predicted 3-D model. Results of SWISS-MODEL are, however, only sent by email (Figs. 23.1b, 23.1c, 23.1d and 23.1e).

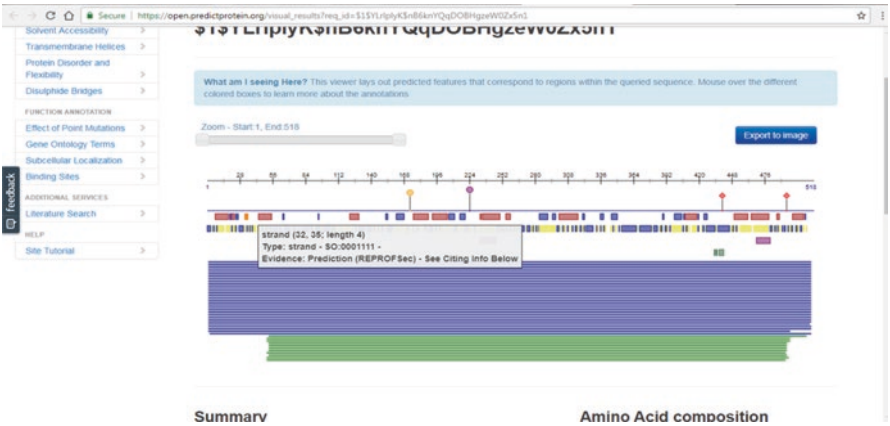

Fig. 23.1c Prediction of various secondary structures (Strand) in a given viral protein using the online tool Predictprotein

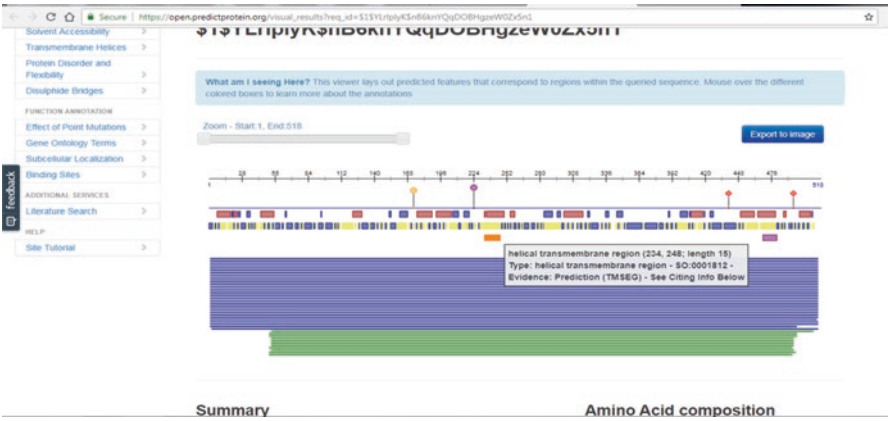

Fig. 23.1d Prediction of various secondary structures (Helical transmembrane region) in a given viral protein using the online tool Predictprotein

23.2 Virus-Centred Bioinformatics Tools

For long, bioinformatic analysis of viruses utilized common bioinformatics tools developed for other organisms. However, analysing viral genomes using general bioinformatics tools could compromise the accuracy and sensitivity of analysis. Virus genomes are too small (e.g. < 10 kb) to compute statistics with their codon usage. To maximize the coding potential, viruses work with unusual codon usage patterns comprising of overlapping coding and non-coding functional elements. Additionally, viruses also rely on other translational mechanisms such as stop codon read-through, frameshifting, leaky scanning and internal ribosome entry sites.

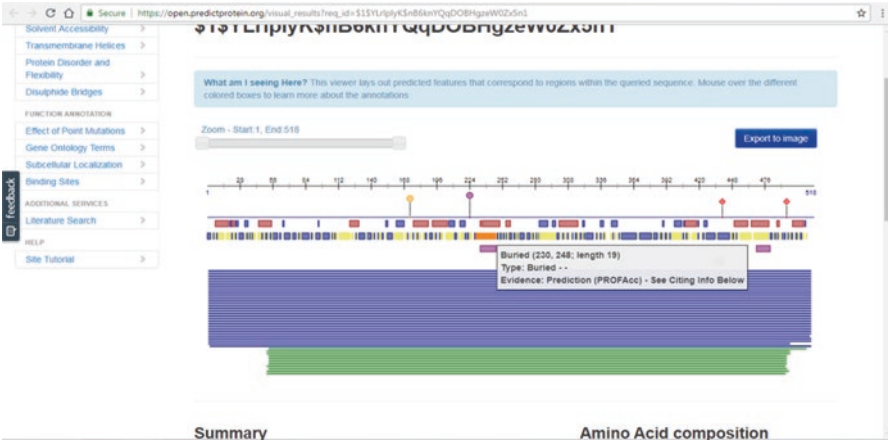

Fig. 23.1e Prediction of various secondary structures (buried sequence motifs) in a given viral protein using the online tool Predictprotein

