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Emerging and Re-emerging Viral Infections

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Preface – Emerging Viruses: From Early Detection to Intervention

Giuseppe Ippolito and Giovanni Rezza

Keywords
Diagnostics • Emergence • Emerging infections • Quarantine • Vaccine

In the last decades, several viruses emerged, after cross-species passage from animal reservoirs and then spreading in human populations; the Ebola virus, two different coronaviruses causing the severe acute respiratory syndrome (SARS-CoV) and the Middle-east respiratory syndrome (MERS-CoV), and the Nipah virus, are paradigmatic examples of biological agents completely new for humans, with high epidemic potential, but also prone to disappear in case early detection and intervention are ensured (Morse 1993; Fauci and Morens 2012).

Other viruses expanded their geographical area of activity from the original ecological niche to new lands and continents, as recently demonstrated by the large outbreaks of Zika or Crimean-Congo haemorrhagic fever in Spain, and dengue in Madeira (Portugal). Several arboviruses represent paradigmatic examples of microorganisms which found the conditions for their spread in previously unaffected areas inhabited by completely susceptible populations, increasing their epidemic potential. In this group, we find several agents transmitted by *Aedes spp*. mosquitoes, from dengue to chikungunya and Zika (McCloskey et al. 2014).

Vector-borne viruses are not the only emerging agent which represent a threat for human health, and other zoonotic viruses are increasingly impacting on the burden of disease at the global level (Morens and Fauci 2013). To this regard, zoonoses account for nearly two-thirds of human infectious diseases, in part due to the increasing anthropogenic pressures on the environment. Leading drivers of infectious disease emergence in humans from wildlife are multiple and complex, and broad and novel approaches are required to tackle them. The "One Health" approach, for example, considers the human-animal-environment interface with a single perspective (IOM 2015). The aim is to promote synergies among public health, information and communication, human and animal health, veterinary and medical approaches, environmental and ecological sciences, mathematical modeling and geographic information systems,

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anthropological and behavioral expertise (Zumla et al. 2015).

Emerging viruses represent an important challenge for global public health, and prompt intervention is needed in order to put outbreaks under control (McCloskey et al. 2014). First of all, early diagnosis of the agent is extremely important to rapidly identify the viral threat and to start the intervention as soon as possible (Memish et al. 2014). To this end, a syndromic approach and the use of an appropriate case-definition may be useful to hypothesize the nature of the disease. However, as demonstrated with Ebola in the large outbreak occurred in West Africa in 2014, only a small proportion of cases had hemorrhagic manifestations, thus relying on bleeding did not provide a valid clue to diagnosis. Laboratory diagnosis is more specific and represents the gold standard for the diagnosis of an emergent virus. However, in certain contexts, it may be difficult to perform relatively sophisticated tests under adverse environmental conditions. Moreover, the lack of protective equipment and high security level laboratories is an obstacle to handling potentially infected samples. To overcome this problem, mobile BSL4 labs have been extensively provided by the international community to allow Ebola virus infection diagnosis during the recent outbreak of Ebola in West Africa.

Response capacity, especially by resource poor countries, and rapid intervention in the context of explosive outbreaks is key to mitigate or control epidemic events (Anema et al. 2014). With diseases like Ebola, that are transmitted through direct contact with diseased persons, dead bodies, or bodily fluids, and are amplified by the family and the hospital setting or burial ceremonies, avoiding contact with physical barriers is rather efficient and productive. Measures as the availability of a large number of hospital beds to keep infected patients away from the community, protective equipment and training of health care workers to avoid direct contact with patient fluids, restriction of movements to minimize the risk of introduction of the infection to naïve areas are likely to succeed when complemented by correct information, lab evidence based decision making for keeping patients under isolation, high quality care and treatment of confirmed patients.

With mosquito-transmitted diseases, which recently caused several large outbreaks in many poor resource countries, prevention also play a major role. A paradigmatic example is represented by the spread of chikungunya and Zika in Latin America and Caribbean, where dengue was already present. However, mosquito control activities may be successful in controlling local outbreaks occurring in temperate areas but do not appear able to mitigate large epidemics in tropical areas. For this reason, the availability of safe and effective vaccines is essential in order to keep virus circulation under control.

There are no vaccines available against most emerging infections, and this may be explained by a series of factors. First, for their own nature, emerging infections have often epidemic patterns that minimize the feasibility of large efficacy trials, which are now considered the gold standard for vaccine evaluation. In fact, the conduction of large studies is limited by the unpredictability of large outbreaks where vaccines may be tested on large population groups; secondly, for the same reason, vaccine demand may be difficult to assess; thirdly, limited resources are allocated to vaccine research and development when economic return is not ensured.

For example, identifying the target populations for vaccination campaigns is not an easy task. For Ebola, health care workers in high risk areas might be a target, as well as health professionals who intervene in case of outbreaks. Finally, ring vaccination of direct and indirect contacts of infected patients might be vaccinated to reduce the risk of disease and transmission in an affected area.

Vaccines against a few arboviral diseases, such as those against yellow fever and Japanese encephalitis, have been extensively used. In

particular, the live attenuated vaccines against yellow fever, which was created in the 1930s, has contributed to the control of the disease both in Africa and in South America. However, a vaccine against dengue, whose target is represented by the local communities in affected area of the world, has proven to be only partially effective, and vaccines against chikungunya and Zika are still lacking.

Nevertheless, making vaccines and effective drugs to be used as prophylaxis in case of detection of early chains of transmission of emerging viruses, as it may happen with a mutated strain of avian influenza (i.e., a "humanized" H5N1 or H7N9 flu virus) would be very useful if complemented by effective molecular surveillance (Hui and Zumla 2015; Marston et al. 2014).

In this volume, we present a series of article on mechanisms and drivers of emergence of novel virus infections in human population, trying to focus the attention on aspects which have not frequently addressed before.

For this special issue, the articles written by internationally renowned experts cover several areas of research. In the paper by Busani et al., the application of the theory of focality of diseases to infectious disease is discussed, providing paradigmatic examples of viral diseases (Busani et al. 2016). The proposed approach is represented by detailed mapping of the areas of activity of biological agents causing natural focal diseases along with evidence-based interventions, such as targeted vaccination.

In Castrucci review, the Human-animal interface is discussed, with a a speficic focus on influenza. The topic is particularly important, since "humanization" of avian viruses represents a persistent threat to human health (Donatelli et al. 2016).

In the Serra-Cobo and López-Roig paper, the roles played by bats in emerging infections is presented and discussed. Maintenance mechanisms and transmission of bat viruses were analyzed, taking into account the phylogenetic history, coevolution processes, bat adaptation to live in different environments, and specific behaviour of different species. These factors allow to assess the epidemiological risk for humansand to plan preventive measures (Serra-Cobo and López-Roig 2016).

In Azhar et al. report, an overview of Middle East Respiratory Syndrome Coronavirus (MERS-CoV) epidemiology and its clinical features is provided. The paper highlights the knowledge gaps and the epidemic risk potential for global spread of this emergent coronavirus (Azhar et al. 2016).

The current Zika virus large outbreak occurring in Pacific Ocean and the Americas, including the critical aspect for a coordinated response, is described by Bordi et al. Several aspects, such as the mode of transmission, the risks associated with pregnancy in infected mothers, the association of the virus with severe consequences, including fetal/newborn microcephaly and Guillain-Barré Syndrome in adults, are discussed in the paper (Bordi et al. 2016).

Animal models are essential for the study of emerging infections, to improve disease knowledge and for developing therapeutic drugs. Warner et al. describe the use of small animal models for the study of infectious diseases, with special focus on the Syrian golden hamsters emerged as an ideal animal model, due to their low cost, small size, ease of handling, and ability to accurately reflect disease progression in humans. In the paper, valuable information to researchers who are deciding whether to use hamsters as an animal model is provided (Warner et al. 2016).

The 2014–2015 Ebola virus outbreak in western Africa illustrates the threat coming from emerging infectious diseases and is perceived by the public as a preeminent global health problem. Nicastri et al. present the activities and the challenging issues encountered in terms of medical management of the patients, preparedness and response to the outbreaks, diagnostic and research challenges (Nicastri et al. 2016).

Highly infectious diseases can spread rapidly across borders through travel or trade, and international coordination is essential to a prompt and

efficient response by bio-containment laboratories. A prioritization of high consequence viruses is essential to improve European laboratory preparedness for cross-border health threats. The strategy to identify priorities for a rational allocation of resources for research and surveillance has been the focus of a large body of research in recent years. The activities and the the strategy used by EMERGE, an European-wide consortium funded by the European Commission, are described in the paper by Nisii et al. (2016).

New emerging technologies are useful for detecting, tracking, reporting, forecasting, and improving early warning systems and proper response. To this regard, Al-Surimi et al. highlights and discuss the potential role of social media in preventing and fighting infectious diseases, summarizing the advantages and limitations of social media and Internet-based data for public health surveillance, in order to identify the gaps that still require further research and improvement (Al-Surimi et al. 2016).

The papers published in this special issue present exciting, insightful observations on emerging viral infections. In this rapidly developing field of study, interdisciplinary and challenging research, performed in both industrialized and resource-limited countries, can bring critically important information for life and social sciences, for public health, and for health care overall. The aim of this special issue is to contribute to the development of knowledge on emerging infection in the endless warfare between viruses and man. However, the articles included in this volume are not representative of the whole field of emerging infections, since they have been selected with the aim of presenting a point of view on uncovered issues or providing insights on key/hot topics for which scientific knowledge is rapidly evolving knowledge. Thus, our intention was not to conduct a systematic collection of all emerging infections, but to address specific issues that may be important in order to understand why and when a new agent may emerge, and how we may intervene in an effective way. We hope we succeeded, at least in part, in this difficult task.

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How to Tackle Natural Focal Infections: From Risk Assessment to Vaccination Strategies

Luca Busani, Alexander E. Platonov, Onder Ergonul, and Giovanni Rezza

Abstract

Natural focal diseases are caused by biological agents associated with specific landscapes. The natural focus of such diseases is defined as any natural ecosystem containing the pathogen's population as an essential component. In such context, the agent circulates independently on human presence, and humans may become accidentally infected through contact with vectors or reservoirs. Some viruses (i.e., tick-borne encephalitis and Congo-Crimean hemorrhagic fever virus) are paradigmatic examples of natural focal diseases. When environmental changes, increase of reservoir/vector populations, demographic pressure, and/or changes in human behavior occur, increased risk of exposure to the pathogen may lead to clusters of cases or even to larger outbreaks. Intervention is often not highly cost-effective, thus only a few examples of large-scale or even targeted vaccination campaigns are reported in the international literature. To develop intervention models, risk assessment through disease mapping is an essential component of the response against these neglected threats and key to the design of prevention strategies, especially when effective vaccines against the disease are available.

Keywords

Natural Focal Diseases • Viruses • TBE • CCHF • Vaccination

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A "natural focus of an infectious disease" is a concept deriving from the theory of focality of diseases, proposed by the Russian scientist Eugene Pavlovsky in 1939. According to this theory, some pathogens are associated with specific landscapes, and the natural "focus" or "nidus" of an infectious disease is defined as "any natural ecosystem that contains the population of a pathogen as an essential component" (Korenberg 2010). The determinant feature of natural-focal diseases is that the pathogen circulates in nature independently from human presence. As a rule, humans beings become infected when they get into the focus and have contact with the infectious vector or, in some cases, with the reservoir host (Korenberg 2010).

To develop the concept of natural focality, Pavlovsky, in his original theory, analysed tick-borne pathogens in Russia, and for such pathogens he stated that the focus of infection should have three critical elements:

1. The aetiological agent of the disease;
2. vertebrate hosts playing different roles (infectious and susceptible recipient hosts, reservoirs);
3. environmental factors enabling the circulation and persistence of the agent.

Starting from this original formulation related to vector-borne diseases, the definition of natural focality was applied also to non-vector-borne zoonoses, such as hemorrhagic fever with renal syndrome, Ebola (when restricted to its natural nïche), leptospirosis, and other infectious diseases. Finally, natural focality for a large group of sapronotic infections, whose agents live in soil or aquatic ecosystems, was also described and discussed. For some vector-borne zoonoses, the concept of a focus (nidus) may be implemented as well. Thus, the phenomenon of natural focality is widespread, and includes many natural-focal diseases with different types of transmission.

The natural transmission of a pathogen, in the context of natural focality, should be considered a "continuous interaction of the pathogen population with the populations of its natural hosts and the environment, which provides for the existence of the pathogen" (Korenberg 2010). Such process is a series of consecutive cycles of pathogen reservation (restriction) and spread (circulation), providing the process to be limited in time and space by the presence of specific conditions needed by the pathogen. Moreover, the circulation of the pathogen in the natural foci is independent on human presence, and human infection, with rare exceptions, is a "biological dead end" for the pathogen. The interaction between pathogens and humans is an accidental event and does not have any coevolutionary consequence (WHO 2016). An outbreak directly connected with natural foci is actually the sum of individual disease cases occurring in different places independently on each other, with infection often being acquired from one or several sources not connected with other diseased persons (Korenberg 2010). This concept is important to distinguish zoonoses with natural focality and human infections acquired from domestic animals. Another difference between natural focal diseases and other zoonoses is the relevance of the socio-ecosystem level in the structure of the epidemic process, which may be high for several zoonotic infections but usually does not play any role in case of zoonoses with natural focality.

However, it should be taken into account that biological and social factors, and increasingly intensive human activities, can cause drastic changes in the structure and functioning of parasitic systems, the frequency and forms of human contact with natural foci, and even the pathway of pathogen transmission to humans.

This "basal" interpretation of the epidemic process in infections with natural focality remains unchanged. It should only be taken into account that people themselves create conditions for their exposure, favouring the entrance of pathogens from natural ecosystems into their immediate environment, and for their active reproduction and amplification in this new environment. For example, epidemic outbreaks of hemorrhagic fever with renal syndrome, a typical zoonosis with natural focality, are usually associated with virus shedding by animal

reservoirs (murine rodents) migrating to populated areas, where favourable conditions for virus amplification are created when the abundance of these animals reaches high values (Korenberg 2010).

In the past two decades, views on the diversity, spread, and epidemic significance of infections with natural focality have changed substantially all over the world. Some new pathogens have been discovered, and periodic epidemic manifestations of natural foci have become a matter of great concern. Moreover, increasing human activity (e.g., intensive suburban construction around big cities, expansion and growth of recreational pressure) have led to a significant increase in contact between human populations and natural foci, creating favourable epidemiological conditions for the spread of natural-focal diseases (Malkhazova et al. 2014). The large outbreak of Ebola occurred in West Africa from December 2013 to March 2016, affecting three countries (Guinea, Liberia, and Sierra Leone), is an example of the epidemic potential of natural focal diseases if virus properties (capacity of inter-human transmission), environmental factors, and social conditions concur to increase the force of infection, leading to large-scale virus circulation throughout human communities. At the end of the outbreak, more than 28,000 cases and 11,000 deaths were reported (WHO http://www.who.int/csr/disease/ebola/en/).

Natural-focal disease prevention is one of the most challenging public health problems. Agents and vectors of these diseases are part of natural landscapes and the spread of these diseases, which may represent a serious hazard for people, is determined by natural factors. Such factors can be identified and described in the affected area, in order to identify the "hot spots" of the disease, which are the most suitable places for the agents and vectors. As proxy of the landscape features, also historical data on biocenosis, health records of humans and other vertebrate hosts (domestic and wild animals) can be used. With such information, predictions on the probability that a given biological agent is present in a specific area are possible. Moreover, this information can be plotted, trying to identify the geographical distribution of the natural foci of infection in a given area. Therefore, medical geography has an important task: evaluating the risk of epidemic hazards of natural ecosystems and providing public health authorities with recommendations necessary to prevent disease outbreaks and conduct epidemiological surveillance.

1 Eco-Epidemiology: How to Predict and Control the Occurrence of Natural Focal Infectious Diseases

Current understanding about the global distribution of most infectious diseases is surprisingly limited. In particular, the spatial distribution of the vast majority of natural focal diseases remains largely unknown and many questions are still unsolved, mainly because of the poor knowledge of their local variations and of the characteristics of the ecological niches that allow the permanence of such diseases in their natural environment. Due to their intrinsic features, the transmission of focal diseases to humans is highly heterogeneous in space and time. At the micro-epidemiological scale, numerous factors influence the transmission dynamics of the diseases in endemic foci, and variations in the distribution of these factors, even in a small area, can result in spatially heterogeneous transmission and appearance of disease hotspots, where transmission intensity is higher than in the surrounding areas.

There are several reasons for mapping the geographical distribution of infectious diseases. Maps of disease distribution and intensity allow an immediate visualization of the extent and magnitude of the public health problem. These maps can also document the background level of the disease in order to monitor its trend and to evaluate interventions. Another reason is that maps may also provide information on the factors that favour the emergence of infectious diseases.

Hereby we present some innovative approaches to disease mapping which provide useful information and predictions.

1.1 Ecological Niche Modelling

A wide range of approaches has been developed for empirical modelling of species and disease distributions, making use of data on point observations of disease occurrence, with the objective of identifying the fundamental niche of the target organism (Hay et al. 2013). Some of these approaches allow to account for environmental covariates, data on presence of pathogens and natural hosts, landscapes, and climate information.

The concept of ecologic niche was proposed by Joseph Grinnell (1917) who was the first to explore connections between ecological niches and geographic distributions of species. According to his proposal, the ecologic niche of a species is the set of conditions under which species populations may be maintained without immigration of individuals from other areas. The idea behind niche modelling is that known occurrences of species across landscapes can be related to raster geographic information system coverages summarizing environmental variations across those landscapes, to estimate the ecological niche of the species (Peterson et al. 2002). Such a modelling approach can be used to identify potential distributional areas for species on any landscape, which may include unsampled or unstudied portions of the native landscape (López-Cárdenas et al. 2005), areas of actual or potential invasion by species with expanding ranges (Townsend Peterson and Kluza 2003), or changing potential distributional areas as a consequence of change (e.g., land use change or climate change) (Escobar et al. 2015).

Ecological niche modelling, may be used to characterize distributional areas of species in complex, linked geographic and ecologic spaces. It permits researchers to characterize ecological needs of species, interpolate between sampling points to predict full distributions of species, predict species distribution into broadly unsampled areas, predict invasive potential in other regions/continents, predict likely distributional change with changing land use, predict likely distributional change with changing climates, and build scenarios for understanding and characterizing unknown disease behaviour. Hence, ecological niche modelling offers a powerful tool for characterizing ecologic and geographic distributions of species across real-world landscapes (Peterson 2008a, b).

1.2 The Boosted Regression Trees Method

To map the occurrence of a given disease, the boosted regression trees method (Elith et al. 2008; De'ath 2007) is one of the most performing methods; it is flexible in being able to accommodate different types of predictor variables (e.g. continuous or categorical data) and easy to understand, implement and uses reliable. The maps generated with such approach are simple to interpret and include a ranked list of environmental predictors.

Another approach that has recently been more widely applied in infectious disease mapping is the model-based geostatistics (Diggle and Ribeiro 2010) that has important advantages when compared to the boosted regression trees method: (i) it deals explicitly with the spatial (and with extension temporal) autocorrelation of disease data. This is still widely ignored in occurrence mapping; (ii) it offers a much more robust parameterization of factors that can affect disease endemicity (such as age of the individuals sampled, the diagnostic technique used, the influence of covariates etc.); (iii) outputs can also show the full uncertainty of the prediction in all parts of the predicted maps by fitting the models using Bayesian inference.

1.3 Macroecology

The "macroecology" appears to provide a new perspective in identifying drivers of infectious disease patterns and impacts at the broadest

scales of organisation. Macroecology investigates patterns and processes at broad spatial, temporal and taxonomic scales, expanding scientific understanding of global infectious disease ecology. In particular, it could help providing new insights about scaling properties across all living taxa, and new strategies for mapping pathogen biodiversity and infection risk. Macroecology seems a useful framework to more accurately predict global patterns of infectious disease distribution and emergence (Stephens et al. 2016). Research in the relatively new discipline of macroecology covers important findings and advances in computational and statistical methods explaining how macroecological approaches can inform human health and conservation initiatives. The advanced computational techniques are applied to enormous data sets to look for patterns; in the case of disease ecology, this kind of analysis can help scientists understand relationships among parasites, hosts and their environments.

Indeed, the development of the principles and methods of synthesizing information from different sources, including geography, to obtain new knowledge about the spatial distribution patterns of natural-focal diseases using new approaches is a research interest. The scientific and methodological basis of disease mapping, using information on landscape and environment, mathematical methods, and multivariable analysis, is well developed and under continuous improvement; however, practical experience are extremely limited in mapping diseases at a broader level (nation, area, continent).

The ability to map a disease stems largely from the type and quality of data that are available for mapping. The accuracy of maps is then largely determined by the abundance, spatial representativeness and heterogeneity of those data (Hay et al. 2013).

Differences in quality and incompleteness of initial information make it difficult to obtain a complete picture of the distribution of natural-focal diseases within a territory. In particular, details on disease/pathogen presence or absence in a given area is limited, due to the limitation of the surveillance activity in humans, domestic animals and wildlife, and the few ecological studies on diseases with natural focality (Malkhazova et al. 2014).

The knowledge of human distribution in many areas of the World remains also surprisingly poor. For many low income countries of the World, spatially detailed, contemporary census data do not exist. This is especially true for much of Africa, where currently available census data are over a decade old, and at administrative boundary levels just below national-level (Hay et al. 2005; Tatem et al. 2008). This information is of significant importance for deriving populations at risk and infection movement estimates.

Another key factor that may affect the distribution and the prediction of natural focal diseases, together with a large number of other human and animal diseases, is climate change. Species' response to climate change are variable and diverse, yet our understanding of how different responses (e.g. physiological, behavioural, demographic) relate and how they affect relevant population parameters (e.g. population persistence) is lacking. Much of the research on responses to climate change does not consider how population size, population growth rate, or extinction risk varies as a function of climate; consequently, the mechanisms causing climate-induced population changes are still poorly understood (van de Pol et al. 2010). Such lack of knowledge impacts on the capability to make consistent predictions of the population dynamics of both hosts and related pathogens.

2 Natural Focal Diseases Mapping and Control: Two Paradigmatic Examples

Detection of the hotspots of natural focal diseases through the different mapping approaches is a key action to better target control efforts and to reduce/stop infection transmission in those areas where transmission intensity is higher for several reasons. The identification of areas and human populations at risk is essential for better address vaccination campaigns and

other interventions. However, only few natural focal diseases have been studied in detail mapping their foci, and vaccines are available for few of them. Hereby we report available information for two important natural focal viral diseases.

2.1 Crimean-Congo Hemorrhagic Fever: A Mapping Exercise

Crimean-Congo hemorrhagic fever (CCHF) is a tick borne disease characterized by fever and hemorrhagic manifestations, with fatality rates up to 30%. The disease was initially described by Russian scientists in the '40s, while the virus was isolated the first time in the Democratic Republic of Congo some years later. CCHF virus (CCHFV) circulation has been reported throughout broad regions of Africa, Europe, the Middle East, and Asia, with a geographic distribution overlapping that of the *Hyalomma* tick, the main vectors of CCHFV. CCHFV is one of the most geographically widespread tick-borne pathogens of medical importance and may spread to new areas if globalization and climate changes create new opportunities for virus introduction and amplification in suitable ecological niches (Hewson 2007) (Papa et al. 2015).

CCHF is considered a disease with natural focality, since the CCHFV is maintained in active foci through a complex cycle that involves ixodid ticks, mainly of the genus *Hyalomma* (the role in nature of other tick species in the natural transmission or maintenance of CCHFV is not clearly demonstrated) and reservoir hosts (e.g. wild and domestic ungulates, domestic livestock), on which adult ticks feed. Also other mammals (rodents, pets) and birds can play a role in the spread and maintenance of the virus transmission cycle.

Little is known about the infection rates in both vectors and hosts in nature. In *Hyalomma* ticks, prevalence of infection is estimated to be about 5%, but large geographical variability exists, due to local environmental conditions, and to presence and abundance of the different types of hosts.

In mammal hosts, the prevalence of infection is poorly investigated, especially in wildlife. Domestic ruminants play a crucial role in the life cycle of the vector ticks and the transmission and amplification of the virus. In most livestock species viremia can lasts up to 14 days, thus immune response starts, and the antibody prevalence in those animals is a good indicator for the presence of CCHFV in a region. Recent studies conducted in different regions of Bulgaria and Turkey showed an overall prevalence in domestic ruminants between 26 and 57%, but in some areas the prevalence was up to 90% (Mertens et al. 2016). The potential usefulness of small ruminants as indicator animals to determine the presence or absence of CCHFV in a given region is also highlighted by Schuster et al. (2016), who pointed out also the limited knowledge about the mechanisms governing the dynamics of CCHFV circulation in a suitable habitat and the role of the various animals. Such circulation is linked variables like age of the animals, with dimostration of increasing antibody prevalence by increasing age of the tested animal population (Wilson et al. 1990; Barthel et al. 2014) husbandry conditions, usage of repellents, host-preferences of the ticks and susceptibility of animal species and breeds for CCHFV.

Large ungulates and livestock are usually asymptomatic and only active testing can show infection in these species. Because the lack of symptoms in animals and the short life-cycle of *Hyalomma* ticks, without active virological and serological surveillance in animals and ticks it is unlikely to detect infection in animals earlier than in humans. Thus, the detection of human cases is often the first sign of CCHFV circulation in an area.

Mapping of the human cases is a way to represent the CCHF distribution. The World Health Organization (WHO) produced maps of the disease at global scale, (http://www.who.int/csr/disease/crimean_congoHF/Global_CCHFRisk_20080918.png?ua=1), but this map represents merely the reported occurrence of human disease rather than the distribution of the virus. This is due to the characteristic of the surveillance (capacity to detect human cases in different

countries/areas, underreporting, underdiagnosis); the disease (a variable but relevant proportion of cases are subclinical, and this proportion may vary in different geographical areas); specific local conditions, like in Spain, where virus circulation was detected in ticks since 2010, but no human cases were observed until the summer of 2016 (Estrada-Peña et al. 2012; García Rada 2016).

A new perspective on the use of occurrence data, which was firstly developed for dengue by Bhatt et al. (2013), has been then applied to CCHF by Messina et al. (2015). This approach is based on the creation of a large database by assembling contemporary data on CCHF occurrence together with geographical location and a suite of environmental covariates. Such data have been collected from many different sources of information, including the reporting of official surveillance systems, the scientific and technical literature and informal online resources. New modelling approaches are then applied to the large dataset to maximise the predictive power of occurrence data. As a result, high resolution spatial map of the probability of occurrence of human CCHF infection can be derived at global level (Messina et al. 2015).

An example of successful modelling approach is the prediction of CCHF expansion in Western Palearctic made by Estrada-Peña et al. They developed a dynamic model for CCHFV transmission in western Palearctic that considered the tick vector, *Hyalomma marginatum* and the effects of variations in temperature and water vapour on hte tick survival (Estrada-Peña et al. 2013). The main outcome was that increase of the temperature is compatible with the spread of CCHFV in the western Palearctic, because expansion of the habitat suitable for tick vectors. According to this scenario, increased virus circulation would happen in sites where high tick populations may already exist. This scenario was confirmed by the occurrence of a human case in Spain in 2016 (García Rada 2016).

Combination of surveillance in humans, host animals, and vectors is much more informative of the distribution of the virus and the areas at risk of human exposure; however, it is rarely carried out due to the costs and the difficulties to establish reliable surveillance activity in animals, especially in wildlife. Serological surveys in livestock have been conducted in different countries, providing snapshots of the circulation of the virus in domestic animals (Adam et al. 2013; Lotfollahzadeh et al. 2011).

Surveys in ticks have also been carried out to identify areas with potential virus circulation. Recent experience of systematic tick surveillance demonstrated the recent colonization of the continental France by *H. marginatum*; this region was considered free from the tick (Vial et al. 2016).

In Europe, ticks are the most important vectors of human and animal infectious diseases, and transmit more pathogens than any other arthropod (Jongejan and Uilenberg 2004; Colwell et al. 2011). Monitoring of ticks requires integrated approach, with expertise in environmental science and entomology as a complement to the human and animal health competencies.

2.2 A Vaccine for CCHF: Give Prevention a Chance

Initial attempts to develop CCHF vaccines goes back to the 1960s, when Soviet scientists advocated the immunization of local populations in endemic areas. In 1974, the Soviet vaccine was licensed in Bulgaria. This inactivated virus vaccine is the currently only available CCHFV vaccine, however its clinical efficacy was not clearly demonstrated (Mousavi-Jazi et al. 2012). More modern approaches, such as DNA vaccines, recombinant viral protein-based vaccines, and virus-like particle vaccines, are under development. The lack of suitable animal models in the past has hampered the development of new, preventive, and therapeutic measures. In a recent study, IFNAR-/- mice was found to be highly susceptible to the Turkey-Kelkit06 strain of CCHFV. Immunization with the cell culture based vaccine elicited a significant level of protection against high dose challenge (1000 PPFU) with a homologous CCHFV in IFNAR-/- mice (Canakoglu et al. 2015). The

Bulgarian vaccine was used in CCHFV-endemic areas of the country for military personnel and medical and agricultural workers over 16 years of age. None of the vaccinated military personnel has contracted CCHF, and none of the vaccinated laboratory personnel working with CCHFV became infected even after occasional exposures by needle (Keshtkar-Jahromi et al. 2011). However, detailed information on vaccination strategies adopted to reduced the burden of disease in endemic foci and their possible outcome are not available. The availability of other effective and safe vaccines would represent a great opportunity and needs to be considered in preparedness plans and control strategies, along with public information and behavioral prevention.

2.3 Tick Borne Encephalitis: Vaccine Use to Control a Natural Focal Disease

Tick-borne diseases (TBDs) are among the most rapidly expanding infections worldwide. Many new human tick-borne pathogens are discovered and several novel TBDs are recognized. Increasing burden of TBDs shows that current available public health interventions and approaches are not effective enough. Vaccination could be a highly cost-effective intervention for preventing TBDs (Šmit and Postma 2016). Among TBDs for which vaccines are currently available, tick-borne encephalitis (TBE) is one of the most widespread in Europe. TBE can affect the central nervous system, which may result in long-term/permanent neurological sequelae or even death (Dumpis et al. 1999). At the European level, TBE presents an increasing public health concern with vaccination against TBE less widely used than possible to reduce the disease burden (Šmit and Postma 2016). TBE incidence shows strong annual variations as well as long fluctuations over time in affected countries, and an overall upsurge has been reported in certain parts of Europe. These changes have been related to climatic, ecological, environmental and socioeconomic factors that can lead to an increased risk of human exposure to infected ticks. In addition, however, the establishment of new natural foci of TBE virus circulation has been described in areas previously considered free of TBE. In Europe, Austria had the highest recorded morbidity for TBE, with several hundred hospitalized patients per year and several deaths (Kunz 2003). A vaccine against TBE became commercially available in 1976 and was administered to those at higher risk (e.g., people handling the infectious virus in the laboratory and professional people working in forests in highly endemic regions). Following the evidence of a limited impact of vaccination of at risk groups only, mass vaccination campaign organized by the Austrian Health authorities began in 1981. The vaccination coverage of the Austrian population increased from 6% in 1980 to 82% in 2013 and has exceeded 90% in some of the high-risk areas. The increasing vaccination coverage led to a steady decline in the number of TBE cases, that are ten times less than the 1976, in addition, between 2000 and 2011, an estimate of 4000 hospitalized TBE cases were prevented by vaccination (Heinz and Kunz 2004; Heinz et al. 2013). These results have been achieved thanks to the high awareness among the Austrian population and the large use of an effective and well-tolerated vaccine (Kunz 2002). However, it could be challenging to maintain a high vaccination coverage in the future. Moreover, recommendations to people visiting affected areas should be delivered, since pathogens and vectors are still there.

3 Conclusions

The importance of natural focal diseases has been largely neglected for a long time. However, the expansion of foci characterized by intense viral activity, even in previously free areas, has raised the attention on this threat. Viral diseases like CCHF and TBE, initially restricted into small geographical niches, have now an important impact on human health in several areas of the world. In some cases, although it may appear paradoxical, mass vaccination campaigns have

been successfully used to prevent and control focal diseases. However, mapping the areas of activity of biological agents causing natural focal diseases, and assessing the population effect of interventions, especially vaccines, is the best strategy to better address interventions against these emerging infections.

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Human–Animal Interface: The Case for Influenza Interspecies Transmission

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Abstract

Since the 1990s, the threat of influenza viruses to veterinary and human public health has increased. This coincides with the larger global populations of poultry, pigs, and people and with changing ecological factors. These factors include the redistribution of the human population to cities, rapid mass transportation of people and infectious agents, increased global land use, climate change, and possible changes in viral ecology that perpetuate highly pathogenic influenza viruses in the aquatic bird reservoir. The emergence of H5N1, H7N9, and H9N2 subtypes of influenza A virus and the increased genetic exchange among influenza viruses in wild aquatic birds, domestic poultry, swine, and humans pose a continuing threat to humanity. Here we consider the fundamental and practical knowledge of influenza A viruses at the human–animal interfaces to facilitate the development of novel control strategies and modified agricultural practices that will reduce or prevent interspecies transmission.

Keywords

Avian influenza • H5N1 • H7N9 • Zoonosis

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1 Introduction

Influenza A viruses are of major concern to both veterinary and human public health because they continue to emerge and cause high mortality in domestic poultry and represent an ongoing threat to human health. With the recognition that aquatic birds are the major reservoir of influenza viruses that sporadically transmit to domestic poultry, swine, and humans, there is acceptance of the "one world" concept of influenza (Jernigan and Cox 2013). When influenza A viruses spread from the aquatic bird reservoir to domestic poultry, pigs, and people, they can evolve into viruses that cause mild or catastrophic diseases. For influenza viruses to spread from the aquatic bird reservoir to humans, they must evolve from intestinal tropism in wild aquatic birds with a body temperature of 42 °C to respiratory tract tropism in humans with a body temperature of 37 °C. Additionally, the virus must change its receptor specificity on the hemagglutinin (HA) from a binding preference for sialic acid (SA)-α2,3 in wild birds to SA-α2,6 in humans (Klenk et al. 2013). Therefore, multiple genetic changes are required. The RNA genome of influenza has a high mutation rate, no proofreading mechanism, and is segmented, which enables the virus to be highly variable, continually acquire mutations, and frequently reassort. Agricultural practices that inadvertently facilitate the exchange of influenza A viruses at the wild bird–domestic bird interface and at the domestic bird–mammalian interface have been adopted as the need to provide additional animal protein to an increasing human population has grown.

Here we consider our basic knowledge of the properties of influenza A viruses that permit them to be so variable and the molecular determinants of host range and pathogenicity involved in the genesis of pandemic influenza viruses of domestic poultry, pigs, and humans. Since the mid-1990s, the number of influenza threats to both veterinary and human public health has increased, including the genesis of triple-reassortant viruses that contain gene segments from wild aquatic birds, swine, and humans. These include a pandemic H1N1 influenza virus in 2009 that spread globally to humans and the H5N1 and H7N9 influenza viruses that continue to evolve and spread. Although neither the H5N1 nor the H7N9 viruses have transmitted consistently among humans, the pandemic potential of these viruses cannot be overemphasized. Our challenge is to use our fundamental knowledge of influenza A viruses to prevent and/or control the emergence of influenza viruses at the aquatic bird–domestic bird–mammalian interface.

2 The Viruses

Influenza viruses are enveloped, negative-sense, single-stranded RNA viruses of the family Orthomyxoviridae, and they exist as 3 different types: A, B, and C. Influenza A and B viruses are associated with seasonal epidemics in humans, and influenza C viruses generally cause sporadic infections. Only influenza A viruses are found in a number of mammalian and bird species (Wright et al. 2007; Webster et al. 1992), and they are further classified into subtypes based on the antigenic properties of their spike-like surface glycoproteins, HA and neuraminidase (NA). At present, 18 HA subtypes (H1–H18) and 11 NA subtypes (N1–N11) have been identified. Each virus contains 1 HA and 1 NA subtype, and most of the influenza A subtypes can be found in numerous possible combinations in aquatic bird populations, except H17, H18, N10, and N11, which have been found only in bats (Tong et al. 2012, 2013). In particular, wild waterfowl are considered the main natural reservoir for influenza A viruses; thus, they play a central role in influenza A virus ecology (Fig. 1). Conversely, only a few influenza subtypes have become established in mammals, and H1, H2, and H3 are the only ones that have caused epidemic and pandemic influenza in humans.

The genome of influenza A virus is approximately 13.6 kb and consists of 8 RNA segments that encode at least 10 proteins, though a few other proteins have been recently described with as-yet unknown functions (Palese and Shaw 2007; Medina and Garcia-Sastre 2011). Viral particles

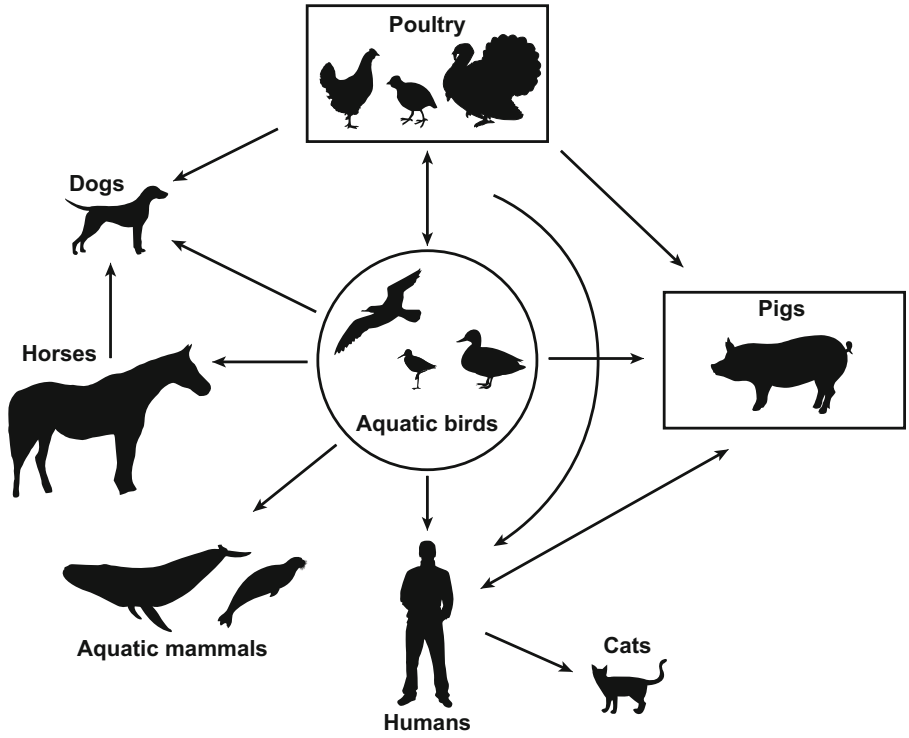

Fig. 1 Schematic illustration of influenza A virus cross-species transmission. *Solid black lines* indicate interspecies transmission events. The *circle* includes the wild aquatic birds that are the natural reservoir of most influenza A viruses. The *boxes* indicate the species most likely involved in the emergence of zoonotic viruses with pandemic potential. Influenza viruses have been isolated from bats but their role in interspecies transmission is not known

possess a host cell–derived lipid envelope, which contains transmembrane glycoproteins HA and NA and matrix proteins (M1 and M2) and surrounds the 8 ribonucleoprotein (RNP) complexes. Each RNP complex contains a single RNA segment encapsulated by the nucleoprotein (NP) and the 3 polymerase proteins (PB1, PB2, and PA) (Arranz et al. 2012; Noda et al. 2006). HA, which is responsible for virus attachment to the host cell, and NA, which assists virus maturation and release by acting as a sialidase, are the major targets of the humoral immune response (Wright et al. 2007).

Influenza A viruses are continuously evolving, and 2 key mechanisms contribute to their variability: antigenic drift and antigenic shift. Antigenic drift is the accumulation of mutations resulting from the infidelity of the virus-encoded polymerase; thus, genetic variants with selective advantages to escape the host's immune response result (Ferguson et al. 2003). Recurrent annual influenza epidemics in humans are the consequence of this gradual, progressive antigenic variation. Antigenic shift involves major antigenic changes by introducing a novel HA and/or NA subtype into the immunologically naïve human population, which leads to a pandemic. This event is caused by reassortment, typically between human and avian viruses in an intermediate host or by direct transmission of avian or swine influenza viruses to humans (Medina and Garcia-Sastre 2011; Horimoto and Kawaoka 2005) (Fig. 1).

3 Molecular Determinants of Host-Range Restriction and Pathogenesis

Influenza A viruses display host-range specificity; however, viruses from the wild bird reservoir sporadically infect humans. For this to occur,

reassortment events or evolutionary adaptation of viral molecular determinants in an intermediate host are needed for the virus to acquire mutations that allow its transmission to humans. If this happens, zoonotic diseases can occur through direct contact with infected animals, as frequently described for H5, H7, and H9 viruses (Horimoto and Kawaoka 2005; Kuiken et al. 2006). Nonetheless, zoonotic influenza viruses usually cause self-limiting illness in humans, and additional mutations are required for them to become transmissible between mammals via respiratory droplets (Neumann and Kawaoka 2015; de Graaf and Fouchier 2014). Although it is a rare event, when this happens, worldwide spread and introduction of a novel virus into the naïve human population will cause a pandemic. Major determinants in the host-range restriction and pathogenicity of these viruses have been identified and are described below, though additional studies are needed to fully understand all of the adaptive mutations that contribute to the transmission and establishment of new influenza virus lineages.

3.1 Hemagglutinin and Receptor-Binding Specificity

The viral HA is most likely the major host-restriction factor that limits infection and interspecies transmission because the cell receptor–binding requirements of the protein are determined by the sialyl sugar structures on glycoproteins or glycolipids, which are on the cell surface and differ across species (Klenk et al. 2013; Imai and Kawaoka 2012; de Graaf and Fouchier 2014). The most common form of SA is the N-acetylneuraminic acid with an α2,3 linkage or an α2,6 linkage to galactose, herein reported as SA-α2,3 and SA-α2,6, respectively. Human influenza A viruses preferentially recognize SA-α2,6, which predominates on ciliated epithelial cells that line the upper respiratory tract. Most avian influenza A viruses preferentially recognize SA-α2,3, which predominates on epithelial cells in the intestine and respiratory tracts of wild birds and domestic birds.

Specific amino acid residues at the receptor-binding pocket of the HA mediate this apparent host preference, and as few as 1 amino acid mutation can significantly change receptor-binding specificity and influence virulence. In particular, amino acid changes of glutamine-to-leucine at position 226 (Q226L) and glycine-to-serine at position 228 (G228S) affect these traits in the H2 and H3 virus subtypes, and changes of glutamic acid–to–aspartic acid at position 190 (E190D) and aspartic acid–to-glycine at position 225 (D225G) change the receptor-binding affinity of the H1 virus subtype from avian-to-human receptors (Rogers and Paulson 1983; Matrosovich et al. 2000; Glaser et al. 2005). Moreover, the distribution of the receptor type also varies by tissue location, including upper versus lower respiratory tract, cell type, and species. In humans, SA-α2,3 is found on certain alveolar cells, and the D225G substitution in HA of influenza H1N1pdm09 virus, which was observed in severe and fatal cases, enables the infection of ciliated bronchial cells in the lower respiratory tract by this virus and by viral strains of avian origin (Shinya et al. 2006; Yamada et al. 2006; Kilander et al. 2010; Chutinimitkul et al. 2010). Nevertheless, the SA-α2,6 receptor–binding preference is considered essential for an influenza virus to infect and spread easily among humans; thus, this preference limits avian viruses from transmitting from birds to humans. Recent H5N1 avian isolates from Egypt also bind SA-α2,6, and the appearance of this sublineage in the local bird population has been correlated with an increase in the bird-to-human transmission efficiency, and thus with an increase in human H5N1 virus infections (Watanabe et al. 2011). Moreover, the H7N9 viruses that emerged in China in 2013 possess the avian-type residue at position 228, but the human virus–type Q226L substitution, which confers a dual avian/human virus receptor–binding specificity (Gao et al. 2013; Shi et al. 2013). Although this most likely explains the efficient transmission of these viruses through direct contact, which has been seen in the ferret and guinea pig models, these viruses still require additional adaptive mutations

for sustained airborne transmission between mammals (Watanabe et al. 2013, Zhu et al. 2013; Belser et al. 2013; Zhang et al. 2013; Richard et al. 2013).

Recent evidence shows that the acquisition of human-type receptor–binding specificity by avian viruses requires compensatory mutations in the stem region of HA to guarantee HA stability and the optimal pH for fusion and thus determine virus transmissibility (Imai et al. 2012; Herfst et al. 2012). Importantly, amino acid substitutions that lower the pH threshold for fusion may increase the replication of A/Vietnam/1203/2004 (H5N1) in the upper respiratory tract of ferrets and play a role in airborne transmission between mammals but only in the presence of an appropriate human-type receptor–binding specificity (Zaraket et al. 2013). More than 70 mutations in H1, H2, H3, H5, and H7 HAs that affect this phenomenon have been described, which highlights how complex these processes can be (Russell 2014).

HA glycosylation also affects a variety of biological properties, including receptor-binding specificity. In particular, the loss of a glycosylation site at position 158–160 in the HA of H5N1 facilitates virus binding to the human-type receptor (Wang et al. 2010). This site is crucial for H5N1 virus virulence in mice and for airborne transmission between mammals (Imai et al. 2012; Herfst et al. 2012; Gao et al. 2009). Moreover, Neumann et al. (2012) recently reported that H5N1 viruses isolated from humans in Egypt lack this glycosylation site, which may contribute to mammalian transmissibility of avian H5 viruses. The loss of this glycosylation site is also reported in H7N9 viruses, thus supporting the virus ability to efficiently infect humans (Kageyama et al. 2013).