Comparative genomic analysis of viruses is complicated by the fact that highly conservative sequences may not be coding for anything. Presence of overlapping pairs may be indicated by conservation for the sequences where there is overlapping of CDSs and/or non-coding functional elements. Novel virus types comprise of new CDSs that are different than previously known CDSs. There are multiple databases and tools available for analysis of human viruses; however, there are still only a limited number of resources designed specifically for veterinary viruses. In this section, some of the databases and resources useful for the analysis of veterinary viruses are discussed (Table 23.1).

23.2.1 Comparative and Diversity Analysis of Viral Sequences

Viruses are one of the most diversified and dynamic microorganisms. With increasing viral genome sequencing, there was a need to develop bioinformatics tools to compare and analyse the voluminous data. To meet this requirement, one such downloadable software package is Base-By-Base, which aids in analysis of whole viral genome alignments at single nucleotide level (Brodie et al. 2004). Moreover, with the online resource Genome Information Broker for Viruses (GIB-V), comparative studies can be made using the generic tools such as ClustalW, BLAST and Keyword Search algorithms (Hirahata et al. 2007). Another downloadable web server tool, ViroBLAST, is an exclusive BLAST tool that can be used for queries against multiple databases (Deng et al. 2007). Sequences from a variety of viral strains can be analysed simultaneously using the Alvira software, which is a multiple sequence alignment tool that provides graphical representation as well (Enault et al. 2007). Furthermore, comparative analysis of genes and genomes of coronavirus can be carried out by using the CoVDB (coronavirus database) (Huang et al. 2008).

Table 23.1 Virus-specific bioinformatics tools

S. no.	Applications	Name of the tools
1.	ORF gene finding	ORF Finder by NCBI SMART BLAST GeneMark VGO VIDA VIGOR
2.	Epitope recognition	MHCPEP SYFPEITHI FIMM MHCBN NetMHC Epitome
3.	Comparative and diversity analysis of viral sequences	GIB-V ViroBLAST ViralZone ViralORFeome PriSM PHACCS VIROME VMGAP Metavir Wommack
4.	Viral recombination and integration-specific resources/ tools	jpHMM ViReMa VIPR HMM SeLOX VIRAPOPS SeqMap VirusSeq ViralFusionSeq VirusFinder
5.	Small-RNA analysis tools	ViTa VIRsiRNAdb siVirus Paparazzi Visitor VIROME
6.	Virus-host interaction tools	PhEVER Virus-PLoc iLoc-virus pLoC-mVirus
7.	Genome annotation tools	NCBI genotyping tool VIGOR VGO GATU ZCURVE_V STAR

(continued)

Table 23.1 (continued)

S. no.	Applications	Name of the tools
8.	Primer design tools	PrimerHunter PhyloType RotaC VirOligo
9.	Virus structural modelling tools	PredictProtein SWISS-MODEL LearnCoil-VMF VIPERdb Jmol STRAP
10	Phylogeny and molecular epidemiology	PHYLIP

The digital resource ViralZone is designed specifically to comprehend viral diversity and acquire information on viral molecular biology, hosts, taxonomy, epidemiology and structures (Hulo et al. 2011). The Simmonics program was upgraded to the simple sequence editor (SSE) software package, wherein the user-given sequences can be aligned and annotated and further can be analysed for diversity and phylogeny (Simmonds 2012). Evolutionary changes in viral genome lead to polymorphisms in their proteins, which in turn result into changes in viral phenotype such as viral virulence, viral-host interactions, etc. The digital database, ViralORFeome, not only stores all variants and mutants of viral ORFS, but also provides tools to design ORF-specific cloning primers (Pellet et al. 2010). Further, degenerate primer pairs can be selected and matched to amplify user-defined viral genomes using the online tool PriSM (Yu et al. 2011). The recent advances in next-generation sequencing and technologies have facilitated to study viral population at an advanced level. The viral population biodiversity and dynamics can be studied using the first such tool developed, PHACCS (Phage Communities from Contig Spectrum), that can analyse the shotgun sequence data to estimate the structure and diversity of phages (Angly et al. 2005). Later on, more tools/resources were developed to analyse viral metagenomics sequences, such as Viral Informatics Resource for Metagenomic Exploration (VIROME), Viral MetaGenome Annotation Pipeline (VMGAP) and Metavir (Lorenzi et al. 2011, Roux et al. 2011, Wommack et al. 2012). Novel viruses can be identified from a pool of specimen types using a specific computational pipeline, VirusHunter (Zhao et al. 2013).