3.2 Other Virulence Determinants

Factors other than receptor specificity influence host susceptibility and the pathogenicity of avian influenza viruses. After a virus particle attaches to the cell receptor and is internalized into an endosome, the fusogenic activity of HA subsequently releases viral RNP complexes into the cytoplasm. To that end, mature HA must be cleaved into 2 subunits, HA1 and HA2, by tissue-specific proteases (Klenk and Garten 1994). The cleavage site of the HA of human seasonal influenza A viruses is composed of a single arginine and trypsin-like proteases that are produced by respiratory cells to recognize and cleave this motif. Most avian viruses also possess 1–2 basic amino acids at the cleavage site, and virus replication is restricted to the intestinal and respiratory tracts, thus causing mild or subclinical infection in poultry. For this reason, these viruses are referred to as "low-pathogenic" avian influenza viruses. In contrast, the HAs of highly pathogenic avian influenza viruses (HPAIVs) of H5 and H7 subtypes that emerge in gallinaceous poultry contain a polybasic cleavage-site motif that can be cleaved by ubiquitous proteases, thereby causing severe systemic infections and death in those species (Horimoto et al. 1994). Although HA cleavability represents a major virulence determinant of avian influenza viruses, its role in mammals is still unclear, as none of the human viruses possess this polybasic motif.

Although HA binds SA-containing receptors on target cells to initiate virus infection, NA facilitates the release of virus particles by cleaving the SA residues from the cell membrane. Thus, a functional balance between the HA-mediated receptor binding and fusion and the NA-mediated sialidase activity is required for efficient virus replication (Baum and Paulson 1991; Castrucci and Kawaoka 1993). In-frame deletions in the stalk region of the NA are frequently found in viruses isolated from poultry, upon transmission from wild waterfowl, and the shortened stalk length of NA is associated with enhanced replication in the intestine and respiratory tract of those species (Campitelli et al. 2004; Li et al. 2011; Zhou et al. 2009). Recent experimental findings suggest that this motif also influences influenza virus transmission (Blumenkrantz et al. 2013).

Polymerase activity also determines the host restriction of influenza viruses, and some adaptive mutations must occur during replication in mammals for an avian influenza virus to

overcome this restriction. In particular, PB2 protein plays a key role in influenza virulence and adaptation of avian influenza viruses to growth at 37 °C (the temperature of the mammalian respiratory tract). A glutamic acid–to–lysine mutation at position 627 (E627K) enables polymerase activities and viral replication in mammals (Subbarao et al. 1993; Hatta et al. 2007). Thus, this mutation is frequently selected during replication of avian viruses in humans and poultry. In addition, an aspartic acid–to–asparagine mutation at position 701 (D701N) and a threonine-to-alanine substitution at position 271 (T271A) of PB2 affect the replicative ability of avian viruses and are involved in influenza virus adaptation and transmissibility to novel hosts (Li et al. 2005; Bussey et al. 2010; Zhou et al. 2013). Several studies have shown the role of PB2 in influenza virus transmissibility and replacement of the key PB2 residues in the 2009 pandemic H1N1 virus, H5N1 strains, or the 1918 pandemic H1N1 has been directly related to higher replication in mammals or respiratory-droplet transmission (Imai et al. 2012; Herfst et al. 2012; Zhou et al. 2013; Van Hoeven et al. 2009; Zhang et al. 2012). Moreover, most of the H7N9 viruses isolated from humans possess the E627K mutation, whereas the strains isolated from avian species maintain the typical avian residue, suggesting that this mutation emerges during virus replication in humans (Kageyama et al. 2013; Lam et al. 2013; Wang et al. 2014). Besides the mutations in key PB2 residues, those in PB1, PA, and NP of the viral RNA polymerase complex also influence the host range of influenza viruses (Gabriel et al. 2005; Watanabe et al. 2009; Manz et al. 2013; Yamayoshi et al. 2014; Cheng et al. 2014; Taft et al. 2015).

Finally, PB1-F2 and NS1 proteins contribute to viral pathogenicity. In particular, PB1-F2 induces apoptosis, and an asparagine-to-serine substitution at position 66 (N66S) in the 1918 pandemic H1N1 and H5N1 viruses is associated with increased virulence (Conenello et al. 2007). NS1 antagonizes interferon production in infected cells, and the aspartic acid–to–glutamic acid substitution at position 92 (D92E) increases virulence of the HPAI H5N1 virus in mice (Seo et al. 2002). Several other amino-acid substitutions and the presence of a PDZ-ligand domain at the C terminus of NS1 enhance viral replication and thus act as determinants of virulence (Jackson et al. 2008; Twu et al. 2007).

In summary, several molecular factors may contribute to pathogenesis, host-range restriction, and transmission of influenza A viruses. Wild waterfowl are the main reservoir of most influenza A viruses, and acquisition of the adaptive mutations described above, during circulation in terrestrial poultry, may facilitate transmission to humans. Importantly, viruses isolated from domestic poultry have low affinity for SA-α2,3 compared to that of viruses isolated from wild aquatic birds, suggesting a role of these species in the emergence of new influenza viruses (Kimble et al. 2010; Perez et al. 2003; Wan and Perez 2006; Costa et al. 2012). In addition, pigs have both human-type and avian-type receptors on their tracheal epithelial cells, and the pig's role in the emergence of novel viral strains by reassortment events between viruses of different animal origins has been largely documented (Ito et al. 1998; Smith et al. 2009). Thus, poultry, quail, and pigs may serve as necessary intermediate hosts for adaptation of avian influenza viruses from their primary natural reservoir to humans.

4 Pandemic Influenza

Influenza pandemics occur when new strains of influenza viruses emerge and acquire the ability to efficiently sustain human-to-human transmission to spread worldwide. Unlike regular seasonal epidemics of influenza, pandemics occur at unpredictable intervals and can cause high levels of mortality. Three influenza pandemics occurred during the twentieth century, and the emergence of a new pandemic virus completely replaced the previous subtype virus. The 1918 influenza pandemic, also known as the Spanish flu, was the most severe, causing the deaths of approximately 50–100 million people. That pandemic was caused by an H1N1 subtype of

influenza A virus, which was probably of avian origin (Smith et al. 2009; Taubenberger and Morens 2006). Subsequent pandemics in 1957 and 1968, also known as the Asian flu and Hong Kong flu, respectively, were associated with high morbidity but killed many fewer people. The 1957 pandemic virus (H2N2) contained the HA, NA, and PB1 genes of avian virus origin from reassortment between human and avian viruses. The 1968 pandemic virus (H3N2) contained an avian HA protein of the H3 subtype and a novel PB1 protein of avian origin (Scholtissek et al. 1978; Kawaoka et al. 1989).

In 1977, the re-emergence of H1N1 viruses after a 20-year absence of the virus from circulation, caused several outbreaks worldwide that were almost exclusively among persons younger than 25 years. This suggested that older individuals were protected by pre-existing immunity (Wright et al. 2007; Nakajima et al. 1978). Since then, viruses of H1N1 and H3N2 antigenic subtypes continue to circulate and cause annual epidemics in the human population.

In 2009, the world experienced the first pandemic of the twenty first century, which was initially known as "the swine flu" and caused by a novel swine-origin influenza virus (H1N1pdm09) that rapidly replaced the previously circulating seasonal H1N1 viruses [Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team 2009]. Although the pandemic virus belonged to the H1N1 subtype, the antigenic divergence between it and the seasonal H1N1 HAs and thus the lack of cross-immunity in a large fraction of the human population, caused a rapid global spread of the novel virus, resulting in infection of 20–30 % of the world's population. The H1N1pdm09 virus is genetically related to viruses that circulate in pigs and was a product of reassortment of influenza virus genes from North American and Eurasian swine, avian, and human viruses that may have occurred years before emergence in humans (Garten et al. 2009; Smith et al. 2009). Mostly, the H1N1pdm09 virus caused a relatively mild disease, though the numbers of hospitalizations and deaths were higher among younger people and patients with an underlying medical condition who were infected with H1N1pdm09 than among those infected with seasonal influenza (Domínguez-Cherit et al. 2009; Vaillant et al. 2009). Since its appearance in 2009, the H1N1pdm09 virus has established in the human population and continues to circulate as a seasonal H1N1 influenza A virus. At present, the frequent transmission of H1N1pdm09 virus from humans into swine, and the high prevalence of reassortants with cocirculating swine influenza viruses detected in pig herds in several countries continue to pose a serious threat to public health (Simon et al. 2014; Nelson et al. 2015).

A number of findings have suggested a role for pigs in the emergence of pandemic influenza viruses, as intermediate hosts in which avian viruses adapt to mammals before they transmit to humans. The last pandemic provided clear evidence of the role of these animals in the epidemiology of influenza viruses of pandemic potential; multiple lineages of influenza A viruses cocirculate in pigs and undergo frequent reassortment. Moreover, the last pandemic highlighted the continued challenges of influenza, in terms of unpredictability. When the influenza community was alerted to a potential H5N1 pandemic, the emergence of an H1N1 was completely unexpected. For this reason, the H2N2 virus that disappeared with the emergence of the H3N2 virus in 1968 but continues to circulate in aquatic birds still poses a pandemic risk for those people born after 1968 who lack H2N2-specific immunologic memory (Jones et al. 2014). Our inability to predict the next pandemic is a public health concern, and only a continuous surveillance program to enhance preparedness will help mitigate the effects of such an unpredictable and potentially severe disease.

5 Animal Influenza

The available information indicates that the wild aquatic birds of the world, particularly ducks, shorebirds, and gulls (Anseriformes and Charadriiformes) are the natural reservoirs of

the majority of influenza A viruses (Webster et al. 1992; Fouchier and Guan 2013). Sixteen hemagglutinin (H1–H16) and 9 neuraminidase (N1–N9) subtypes and the majority of possible combinations have been isolated (Fouchier and Guan 2013; Hoye et al. 2010). Recent studies have described 2 additional subtypes of influenza A viruses from bats (H17N10 and H18N11) (Tong et al. 2012, 2013), raising the likelihood that additional subtypes will be found and that other reservoirs of influenza in nature should be considered.

In aquatic birds, the vast majority of influenza viruses are low pathogenic and replicate predominantly in the intestinal tract (Webster et al. 1978) with inapparent disease signs and are spread by the fecal–oral route (Olsen et al. 2006) and a preening-mediated way of infection (Delogu et al. 2010, 2012; De Marco et al. 2014). Although influenza A viruses have been isolated from more than 100 species of waterfowl, the predominant source is from dabbling ducks, especially mallards (*Anas platyrhynchos*) (Olsen et al. 2006; Krauss et al. 2004; Munster et al. 2007).

5.1 Wild Bird–Domestic Bird Interface

Domestic ducks and geese raised on an open range with access to comingling wild waterfowl are the obvious means of spreading influenza viruses between wild and domestic animal species (Shortridge 1992). When untreated water is used in domestic poultry houses or biosecurity is minimal, fecal droppings containing influenza viruses from wild aquatic birds or domestic ducks can be spread to the high-density poultry-raising operations (e.g., mega-chicken and -turkey operations). Other agricultural practices that provide an alternative mechanism for spreading influenza include backyard poultry raising with a mixture of ducks, chickens, quails, etc., or open-range raising of chickens, turkeys, or other domestic fowl. The ultimate man-made spreaders are the live poultry markets (wet markets) (Webster 2004; Fournie et al. 2013). These markets provide optimal conditions for ducks and geese, which have contact with the wild aquatic bird reservoir, to mix with chicken, quail, and other terrestrial domestic poultry. Traditional live poultry markets are rarely thoroughly cleaned and are topped-up daily with new birds, thereby providing the ultimate system for generating influenza virus reassortants. Such markets can be thought of as polymerase chain reaction generators and spreaders of influenza reassortants.

5.2 Domestic Bird–Mammalian Interface

Domestic poultry and swine raising place them in direct contact with human care-takers. As more animals are raised under closed conditions they increase the contact with humans. The interface between domestic birds and pigs with the general public occurs at live poultry markets and animal fairs. In this section we consider the different interfaces.

5.2.1 Domestic Poultry

All of the 16 HA subtypes of influenza A viruses detected in wild aquatic birds, including the H5 and H7 subtypes, cause asymptomatic infection in their natural hosts. However, H5 and H7 influenza subtypes are unique in that they can evolve into highly pathogenic strains in domestic chickens and other gallinaceous poultry (Klenk et al. 2013). These outbreaks of HPAIVs occur sporadically and can be traced genetically to wild-bird reservoirs (Capua and Marangon 2000). H5 and H7 HPAIVs can cause up to 100 % mortality in domestic gallinaceous poultry and can spread sporadically to mammals, including humans. The H5N1 HPAIV that emerged in Hong Kong in 1996 (A/Goose/Guangdong/1/96) and spread sporadically to humans was initially stamped-out by closing the live poultry markets and culling all poultry in Hong Kong. However, other genotypes of this HPAIV H5N1 emerged from domestic ducks in Southern China, and between 2003 and early 2004 spread to Russia and many countries in

Asia and Europe (Fouchier and Guan 2013, Medina and Garcia-Sastre 2011) The H5N1 HPAIVs have continued to evolve with several different clades and subclades (Bourouiba et al. 2010; Sonnberg et al. 2013) and are now endemic in multiple countries, including China, Vietnam, Indonesia, Bangladesh, and Egypt.

Despite the annual migration of millions of aquatic birds between Eurasia and Alaska, the H5N1 HPAIV failed to spread to the Americas between 1997 and 2013. This changed dramatically after the emergence of the H5N8 clade 2.3.4.4 influenza virus in South Korea in 2014 (Jeong et al. 2014; Lee et al. 2015). This H5N8 HPAIV was spread by migratory waterfowl to Russia, Europe, and Canada in 2014 (Lee et al. 2015). After the H5N8 HPAIV arrived in the Americas, it reassorted with H?N1 and H?N2 influenza viruses of North American lineage to generate H5N1 and H5N2 HPAIVs (Pasick et al. 2015). During the spring of 2015, these H5N1 and H5N2 HPAIVs were transmitted to and spread by wild aquatic birds and raptors to domestic poultry throughout the Mississippi Valley. This caused a catastrophic loss of domestic poultry, with more than 250 detections and the culling of more than 50 million birds (OIE WAHIS 2015). Although the H5N1 clade 2.3.4.4 viruses have reportedly infected humans in China, there is no evidence to date that the H5N1, H5N2, and H5N8 HPAIVs in the Americas have transmitted to humans or lower mammals.

The H9N2 influenza that emerged in Asia in the early 1990s evolved into two clades represented by A/Quail/Hong Kong/G1/97 (G1 lineage) and A/Chicken/Beijing/1/94 (Y280/G9) (Guan et al. 1999). Since 1998, there have been sporadic mild infections of immunocompromised humans but without evidence of human-to-human transmission. Transmission of the G1 lineage to swine has also been reported (Peiris et al. 2001). These viruses have become widespread in chickens in Eurasia but to date have not spread to the Americas. Although H9N2 viruses cause inapparent infection in chickens experimentally, they can cause severe weight loss with markedly reduced egg production and increased respiratory disease when coinfections with other disease agents occur in the field. Consequently, inactivated H9N2 vaccines are used in endemic areas (Zhang et al. 2008). The major concern about H9N2 viruses is their contribution of internal gene segments to reassortant influenza viruses that have become transmissible in humans, including the H5N1 HPAIV and the H7N9 influenza viruses. Other influenza viruses that have acquired H9N2 gene segments include H5N2, H7N7, and H10N8 (To et al. 2014) that have been isolated from avian, swine, and human hosts.

The H7N9 influenza virus that transmitted more efficiently to humans than did the H5N1 HPAIV was again a triple reassortment with 6 internal gene segments from the dominant H9N2 influenza virus in domestic poultry in Asia and the HA and NA from different Asian H7N? and H?N9 influenza viruses circulating in wild aquatic birds (Gao et al. 2013; Lam et al. 2015). This H7N9 reassortant that caused severe disease and lethality in humans differs from the cocirculating H5N1 HPAIV in that it is low pathogenic in domestic poultry. Live poultry markets served as the main interface between domestic poultry and humans. As of July 2016, 793 cases of H7N9 were reported in humans, and 319 deaths had occurred. To date, all H7N9 human infections have occurred in China, including the cases in travelers in Taiwan, Canada, and Malaysia. The 20 human clusters of H7N9 attest to the transmissibility of the virus in humans, though consistent human-to-human transmissibility has not occurred, and close monitoring of cases is ongoing.

The role of domestic poultry in the transmission of influenza viruses to humans was clearly demonstrated in studies of live poultry markets. Such markets provide optimal conditions for the transmission of avian influenza viruses to humans. This was clearly demonstrated when live poultry markets in Hong Kong were closed in 1997, and the number of human cases of H5N1 infection fell from 18 to 0. Following the emergence of the H7N9 influenza virus in China in

2013 (Gao et al. 2013) that caused as much as 30 % lethality in humans, the closure of live poultry markets again was correlated with a reduction in the number of human cases (Wu et al. 2014).

5.2.2 Swine Influenza

Although many avian influenza A viruses have been isolated from swine, including H2N3, H3N3, H4N6, H5N1, H9N2 (reviewed in Webby and Richt 2013), only H1N1, H3N2, and 2 reassortants (H1N2 and H3N1) have become established in these animals globally during the past century. Swine, which are described as an intermediate host for transmission of influenza viruses from the avian reservoir to other mammalian species, express dual receptors for avian and mammalian influenza viruses (both SA-α2,3 and SA-α2,6) and have a lower core body temperature than avian species (Scholtissek 1990). The role of the pig in the exchange of influenza virus genes between species was clearly demonstrated in Europe in 1979, when an influenza virus from wild birds transmitted to and became established in swine (Pensaert et al. 1981). Subsequently, this avian influenza virus reassorted with human influenza viruses in swine and caused disease (Castrucci et al. 1993, Campitelli et al. 1997). The role of the pig in the genesis of influenza viruses was also clearly illustrated in the emergence of triple reassortants in swine in the U.S. (Zhou et al. 1999; Webby et al. 2000), with 5 gene segments (HA, NP, NA, M, NS) from classical swine influenza virus, 2 from North American wild aquatic birds (PB2, PA), and 1 from humans (PB1). Subsequently, the triple reassortants from swine in the U.S. reassorted with a swine influenza virus of Eurasian avian origin to acquire its NA and M genes and produced the 2009 pandemic H1N1 influenza that spread globally. It is noteworthy that the human pandemic 2009 H1N1 virus transmitted freely back to pigs, where it reassorted with a human-like H3N2 influenza virus. The H3N2 variant in swine acquired the M-gene segments and is the virus that readily infects children at U.S. county fairs (Bowman et al. 2012). Therefore, influenza A viruses are truly zoonotic, and the human–lower mammal interface shows increasingly frequent genetic exchanges.

6 Increasing Threats of Influenza at the Human–Animal Interface

Since the 1990s, more influenza viruses have appeared to sporadically transmit to intermediate animal hosts and humans. Influenza viruses in poultry and pigs have shown modest antigenic drift and shift for most of the twentieth century and were genetically conserved with limited reassortment. After the emergence of the 1918 H1N1 Spanish influenza, the virus transmitted to swine and caused classical H1N1 swine influenza. In the U.S. pig population, this H1N1 virus remained remarkably genetically conserved with minimal antigenic drift and no detectable reassortment for more than 70 years (Shu et al. 1994). This changed after the emergence of the triple reassortant, the 2009 H1N1 human pandemic, and the continuing generation of multiple reassortants.

The greatest number of challenges at the human–animal interface comes from avian influenza viruses. This includes the highly pathogenic H5N1 that is now endemic in China, Vietnam, Indonesia, Bangladesh, and Egypt; the H7N9 that is low pathogenic in poultry but is up to 30 % lethal in humans; and the H9N2 influenza viruses that are low pathogenic and spread sporadically to humans, pigs, and dogs.

It is instructive to examine the historical records of human infections with H5 and H7 influenza viruses. Prior to 1996, there are no recorded infections of humans with H5 influenza viruses and only sporadic infections of humans with H7 viruses (Subbarao and Katz 2000), including the death of a veterinarian in the Netherlands and serological evidence of human infection (Fouchier et al. 2004). Since 2003, there have been 854 laboratory-confirmed human cases of H5N1 influenza infection with 450 deaths and 793 cases of H7N9 infection with 319 deaths (WHO website 2016).

Table 1 Increases in the global populations of humans, poultry, and pigs

Species	Global populations		Approximate increase in the population (fold)	Top 3 countries with the largest population increase		
	1961	2013		#1	#2	#3
Human	3 billion	7.1 billion	2.4	China	India	USA
Chicken	3 billion	20 billion	6.5	China	USA	Indonesia
Duck	200 million	1 billion	5	China	Vietnam	Malaysia
Swine	406 million	977 million	2.2	China	USA	Brazil

Changing Conditions that Favor Influenza Transmissibility

1. Global land use is increasing, with expansion into forests and wetlands.
2. More than two-thirds of the terrestrial biosphere is transformed into anthromes.
3. Human life expectancy is increasing.
4. About 75 % of the global human population lives in cities.
5. Genetic engineering of plants and animals has increased food supplies.
6. Global warming and overuse of fresh water will increase interspecies contact.
7. Rapid mass transportation of people and products has increased the global spread of emerging infectious agents.
8. The ecology of influenza viruses in the aquatic bird reservoir may be changing in that highly pathogenic H5 and H7 influenza viruses are more frequently spread by migratory birds but the evidence indicates that the highly pathogenic H5 and H7 influenza viruses are not perpetuated in the aquatic bird reservoir.

Multiple ecological factors at the human–animal interface influence the increased number of human fatalities caused by influenza. One factor is the increase in global populations of animals that are considered intermediate hosts between the aquatic bird reservoir and humans (FAOstat website 2015) (Fig. 1). The global population of ducks and chickens has increased by approximately 5 fold and 6.5 fold, respectfully, over the past 52 years, while the global population of swine and humans has approximately doubled (Table 1) (FAOstat website 2015). These increases have occurred primarily in China, India, and the U.S. Multiple ecological changes continue to occur with increased global land use, human life expectancy, and rapid transport of people and pathogens (Jernigan and Cox 2013).

From a viral ecological perspective, the following three events are associated with increased animal-to-human transmission of influenza viruses: (1) The transmission of avian influenza virus to swine in Europe and the subsequent reassortants in the 1990s with human H3N2 viruses (Campitelli et al. 1997) to generate a virus with avian and human influenza genes in swine. (2) In the U.S. in the early 1990s, double- and triple-reassortant influenza viruses emerged in pigs. (3) In China, triple reassortants of H5N1 containing 6 gene segments from H9N2 influenza virus (Guan et al. 1999) and the surface glycoproteins from different avian influenza viruses emerged in the 1990s and were highly pathogenic in poultry and people. Each of these three events represented the transmission of genetic properties from the aquatic bird reservoir to pigs, poultry, and humans. The molecular basis of the increased transmissibility of these viruses contributed by gene segments from wild birds has not been fully resolved. As the global population of humans continues to increase, it will impinge increasingly on the use of land and water to provide the food supplies required. It is inevitable that the frequency of exchange of influenza viruses and influenza genetic information between natural reservoirs and humans will increase. The challenge for humanity will be to rapidly identify those influenza viruses that have the ability to not only transmit and cause disease in humans but also transmit efficiently among humans.

7 Conclusions

The realization that the aquatic birds of the world are the reservoir for most of the influenza viruses that are ultimately a threat to veterinary and human public health raises the possibility of designing strategies to reduce influenza virus exchange at the aquatic bird–domestic bird–swine–human interface. A first requirement is knowledge of the fundamental properties of the influenza viruses that will provide insight into designing such strategies. At the practical level, we need to continue biosecurity education for farmers who raise domestic poultry and pigs, so that they are aware of biosecurity and the increasing risk of open-range poultry and pig farming as the global populations of those animals increase. Perhaps the most egregious practice is live poultry and livestock markets, which optimally should be phased-out. This is already happening in China. The Food and Agricultural Organization of the United Nations (FAO 2015) has also recently recommended implementing a policy of no carryover of animals (i.e., housing the same animals in the marketplace for multiple days), which improves live-market design to reduce influenza virus exchange. However, many issues and questions still need to be addressed:

- Influenza viruses in their natural reservoirs most likely cannot be eradicated. One conceivable approach to this issue would be to genetically engineer domestic animals to be completely resistant to influenza; however, acceptance of genetically engineered animals is problematic.
- The role of the host in determining influenza susceptibility is being elucidated. For example, the *RIG-1* gene that is involved in interferon signaling and modulates influenza in ducks is absent in chickens (Barber et al. 2010). Can influenza-resistant domestic animals be developed as more influenza host–resistance genes are elucidated?
- On the basis of genomics analysis, can we predict which influenza viruses in the aquatic bird reservoir have the potential to spread among humans? Although we know some of the molecular determinants for virulence of H5 and H7 viruses, how these viruses transmit to mammals (including humans) is not fully understood. Similarly, information on H1, H2, H3 viruses does not allow us to predict which influenza viruses in the aquatic bird reservoir have pandemic potential.
- Is the ecology of influenza changing? Are the highly pathogenic H5 and H7 viruses perpetuated in the wild aquatic bird reservoir? In the aquatic bird reservoir, only the non-pathogenic influenza viruses are perpetuated and spread from one generation to the next. There is little doubt that the highly pathogenic H5N1 from [Asia?] was spread to Europe by migratory waterfowl and that H5N8 from Asia was spread to the Americas by migratory waterfowl, but there is no evidence that the highly pathogenic H5N2 virus that evolved in the Americas is now being perpetuated in the aquatic bird reservoir? This is a controversial issue but 43 years of surveillance in aquatic birds and the absence of HP H5N2 influenza viruses in The Americas after the severe outbreaks in domestic poultry in 2015 supports the conclusion that HP influenza viruses are not perpetuated in aquatic birds and transmitted to the next generation. Thus the ecology of influenza in wild aquatic birds is not changing.
- Will H5, H7, and H9 influenza virus reassortants acquire consistent human-to-human transmissibility? Studies in ferrets indicate that a limited number of mutational changes in the HA facilitates aerosol transmissibility (Imai et al. 2012; Herfst et al. 2012). While the H5NX, H7NX, and H9N2 viruses continue to circulate in domestic poultry, the threat to humanity will persist.
- The use of influenza vaccines at the domestic poultry–mammalian interface has many pros and cons. Although efficacious influenza vaccines for domestic poultry are available

(Swayne and Kapczynski 2008), they should be used only as a tool towards eradication and then discontinued. In those countries that continue to use poultry vaccines to control HPAI H5N1 or low-pathogenic H9N2 influenza, the viruses are now endemic and a continuing threat to humanity.

- The possibility of developing universal vaccines for influenza (Krammer and Palese 2015) could change current approaches to influenza control, at least in mammals. Such vaccines would probably not provide sterilizing immunity, which is a problem with current poultry vaccines (Swayne and Kapczynski 2008).

Our overall challenge is to use our fundamental knowledge of influenza viruses to prevent and/or control the emergence of influenza viruses from the aquatic bird reservoir and to change agricultural practices that facilitate interspecies transmission.

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Bats and Emerging Infections: An Ecological and Virological Puzzle

Jordi Serra-Cobo and Marc López-Roig

Abstract

More than 200 viruses have been detected in bats. Some unique bat characteristics can explain the roles played in the maintenance and transmission of viruses: long phylogenetic history can have originated coevolution processes, great number of species are adapted to live in different environments, big mobility, long lifespan and gregarious behaviour of many species.

To analyse zoonoses long longitudinal studies are needed with a multidisciplinary approximation to obtain the following eco-epidemiological data: colony size, number of bats per species, population structure, behaviour of each species, degree of contact between bats, social structure, remaining time of bats in the colony, colony type, foraging area, turnover rate of individuals, shelter temperature, relationship with other colonies and co-infection processes. These data allows assessing the epidemiological risk and which preventive measures are necessary to take.

The structure and functionality of ecosystems are changing worldwide at an unprecedented rate and can modify the interactions between humans and infected bats. There are more or less local factors that can affect the emergence and spread of diseases (environmental alterations, changes in land use, human population growth, changes in human socioeconomic behavior or social structure, people mobility increase, trade increase, forest fires, extreme weather events, wars, breakdown in public health infrastructure, etc.).

Twenty-three percent of all bat species in the world are decreasing. How does the regression of bat species affect the dynamic of viruses? The

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dichotomy between health risk and bat preservation is compatible with a preventive task based on more information and training.

Keywords
Bats • Zoonoses • Emergent viruses • Bat ecology • Public health

1 Introduction

The result of millions years of evolution has given rise to an extraordinary biodiversity with complex inter and intraspecific interrelations of the species and also with the environment in which they live. Each host species performs a specific ecological function, followed by a particular story and interacts with other species and their environment. The million years of evolution also has given rise to the co-evolution processes between host and pathogen, very important to understand the virus dynamic in host populations. All these complex interrelations between host-pathogen-environment require analysis of multiple biological and environment factors and this is only possible with a multidisciplinary approach. All these factors must be taken into account in eco-epidemiological studies and are very important in order to find preventive measures that reduce the risk of transmission to the human population, livestock and pets.

The evolution of bats is a very successful singular history amongst the mammals that has produced an enormous diversity of species adapted to a great spectrum of environments.

In recent years, bats have been implicated in numerous emerging infectious disease events and have been recognized as important reservoir hosts for viruses that can cross the species barrier to infect humans and other domestic and wild mammals (Calisher et al. 2006; Hayman et al. 2013; Moratelli and Calisher 2015). More than 200 viruses of 28 families have been isolated or detected in bats, distributed in the two suborders, 11 families and 37 genera of bats (Table 1). These viruses apparently cause little or no pathology in bats. A comparative analysis (Luis et al. 2013) showed bats to be more likely to be infected with more zoonotic viruses per host species than rodents, thus adding weight to the importance of bats as reservoir and they play a key role in dynamic of viruses (O'Shea et al. 2014). For example, numerous bat species can be infected by lyssaviruses. Bats serve as reservoirs of 13 out of the 15 lyssavirus species described (the only lyssavirus species that have not been isolated from bats, to date, are Mokola virus and Ikoma virus). Furthermore, recently described lyssavirus species enlarged the genetic diversity of lyssaviruses found in bats, suggesting that the lyssaviruses originated in these mammals and progressively diverged from a common ancestor (Badrane and Tordo 2001; Delmas et al. 2008).

The epidemiological studies made about bats raise different questions. Why are bats good virus reservoirs? What is the dynamic of viruses in bat populations? Are there processes of co-evolution between bats and pathogens? Can the environmental changes influence the dynamic of viruses and the risk of transmission to humans? What are the main gaps in the study of bat zoonosis? Which are the risk factors and the prevention tasks? These questions have no simple answers because it requires a multidisciplinary approach that should take into account a diverse range of abiotic and biotic factors. The world in which we live is extremely complex, with a multitude of relationships between living organisms and the environment where they live. For example, the emergence of a viral epidemic will depend on the dynamics of the virus, which in turn will be influenced by external environmental factors. The dynamics of the reservoir species influence the virus dynamics. But the reservoir depends on factors such as temperature, rainfall, state of conservation of the habitat, the situation of stress affecting the species, etc. The aim of this paper is to contribute to elucidate this ecological and virological puzzle.

Table 1 Viruses found in bats

Virus	Genus
Family *Adenoviridae*	*Mastadenovirus*
Family *Arenaviridae*	*Arenavirus*
Family *Astroviridae*	*Mamastrovirus*
Family *Bornaviridae*	*Unnamed genus*
Family *Bunyaviridae*	*Orthobunyavirus*
	Hantavirus
	Phlebovirus
	Nairovirus
Family *Caliciviridae*	*Sapovirus*
Family *Circoviridae*	*Circovirus*
	Cyclovirus
Family *Coronaviridae*	*Alphacoronavirus*
	Betacoronavirus (SARS, MERS)
Family *Dicistroviridae*	
Family *Filoviridae*,	*Cuevavirus*
	Ebolavirus
	Marburgvirus
Family *Flaviviridae*	*Flavivirus*
	Hepacivirus
	Pegivirus
	Pestivirus
Family *Hepadnaviridae*	*Orthohepadnavirus*
Family *Hepeviridae*	*Unnamed genus*
Family *Herpesviridae*	*(Alpha-herpesvirinae) Simplexvirus*
	(Beta-herpesvirinae) unnamed genus
	Cytomegalovirus
	Percavirus
	Rhadinovirus
	Macavirus
Family *Nodaviridae*	*Nodavirus*
Family *Orthomyxoviridae*	*Influenzavirus A*
Family *Papillomaviridae*	*Omegapapillomavirus*
Family *Paramyxoviridae*	*Morbillivirus*
	Henipavirus
	Rubulavirus
	Pneumovirus
Family *Parvoviridae*	*Dependovirus*
	Bocavirus
Family *Picobirnaviridae*	*Picobirnavirus*
Family *Picornaviridae*	*Kobuvirus*
Family *Polyomaviridae*	*Unnamed genus*
Family *Poxviridae*	*Molluscipoxvirus*
Family *Reoviridae*	*Orbivirus*
	Orthoreovirus
	Rotavirus
Family *Retroviridae*	*Betaretrovirus*
	Spumavirus
	Gammaretrovirus
Family *Rhabdoviridae*	*Lyssavirus*
	Vesiculovirus
Family *Togaviridae*	*Alphavirus*
Family *Totiviridae*	*Totivirus*
Unassigned family	*Hepevirus*

2 Why the Bats Are Good Virus Reservoirs?

The roles played by bats in the maintenance and transmission of viruses requires consideration of the unique characteristics that distinguish bats from all other mammals.

2.1 Evolution and Phylogeny of Bats

The origin of bats is estimated in about 64 million years ago at or following the Cretaceous-Tertiary boundary. The Order *Chiroptera* is classified in *Yinpterochiroptera* (*Rhinolophoidea* and *Pteropodidae*) and *Yangochiroptera* (all other bat families) (Teeling et al. 2005). Hence this long period of time can have originated coevolution processes between bats and viruses. The analysis done by Luis et al. (2013) indicated that bats host more zoonotic viruses and more total viruses per species than rodents, despite there are a lot more of rodent species in the world. Zhang et al. (2013) did an extensive genomic analysis of two distantly related species of bats and found a concentration of positively selected genes in the DNA damage checkpoint and nuclear factor kB (protein complex that controls transcription of DNA) pathways that may be related to the origin of flight. These authors propose that the flight evolved in tandem with concomitant genetic changes to their innate

immune systems. These changes were related to the need of DNA damage repair because of high metabolic rates that are produced during flight. Baker et al. (2013a, b) suggested the possibility that bats might be able to control viral replication through innate immunity.

2.2 Species Richness

The bat evolution has led to a great number of species adapted to fly, to consume a wide range of food resources and live in very different environments, characteristics that allowed them to colonize much of the terrestrial ecosystems. We find bats in deserts oasis, tropical and subtropical rainforests, plains near the sea, mountains relatively high, islands far from continents, temperate regions, boreal regions, etc. There are insectivorous, frugivorous, carnivorous, piscivorous, haemtophagous, nectarivorous, and scavengers bat species, and there are also bats that eat scorpions. Bats are the second largest order of mammals and currently, there are ~1200 worldwide recognized bat species, accounting for approximately 21 % of all mammalian species. Every year new bat species from around the world are described, found not only in tropical and subtropical regions but also in temperate regions. The species richness increases towards the tropics and in most tropical areas bat diversity is higher than in any other group of mammals (Moratelli and Calisher 2015).

They are the only mammals that can fly and they play a major ecological role as insect predators, seed dispersers and pollinators. Approximately 75 % of microchiroptera species are insectivorous and make an important control about the insect populations. In a longitudinal study done in Natural Park of Sant Llorenç del Munt i l'Obac (Barcelona, Spain), we estimate that *Miniopterus schreibersii* population in about 17,000 individuals that could annually consume between 15 and 30 tonnes of insects. McCraken estimated in about 10 tonnes of insects the daily consume of a big breeding colony constitute by a million of *Tadarida brasiliensis* that lived in Texas (McCracken and Wilkinson 2000).

The diversity of bat species and their worldwide distribution contribute to the biodiversity of their pathogens.

2.3 Ability to Fly

Bats have a high mobility and have the potential to spread rapidly and widely the viruses. The flight provides them more mobility than the greatest part of terrestrial mammals have, including rodents. Some species can do long seasonal movements, behaviour that can enable the virus spread into regions that are more or less far away. The migration is an important component in the life cycle of numerous animals, especially in a changing world in space and time. The seasonal movements not only allow to escape from the adverse conditions, but also possibilities the exploitation of news habitats and contribute to the gen flow between colonies. Cross et al. (2005) showed that the probability of a pandemic event depended on the interaction between colony size and movement of hosts amongst groups during their infectious lifetime. They suggested that the gregarious hosts that form large groups and frequent movements are more heavily impacted by acute diseases than hosts with small groups and infrequent movement. It is possible to find examples of long bat migrations in all continents. Some African species perform seasonal migrations between rain forest areas, where they remain during the dry season, and savannah areas where they frequent during the rainy season (Ossa et al. 2012). *Eidolon helvum* is an African bat species on which it's found neutralizing antibodies against *Zaire ebolavirus*. This species forms big colonies and does seasonal migrations that can exceed the 2500 km (Sørensen et al. 2001; Richter and Cumming 2008; Hayman et al. 2013). *E. helvum* can play a role in the spread of filoviruses. In this sense, Ogawa et al. (2015) suggest in their work on *E. helvum* the introduction of multiple species of filoviruses in the migratory bat population and point to the need for continued surveillance of filovirus infection of wild animals in sub-Saharan Africa, including

hitherto nonendemic countries. Pons-Salort et al. (2014) show the importance that can have *Miniopterus schreibersii* in the dynamic of European Bat lyssavirus on the bat populations of Balearic Islands. They found that EBLV-1 could not be sustained if transmission between *M. schreibersii* and other bat species was eliminated. This species can do seasonal movements of some 100 km and can move between Mallorca and Menorca islands (Amengual et al 2007a, b).

Bats, except for most species of megachiroptera, emit ultrasounds to orient themselves during the flight, to communicate between them and also to locate and capture their prey. Lazzaro Spallanzani, considered one of the fathers of experimental biology, observed in 1793 that bats that were bereft of vision were able to orient themselves without any problem. Later, Spallanzani and Jurine found that bats need the sense of hearing to navigate and orient themselves during flight. It was not until 1941 that Griffin and Galambos showed that bats use ultrasound for orientation. Griffin proposed the term echolocation to define the process by which bats locate objects that can not see or touch thanks to the emission of acoustic signals and its echo analysis. We can distinguish two groups according to the type of bats that emit ultrasonic signals: the species that emit ultrasonic signals of constant frequency and narrow frequency band (these bats use as a nasal cavity as a resonator organ); species that emit FM ultrasonic signals with a frequency of wideband (these bats use buccal cavity as a resonator organ), where the frequency is modulated by the position of the tongue and lips.

Production of such loud sounds could generate droplets or small-particle aerosols of oropharyngeal fluids, mucus, or saliva, enabling transmission of viruses between individuals in close proximity. Airborne rabies virus transmission was documented in a large colony of Mexican free-tailed bats constituted by several million of individuals (Constantine 1967). Furthermore, the isolation of rabies virus from mucus obtained from naturally infected same species of bats could support the hypothesis that rabies virus could be expelled from the nostrils of echolocating bats (Constantine et al. 1972; Winkler 1968).

2.4 Long Lifespan and Bat Ecology

The existence of a trade-off between lifespan and reproduction is central to the concept of life history strategy (Partridge and Harvey 1988; Stearns 1992). Organisms cannot simultaneously maximize both of these traits in the nature (resources are limited) but must balance investment in survival versus offspring to maximize its lifetime reproductive fitness. Moreover, in stable populations, survival and birth rates must be inversely related (Sibly and Calow 1987). The evolution of life history therefore is constrained along a slow–fast continuum strategies, in which species with slow life histories generally have higher survival rates, live longer maximum lifespans, mature at older ages and produce fewer young per year compared with species with fast life histories. The bats belong to this type of life history and they have a long lifespan greater than most of mammal species of the same size (for example mice and shrews). The greater longevity observed in a bat is 41 years in a *Myotis brandtii* (Wilkinson and South 2002). However, the longevity of most species is much lower. The greater lifespan observed in our studies ranged from 8 years in *Pipistrellus khulii* to 17 years in *R. ferrumequinum*. The extreme longevity of bats, together with the possibility that they might develop persistent infections with certain viruses, may help to maintain the viruses and transmit them to other vertebrates.

Lifespan is generally longer in heterothermic mammals (such as bats) than in related homeotherms. Heterotherms can employ several strategies (as hibernation) to withstand adverse periods and then repopulate when circumstances improve. Hibernation is associated with high rates of overwinter and annual survival and an increase in survival in hibernating species is linked to the coevolution of indicative traits of relatively slow life histories (Turbill et al. 2011). Endothermic mammals have the ability to

maintain a constant high body temperature (Tb) over a wide range of ambient temperatures (Ta). However, keep a constant high Tb can have a high energetic cost. This is especially critical for small mammals because heat loss increases with decreasing size as a result of increasing relative surface area. Small size also limits the relative amounts of fat that can be stored. As heat loss is a function of the Tb-Ta differential, thermoregulatory costs at low Ta may become prohibitively expensive in small endotherms, especially when energy availability is low (Kronfeld-Schor and Dayan 2013; Geiser 2004).

For this reason, bats use two common patterns of torpor as physiological and behavioral adaptation that permits survival during seasonal periods of low resources: daily torpor and hibernation or multiday torpor.

Daily torpor is widely used in bats and it is an important strategy for coping with fluctuating environments, involves significant plasticity, and may constitute an important part of how endotherms cope with environmental challenges. The daily torpor is not as deep as hibernation, lasts only for hours rather than days or weeks, and is usually interrupted by daily foraging and feeding. Bats used daily torpor in response to adverse conditions such as low food availability and low ambient temperature, mainly when their mass and fat stores are low (Geiser 2004; Geiser and Stawsky 2011).

Hibernation is often seasonal and usually lasts from late autumn to late winter/spring. However, hibernating bats do not remain torpid throughout the hibernation season. Bouts of torpor, during which body temperature (Tb) is low and bodily functions are reduced to a minimum, last for several days or weeks, but are interrupted by periodic rewarming and brief (usually less than 1 day) normothermic resting periods with high Tb and high energy turnover. Over-winter survival of hibernating bats should depend primarily on the size of their energy reserves at the onset of hibernation, the rate at which the energy store is depleted during winter, and the length of the winter. If the size of the reserve is less than the rate of depletion times the length of winter, the hibernator will not survive (Humphries et al. 2003). In this sense, bats exhibit selection in roost choice, showing preferences linked to their ecological requirements, which differ among species, different seasons (Kunz 1982; Kunz and Lumsden 2003) and geographical areas (Rodrigues et al. 2003). Bats spend a considerable part of their life roosting, and thus roost characteristics have important implications for survival and reproductive success (Kunz 1982). Roost location, structure and aspect determine microclimatic conditions, which may influence the energetic costs of key stages of life cycle such as hibernation (Humphries et al. 2002), pregnancy and lactation (Sedgeley 2001; Kerth et al. 2001). Consequently, summer and winter shelters often differ in microclimates conditions and bat populations move seasonally between them, thereby connecting seemingly isolated populations. The metapopulation structure, social organization within the refuges (intra- and interspecific interactions), where multiple bat species usually cohabit, can play important roles in the dynamics of virus persistence. Persistent viral infections occurring among long-lived bats together with their often gregarious roosting behaviour, could greatly increase the potential for intra- and inter-species transmission of viruses (Halloran 1998). Some bat species form a very large monospecific or multispecific colonies of thousands individuals tight against each other (Fig. 1). For example, we estimated the density of the hibernation colony of *M. schreibersii* that lives near Barcelona in 1900 bats for square meter. This dense clustering of individuals can provide large opportunities for viral exchange in bat colonies. However, apart from the colony size, bat species richness appear to be another ecological factor strongly associated with European bat lyssavirus type 1 seroprevalence in bat colonies (Serra-Cobo et al. 2013). The bat colonies are often composed by more than one species, which may facilitate the virus transmission between species. For example, in South of Europe there are mixed colonies where *M. schreibersii*, *M. myotis* and *M. capaccinii* have direct physical contact amongst them and also their urine and guano. This suggests that interspecies virus transmission

Fig. 1 Multispecies bat colony of *Myotis punicus* and *Miniopterus schreibersii* shelter in a Moroccan cave. The two bat species are tight against each other

plays an important role in EBLV-1 dynamics. A high number of species might not only increase the rates of contact between bat groups and species, but also could facilitate virus entry or spread through the higher mobility of individuals among colonies, especially if these bats exhibit a migratory behaviour.

3 Virus Dynamic in Bat Populations

The dynamic of viruses is the result of the interaction between the characteristic of pathogen, the life history traits of host population and the environmental factors. The seasonality existing in great part of the world (winter, spring, summer, autumn, dry and rainy seasons) determines the birthing periods, migration, gregarious behavior and the torpor of bat species. Each of these aspects of the bat life may affect population density, rates of contact between individuals and immune response, thus leading to spatiotemporal variation in infection dynamics (George et al. 2011; Hayman et al. 2013).