23.2.2 Viral Recombination and Integration-Specific Resources

The phenomenon of genetic recombination in viruses is responsible for the emergence of new viruses, increased virulence and host range, immune evasion and development of antiviral resistance. This distinct process of viral recombination can be detected by two bioinformatics tools, viz. jpHMM (Jumping Profile Hidden

Markov Model) and ViReMa (Virus Recombination Mapper) genomes (Schultz et al. 2009; Routh and Johnson 2014). The jpHMM, a web server, can be used for predicting recombination in HIV-1 and HBV, whereas ViReMa, a downloadable software, can be used to analyse next-generation sequencing data. Additionally, another software called VIPR HMM (Viral Identification with a PRobabilistic algorithm incorporating hidden Markov model) can detect recombinant and non-recombinant viruses using microbial detection microarrays (Allred et al. 2012). Further, viral genome sequences can be searched for degenerate locus of recombination (lox)-like sites by a web server called SeLOX (Surendranath et al. 2010). A downloadable software, VIRAPOPS, is a forward simulator that allows simulation of RNA virus population (Petitjean and Vanet 2014). With this software, the drastic changes in rapidly evolving RNA viruses such as mutability, recombination, variation, covariation, etc. can be simulated to predict their effects on viral populations. SeqMap is a tool capable of identifying viral integration sites (VIS) from ligation-mediated PCR (LM-PCR), linear amplification-mediated PCR (LAM-PCR) and nonrestrictive LAM-PCR (nrLAM-PCR) reactions and mapping short sequences to the genome (Hawkins et al. 2011). Further, VIS can also be detected by three more distinct tools, VirusSeq, ViralFusionSeq, and VirusFinder (Chen et al. 2013, Li et al. 2013, Wang et al. 2013). For more precise VIS prediction, all four tools can be employed by virologists.

23.2.3 Small-RNA Analysis Tools

miRNAs: A microRNA (miRNA) is a small, regulatory, non-coding RNA molecule that regulates the translation or stability of viral and host target mRNAs, thereby affecting viral pathogenesis. This host-viral regulatory relationship can be investigated by a database called ViTa, capable of curating known viral miRNA genes and known/putative target sites of host miRNA (Hsu et al. 2007). ViTa exploits miRanda and TargetScan to scan viral genomes and determine miRNA targets. ViTa is also capable of annotating the viruses, virus-infected tissues and tissue specificity of host miRNAs. Subtypes of viruses, for example, influenza viruses, and the conserved regions in various viruses can also be compared using the ViTa database. Viral miRNA candidate hairpins can be predicted using the database Vir-Mir. It serves as a platform to query the predicted viral miRNA hairpins (based on taxonomic classification) and host target genes (based on the use of the RNAhybrid program) in human, mouse, rat, zebrafish, rice and *Arabidopsis* (Li et al. 2008).

siRNA: A siRNA is similar to miRNA that operates within the RNA interference (RNAi) pathway. It interferes in expression of specific genes and, therefore, is used in post-transcriptional gene silencing. VIRsiRNAdb is an online curated repository that stores experimentally validated research data of siRNA and short hairpin RNA (shRNA) targeting diverse genes of 42 important human viruses, including influenza virus (Tyagi et al. 2011, Thakur et al. 2012). The current database includes experimental information on siRNA sequence, virus subtype, target gene, GenBank accession, design algorithm, cell type, test object, method, efficacy, etc. A

web-based software, siVirus, is an antiviral sRNA design software that allows analysis of influenza virus, HIV-1, HCV and SARS coronavirus (Naito et al. 2006). Further, viral siRNA sequence data sets can be analysed using the softwares Visitor and VIROME (Antoniewski 2011; Watson et al. 2013). A Perl script, called Paparazzi, enables reconstitution of viral genome using a viral siRNA in a given sample (Vodovar et al. 2011).

23.2.4 Virus-Host Interaction and Miscellaneous Softwares

Host-pathogenic interactions play an important role in determining the pathogenicity of a pathogen or immune evasion mechanism of a host. To comprehend such interactions between viral and host cellular proteins, various databases and softwares are available. One such database is PhEVER that enables to explore virus-virus and virus-host lateral gene transfers by providing evolutionary and phylogenetic information (Palmeira et al. 2011). This distinct database catalogues homologous families between different viral sequences and between viral and host sequences. It compiles the extensive data from completely sequenced genomes (2426 non-redundant viral genomes, 1007 non-redundant prokaryotic genomes, 43 eukaryotic genomes ranging from plants to vertebrates). Thus, it enables compiling of various proteins into homologous families by selecting at least one viral sequence, related alignments and phylogenies for each of these families.