Some authors suggest that the pathogen replication in the host is generally very temperature-dependent (Sadler and Enright 1959; Sulkin et al. 1960; Luis and Hudson 2006; Bouma et al. 2010). Different studies demonstrated that bat lyssavirus dynamics exhibit a strong seasonal pattern and that the breeding period could favor bat infection (George et al. 2011; Serra-Cobo et al. 2013). We analyzed ecological and epidemiological factors that might be associated with the infection dynamics of EBLV-1 observed in Spanish bat colonies. The analyses revealed that the colony size and species richness were two important ecological factors and showed their relevant roles in seroprevalence variability. Higher seroprevalence was observed in multispecies colonies compared to monospecific colonies, suggesting that interspecific virus transmission plays an important role in EBLV-1 dynamics. The results suggested that EBLV-1 seroprevalence was strongly affected by the colony size and species richness, and indicated that multispecies, large colonies, especially those with three or more different bat species, had a higher probability of EBLV-1 infection (Serra-Cobo et al. 2013). Large colonies and multispecies associations occurred frequently among cave-dwelling bats, principally during the maternity period. This colonial behaviour confers thermodynamic and social advantages to reproductive females during pregnancy and lactation (Willis and Brigham 2007).

In order to know the virus dynamic is necessary to carry long longitudinal studies with a multidisciplinary approximation. The long active surveys can provide information about the

dynamic of the host population and the cycles followed by virus infection. The work of monitoring the bat colonies usually is not easy. Their shelters have often difficult access, it's necessary to work during night and are animals with a great mobility, factor that difficult their recapture. The long longitudinal studies of bat colonies using capture-mark-recapture techniques can provide the infection history at individual level in species with long lifespan. These studies also allow obtaining demographic and epidemiological parameters (mortality, survival and turnover rates, colony size, immune lifespan, period of infections cycles, distribution of individuals infected in the colony, basic reproductive rate of virus, threshold population for infection).

The basic reproductive number (R_0) is an important parameter in the diseases dynamic and is the average number of new infections that would arise from a single infectious host introduced into a population of susceptible hosts. The immunity induced by many viral infections reduces the number of susceptible individuals, which reduces R_0, which therefore tends to lead to a decline in the incidence of the infection itself in the colony. However and before the infection disappears from the population, it is likely there is an influx (births, immigration and loss of immunity) of new susceptible into the population that will produce a subsequent increase in susceptible and R_0, and so on. Theoretical works about disease invasion have assumed large and well-mixed host populations. However, many wildlife systems have small groups with limited contacts amongst them. In these situations, the R_0 is likely to be a poor predictor of a disease pandemic because it typically does not account for group structure and contacts of individuals amongst groups. For example, Kerth et al. (2011) detected two stable subunits in a larger colony of *Myotis bechsteinii* and each of them comprised bats from several family lineages. These authors showed that the links between these subunits were mainly maintained by older bats and persisted over all years.

The immunity lifespan is a parameter that should be taken into consideration in epidemiological studies because influences the disease dynamic in a bat colony. The immunity response and the lifespan of immunity in front of a virus infection can differ between bat species. So, it is more likely to find seropositive bats in the species with long lifespan immunity. This is a factor to consider in the serological active survey. For example, EBLV-1–seroprevalence differences were found between bat *Vespertilionidae* and *Rhinolophidae* families. This variability might be explained by different immunological responses of the bat species to EBLV-1 virus. These differences might rather suggest different seroconversion rates in these two families (Serra-Cobo et al. 2013). Despite his interest, there is very few information about the lifespan of immunity of bat species in front of viruses infection.

The threshold levels of host abundance for invasion or persistence of infectious diseases are an important epidemiological parameter. The notion of a threshold population for invasion is a founding principle of epidemiological theory (Lloyd-Smith et al. 2005). This parameter is important in the gregarious bat species. But in order to be applicable is necessary to know the spatial structure and dynamic of the population. Most of gregarious species of bats have a metapopulation structure (consisting of periodically interacting, spatially discrete subpopulations) with variations in their subpopulations. The total number of individuals in the various subpopulations must be sufficient to maintain virus circulation in the metapopulation over time, while immunity or death due to viral infection extinguishes transmission chains within individual subpopulations. We observed this phenomenon of local virus extinctions in bat colonies from Spain that follow a metapopulation structure. All efforts to eradicate wildlife diseases by reducing the numbers of susceptible hosts through controversial methods such as culling, sterilization, or vaccination are based in the threshold concept. However, the general applicability of standard threshold concepts in wildlife disease systems is very difficult and often forgets many factors relevant to natural populations (turnover rate of population, migrations, immunity loss).

A long longitudinal study done from 1995 to 2006 demonstrates cyclic EBLV-1 infection in spatial discrete subpopulations of *M. myotis* in Spain, without any significant increase in

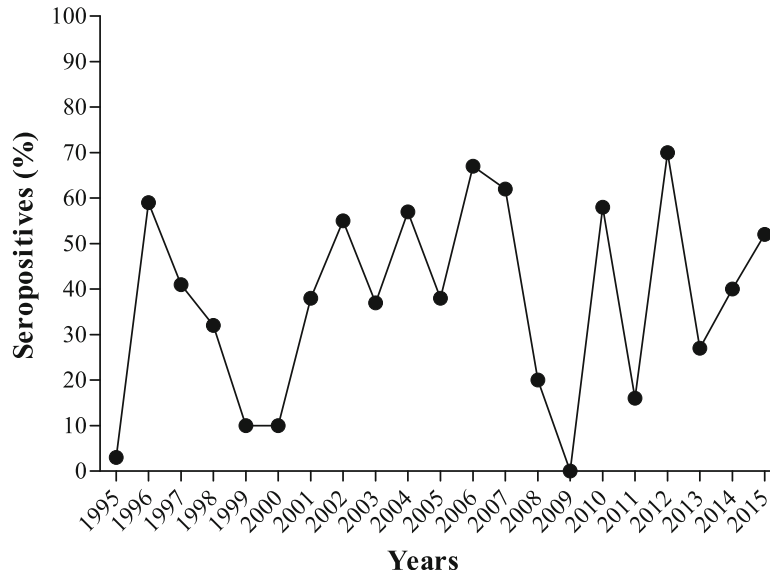

Fig. 2 Interannual variations of seropositive bats observed in a *Myotis myotis* colony from Mallorca (Spain). Results obtained together with H. Bourhy team from Institut Pasteur (Paris)

associated mortality. The data provided by this longitudinal study was the first evidence that mortality of *M. myotis* in their natural environment does not increase significantly after episodes of EBLV-1 infection (Amengual et al. 2007a, b). The monitoring of these colonies until 2015 showed that the *M. myotis* immunity could persist for 2 years (Fig 2). During the 20 years of survey we observed periodic oscillations in the number of susceptible, immune and infected bats. The delay between the waves was dependent on the rate of inflow of susceptible bats into the colonies as a consequence of new births, immigration of naive animals from neighbouring colonies, and expiration of EBLV-1specific immunity in previously infected animals. When a sufficient fraction of susceptible individuals in the bat population was reached, the virus spreads again if infected individuals joined the colony. Here we showed the cycles observed in *M. myotis* seroprevalence for EBLV-1, but the characteristics of these cycles can change according to the zoonotic diseases and the host species.

Only a few studies have addressed the interannual dynamics of lyssavirus amongst bat multispecies that are roosting in the same refuge despite these studies are giving a better understanding of the dynamics of bat lyssaviruses and facilitate information about the virus transmission between species. The longitudinal study of a *T. teniotis* and *Plecotus austriacus* colony showed significant inter-annual fluctuations in percentage of seropositive bats. However, significant differences were observed in the temporal patterns of the seroprevalence modelling of *T. teniotis* and *P. austriacus*. The behavioural ecology of the species involved could explain the different annual fluctuations in EBLV-1 seroprevalence (López-Roig et al. 2014).

Works that consider co-infection processes in hosts that are produced by more than one parasite and their interactions are not abundant. The epidemiological studies usually analyse a single pathogen. The results obtained by Munson et al. (2009) in a study done in two lion populations from Serengeti and Ngorongoro Crater shows the importance that might have the processes of co-infections in wildlife. These authors observed a high mortality in lion populations affected by a canine distemper virus epidemic. They show that lions were infected by unusually high numbers of *Babesia*, infections that were magnified by the immunosuppressive effects of coincident canine distemper virus epidemic. Complex interplay between epidemic and endemic pathogens that are normally tolerated in isolation, but with

co-infection, result in unusually mortality. Bats have been recognized as important reservoir hosts for emerging viruses and despite this, there is a big lack of knowledge about co-infection processes. Studies about co-infection processes might contribute to understand the viruses dynamic in the colonies and possible outbreaks. On the other hand, the studies of a single parasite may drive incorrect or incomplete epidemiological conclusions.

In summary, the knowledge of virus and host dynamics is relevant in terms of public health because it allows assessing the epidemiological risk and also to take preventive measures. Analysing the virus dynamic in bat colonies is important to obtain the following eco-epidemiological data: the number of species that form the colony, how many bats there are of each species, the structure of the population, the behaviour of each species (for example, is important to know if there are one or more migratory species that might spread the virus), the degree of contact between them, the social structure, how much time the individuals of the colony rest together, colony type (reproduction, mating, hibernation), the foraging area of species, the turnover rate of individuals, the shelter temperature and the relationship with other colonies, the co-infection processes.

4 Changes in Ecology and Management of Bat Populations

Wildlife plays a key role in emerging infectious diseases by providing a "zoonotic pool" from which the pathogens may emerge (Daszak et al. 2000). Zoonotic pathogens represent approximately 60 % of all known pathogens able to infect humans. Many emerging viral zoonoses arise from greater contact between human populations, livestock and pets with wildlife reservoirs of pathogens. The risk of zoonotic viruses in a given region depends largely on three factors: (1) the prevalence of the virus in wild species that inhabit the region; (2) the effects of environmental changes on the prevalence of pathogens in wild populations; and (3) the frequency of human contact and pets with wild animals potentially zoonotic reservoirs (including not only direct contact with the animals but also with their droppings, saliva or urine). Moreover, the structure and functionality of ecosystems are changing worldwide at an unprecedented rate (Jones et al. 2009) and affect the three factors exposed. These changes can modify the interactions between humans and infected animals, in our case bats. The emergence of a disease is often the result in changes from ecology of the host or the pathogen, or in both. In this sense, various authors assert that environmental changes and ecological disturbances, due to natural phenomena and human activities, have a strong influence on emerging diseases (Patz and Wolfe 2002; McMichael 2004). Interest has grown in knowing how and to what extent environmental changes, especially global climate change, affect the dynamics of infectious diseases. Much attention has been accorded to the role and potential impact of global environmental changes on the dynamics of infectious diseases. But there are other more or less local factors that can affect the emergence and spread of diseases. For example, environmental alterations, changes in land use of a region, human population growth, changes in human socioeconomic behavior or social structure, people mobility increase, trade increase, forest fires, extreme weather events, wars, breakdown in public health infrastructure, etc. Understanding infectious diseases beyond the scale of individual clinical cases requires assessment of ecological and evolutionary perspectives. Changes in abundance of reservoir hosts can increase transmission risk of zoonotic virus for humans, livestock and pets. Land modification, changes vegetation paterns, disturbances in vector and host species dynamics and microclimates changes can increase the contact between human or livestock and wildlife (Karesh et al. 2012). For example, local episodes of Ebola diseases have taken place regularly for years in Africa. Studies based on analysis of poleovirus estimate the age of the divergence between Marburgvirus and Ebolavirus at early

Miocene (Taylor et al. 2014). It is therefore likely that the Ebola virus has been present in Africa for many years. Also is likely that the virus were in bats for many years. However, unlike the epidemic that occurred in 2014–2015, in previous outbreaks the number of cases was relatively small. Why an epidemic of colossal proportions not comparable with earlier outbreaks occurred in 2014? What has changed? Probably there has been more human contact with bat species or contact with others infected mammals and higher mobility of people, factors that may have contributed to this epidemic. In this regard, in March 2014 Ebola infections had already spread outside the village where there was the initial case and had arrived to Guinea capital, Conakry, a city with over a million and half inhabitants. The current people mobility has no precedent and is a very important epidemiological factor to take into account, because it increases the risk of diseases spread. Hence to determine which are the causes of epidemic Ebola whose origin seem to be the bats, it is necessary to conduct a multidisciplinary approach. This study needs to provide information on the dynamics of pathogens in wildlife, interactions between humans and wildlife, anthropogenic pressures on wildlife populations and socio-economic changes that have occurred in human societies that live in the region where the epidemic originated (Serra-Cobo 2016). The deforestation of areas to perform human activities, either to find new resources, install farms, crops, houses, roads,... is generally analysed in terms of loss or alteration of biological diversity (loss or reduction of species). However, the consequences of deforestation can be much more important and unpredictable than it might seem at first glance. Some of the animals leave the area deforested toward new areas while others remain in the area where they lived before the alteration. The species that continue to develop its activity in the deforested area, unlike what happened previously, are much more likely to come into contact with the human population, either directly or indirectly through livestock or pets. So, they continue searching for food and shelter, and may enter farms and houses of the new inhabitants of the area. Some of the animals that hardly had contact with humans now can have it. The contacts may be important if any of the animal species is the reservoir of zoonotic viruses and can infect locals, their livestock or pets. Therefore, it is possible the contact between the human species and certain pathogens that remained more or less isolated in their animal reservoirs. An example is the Nipah virus outbreak occurred in 1998–1999 in Malaysia. The outbreak has been linked to intensification of pig farming. More than 100 people died during this outbreak and more than one million pigs were killed to control the disease (Chua et al. 1999; Karesh et al. 2012; Hayman et al. 2013). The infections were produced by the direct or indirect (urine, guano) interaction between fruit bats and pigs.

The deforestation rate can be very high in certain African regions, such as in Cameroon where tree cover loss is estimated at 800–1000 km^2 annually for road construction and expansion of human settlements. According to Wolfe et al. (2005), deforestation promotes bushmeat trade in Cameroon and increases the contact between hunters and wildlife. The opening of roads for logging also provides at hunters better access to hunting areas that until today were hardly accessible. Exposure to new pathogens is not always the result of a more or less important exploitation of forest areas, poverty also leads people to expand their range of activities to survive into the rainforest in search of new resources.

The overall trend of the populations of bats is evident in the data presented by the IUCN under which 23 % of the all bat species that live in the world are considered to be decreasing (The IUCN Red List of Threatened Species 2015-3 http://www.iucnredlist.org/search). How does the regression of bat species affect the dynamic of viruses? We have little information about this question and it represents an important eco-epidemiological point for analysis. The shelters are usually vulnerable to a wide range of threats, which in recent years have led to the loss and fragmentation of habitats. Our observations, over the past 32 years in Spain,

indicate that bat-shelter alterations are frequent and cause the disappearance or a partial reduction of the bat colonies. Such latter phenomena promote the between-colony exchange of individuals and big changes in metapopulation structures of bat species. The environmental disturbances that affect shelters of large bat colonies have a big impact on the species. These changes have incidence on the ethology and ecology of species, which has resulted in demographic changes in populations of different species and changes in land use. In some species, there is the tendency of bats being concentrated in a smaller number of shelters with largest colonies. In this sense, the formation of relatively large colonies, distributed in fewer shelters, presents a higher risk for species conservation than the distribution of relatively smaller colonies in more numerous shelters. Also, the formation of large colonies may increase the probability of virus presence.

5 Risk Factors and Prevention Tasks

Are bats dangerous? On one hand bats play ecological important roles and place high levels in the ecosystems where their live and this is the reason why is necessary to preserve them. But on the other hand, they are host of emergent zoonosis. Apparently, we are in the dichotomy between the health risk and the bat preservation. What shall we do? The answer is not easy and a lot of factors have to be considered. It is important to take into account that bats can have some zoonotic viruses without having signs of diseases, which can reduce the perception of risk and difficult the prevention tasks. Most bat species are not aggressive if they are not disturbed, which is helpful to reduce the risk of viruses transmission. Another factor to be taken into account is that the health risk of bats for humans is different depending on the region of the world and the species considered. Which are the ways that bats can transmit viruses? The ways can be different depending on the viruses considered. We reckon five different ways: by bite, by inhalation of viral particles, by scratches done due to direct contact with bats, by eating bats (probably this has been the beginning of Ebola epidemic in Africa) or by being in contact with something that had previously been in contact with bats or their urine or guano (fruits, water, etc.). Another factor that should be considered is the synanthropic character of some bat species. Due to important loss of habitat, some bat species have found shelter in human environments (houses, underground, farms, churches, mosques, etc.), increasing exposition of humans to pathogens. Synanthropic species are possible to be found in all continents. In this sense, we should always avoid direct contact with bats or with their urine or guano.

In order to decrease the risk of viral transmission is important to take the following recommendations: do not touch the bats, if you need to handle bats do protect the hands and wash them often with soap, avoid any contact with bats guano (in a lot of shelters there is big amount of guano in which numerous infectious agents for humans live, ie, viruses, bacteria, fungi), develop training about the risks and benefits that involve bats with the objective to learn good practices and preserve the bat populations from disturbances that can change the colony dynamic and the viruses transmission risk.

The bat preservation, especially insectivorous species, is very important in a time where vector species and zoonotic viruses spread in large regions over the world. In the last years different mosquitoes of *Aedes* species have colonized large areas of Europe. These species can be potentially vectors of zoonotic viruses (flavivirus and alphavirus). In this sense, the bat insectivorous species can play a major role in the vector control and in the reduction of transmission risk.

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The Middle East Respiratory Syndrome Coronavirus – A Continuing Risk to Global Health Security

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Abstract

Two new zoonotic coronaviruses causing disease in humans (Zumla et al. 2015a; Hui and Zumla 2015; Peiris et al. 2003; Yu et al. 2014) have been the focus of international attention for the past 14 years due to their epidemic potential; (1) The Severe Acute Respiratory Syndrome coronavirus (SARS-CoV) (Peiris et al. 2003) first discovered in China in 2001 caused a major global epidemic of the Severe Acute Respiratory Syndrome (SARS). (2) The Middle East respiratory syndrome coronavirus (MERS-CoV) is a new corona virus isolated for the first time in a patients who died of severe lower respiratory tract infection in Jeddah (Saudi Arabia) in June 2012 (Zaki et al. 2012). The disease has been named Middle East Respiratory Syndrome (MERS) and it has remained on the radar of global public health authorities because of recurrent nosocomial and community outbreaks, and its association with severe disease and high mortality rates (Assiri et al. 2013a; Al-Abdallat et al. 2014; Memish et al. 2013a; Oboho et al. 2015; The WHO MERS-CoV Research Group 2013; Cotten et al. 2013a; Assiri et al. 2013b; Memish et al. 2013b; Azhar et al. 2014; Kim et al. 2015; Wang et al. 2015; Hui et al. 2015a). Cases of MERS have been reported from all continents and have been linked with travel to the Middle East (Hui

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et al. 2015a; WHO 2015c). The World Health Organization (WHO) have held nine meetings of the Emergency Committee (EC) convened by the Director-General under the International Health Regulations (IHR 2005) regarding MERS-CoV (WHO 2015c). There is wishful anticipation in the political and scientific communities that MERS-CoV like SARS-CoV will disappear with time. However it's been nearly 4 years since the first discovery of MERS-CoV, and MERS cases continue to be reported throughout the year from the Middle East (WHO 2015c). There is a large MERS-CoV camel reservoir, and there is no specific treatment or vaccine (Zumla et al. 2015a). With 10 million people visiting Saudi Arabia every year for Umrah and/or Hajj, the potential risk of global spread is ever present (Memish et al. 2014a; McCloskey et al. 2014; Al-Tawfiq et al. 2014a).

Keywords
Coronavirus • MERS • MERS-CoV • Middle East • Drugs • Infection control • Treatment • Risk • Camels

1 Introduction

Two new zoonotic coronaviruses causing disease in humans (Zumla et al. 2015a; Hui and Zumla 2015; Peiris et al. 2003; Yu et al. 2014) have been the focus of international attention for the past 14 years due to their epidemic potential; (1) the Severe Acute Respiratory Syndrome coronavirus (SARS-CoV) (Peiris et al. 2003) first discovered in China in 2001 which caused a major global epidemic of the Severe Acute Respiratory Syndrome (SARS); and (2) the Middle East respiratory syndrome coronavirus (MERS-CoV) first isolated from a patient who died of severe lower respiratory tract infection in Jeddah (Saudi Arabia) in June 2012 (Zaki et al. 2012). The disease has been named Middle East Respiratory Syndrome (MERS) and it has remained on the radar of global public health authorities because of recurrent nosocomial and community outbreaks, and its association with severe disease and high mortality rates (Assiri et al. 2013a; Al-Abdallat et al. 2014; Memish et al. 2013a; Oboho et al. 2015; The WHO MERS-CoV Research Group 2013; Cotten et al. 2013a; Assiri et al. 2013b; Memish et al. 2013b; Azhar et al. 2014; Kim et al. 2015; Wang et al. 2015; Hui et al. 2015a). Cases of MERS have been reported from all continents and have been linked with travel to the Middle East (Hui et al. 2015a; WHO 2015c). The World Health Organization (WHO) have held nine meetings of the Emergency Committee (EC) convened by the Director-General under the International Health Regulations (IHR 2005) regarding MERS-CoV (WHO 2015c). There is wishful anticipation in the political and scientific communities that MERS-CoV like SARS-CoV will disappear with time. However it's been nearly 4 years since the first discovery of MERS-CoV, and MERS cases continue to be reported throughout the year from the Middle East (WHO 2015c). There is a large MERS-CoV camel reservoir, and there is no specific treatment or vaccine (Zumla et al. 2015a). With 10 million people visiting Saudi Arabia every year for Umrah and/or Hajj, the potential risk of global spread is ever present (Memish et al. 2014a; McCloskey et al. 2014; Al-Tawfiq et al. 2014a).

This chapter gives a succinct overview of MERS-CoV epidemiology, clinical features, and highlights the knowledge gaps and its epidemic risk potential.

2 Epidemiological Features of MERS-CoV

2.1 Discovery and Evolution

At first identification and publication of the isolation of a novel β CoV coronavirus in September 2012 (Zaki et al. 2012), the name EMC/2012 was given to it after the laboratory at the Erasmus Medical Centre (EMC) in the Netherlands. The EMC laboratory had sequenced the virus from clinical samples shipped from a hospital in Jeddah, Saudi Arabia where a patient had died of respiratory failure in June 2012. The virus was renamed MERS-CoV after international consensus and the clinical disease it caused was called Middle East Respiratory Syndrome (MERS) (de Groot et al. 2013). In order to ascertain whether it was a new disease of humans, several retrospective and historical studies were performed on stored biobanks of patient samples in the Middle East. In particular one study showed that in April 2012 there was a hospital MERS cluster of infections in Jordan (Hijawi et al. 2013), predating the Jeddah case. Recent evolutionary studies based on whole-genome sequences and temporal analysis of infection clusters suggested that MERS-CoV most probably emerged between November 2009 and April 2012 (Cotten et al. 2013b, 2014; Penttinen et al. 2013). Ever since its first discovery, intermittent endemic cases of MERS cases are being reported throughout the year from Saudi Arabia as single cases, clusters in the community or hospital outbreaks (WHO 2015c). Furthermore there have been MERS cases reported from all continents and these have been linked to travel to the Middle East (Zumla et al. 2015a; WHO 2015c).

2.2 Geographical Distribution

As of 25th November, 2016, WHO reports that globally there have been 1,832 laboratory-confirmed cases of MERS-CoV with 651 deaths reported (case fatality rate 35 %) (WHO 2015c). Twenty seven countries have reported cases of MERS to the WHO (Fig. 1): Baharain, Iran, Jordan, Kuwait, Lebanon, Oman, Qatar, Saudi Arabia, the United Arab Emirates, and Yemen (Middle East); Austria, France, Germany, Greece, Italy, Netherlands, Turkey, and the United Kingdom (UK) (Europe); Algeria, Tunisia and Egypt (Africa); China, Malaysia, Republic of Korea, the Philippines and Thailand (Asia); and the United States of America (Americas) (WHO 2016). A large proportion of MERS cases have been reported from Saudi Arabia. The largest MERS outbreak outside Saudi Arabia occurred in hospitals in the Republic Korea in mid-2015 where MERS-CoV was imported by a traveler to the Middle East. Poor infection control measures led to spread of MERS-CoV resulting in 184 MERS cases with 33 deaths (WHO 2015c).

2.3 Origin and Transmission of MERS-CoV

Several studies have sought to ascertain the natural reservoir of MERS-CoV. Studies on bat feces from Middle East, Africa and several European countries have reported CoV in Nycteris and Pipistrellus bats (Annan et al. 2013; Memish et al. 2013c). From Saudi Arabia, over a thousand bat samples were tested and only one fragment of MERS-CoV was found in one Taphozous bat which was related to MERS-CoV isolated from humans (Memish et al. 2013c). Several studies have subsequently indicated that MERS-CoV is a zoonotic virus and human infections have been associated with direct or indirect contact with infected dromedary camels (Reusken et al. 2013, 2014; Haagmans et al. 2014; Hemida et al. 2014; Meyer et al. 2014; Muller et al. 2014; Gossner et al. 2016). Strains of MERS-CoV have been identified in camels in several countries, including Saudi Arabia, Egypt, Oman, and Qatar. MERS-CoV antibodies have been found in camels in Africa and throughout the Middle East. Recently at least five lineages of MERS-CoV in Saudi Arabian camels have been found (Sabir et al. 2016; Du and Han 2016). Human to human transmission of MERS-CoV has been

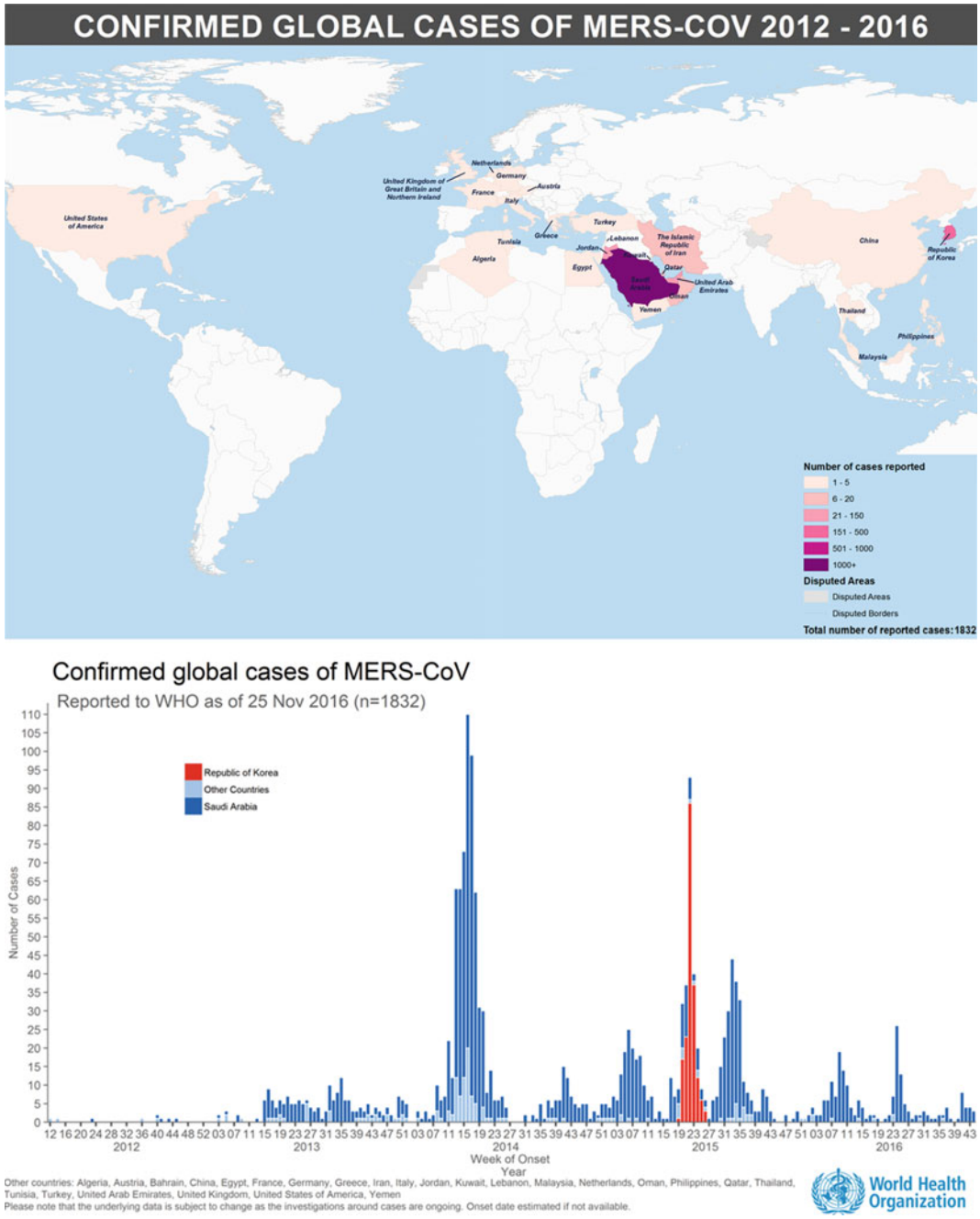

Fig. 1 Global cases of MERS-CoV infection reported to WHO (2012–2015)

documented only for close contacts of infected subjects including transmission among family members and between patients and healthcare worker (Assiri et al. 2013a, b; Cotten et al. 2013a; Memish et al. 2013b; Kim et al. 2016a; Younan et al. 2016). Convincing evidence support the hypothesis that dromedary camel are a natural reservoir of the infection and that this animal species can have a primary role for the transmission of MERS-CoV to human beings. However, only a small proportion of the primary cases have reported contact with

camels. The apparent rarity of MERS-CoV transmission from primary MERS cases apart from hospital settings indicates that the transmission potential and infectivity of such cases is low. The occasional sporadic occurrence of MERS-CoV infection in MERS cases who have any reported animal contact or exposure to MERS cases may be explained by low level infectivity of sub-clinical or asymptomatic cases of MERS-CoV infection (Lessler et al. 2016).

2.4 Natural History and Pathogenesis

The sporadic nature of MERS-CoV infection with new cases or clusters distributed over a wide geographic area and rather heterogeneous social settings, represents a significant issue for designing and for implementing solid prospective studies. Thus, the main questions about the epidemiology, source of infection, natural history of the disease, the transmission patterns and pathogenesis remain largely unanswered, as yet (Hui and Zumla 2014). Furthermore, the appearance of MERS-CoV, in human populations soon after the SARS-CoV pandemic emphasizes the importance of a One Health approach (Rabozzi et al. 2012) to surveillance of zoonotic infections through integration of human, animal, and environmental health programs. Strengthening surveillance and laboratory networks, as well as training of an effective surveillance workforce is required and needs commitment by all stakeholders, particularly Health Authorities in Middle Eastern Countries.

3 Clinical Presentation

There have been several reviews on the clinical aspects of MERS-CoV (Zumla et al. 2015a; Hui and Zumla 2015; The WHO MERS-CoV Research Group 2013; Assiri et al. 2013b; Al-Tawfiq et al. 2014b; ISARIC and Public Health 2014). MERS presents as a clinical spectrum from the asymptomatic, mild, moderate to severe fulminant multisystem disease. There is limited data on pathogenesis due to lack of autopsy or histological studies. MERS-CoV is known to bind to dipeptidyl peptidase 4 (DPP4) receptors (Lu et al. 2013) that are widespread in the body but are primarily located in the lower respiratory tract and thus a typical case of MERS presents with fever, cough, and/or shortness of breath and pneumonia (detailed in Table 1). Severe illness can occur in both immunocompetent and immunocompromised host. In general

Table 1 Clinical and laboratory features of patient with MERS

Clinical/laboratory feature(s)	
Date of first case (place)	April 2012 (Zarqa, Jordan)
	June 2012 (Jeddah, KSA)
Incubation period	Mean: 5.2 days (95% CI:1.9–14.7)
	Range: 2–14 days
Serial interval	7.6 days
Basic reproduction number	<1
Age group	
Adults	Adults (98 %)
Children	Children (2 %)
Age (years):	Range:1–94;
Range, Median	Median: 50
Gender (M,F)	M: 64.5 %, F: 35.5 %
Mortality	
Case fatality rate (CFR)-overall	40 %*
CFR in patients with co-morbidities	60 %
Disease progression	
Time from onset to ventilatory support	Median 7 days
Time from onset to death	Median 11.5 days
Presenting symptoms	
Fever > 38C	98 %
Chills/rigors	87 %
Cough	83 %
	56 %
Dry	44 %
Productive	
Haemoptysis	17 %
Headache	11 %
Myalgia	32 %
Malaise	38 %
Shortness of breath	72 %

(continued)

Table 1 (continued)

Clinical/laboratory feature(s)	
Nausea	21 %
Vomiting	21 %
Diarrhoea	26 %
Sore throat	14 %
Rhinorrhoea	6 %
Co-morbidities (eg obesity, diabetes, cardiac disease and lung disease)	76 %
Laboratory results	
CXR abnormalities	90–100 %
Leukopenia ($<4.0 \times 10^9$/L)	14 %
Lymphopenia ($<1.5 \times 10^9$/L)	32 %
Thrombocytopenia ($<140 \times 10^9$/L)	36 %
Elevated LDH	48 %
Elevated ALT	11 %
Elevated AST	14 %
Risk factors associated with poor outcome (severe disease or death)	Any immunocompromised state, comorbid illness, concomitant infections, low albumin, age $\geq$ 65 years

Compiled from references Zumla et al. (2015), Assiri et al. (2013a, b), Al-Abdallat et al. (2014), Memish et al. (2013a, b), Oboho et al. (2015), The WHO MERS-CoV Research Group (2013), Cotten et al. (2013), Azhar et al. (2014)

progression to respiratory and/or renal failure requires intensive care support. Some patients have multi-organ failure and secondary infections leading to septic shock. Mortality rates are high in older people, immunosuppressed patients and in those with co-morbities such as diabetes, cancer, chronic obstructive pulmonary and heart disease.

4 Laboratory Diagnosis and Diagnostics

Many cases of MERS-CoV can be easily missed since the presentation is that of any community acquired pneumonia (Zumla et al. 2015a; WHO 2015c; Lessler et al. 2016; Al-Tawfiq et al. 2014b; ISARIC and Public Health 2014). Rapid and accurate diagnosis of MERS-CoV infection is important for the clinical management and epidemiological control of MERS-CoV infections. Thus a high degree of clinical awareness of the possibility of MERS-CoV infection is required in all healthcare settings in the Middle East so that an accurate diagnosis can be made and adequate infections control measures promptly implemented (WHO 2015a; ISARIC and Public Health 2014; Zumla and Hui 2014). A history of travel to the Middle East is important for patients presenting in non-Middle Eastern countries (WHO 2015c; ISARIC and Public Health 2014; Zumla and Hui 2014).

Laboratory confirmation of MERS-CoV infection can be obtained by: (a) MERS-CoV specific nucleic acid amplification test (NAAT) with up to two separate targets and/or sequencing; or (b) virus isolation in tissue culture; or (c) serology on serum tested in a WHO collaborating center with established testing methods. Real-time reverse-transcriptase polymerase chain reaction (rRT-PCR) is used (Zumla et al. 2015a; ISARIC and Public Health 2014; Corman et al. 2012, 2014) for specimens collected from the respiratory tract of suspected cases. CDC recommends the collection of three specimen types, lower respiratory, upper respiratory and serum specimens, for testing using the MERS rRT-PCR assay Accurate laboratory molecular diagnostic tests are available (MERS CDC Laboratory testing for MERS-CoV 2016) using highly sensitive and specific Real-time reverse transcription (RT-PCR) assays targeting unique gene regions such as the upE region (gene region upstream to E gene). These assays have been used for viral load quantitation in studies on viral shedding patterns, optimization of treatment and infection control strategies. Serological tests have been developed for surveillance purposes although they require evaluation in field studies (Park et al. 2015).

5 Management of Mers Patients

The clinical management of patients with MERS is largely symptomatic and aimed to reduce the risk of most severe complications, such as secondary infections, and to support renal and respiratory function (Reviewed in Zumla et al. 2015a; WHO 2015b, c; Lessler et al. 2016; Rabozzi et al. 2012; Al-Tawfiq et al. 2014; ISARIC and Public Health England 2014; CDC 2016). Seriously ill patients should receive intensive care. Moreover, the implementation of appropriate infection control measures as soon is possible, is critical for preventing spread of the infection especially in hospitals. Whilst a range of treatments (CDC 2016; WHO 2015b; de Wilde et al. 2013; Falzarano et al. 2013a, b; Chan et al. 2013; Omrani et al. 2014; Shalhoub et al. 2015; Zumla et al. 2016) may be useful (Table 2), currently there are no specific treatments for MERS-CoV infections and no controlled randomized clinical trials of any therapeutic have been conducted to date. A whole range of treatments have been used empirically for serious cases of MERS but there is no solid evidence that any of them can improve the clinical outcome. A range of anti-MERS-CoV drugs and host-directed therapies are in the pipeline (Zumla et al. 2014, 2016; [61]), properly designed, randomized, controlled clinical trials are required to be performed.

Table 2 Potentially useful antiviral agents for Middle East respiratory syndrome Coronavirus (MERS-CoV) infection

Neutralizing Antibodies[a]:
Convalescent plasma
Polyclonal human immunoglobulin from transgenic cows,
Equine F(ab')2 antibody fragments,
Camel antibodies,
Anti-S monoclonal antibodies
Interferons[a]:
Interferon alfa,
Interferon beta
Repurposed drugs:
Ribavirin monotherapy[b] (±interferon),
HIV protease inhibitors (lopinavir[a], nelfinavir),
Cyclophilin inhibitors (ciclosporin, alisporivir),
Chloroquine (active in vitro),
Mycophenolic acid,
Nitazoxanide
Recombinant human mannose-binding lectin
siRNA to key MERS-CoV genes

Compiled from references Zumla et al. (2015a), Hui and Zumla (2015)

[a]Treatment benefits likely to exceed risks

[b]Risks likely to exceed benefits

6 Infection Control and Transmission Risk

There have been several nosocomial outbreaks of MERS-CoV infection within Saudi Arabia (Assiri et al. 2013a; Oboho et al. 2015; Memish et al. 2013b). The largest nosocomial outbreak outside Saudi Arabia occurred in mid-2015 in the Republic of Korea (Petersen et al. 2015; Hui et al. 2015b; Zumla et al. 2015c; Kim et al. 2016b) where the index case was 68-year-old male from Korea who visited several Middle Eastern countries (Saudi Arabia, UAE, Bahrain and Qatar) and developed symptoms upon return to Korea and due to lack of isolation and patient consulting several hospitals, a major outbreak ensued involving several hospitals.

Early recognition of MERS cases and rapid implementation of infection control guidance is necessary to prevent nosocomial outbreaks of MERS-CoV. Implementation of effective infection control measures at the first consideration of the diagnosis of MERS-CoV is crucial for prevention of MERS-CoV outbreaks. The first major nosocomial outbreak of MER-CoV in 2013 occurred at Al-Hasa, Saudi Arabia in four hospitals where 21 cases of hospital acquired MERS-CoV infection were confirmed by sequence analyses (Assiri et al. 2013a).

Global public health authorities guidelines (CDC 2016; WHO 2015b) recommend to use, whenever it is possible airborne infection control

measures for all patients with suspected or confirmed MERS-CoV infection. Moreover airborne infection control measures are mandatory for healthcare workers dealing with patients who undergo aerosol-generating procedures. Several outbreaks of MERS-CoV in Saudi Hospitals in Jeddah, Al-Hasa, and Riyadh were attributed to overcrowding in the emergency departments, uncontrolled patient movement, and high traffic of visitors, lack of infection control stewardship. Effective triage is required at the first suspicion of MERS-CoV and in ill patients with a history of travel to the Middle East. Tracing, screening for symptoms and MERS-CoV, and follow up of all contacts, (family, workmates, patients and visitors) is important in preventing further spread. The implementation of extensive contact tracing in order to rapidly diagnose suspected MERS cases and isolate infectious individuals to break the chain of infections is important.

7 Surveillance, Prevention and Control

There is currently no licensed vaccine available, although several experimental candidate MERS-CoV vaccines are being developed. For example, researchers at the National Institute of Health in collaboration with other investigators, including the Public Health Agency of Canada, developed an experimental synthetic DNA based vaccine that can generate protective MERS-CoV antibodies in mice, monkeys, and camels (Muthumani et al. 2015). Whilst we await the development of effective MERS-CoV vaccines, public health systems in Western and Middle Eastern countries have put in place surveillance systems for the prompt detection and investigation of new cases and contact tracing. The MERS outbreak in South Korea highlights the potential of MERS-CoV to spread across the globe and cause local outbreaks (Petersen et al. 2015; Hui et al. 2015b). Whilst cases of MERS related to travel to the Middle East have been reported from a wide geographical area, of note is the absence of any significant number of MERS cases (primary or travel related) reported from sub-Saharan African (SSA) countries (WHO 2015c; Zumla et al. 2015c). The reasons why MERS-CoV predominantly affects humans in the Middle East and is not endemic in Africa or Asia where MERS-CoV infected camels and bats are present requires further study (Zumla et al. 2015d). However this observation may reflect the lack of clinical awareness of MERS and that diagnosis and treatment of respiratory tract infections largely remains empiric, without laboratory confirmation.

An estimated 10 million visitors from over 184 countries travel to Saudi Arabia to participate in Hajj pilgrimage, the mini-pilgrimage Umrah or during the month of Ramadan, the vast majority come from developing countries (Memish et al. 2014a). If MERS-CoV was a major public health risk, 4 years after its first discover one would have expected cases of MERS-CoV infection in pilgrims. There were no cases of MERS reported during the 2012, 2013, 2014 and 2015 among Hajj pilgrimages (Waldron and Doherty 2015; Lessler et al. 2014). It is possible that like SARS-CoV, MERS-CoV will die out with time. Conversely it is also possible that MERS-CoV will mutate and increase its transmission potential and the risk of MERS-CoV spreading globally remains. Coker and colleagues (Soliman et al. 2015) estimated the potential risk of MERS-CoV infection to pilgrims who visit Saudi Arabia from different regions of the world based on the most likely scenario using recent pilgrim numbers for sub-Saharan Africa. They predict that there will be at most ten returning pilgrims each year with MERS-CoV infections. As the recent Ebola Virus Disease epidemic in West Africa illustrates, African and Asian countries are vulnerable to a Korea-like MERS-CoV outbreak (Zumla et al. 2015e).

A recent study published in Science by Sabir and colleagues (Sabir et al. 2016) found that at least five lineages of MERS-CoV are circulating in Saudi Arabian camels. These results suggest that multiple lineages of MERS-CoV have been co-circulating in Saudi Arabia confirming what

was suspected before (Cotten et al. 2013b, 2014). This is a pre-requisite for recombination to occur and it is no surprise that Sabir et al. identified at least six recombination events, showing that recombination is frequent in MERS-CoV. Of interest was that one lineage sequenced by Sabir et al (Sabir et al. 2016) was associated with the 2015 Riyadh nosocomial outbreak (Balkhy et al. 2016), and the MERS-CoV sequenced from the Republic of Korean outbreak also had a recombinant origin. It's been suggested that the recombinant lineage originated between December 2013 and June 2014, and has rapidly become the predominant lineage in Saudi Arabian camels since November 2014.

MERS-CoV remains a major threat for global health. With recent outbreaks of Ebola virus and Zika virus a coordinated global response is needed to tackle emerging and re-emerging infectious diseases with epidemic potential (Zumla et al. 2015e; Petersen et al. 2016; Memish et al. 2014b). Meanwhile there are critical knowledge gaps related to MERS-CoV which, require to be filled (The WHO MERS-CoV Research Group 2013; Hui and Zumla 2014).

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Emerging Zika Virus Infection: A Rapidly Evolving Situation

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Abstract

Zika virus is a mosquito-borne flavivirus, firstly identified in Uganda and responsible for sporadic human cases in Africa and Asia until recently, when large outbreak occurred in Pacific Ocean and the Americas. Since the main vectors during its spread outside of Africa have been *Ae. albopictus* and *Ae. aegypti* mosquitoes, which are widely distributed all over the world, there is urgent need for a coordinated response for prevention and spread of ZIKV epidemics.

Despite clinical manifestation of Zika virus infection are usually mild and self limiting, there are reports suggesting, during the recent epidemic, an association of ZIKV infection with severe consequences, including fetal/newborn microcephaly, due to vertical in utero transmission, autoimmune-neurological presentations including cranial nerve dysfunction, and Guillain-Barré Syndrome in adults. The primary mode of transmission of Zika virus between humans is through the bite of an infected female mosquito of the *Aedes* genus, but also sexual and blood transfusion transmission may occur. Moreover, a case of non-sexual spread from one person to another has been described, indicating that we still have more to learn about Zika transmission.

Biological basis for pathogenetic effects are under investigation. Laboratory diagnosis is challenging since, so far, there are no "gold standard"

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diagnostic tools, and the low and short viremia in the acute phase, and together with the high cross-reactivity among the members of flavivirus genus are the most challenging aspects to be overcome.