With increasing availability of viral genome sequences, data mining, curation and genome annotation have become essential components to better comprehend the structure and function of genome components. This information can further be exploited to develop diagnostics, vaccines and therapeutics.

There are a number of tools available capable of annotation and classification of viral sequences, such as NCBI genotyping tool (Rozanov et al. 2004), VIGOR (Viral Genome ORF Reader) (Wang et al. 2010), Viral Genome Organizer (VGO) (Upton et al. 2000), Genome Annotation Transfer Utility (GATU) (Tcherepanov et al. 2006), Virus Genotyping Tools (Alcantara et al. 2009), ZCURVE_V (Guo and Zhang 2006) and STAR (Subtype Analyser) (Myers et al. 2005).

VGO is a web-based genome browser that allows viewing and predicting genes and ORFs in one or more viral genomes. It also allows performing searches within viral genomes and acquiring information about a genome such as locating genes, ORFs, start/stop codons, etc. Within genome, the sequences can be searched for regular expression, fuzzy motif pattern, genes with highest AT composition, etc. Using VGO, comparative analyses can be made between different viral genomes. VGO uses the graphical user interface (GUI) for constructing alignments and display orthologues in a set of genomes. It also allows searching the translated genome for matches to mass spec peptides.

VIGOR is a gene prediction online tool that was developed by J. Craig Venter Institute in 2010. It started with gene prediction in small viral genomes such as coronavirus, influenza, rhinovirus and rotavirus. With the updated version in 2012 (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3394299/), VIGOR is now

capable of gene prediction in 12 more viruses: measles virus, mumps virus, rubella virus, respiratory syncytial virus, alphavirus and Venezuelan equine encephalitis virus, norovirus, metapneumovirus, yellow fever virus, Japanese encephalitis virus, parainfluenza virus and Sendai virus. With VIGOR, based on sequence similarity searches, users are able to predict protein coding regions, start and stop codons and other complex gene features such as RNA editing, stop codon leakage and ribosomal shunting. Further, various features such as frameshifts, overlapping genes, embedded genes, etc. can be predicted in the virus genome. Additionally, a mature peptide can be predicted in a given polypeptide open reading frame. VIGOR is also capable of genotyping influenza virus and rotavirus. Four output files – a gene prediction file, a complementary DNA file, an alignment file, and a gene feature table file – are produced by VIGOR. GenBank submission can be directly done using the gene feature table.

Genome Annotation Transfer Utility (GATU) facilitates quick and efficient annotation of similar target genome using the reference genomes that have already been annotated. Later, the users can manually curate the annotated genome. The newly annotated genomes can be saved as GenBank, EMBL or XML file format. Although it doesn't provide a complete annotation system, GATU serves as a very useful tool for the preliminary work in genome annotation. GATU utilizes tBLASTn and BLASTn algorithms to map genes onto the new target genome by using an annotated reference genome. As a result, majority of the new genome's genes are annotated in a single step. With GATU, users can also identify open reading frames present in the target genome and absent from the reference genome. These ORFs can further be scrutinized by using other bioinformatics tools such as BLAST and VGO, which can determine if the ORFs should be included in the annotation. Multiple-exon genes and mature peptides can also be analysed using GATU.

A primer design tool, PrimerHunter, allows to design highly sensitive and specific primers for virus subtyping by PCR (Duitama et al. 2009). PrimerHunter allows predicting specific forward and reverse primers with respect to a given set of DNA sequences. PhyloType is a web-based as well as downloadable software that uses parsimony to reconstruct ancestral traits and to select phylotypes (Chevenet et al. 2013). RotaC is an automated genotyping tool for group A rotaviruses (Maes et al. 2009). It works by comparing a complete ORF of interest to other complete ORFs of cognate genes available in the GenBank database by performing BLAST searches.

VirOligo is a database of virus-specific oligonucleotides. The VirOligo database acts as a repository for virus-specific oligonucleotides for virus detection (Onodera and Melcher 2002). The database comprises of Oligo data and Common data tables. The Oligo data table enlists PCR primers and hybridization probes that are used for viral nucleic acid detection, while Common data table contains PCR and hybridization experimental conditions used in their detection. Each Oligo data entry provides information on the name of the oligonucleotide, oligonucleotide sequence, target region, type of usage (PCR primer, PCR probe, hybridization or other), note and direction of the PCR oligonucleotide (forward or reverse). Each oligonucleotide entry also contains direct links to PubMed, GenBank, NCBI Taxonomy databases and

BLAST. On the updated version of VirOligo as of September 2015, the database contains complete listing of oligonucleotides specific to various animal viruses. The viruses are vaccinia virus; canine parvovirus; porcine parvovirus; rodent parvovirus; tobamovirus; potyvirus; borna virus; bovine herpesvirus types 1, 3, 4 and 5; bovine viral diarrhoea virus; bovine parainfluenza 3 virus; bovine respiratory syncytial virus; bovine adenovirus; bovine rhinovirus; bovine coronavirus; bovine reovirus; bovine enterovirus; foot-and-mouth disease (FMD) virus; and alcelaphine herpesvirus.