Keywords
Zika Virus • Spread • Diagnosis • Pathogenetic Effects • Phylogenesis • Transmission • Clinical Manifestation

Abbreviations

ZIKV	Zika virus
YFV	Yellow fever virus
DENV	Dengue virus
JEV	Japanese encephalitis virus
WNV	West Nile virus
TBEV	Tick-borne encephalitis virus
	SLEV
	St. Louis encephalitis virus
MVEV	Murray Valley encephalitis virus
ROCV	Rocio virus
KFDV	Kyasanur forest virus
ALKV	Alkhurma virus
OHFV	Omsk Hemorrhagic fever virus
POW	Powassan virus
C	capsid protein
prM	the precursor of membrane protein
E	the envelope protein
RdRP	RNA-dependent RNA polymerase
NSP	non structural proteins
ER	endoplasmic reticulum
CHIKV	Chikungunya virus
GBS	Guillain-Barré syndrome
hNPCs	human neural progenitor cells
hESC	human embryonic stem cells
DC-SIGN	adhesion molecule of dendritic cells
IFN-α	interferon-α
IFN-β	interferon-β
PRRs	pattern recognition receptors
PAMPS	pathogen-associated molecular patterns
CPE	cytopathic effect
DC-SIGN	adhesion molecule of dendritic cells
TLR	Toll like receptor
WHO	World Health Organization

1 Introduction

Zika virus (ZIKV) is a mosquito-borne flavivirus, belonging to the *Flaviviridae* family. This infection was a rare tropical disease before 2015, when the rapid spread of the virus in the Americas has raised the attention of the global audience. The virus was identified in 1947, when fever developed in a rhesus monkey that had been placed in a cage on a tree platform in the Zika Forest of Uganda (Dick et al. 1952). The monkey, Rhesus 766, was a sentinel animal in the Rockefeller Foundation's program for research on jungle yellow fever. Two days later, Rhesus 766, still febrile, was brought to the Foundation's laboratory at Entebbe and its serum was inoculated into mice. After 10 days all mice that were inoculated intracerebrally were sick, and a filterable transmissible agent, later named Zika virus, was isolated from the mice brains. In early 1948, ZIKV was also isolated from *Ae. africanus* mosquitoes trapped in the same forest (Macnamara 1954). Serological studies performed on several individuals living in the area in the same period showed the presence of antibodies against ZIKV, thus suggesting that the virus was already circulating in human population (Dick 1952).

2 Geographic Distribution

After its first description in the Zika forest in Uganda, serological and entomological data indicated ZIKV circulation in the African continent (Fig. 1a) in Nigeria in 1971 and 1975 (Fagbami 1979), Sierra Leone in 1972 (Robin and Mouchet 1978), Gabon in 1975 (Jan et al.

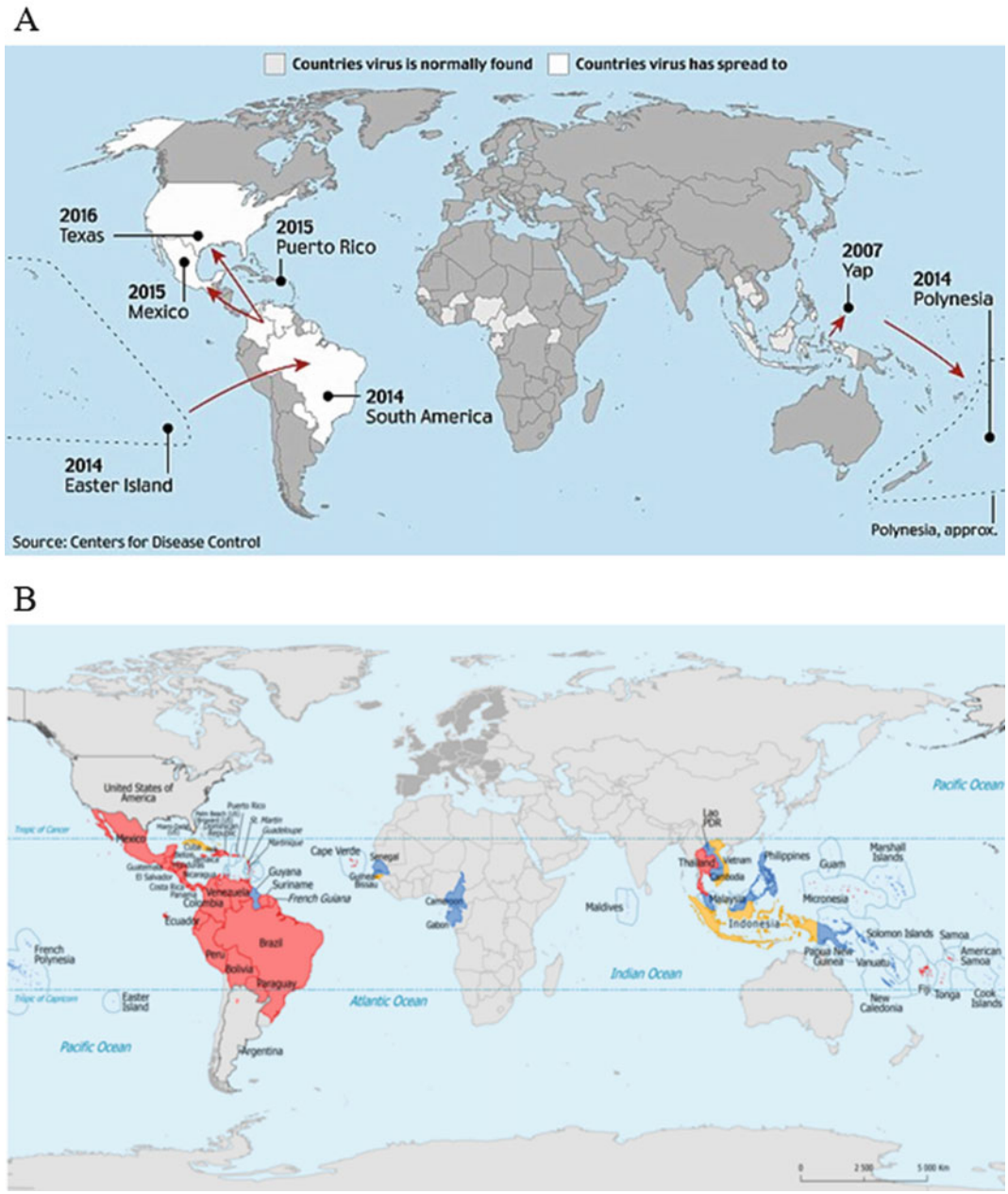

Fig. 1 ZIKV geographic distribution (**a**) ZIKV global spread from East Africa to both West Africa and Asia during the years (source: centers for disease control www.cdc.gov/zika/); (**b**) Countries and Territories with Active ZIKV Transmission (http://www.cdc.gov/zika/geo/active-countries.html, updated: August 19, 2016)

1978), Uganda in 1969 and 1970 (McCrae and Kirya 1982), Central African Republic in 1979 (Saluzzo et al. 1981), Senegal from 1988 to 1991 (Monlun et al. 1993) and Cote D'Ivoire in 1999 (Akoua-Koffi et al. 2001). A small number of cases were also reported in Asia (Marchette et al. 1969; Olson and Ksiazek 1981; Darwish et al. 1983) until 2007, when a large outbreak on Yap Island in the Federated States of Micronesia occurred (Lanciotti et al. 2008; Duffy et al.

2009). During the epidemic in Yap, three quarters of the local population are estimated to have been infected, but no deaths, hospitalizations or severe complications were noted or reported (Duffy et al. 2009). The largest known ZIKV outbreak reported started in October 2013 in French Polynesia, South Pacific (Cao-Lormeau et al. 2014), a territory of France comprising 67 inhabited islands; an estimated 28,000 persons (11% of the population) sought medical care for the illness (Musso et al. 2014a, b). The affected areas in the Pacific have expanded to include the Cook Islands, New Caledonia, and Easter Island (Kwong et al. 2013; Tappe et al. 2014; Pyke et al. 2014; Fonseca et al. 2014), thus emphasizing the capacity of ZIKV to spread to new areas where the proper mosquito vector might be present. In this large outbreak the presence of ZIKV has been correlated with severe neurological and autoimmune complications never observed before (Ioos et al. 2014). The first ever case of ZIKV disease in Brazil was reported in May 2015 (Campos et al. 2015; Zanluca et al. 2015) and, now, has rapidly spread to more than 54 countries and territories among Americas, Africa, Asia and the Pacific. (European Centre for Disease Prevention and Control. Available from: http://ecdc.europa.eu/en/healthtopics/zika_virus_infection/zika-outbreak/Pages/Zika-countries-with-transmission.aspx Centers for Disease Control and Prevention. Zika Virus. http://www.cdc.gov/zika/ accessed August 19th, 2016, Heymann et al. 2016) (Fig.1b).

3 ZIKA Virus as Health Emergency of International Concern

The rapid spread of ZIKV in America and the increasing reports of cases of congenital abnormalities temporally associated with ZIKV infection led WHO to announce on February 1st 2016 that the ZIKV outbreak constituted a 'Public Health Emergency of International Concern'.

A heightened state of global alert is in place in Europe and USA to screen for ZIKV in travellers with fever returning from ZIKV endemic countries (WHO 2016. WHO statement on the first meeting of the International Health Regulations (2005) (IHR 2005) Emergency Committee on Zika virus and observed increase in neurological disorders and neonatal malformations http://www.who.int/mediacentre/news/statements/2016/1st-emergency-committee-zika/en/-accessed February 1st).

Increasing number of cases of ZIKV detectable in travellers returning from epidemic areas have been reported also in Europe (Table 1). No autochthonous ZIKV transmission has been reported from EU countries until very recently, when Venturi and colleagues described a case of ZIKV infection imported in Florence, Italy, from Thailand, leading to a secondary autochthonous case, probably due to sexual transmission (Venturi et al. 2016). Since then, the number of ZIKV infections diagnosed in Italy, virtually all imported cases, dramatically increased.

The World Health Organization (WHO) has recommended keeping the public health emergency in place, and announced that, considering that the virus continues to spread to new regions and that there are still major knowledge gaps, the event still constitutes a public health emergency of international concern (PHEIC). Moreover, the US Centers for Disease Control and Prevention (CDC) announced $2.4 million in funding to help five of the nation's most populated cities detect and manage Zika-related birth defects (http://www.cidrap.umn.edu/news-perspective/2016/09/study-zika-could-reach-26).

4 Phylogenesis

In order to understand ZIKV evolutionary biology, different research groups analysed ZIKV strains circulating in Africa and Asia (namely Nigeria, Uganda, Senegal, Yap islands, Cambodia and Malaysia) during the period 1947–2010, comparing their sequences with those available in GenBank (Haddow et al. 2012; Faye et al. 2014). Those studies reported 3 main ZIKV lineages, 2 from Africa and 1 from Asia. More in detail, authors reported that ZIKV

Table 1 European ZIKV imported and autochthonous cases in travellers returning from epidemic areas

European imported cases	European autochthonous cases	References
Italy	Italy	ECDC. Epidemiological update: Outbreaks of Zika virus and complications potentially inked to the ZIKV infection; 19th August 2016 ECDC; 2016
Austria		
Germany		
Finland		
France		
Denmark		
Norway		
Ireland		
Portugal		
Spain		
Sweden		
Switzerland		
U.K.		
Belgium		Tappe et al. (2014)
Czech Republic		Zammarchi et al. (2015)
Luxembourg		Venturi et al. (2016)
Malta		Bachiller-Luque et al. (2016)
Netherlands		
Romania		
Slovenia		
Slovakia		

had his origin in East Africa (MR766 prototype cluster), and subsequently (50–100 years ago) spread to both West Africa and Asia, originating the Nigerian and the Asian cluster, respectively (Faye et al. 2014). Two independent ZIKV introductions into West Africa from East Africa have been described: the first was related to MR766 cluster which moved form Uganda to Cote D'Ivoire and Senegal; the second was related to the Nigerian cluster, which moved from Uganda to Central African Republic and Nigeria (Faye et al. 2014). Moreover an additional lineage form Uganda spread to Malaysia and Micronesia, originating the Asian Cluster (Haddow et al. 2012) (Fig. 1a). The virus has circulated in South East Asia for at least 50 years, then it was introduced in Yap island during the 2007 epidemic and in 2010 in Cambodia (Haddow et al. 2012).

The complete genome sequence of ZIKV that was recovered in fetal brain tissue of fetus from an expectant mother with diagnosed microcephaly was consistent with the observation that the strain circulating in Brazil has emerged from the Asian lineage, with the highest identity (99.7%) with ZIKV isolated from a patient from French Polynesia in 2013, followed by a strain isolated in Cambodia (98.3% identity) and finally by a strain isolated from the 2007 outbreak in Micronesia (Mlakar et al. 2016). More in detail, in this study 5 polymorphisms were detected in ZIKV polyprotein in comparison with the one isolated from French Polynesia: 3 amino acid changes were found in the NS1 region (K940E, T1027A, M1143V), 1 in the NS4B region (T25091) and the last one in the FtsJ-like methyltransferase region (M2634V) (Mlakar et al. 2016; Calvet et al. 2016).

Moreover complete genome sequence of ZIKV obtained in serum specimen from person of Puerto Rico and Guatemala revealed that they all originated from the Asian lineage gradually evolving and spreading throughout Asia and Pacific Islands (Faye et al. 2014; Lanciotti et al. 2016; Cunha et al. 2016). In a study from Barzon and colleagues the phylogenetic tree of

full genome sequences of ZIKV of a case imported to Italy from the Dominican Republic demonstrated that the virus belonged to the Asian lineage and clustered with ZIKV from Latin America, showing 99.6% identity with strains isolated in French Poynesia and Brazil (Barzon et al. 2016). Moreover, phylogenetic analysis performed on ZIKV strain recently isolated from patient returning from Samoa revealed that also the strain circulating in China belongs to Asian lineage (Deng et al. 2016). A phylogenetic molecular clock approach was used to analyse seven Brazilian ZIK genomes, sampled from four self-limited cases, one blood donor, one fatal adult case and one newborn with microcephaly and congenital malformations, to further explore the epidemiology of virus in the Americas (Faria et al. 2016). Results obtained from these analysis showed that the explosion of ZIKV cases in the Americas followed a single introduction, probably occurred between May-Nov 2013. This is more than 12 months earlier than the detection of ZIKV in Brazil, and coincides with the time of occurrence of the outbreak in Pacific Islands, when an increase in air passengers from these areas to Brazil occurred, fuelled by international sports events. Moreover, ZIKV genomes from Brazil resulted phylogenetically interspersed with those from South America and Caribbean countries, forming a robust monophyletic cluster within the Asian genotype and sharing a common ancestor with the ZIKV strain that circulated in French Polynesia in 2013 (Faria et al. 2016). The sequence obtained from a microcephaly case in Brazil in this study showed 9 aminoacid changes, none of them shared with the other recently published microcephaly-cases genomes (Mlakar et al. 2016), and the authors speculate that viral determinants of severe infant disease should probably rely on amino acid substitutions established before the recognition of fetus development defects.

Faye and colleagues presented evidences that ZIKV have experienced several recombination events, among which the recurrent loss and gain of the N-linked glycosylation site around position 154 of E protein, which could be related to infectivity for mosquito cells (Lee et al. 2010). Changes in glycosylation patterns of E and vector competence seemed to be strictly correlated, thus suggesting a possible role in the pathogenesis of the clinical features (Faye et al. 2014; Chang et al. 2016). Moreover, a comparative analysis of genetic differences among Africans and Asian lineages highlighted that recent Asian epidemic lineages had stronger codon bias on NS1 and NS4A genes and resulted more adapted to humans (de melo Freire et al. 2015). These findings suggest that these two proteins could account for the higher pathogenicity of the Asian lineages, also considering that one of the relevant function of NS1 protein is to assist flaviviruses towards immune evasion (Muller and Young 2013; Wang et al. 2016).

Many authors speculated the phylogeny and movement of ZIKV and Chikungunya virus (CHIKV) are strikingly similar.

CHIKV is an alphavirus transmitted in an urban epidemic cycle by the mosquitoes *Ae. aegypti* and *Ae. albopictus* first identified in Tanzania in 1952 (Lumsden 1955). The virus was later found to cause sporadic, mostly small outbreaks in Africa and Asia through the 1960s and 1970s and little activity was reported from the mid-1980s until June 2004, when an epidemic occurred on Lamu Island, Kenya; subsequent spread to Comoros, La Reunion, and to other Indian Ocean islands occurred during 2005, causing ≈500,000 cases (Powers 2010). This was followed in 2006–2009 by an epidemic in India that produced >1.5 million cases in 17 of the country's 28 states, and subsequently spread through Southeast Asia to the islands of the Pacific Ocean (Staples et al. 2009) and to the South, Central, and North America (Leparc-Goffart et al. 2014). Three CHIKV genotypes (East-Central-South African, West African, and Asian) have been described, being the Asian genotype associated with outbreaks in the Pacific region (Leparc-Goffart et al. 2014).

On the whole, CHIKV and ZIKV share a number of features, suggesting that similar ecologic and human/social factors could be responsible for the spreading of CHIKV and ZIKV into the New World at approximately the same time

(Musso et al. 2015a, b; Higgs 2016; Lanciotti et al. 2016). Particularly, both viruses use the same vector(s) (see below); the spread of the infection followed for both viruses the same path, from East Africa to Asia, Pacific Ocean and New World (Volk et al. 2010); finally, the geographic distribution pattern was similar, and 3 genotypes evolved for both viruses according with the geographical spread.

5 Vectors and Reservoirs

ZIKV was firstly isolated in 1948 from *Ae. africanus* mosquitoes, which were abundant in the Zika Forest, but there was no clear evidence that these mosquitoes were actually the vector for enzootic ZIKV transmission to monkeys (Dick et al. 1952).

Subsequently, Boorman and Porterfield demonstrated the transmission of ZIKV to mice and monkeys by *Ae. aegypti*, by infecting mosquitoes with ZIKV employing a mouse skin membrane and heparin-treated blood (Boorman and Porterfield 1956). Virus content in the mosquitoes was high on the day of artificial feeding, dropped to undetectable levels through day 10 after feeding, increased by day 15, and remained high from days 20 through 60, thus suggesting an extrinsic incubation period for ZIKV of ≈10 days in mosquitoes. Successful infection of a rhesus monkey by mosquito bite was demonstrated 72 days after an infected blood meal. These results, along with the viral isolations from wild mosquitoes and monkeys and the phylogenetic proximity of ZIKV to other mosquito-borne flaviviruses, made it reasonable to conclude that ZIKV is transmitted through mosquito bites. Surprisingly, previous studies investigating the vector competence for ZIKV have neglected other mosquito species, such as *Culex* species, very abundant in the tropical areas where the virus has spread and able to transmit arboviruses closely related to ZIKV, such as West Nile virus. ZIKV has been lately isolated from *Mansonia uniformis*, *Anopheles coustani,* and *Culex perfuscus,* suggesting that these mosquito species could probably contribute to the zoonotic cycle of ZIKV transmission (Ayres 2016). However, the simple detection of a virus in a mosquito sample does not incriminate it as a vector. It is important to prove in laboratory conditions that an organism is able to acquire the pathogen and maintain and transmit it to other hosts. A recent study from Zika research team at The University of Texas reported that the *Culex quinquefasciatus* and *Culex pipiens* colonies were unable to transmit ZIKV either up to 21 days post an infectious blood meal or up to 14 days post intrathoracic inoculation, thus suggesting that it is unlikely that *Culex* mosquitoes are involved in the rapid spread of ZIKV (Amraoui et al. 2016).

Many other *Aedes* species have been surveyed for the detection of ZIKV and, thus far, the virus has been detected by RT-PCR or isolated from *Ae. apicoargenteus*, *Ae. luteocephalus*, *Ae. aegypti*, *Ae. vitattus, Ae. dalzieli* and *Ae. furcifer* mosquitoes (Fagbami 1979; Marchette et al. 1969; Akoua-Koffi et al. 2001; McCrae and Kirya 1982). *Ae. hensilii* was the predominant mosquito species present on Yap during the ZIKV disease outbreak in 2007 (Duffy et al. 2009). However, it has to be considered that the detection of virus does not automatically make the species an efficient vector for the disease. The first report of ZIKV outside of Africa or Asia was followed by another novel event, when an outbreak in Gabon in 2007 was attributed to the Asian tiger mosquito, *Ae. albopictus* as the primary vector, while *Ae. aegypti* was considered as the main vector for human epidemics prior to 2007 (Grard et al. 2014).

This phenomenon has been already observed for another arbovirus, CHIKV, whose spreading outside of Africa and Asia has been associated with the acquisition and fixation of the mutation A226V in E1 protein, able to increase CHIK viral fitness in *Ae. albopictus* vector (Tsetsarkin et al. 2007; Bordi et al. 2008, 2011).

The capacity to be transmitted by two mosquito species that preferentially feed on people exacerbates an already difficult situation. Both species thrive in close proximity to people but they differ in their behavior and biology, so that they occupy different niches (Eisen and Moore

2013). *Ae. aegypti* mosquito feeds almost exclusively on humans in daylight hours and typically rests indoors (Scott and Takken 2012). In contrast, *Ae. albopictus* mosquito is usually exophagic, feeds outdoors and bites humans and animals opportunistically (Paupy et al. 2009) but has also been shown to exhibit strongly antropophilic behaviour (Ponlawat and Harrington 2005; Delatte et al. 2010). This means that control methods for one species do not necessarily control the other. Furthermore, when populations of *Ae. aegypti* are reduced, the invasive *Ae. albopictus* may rapidly move opportunistically into the same area (Higgs 2016). The unique climatic conditions created during the severe El Nino event have been considered contributing factors in the spreading of ZIKV in the Americas (Paz and Semenza 2016) since, elevated temperature have been demonstrated to expand the geographic vector range, increasing the female mosquito biting rate (Morin et al. 2013).

A recent study highlights the importance to work out where these mosquitoes are found around the globe to identify the areas at risk and to predict where these species could become established if they were introduced, identifying areas that could become at risk in the future (Kraemer et al. 2015). In this study authors have provided predictions about these two mosquitoes species, showing that, unlike in the United States, many of the areas in Europe and China that could support the mosquito species do not appear to have been colonized yet (Kraemer et al. 2015), thus highlighting the urgent need for a coordinated response for prevention and spread of ZIKV epidemics. The global risk of ZIKV to spread from infected travelers arriving at airports in new regions has been also calculated and seems to depend on vector status of *Ae albopictus*: if *Ae aegypti* is considered the only competent vector, the risk is geographically restricted while in presence of both vectors the risk of autochthonous transmission is high in Canada, Chile, Europe and Asia (Gardner et al. 2016). To support public health readiness, a recent study proposed a model combined transportation network analysis, ecological modelling of mosquito occurrences, and vector competence for flavivirus transmission, using data from the International Air Transport to identify the areas around the world most at risk for ZIKV. Based on this model, an estimated 2·6 billion people live in areas of Africa and the Asia-Pacific region (1.2 billion people in India, 242 million in China, and 197 million in Indonesia) where the presence of competent mosquito vectors and suitable climatic conditions could support local transmission of ZIKV. Those countries have been described to have four factors that put them at risk: high-frequency travel by people from Zika-endemic areas, *Aedes* mosquito vectors, a climate that sustains mosquito-borne disease, and low public health resources (Bogoch et al. 2016).

ZIKV is most likely maintained in a sylvatic cycle involving non-human primates and mosquitoes (Dick 1952; Boorman and Porterfield 1956), with cyclic epizootics in monkeys reported in Uganda (Kirya and Okia 1977; McCrae and Kirya 1982). In the sylvatic transmission cycle, humans likely serve as incidental hosts. However, in areas without non-human primates, humans probably serve as primary amplification hosts and potentially as reservoir hosts if their viremia is sufficient in duration and magnitude (Duffy et al. 2009).

Although it is thought that enzootic ZIKV is maintained primarily in a monkey/mosquito transmission cycle, antibodies have been detected in numerous other animal species including water buffalo, elephants, goats, hippos, impala, kongoni, lions, sheep, rodents, wildebeest, and zebras (Darwish et al. 1983).