Virus-PLoc is a web server for prediction of subcellular localization of viral proteins within host and virus-infected cells (Shen and Chou 2007). Another web server developed a little later, iLoc-Virus, is a multi-label learning classifier that predicts the subcellular locations of viral proteins with single and multiple sites (Xiao et al. 2011). Similarly, a most recent web server, pLoC-mVirus (Cheng et al. 2017), is a new predictor that identifies subcellular localization of viral proteins with both single and multiple location sites. It works by extracting information from the Gene Ontology (GO) database and is claimed to be more successful than the state-of-the-art method, iLoc-Virus, in predicting subcellular localization of viral proteins. AVPpred is an antiviral peptide prediction algorithm that contains the peptides with experimentally proven antiviral activity (Thakur et al. 2012). The prediction is based on peptide sequence features, peptide motifs, sequence alignment, amino acid composition and physicochemical properties. VIPS is a viral internal ribosomal entry site (IRES) prediction system that can predict IRES secondary structures (Hong et al. 2013). VIPS uses the RNA fold program that predicts local RNA secondary structures, RNA align program that compares predicted structures and pknotsRG program (Reeder et al. 2007) that calculates the pseudoknot structures. VaZyMolO, a database that deals with viral sequences at protein level, defines and classifies viral protein modularity (Ferron et al. 2005). It extracts information of complete genome sequences of various viruses from GenBank and RefSeq and organizes the acquired information about modularity on viral ORFs (Fig. 23.1f).

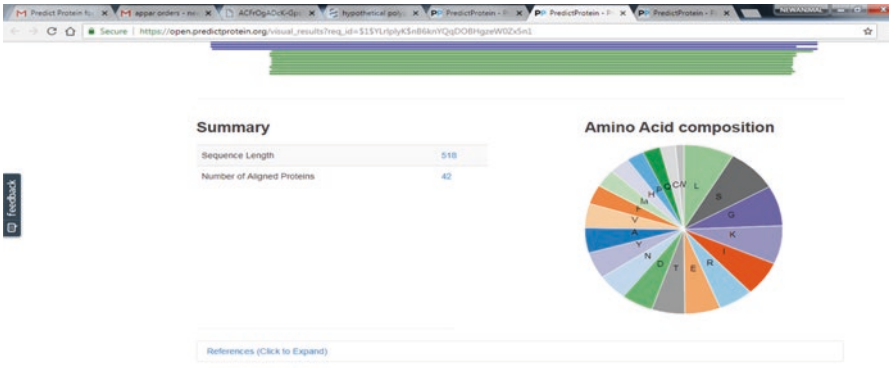

Fig. 23.1f Representation of amino acid composition in a given viral protein using the online tool Predictprotein

There are web-based tools available to predict and analyse structural aspects of viruses. The LearnCoil-VMF is a computational tool that allows to predict coiled-coil-like regions in viral membrane fusion proteins (Singh et al. 1999). The membrane fusion proteins are known to be diverse and share no sequence similarity between most pairs of viruses in the same or different families. The LearnCoil-VMF is also capable of characterizing the core structure of these membrane fusion proteins.

VIPERdb (Virus Particle Explorer database) is a web-based database that enables manual curation of icosahedral virus capsid structures (Carrillo-Tripp et al. 2009). This database serves as a comprehensive resource for specific needs of structural virology and comparatives of data derived from structural and computational analyses of capsids. With the updated version, VIPERdb (2), capsid protein residues in the icosahedral asymmetric unit (IAU) can be deduced using Phi-Psi (Phi-Psi) diagrams (azimuthal polar orthographic projections) (Ref: https://www.ncbi.nlm.nih.gov/pubmed/18981051). These diagrams can be depicted as dynamic interface and surface residues and interface and core residues and can be mapped to the database using a new application programming interface (API). This aids in identifying family-wide conserved residues at the interfaces. Additionally, Jmol and STRAP are built in the system to visualize an interactive model of viral molecular structures.

VIDA is a database that organizes animal virus genome open reading frames from partial and complete genomic sequences (Alba et al. 2001). Presently, VIDA includes a complete collection of homologous protein families from GenBank for *Herpesviridae*, *Papillomaviridae*, *Poxviridae*, *Coronaviridae* and *Arteriviridae*. The homologous proteins in VIDA include both orthologous and paralogous sequences. VIDA retrieves virus sequences from GenBank and the files are parsed into subfields. The parsed fields contain all the information such as GenBank accession number, GenBank identifier (GI numbers), protein sequence source, sequence length, gene name and gene product. In order to eliminate 100% redundancy, the virus protein sequences thus retrieved are filtered and a list of synonymous GIs is created for reference. The ORFs from complete and partial virus genomes are further organized into homologous protein families, on the basis of sequence similarity. Furthermore, the structure of known viral proteins or homologous to viral proteins is also mapped onto homologous protein families. VIDA also provides functional classification of virus proteins into broad functional classes based on typical virus processes such as DNA and RNA replication, virus structural proteins, nucleotide and nucleic acid metabolism, transcription, glycoproteins and others. This database also provides alignment of the conserved regions based on potential functional importance. Apart from functional classification, VIDA also provides a taxonomical classification of the proteins and protein families. The protein families serve as a tool for functional and evolutionary studies, whereas alignments of conserved sequences provide crucial information on conserved amino acids or construction of sequence profiles.

23.3 Virus Bioinformatics Databases

23.3.1 Viral Bioinformatics Resource Center (VBRC)

The Viral Bioinformatics Resource Center (VBRC) is one of eight NIH-sponsored Bioinformatics Resource Centers (http://www.oxfordjournals.org/nar/database/summary/798). It is an online platform that provides informational and analytical tools and resources to scientific community. The VBRC is oriented to conduct basic and applied research to better comprehend the viruses included on the NIH/NIAID list of priority pathogens. These viruses are selected based on their possibility of bioterrorism threats or as emerging or re-emerging infectious diseases. The VBRC focuses specifically on large DNA viruses. It includes the viruses that belong to the *Arenaviridae*, *Bunyaviridae*, *Filoviridae*, *Flaviviridae*, *Paramyxoviridae*, *Poxviridae* and *Togaviridae* families. It serves as a relational database and web application tool that allows data storage, annotation, analysis and information exchange of the data. The current version (V 4.2) consists of 369 complete genomic sequences.

Using the VBRC, each of the viral gene and genome can be curated. As a result, a comprehensive and searchable summary is acquired that details about the genotype and phenotype of the genes. The role of the genes in host-pathogen relationships is also being emphasized in these curations. Additionally, the VBRC also houses multiple analytical tools such as tools for genome annotation, comparative analysis, whole genome alignments and phylogenetic analysis. Further, this database also looks forward to include high-throughput data derived from other studies such as microarray gene expression data, proteomic analyses and population genetics data.

23.3.2 Poxvirus Bioinformatics Resource Center (PBRC)

The Poxvirus Bioinformatics Resource Center (PBRC, now merged into VBRC) is an online platform that serves as an informational and analytical resource to better comprehend the *Poxviridae* family of viruses. It allows data storage, annotation, analysis and information exchange of the data.

23.3.3 Influenza Virus Database (IVDB)

Influenza virus is one the major global concern. It gained attention after the emergence of pandemic influenza A virus (H1N1, swine flu) in 2009. There are a total of 11 web portals and tools that focus only on influenza virus. This includes the Influenza Virus Database (IVDB), Influenza Research Database (IRD) and NCBI Influenza Virus Resource (NCBI-IVR) (Chang et al. 2007; Bao et al. 2008; Squires et al. 2008). Researchers can exploit all the three websites mentioned for sequence databases as well as various basic tools such as BLAST, multiple-sequence alignment, phylogenetic tree construction, etc.

IVDB provides access to additional tools such as (i) the Sequence Distribution Tool, which provides global geographical distribution of a given viral genotype as well as correlates its genomic data with epidemiological data, and (ii) the Quality Filter System, which according to their sequence content (coding sequence [CDS], 5'untranslated region [5'UTR], and 3'UTR) and integrity (complete [C] or partial [P]) categorizes a given viral nucleotide sequence into either of the seven categories of C1 to C4 and P1 to P3, respectively. NCBI-IVR is the most widely used and cited online resource. With NCBI-IVR, the given viral genomic sequences can be annotated using a genome annotation tool and Flu ANnotation (FLAN) tool. Additionally, large phylogenetic trees may be constructed and can be visualized in aggregated form with sub-scale details (Bao et al. 2007; Bao et al. 2008; Zaslavsky et al. 2008). IRD provides tools for genomic and proteomic intervention, immune epitope prediction and surveillance data for viral nucleotide sequences (Squires et al. 2012). Furthermore, this resource is also equipped with tools that provide insight into host-pathogen interactions, type of virulence, host range and a correlation of sequence variation and these processes. There are other repositories available: Global Initiative on Sharing Avian Influenza Data (GISAID) consortium that mediated the EpiFlu database and FluGenome database that exclusively provides genotyping of influenza A virus and aids in detecting reassortments taking place in divergent lines (Lu et al. 2007). Furthermore, reassortment events in influenza viruses exclusively can be identified by a program GiRaF (Graph-incompatibility-based Reassortment Finder) that can be downloaded (Nagarajan and Kingsford 2011). Another distinct repository, Influenza Sequence and Epitope Database (ISED), provides viral sequences and epitopes from Asian countries; the information could be exploited to understand and study evolutionary divergence and migration of strains (Yang et al. 2009). The web server ATIVS (Analytical Tool for Influenza Virus Surveillance) provides an antigenic map for conducting surveillance and selection of vaccine strains by scrutinizing the serological data of haemagglutinin sequence data of influenza A/H3N2 viruses and influenza subtypes (Liao et al. 2009). There is another online repository OpenFluDB (an isolate-centred inventory), where information of an isolate such as virus type, host, date of isolation, geographical distribution, predicted antiviral resistance, enhanced pathogenicity or human adaptation propensity may be obtained (Liechti et al. 2010). For influenza viruses, primers and probes can be designed using the Influenza Primer Design Resource (IPDR) (Bose et al. 2008). Further, prospective influenza seasonal epidemics or pandemics can be predicted using a stochastic model, FluTE (Chao et al. 2010) (Table 23.2).

23.3.4 Virus Variation Resource (NCBI-VVR)

The NCBI Virus Variation Resource (NCBI-VVR) is a web-based database of a set of viruses, viz. influenza virus, dengue virus, rotavirus, West Nile virus, Ebola virus, Zika virus and MERS coronavirus (Resch et al. 2009). It enables the user to submit

Table 23.2 Virus-specific online databases/repositories

S. no.	Name of the databases/ repositories	Available information
1.	VirOligo	Virus-specific oligonucleotides
2.	VIDA	Animal viruses comprising of the families of *Herpesviridae*, *Papillomaviridae*, *Poxviridae*, *Coronaviridae* and *Arteriviridae*
3.	VBRC	Large DNA viruses belonging to the families of *Arenaviridae*, *Bunyaviridae*, *Filoviridae*, *Flaviviridae*, *Paramyxoviridae*, *Poxviridae*, *Togaviridae*, etc.
4.	PBRC (now merged into VBRC)	*Poxviridae* family of viruses
5.	IVDB, IRD, NCBI-IVR, FluGenome, ISED, ATIVS, OpenFluDB, GISAID	Influenza group of viruses
6.	NCBI-VVR	Influenza virus, dengue virus, rotavirus, West Nile virus, Ebola virus, Zika virus and Corona virus

their viral sequences along with relevant metadata such as sample collection time, isolation source, geographic location, host, disease severity, etc. It further allows integrating and analysing the viral sequences using the generic tools such as multiple sequence alignment and phylogenetic tree construction.

23.3.5 Web-Based Genotyping Tools

Rotavirus A (RVA) is the most frequent cause of severe diarrhoea in human and animal infants worldwide and remains as a major global threat for childhood morbidity and mortality (Minakshi et al. 2005; Basera et al. 2010). In recent years, extensive research efforts have been done for the development of live, orally administered vaccines. In India, an orally administered vaccine ROTAVAC was also introduced after successful clinical trials in 2014 which became available to clinicians in 2016, although these vaccines will have to be scrutinized and have to be updated regularly to accommodate the emerging rotavirus genotype variations, following which molecular and genetic characterization of new circulating and emerging genotypes of rotavirus strains in humans and animals becomes necessary. Recently, a classification system for RVAs has been described by the Rotavirus Classification Working Group (RCWG) in which all the 11 genomic RNA segments are assigned a particular alphabet followed by the particular genotype number. The classification system will be helpful in explaining the importance of genetic reassortments among RVAs, host range, transfer of gene segments among two different genotypes and adaptation to different hosts. To differentiate between different gene segments of RVAs, an online web-based tool RotaC was developed by the leading researchers from Rega Institute, KU Leuven, Belgium, in 2009 (Table 23.3). It's an easy-to-use

Table 23.3 A list of virus database and sequence-based typing tools that are used globally

S. no.	Typing tool	Viruses	References	Web link
1	*RotaC*	Group A rotaviruses	Maes et al. (2009)	http://rotac.regatools.be
2	LANL HCV database	HCV		http://hcv.lanl.gov
3	PaVE	Papillomavirus		http://pave.niaid.nih.gov
4	REGA HPV subtyping tool	Human papillomavirus (HPV)	Alcantara et al. (2009)	http://bioafrica.mrc.ac.za/rega-genotype/html/
5	REGA HIV subtyping tool	Human immunodeficiency virus 1 and 2		http://dbpartners.stanford.edu:8080/RegaSubtyping/stanford-hiv/typingtool
6	BioAfrica HHV8 automated tool	Human herpesvirus type-8	Alcantara et al. (2009)	http://bioafrica.mrc.ac.za/rega-genotype/html/
7	LASP HTLV-1 subtyping tool	Human T-lymphotropic virus type-1	Alcantara et al. (2009)	http://bioafrica.mrc.ac.za/rega-genotype/html
8	Oxford HBV subtyping tool	Hepatitis B virus	Alcantara et al. (2009)	http://bioafrica.mrc.ac.za/rega-genotype/html
9	Oxford HCV subtyping tool	Hepatitis C virus	Alcantara et al. (2009)	http://bioafrica.mrc.ac.za/rega-genotype/html
10	Dengue, Zika and chikungunya viruses typing tool	Dengue, Zika & Chikungunya Viruses		http://bioafrica.mrc.ac.za/rega-genotype/html
11	CoVDB	Coronavirus genes/genomes		http://covdb.microbiology.hku.hk
12	FLAVIdB Flavitrack	Flaviviruses		http://cvc.dfci.harvard.edu/flavi/ http://carnot.utmb.edu/flavitrack
13	FluGenome	Influenza A virus	Lu et al. (2007)	http://www.flugenome.org/
14	HERVd	Repository of human endogenous retroviruses		http://herv.img.cas.cz
15	HVDB	Hepatitis A, B, C, D and E virus sequences		http://s2as02.genes.nig.ac.jp
16	RetroTector	Retroviral sequences in vertebrate genomes		http://retrotector.neuro.uu.se/
17	SCUEAL	HIV-1 subtypes		http://www.datamonkey.org/dataupload_scueal.php

18	SeqHepB	Relational database for HBV		http://www.seqhepb.com
19	STAR	Subtyping tool for HIV-1 and HBV	Myers et al. (2005)	http://pgv19.virol.ucl.ac.uk/download/star_linux.tar
20	Norovirus typing tool – RIVM	Norovirus		www.rivm.nl/mpf/typingtool/norovirus/
21	Enterovirus typing tool – RIVM	Enterovirus		https://www.rivm.nl/mpf/typingtool/enterovirus/
22	Hepatitis E virus genotyping tool – RIVM	Hepatitis E virus		https://www.rivm.nl/mpf/typingtool/hev/
23	Yellow fever virus phylogenetic typing tool	Yellow fever virus		http://bioafrica2.mrc.ac.za/rega-genotype/typingtool/yellowfevervirus/
24	FluTE	Influenza A virus	Chao et al. (2010)	http://www.cs.unm.edu/_dlchao/flute/
25	OpenFluDB	Influenza A virus	Liechti et al. (2010)	http://openflu.vital-it.ch

and reliable classification tool for RVAs and works on the agreement with RCWG. It's a platform-independent tool which works on any web browser by simply going to its URL (http://rotac.regatools.be/) and has been released without any restriction of use by academicians or anyone else. As claimed, the RotaC web-based tool will be updated regularly to reflect the established as well as newly emerging genotypes announced by the RCWG from time to time.

23.4 Conclusions and Future Prospects

Various researches in animal viral diseases are being conducted at the genomic level. Often, handling an enormous data obtained from sequencing is daunting to researchers. The chapter categorically provides a list of bioinformatics approaches that are useful in data mining. There are tables that list all such bioinformatics programs as per the applications. The tables also list databases that organize information on human and animal viruses such as genomic data, ORFs, oligonucleotides, etc. An illustration has also been provided in the chapter showing the application of the tool PredictProtein, which is used for prediction of three-dimensional structures of viral proteins. The major goal of the chapter has been to provide a roadmap to bioinformatics approaches in the field of animal viral diseases.

Although the chapter elaborates on viruses-specific bioinformatics programs, most of these programs are designed for human viruses. Nevertheless, there are bioinformatics tools that are animal-virus specific, but these are limited in number. Henceforth, in many cases, researchers have to switch to either human virus-specific tools or other generic tools. Application of such tools for studying animal viruses or animal diseases, in many situations, may not be as accurate as with specialized tools. The users should take precautions while using the settings of such tools. Furthermore, the results, thus obtained, also need to be scrutinized. Therefore, development of new bioinformatics programs/tools that are specifically designed for animal viruses/diseases should be taken up robustly. Specialized tools will provide much accurate results and predictions, thereby accelerating the bioinformatics researches in the field of animal viral diseases.

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