6 Flavivirus Structure

Flaviviruses comprise more than 70 different viruses, many of which are arthropod-borne and transmitted by either mosquitoes or ticks (Gubler et al. 2007). Taxonomically, they form a genus in the family *Flaviviridae* (Thiel et al. 2005; Gubler et al. 2007) (Table 2).

As all flaviviruses, ZIKV is an enveloped, icosahedral virus and its genome consists of

Table 2 Schematic representation of Flavivirus species, vector and peculiarities

Family	Genus	Species	Vector	Peculiarities
Flaviviridae	Flavivirus	Yellow fever virus (YFV)	Mosquito	Flavivirus of public health importance
		Dengue virus (DENV)	Mosquito	
		Japanese encephalitis virus (JEV)	Mosquito	
		West Nile virus (WNV)	Mosquito	
		Tick-borne encephalitis virus (TBE)	Tick	
		St. Louis encephalitis virus (SLEV)	Mosquito	Flavivirus causing also severe disease in humans but with limited potential exposure and small number of cases reported
		Murray Valley encephalitis (MVEV)	Mosquito	
		Rocio virus (ROCV)	Mosquito	
		Kyasanur forest virus (KFDV)	Tick	
		Alkhurma virus (ALKV)	Tick	
		Omsk Hemorragic fever virus (OHFV)	Tick	
		Powassan virus (POW)	Tick	

positive single-stranded RNA. The ZIKV genome, 10,794 kb long, contains a single open reading frame encoding a polyprotein that is cleaved into three structural proteins, i.e., the capsid (C), the precursor of membrane (prM), and the envelope (E) proteins, and seven non-structural proteins, i.e. NS1, NS2A, NS2B, NS3, NS4A, 2K, NS4B, and NS5 (Kuno et al. 1998; Chambers et al. 1990; Kuno and Chang. 2007). The E protein is the major virion surface glycoprotein and is involved in various aspects of the viral cycle, mediating binding and membrane fusion with the host cell.

After uncoating, translation and genome replication, assembly of viral particles occurs in the membranes derived from endoplasmic reticulum (ER). Immature virions containig prM and E proteins bud into the lumen of ER and are transported to the cell membrante through the secretary pathway (Lindenbach et al. 2007). Following cleavage of prM protein on immature virions by furin or furin-like cellular protease in the trans-Golgi, mature virions are generated and released from cells by exocytosis which express M protein (Keelapang et al. 2004).

Among non structural proteins, NS5 is the largest viral protein whose C-terminal portion has RNA-dependent RNA polymerase (RdRP) activity and the N terminus is involved in RNA capping by virtue of its processing due to methyl transferase activity (Lindenbach and Rice 2003). NS1 is a glycoprotein existing in multiple oligomeric forms and found at different cellular locations. It can be found associated with cell membrane (mNS1), both within cells and at cell surface, or as a secreted extracellular hexamer (sNS1). Intracellular NS1 plays an essential cofactor role in virus replication and has been

shown to co-localize with dsRNA and other components of the viral replication complex (Westaway and Goodman 1987; Westaway et al. 1997). Shed NS1 may play a role in serological diagnosis of ZIKV infection.

7 Mode of Transmission

7.1 Mosquitoe-Borne ZIKV Transmission

The primary mode of transmission of ZIKV between humans is through the bite of an infected female mosquito of the *Aedes* genus (WHO 2016; Campos et al. 2015; Zanluca et al. 2015), as already discussed in the previous paragraph. Noteworthy, the genus *Aedes* mosquitoes are able to transmit other important arboviruses i.e. CHIKV, DENV and YFV.

7.2 Sexual ZIKV Transmission

Among other non-vector means of transmission, sexual transmission has been firstly postulated by Foy DB and colleagues, who described a patient infected with ZIKV in Senegal in 2008 that, after returning to his home in Colorado, experienced common symptoms of ZIKV infection. Four days later, peculiar hematospermia appeared and, on the same day, his wife developed symptoms of ZIKV infection. Since the wife of the patient had not travelled out of the United States during the previous year and had sexual intercourse with him 1 day after he returned, transmission by semen was suggested (Foy et al. 2011). Other cases of sexual transmission associated with hematospermia have been subsequently described in Texas (McCarthy 2016), in Tahiti (Musso et al. 2015a, b) and in Florence, Italy, leading to the first autochthonous secondary case of ZIKV (Venturi et al. 2016). The presence of ZIKV RNA in semen for more than 4 months has been demonstrated (Nicastri et al. 2016a, b), and a recent study highlighted that ZIKV viral load in semen was up to 100.000 times higher than in other districts more than 3 weeks after start of symptoms, being able to replicate in African green monkey cells (Besnard et al. 2014; Hearn et al. 2014; Atkinson et al. 2016; Mansuy et al. 2016). These results, taken together, suggest that virus could replicate specifically in the male genital tract and may persist in semen for a long time, probably for several months, which, in turn, could indicate a prolonged potential for sexual transmission of ZIKV. Although published case reports have documented sexual transmission mainly from infected men to their female sex partners through vaginal sex, male-to-male sexual transmission of ZIKV was also described in Texas through anal sex (Deckard et al. 2016).

Moreover, suspected ZIKV female-to-male sexual transmission through condomless vaginal intercourse has been recently reported (Davidson et al. 2016). The woman was viremic at the time of sexual intercourse because her serum, collected 3 days later, had evidence of ZIKV RNA and 7 days after sexual intercourse (day 6), the woman's male partner developed fever, a maculopapular rash, joint pain, and conjunctivitis. Authors speculated that virus present in either vaginal fluids or menstrual blood might have been transmitted during exposure to her male partner's urethral mucosa or undetected abrasions on his penis. It has to be considered that recent reports document detection of ZIKV in the female genital tract, including vaginal fluid: (i) A study on nonhuman primates found ZIKV RNA detected in the vaginal fluid of three nonpregnant females up to 7 days after subcutaneous inoculation (Dudley et al. 2016); (ii) ZIKV RNA was detected in specimens from a woman's cervical mucous, genital swab, and endocervical swab collected 3 days after illness onset (Prisant et al. 2016). Interstingly, on day 11 after the onset of symptoms, the patient's blood and urinary samples tested negative, whereas her cervical mucus still tested positive for the presence of ZIKV RNA, thus suggesting a persistence in the female genital tract and its clearance after the disappearance of the symptoms.

Considering that current guidance to prevent sexual transmission of ZIKV is based on the assumption that transmission occurs from a

male partner to a receptive partner (Oster et al. 2016; Hills et al. 2016), ongoing surveillance is needed to determine the risk for transmission of ZIKV infection from a female to her sexual partners.

7.3 Blood Transfusion and Transplantation ZIKV Transmission

Musso and colleagues detected an unexpected high number of PCR-positive asymptomatic blood donors (42/1505;3%) during the outbreak, suggesting the potential for ZIKV transmission through blood transfusion (Musso et al. 2014a, b; Marano et al. 2016); following this possibility, the Food and Drug Administration released on February 2016 a new guidance to reduce the risk for blood transmission in the US, recommending the deferral of individuals from donating blood if they have been to areas with active ZIKV transmission (http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Blood/UCM486360.pdf). These recommendation have been implemented by National Blood Transfusion centre. The risk of transmission through organ donation is less documented; however, the National Transplant Networks highlighted that recipients of organs from persons with history of travel in the affected areas should be carefully monitored for symptoms consistent with ZIKV infection (CDC 2016).

The anamnestic surveillance for travel should also be strengthened to sperm donors. The Institutions of Medically Assisted Procreation (PMA) tissues should suspend the removal of sperm from donors for 3 months following the return from affected areas or at least new evidences can clarify the time of permanence of ZIKV in sperm.

7.4 Vertical ZIKV Transmission

Vertical transmission has been firstly postulated due to the potential link between microcephaly and ZIKV in pregnant women. The incidence of microcephaly in Brazil resulted several times higher than in previous years in parallel with the introduction of ZIKV (Kleber de Oliveira et al. 2016). Detection of ZIKV complete genome and ZIKV specific IgM in the amniotic fluid of two pregnant women living in Brazil with microcephalic fetuses supported the hypothesis that the virus can cross placental barrier and possibly infect the fetus (Calvet et al. 2016). The authors revealed the presence of the viral genome in amniotic fluid samples of both pregnant women, several weeks after the acute phase of the disease, when urine and serum resulted negative to the PCR analysis, thus suggesting that the intrauterine viral load could be due to persistent local replication (Calvet et al. 2016). Moreover, Wu and colleagues showed that a contemporary ZIKV strain isolated from the 2015 epidemic area could be vertically transmitted from the infected pregnant mice to their fetuses, where it specifically infected the cortical neural progenitors in the brain. They found that intraperitoneal injection of ZIKV in pregnant mice led to the infection of radial glia cells of dorsal ventricular zone of the foetuses and caused a marked reduction of these cortex founder cells, thus supporting the conclusion that vertically transmitted ZIKV affects fetal brain development (Wu et al. 2016).

Moreover a study performed in French Polynesia described the case of a mother resulted positive to ZIKV in the serum 5 days post-partum with the newborn positive up to 6 days. ZIKV was also detected in breast milk at day 4, which produced cytopathic effect (CPE) on Vero cells, indicating that the virus was able to replicate and suggesting perinatal transmission (Besnard et al. 2014).

7.5 Human-To-Human ZIKV Transmission

Reports of detection of ZIKV in saliva emphasize the important issue of human-to-human transmission through intimate contact during kissing or sexual intercourse. ZIKV has been

cultured from saliva from a patient during acute ZIKV infection on day 6 after symptom onset, and prolonged viral shedding was observed for up to 29 days (Barzon et al. 2016). The risk of human-to- human ZIKV transmission by saliva is difficult to quantify. Specific studies on the viral kinetics of ZIKV, viral load concentrations and duration of infectivity are urgently required for developing prevention guidelines and to establish whether pregnant women or those planning to get pregnant should be advised to avoid kissing anyone in ZIKV endemic areas (Liuzzi et al. 2016a, b). Moreover, a probable case of non-sexual spread from one person to another has been described, indicating that we still have more to learn about Zika transmission (http://www.cdc.gov/media/releases/2016/s0718-zika-utah-investigation.html).

8 Clinical Manifestations

8.1 Typical ZIKV Manifestation

The incubation period is estimated to range from 3 to 12 days after the mosquito bite.

The typical form of the disease associates a low-grade fever (between 37.8 °C and 38.5 °C), arthralgia, notably of small joints of hands and feet, with possible swollen joints, myalgia, headache, retroocular pain, conjunctivitis, and cutaneous maculopapular rash, with frequent post-infection asthenia. This typical clinical picture could easily be misdiagnosed with DENV or CHIKV fevers, two common arboviral infections which produce similar clinical presentations and are often responsible for simultaneous outbreaks (Moulin et al. 2016; Pessoa et al. 2016) (Table 3). Digestive troubles, mucous membrane ulcerations, pruritus and haematospermia have been more rarely observed (Simpson 1964; Filipe et al. 1973; Olson and Ksiazek 1981; Foy et al. 2011; Heang et al. 2012; Musso et al. 2015a, b).

Information on typical laboratory alterations associated with ZIKV infection are scarce, but include leukopenia, thrombocytopenia, subcutaneous bleedings (Karimi et al. 2016) and slight elevation of serum lactate dehydrogenase, gamma-glutamyl transferase, and of inflammatory parameters (C– reactive protein, fibrinogen and ferritin) (Tappe et al. 2014).

8.2 ZIKV-Associated Guillain-Barré Syndrome

Despite clinical manifestation of ZIKV are usually mild and generally resolve within 7 days, a cluster of Guillain-Barré syndrome (GBS) cases was identified during the 2013 French Polynesia outbreak (Ioos et al. 2014; Oehler et al. 2014). Clinically, GBS manifests as progressive weakness, which initiates in the lower limbs and ascends proximally over a few weeks. Accompanying the motor dysfunction and paralysis, patients typically present with reduction or absence of deep tendon reflexes and may also develop cranial nerve disorders. Oehler and colleagues reported the first case of GBS in a women 7 days after an influenza-like illness evoking ZIKV infection during the ZIKA and Dengue fever co-epidemics in French Polynesia, suggesting that the simultaneous epidemics could be a predisposing factor for developing GBS due to a sequential arboviral stimulation (Oehler et al. 2014). Subsequently, a further increase in the incidence of GBS was identified in several American countries where ZIKV epidemics have spread since 2015 (WHO. Situation Report. 19 Feb 2016; http://apps.who.int/iris/bitstream/10665/204454/1/zikasitrep_19Feb2016_eng.pdf?ua=1) ore definitive evidence for this association came from a recently published case-control study performed in French Polynesia in which 42 GBS cases were compared to 98 controls: the presence of anti-ZIKV IgM or IgG was observed in 98% of GBS cases and in 36% of controls, while neutralizing antibodies against ZIKV were found in 100% of GBS cases as compared with 56% of controls. Since the incidence of GBS was estimated to be 2.4 per 10,000 ZIKV infection in French Polynesia, although it is unknown the attack rates of ZIKV epidemics in Latin America.

Table 3 Schematic representation of clinical sign and symptoms in chikungunya, dengue and zika virus diseases

Sign/Symptoms	Dengue	Zika	Chikungunya
Fever (duration)	>38 °C (4–7 days)	Absent or mild (1–2 days)	>38 °C (2–3 days)
Rash (frequency)	Starting from day 4 (30–50% of cases)	Starting from day 1–2 (90–100% of cases)	Starting from day 2–5 (50% of cases)
Myalgia (frequency)	***/***	**/***	*/***
Arthralgia (frequency)	*/***	**/***	***/***
Arthralgia (intensity)	Mild	Mild/moderate	Moderate/severe
Edems joint	Rare	Frequent but mild	Frequent and moderate/severe
Conjunctivitis	Rare	50–90% of cases	30% of cases
Headache	***	**	**
Itch	Mild	Moderate/severe	Mild
Lymphoadenopathy	Mild	Severe	Moderate

Nonetheless, an increase number of GBs cases might be expected in the coming months as the results of this association (Cao-Lormeau et al. 2016).

8.3 ZIKV-Associated Neurological Disorders

ZIKV can cause other neurological syndromes such as myelitis, meningitis and meningoencephalitis, as described in French Polynesia outbreak (Solomon et al. 2016; Broutet et al. 2016; Shakir 2016). A case report described the hospitalization of a 15-years old girl who developed acute lower back pain, paraesthesia on the left side of her body, without fever or signs of meningism. Laboratory analyses were normal, with the exception for raised leucocytes and polymorphonuclear leucocytes. Brain magnetic resonance imaging (MRI) was normal while spinal MRI showed lesions of the cervical and thoracic spinal cord. High concentrations of ZIKV were found in serum, urine, and cerebrospinal fluid of the patient with acute myelitis, thus providing further evidence that this virus might be neurotropic (Mécharles et al. 2016).

Moreover, Carteaux and colleagues have described a case of central nervous system infection with ZIKV in an 81-year-old man febrile and comatose, with hemiplegia of his left side, and paresis of the right upper limb. MRI of the brain was suggestive of meningoencephalitis and several electroencephalograms showed no direct signs that were suggestive also of epilepsy. The patient resulted positive for ZIKV on CSF and the virus was able to growth in culture Vero cell lineage, thus supporting the diagnosis of ZIKV-associated meningoencephalitis (Carteaux et al. 2016).

8.4 ZIKV-Associated Microcephaly

Cluster of microcephaly cases have been described, and the causal relation between in-utero exposure to ZIKV and microcephaly was initially strongly suspected (Victora et al. 2016; Liuzzi et al. 2016a, b). As defined by WHO microcephaly occurs whenever the occipital frontal circumference of the head of the newborn child or fetus is 2 standard deviations smaller than the mean for the same age and sex (de Onis and Onyanngo 2008). Congenital microcephaly can be caused by various factors including genetic disorders, exposure to chemicals and drugs, brain injury, and intrauterine infection with viruses and bacteria (Van der Hagen et al. 2014). A case of microcephaly, with almost complete agyria, hydrocephalus and multifocal dystrophy calcifications in the cortex and

subcortical white matter was described following autopsy performed on fetus at 32 weeks of gestation from an expectant mother living in Brazil and infected with ZIKV during the first trimester of pregnancy (Mlakar et al. 2016). No presence of virus and no pathological changes were detected in any other fetal organs apart from the brain where an high copy number of ZIKV RNA were detected, thus suggesting a strong neurotropism of the virus (Mlakar et al. 2016). Both ZIKV genome and IgM have been also detected in amniotic fluid from fetuses that were diagnosed with microcephaly prenatally, and also in brain and placenta tissues from infected newborn, thus suggesting that ZIKV can cross the placenta barrier (Martines et al. 2016; Calvet et al. 2016). Retrospective studies performed on ZIKV outbreak in French Polynesia showed that of 8 cases of microcephaly reported, 7 occurred in a 4-month period around the end of ZIKV outbreak, thus supporting a temporal association between the two events. Moreover, in the proposed model the baseline prevalence of microcephaly was 2 cases per 10,000 neonates, and the risk of microcephaly associated with ZIKV infection was 95 cases per 10,000 women infected in the first trimester, strongly supporting the hypothesis that infection in first trimester of pregnancy is associated with an increased risk of microcephaly (Cauchemez et al. 2016).

Further support comes from a study in the US on 9 pregnant women returning from affected areas with confirmed ZIKV infection during pregnancy, among which no hospitalization or deaths were reported (Meaney-Delman et al. 2016). More in detail, pregnancy outcomes included two early pregnancy losses, two elective terminations, and three live births (one infant with severe microcephaly and two apparently healthy); the two pregnant women infected later during gestatation, were continuing pregnancy without complication, thus suggesting an association between the early infection during pregnancy and the severe outcome (Meaney-Delman et al. 2016).

The CDC has announced that, on the basis of a review of the available evidence, using both criteria that are specific for the evaluation of potential teratogens (Shepard 1994) and the Bradford Hill criteria (Hill 1965) as frameworks, sufficient evidences have been accumulated to infer a causal relationship between prenatal Zika virus infection and microcephaly and other severe brain anomalies (http://www.theguardian.com/world/2016/apr/13/zika-virus-confirmed-cause-microcephaly-birth-defect-cdc; Rasmussen et al. 2016)

In in vitro study Tang and colleagues showed that MR766 strain of the ZIKV was able to efficiently infect human neural progenitor cells (hNPCs) derived from induced pluripotent stem cells, while human embryonic stem cells (hESC) and immature neurons exhibit lower levels of infection, supporting the suspected link between ZIKV infection and microcephaly. Noteworthy, the effect of ZIKV infection was an increased cell death and a dysregulation of cell-cycle progression of hNPC (Tang et al. 2016). Another interesting hypothesis has been proposed by Gil Mor who suggested that microcephaly resulting from ZIKV infection may be the results of an inflammatory process in the placenta that disrupts the production of neuropeptides and grow factors necessary for normal brain development (Mor 2016).

9 Diagnosis

Laboratory ZIKV diagnosis is challenging since there is no "gold standard" diagnostic tool. Acute phase diagnosis relies on molecular technologies and must take into account that the viremia is low and of short duration. Studies performed during French Polynesia outbreak highlight that ZIKV can be generally detected in serum during the first 5 days after the clinical onset and that the use of saliva samples can increase the rate of molecular detection of ZIKV (Musso et al. 2015a, b). Saliva is of particular interest when blood is difficult to collect, for instance in case of children and neonates. In fact, the presence of viral RNA in saliva and urine has been shown to be more conspicuous and more prolonged than blood viremia (up to 29 days

after onset symptoms), highlighting the importance of saliva as a preferred sample both for nucleic acid detection and for virus isolation (Barzon et al. 2016; Liuzzi et al. 2016a, b).

Urine has been demonstrated to be an useful sample for diagnosis since it resulted positive for ZIKV RNA >20 days after disease onset (Gourinat et al. 2015). In particular situations another useful sample could be semen, since numerous studies indicate prolonged (up to 62 days after clinical disease) presence of virus in semen (Besnard et al. 2014; Hearn et al. 2014; Atkinson et al. 2016). A recently published study demonstrated the presence, in a men returning from Haiti, of ZIKV RNA in urine and saliva 91 days after symptom onset and in semen up to day 134, indicating a prolonged potential risk for sexual transmission, for a period longer than previously reported (Nicastri et al. 2016a, b)

ZIKV RNA can also be detected in amniotic fluid of pregnant women. Indeed, positive results to ZIKV infection have been used to verify the possible infection of the fetus (Oliveira et al. 2016).

Since viremia decreases over time, a negative RT-PCR in blood collected 5–7 days after symptom onset does not exclude ZIKV infection, and serologic testing should be performed. Several serological methods are available to detect antibodies against ZIKV, based on either ELISA or immuniofluorescence, only some of which are commercially available, and often without extended clinical validation (Charrel et al. 2016). However, antibody detection presents several bias, due to the high similarity of viral proteins against which antibody are directed, generating high cross-reactivity among the members of flavivirus genus.

Cross reactivity of ZIKV antibodies with other flaviviruses may require confirmation by neutralization assay (Oehler et al. 2014) and makes rapid serologic confirmation difficult. In addition, the interpretation of neutralization test is complex since, despite neutralization generally improve specificity over immunoassays, it may still yield cross-reactive results in secondary flaviviruses infections and the increase of the titre could not be sufficiently discriminatory for one flavivirus with respect to the others (Lanciotti et al. 2008), thus highlighting that serology interpretation is troublesome and complex.

Virus specific IgM antibodies may be detectable in serum starting from 4 days after illness onset and generally persist for approximately 2–12 week, while IgG antibodies develop a few days after IgM appearance, and can persist for months or years. In a study performed during Yap epidemics Lanciotti and colleagues have demonstrated that ZIKV-infected patients can be positive in an IgM assay for DENVs, particularly if ZIKV is a secondary flavivirus infection, while if ZIKV is the first flavivirus encountered, minimal cross-reactivity is observed. Results from this study suggest that, if ZIKV infections occur in a population with DENV (or other flavivirus) background immunity, extensive cross-reactivity in the dengue IgM assay could occur, leading to the erroneous conclusion concerning the cause of the infection (Lanciotti et al. 2008). As result, misdiagnosis based on IgM assay results is relatively common, and, in fact, DENV infection was first diagnosed on the basis of serological results in an Italian patient returning from Thailand, but a retrospective analysis of this infection turned out to be due to ZIKV (Venturi et al. 2016). Due to the serological cross-reactivity also in case of vaccination against YFV, neutralization assay could be necessary to discriminate between cross-reacting antibodies. Neutralizing antibodies have been described to develop generally as early as 5 days after illness, but this is not always the case. In general, serological diagnostic testing for flavivirus infections should include an acute-phase serum sample collected as early as possible after the onset of illness and a second sample collected 2–3 weeks later, in order to evaluate the possible seroconversion, and in any case interpretation requires careful circumstantial evaluation.

A special group of patients, i.e. pregnant women, should be considered since the timing of appearance of IgM and IgG could be shifted during pregnancy. Moreover ZIKV IgM have

been also detected in amniotic fluid from fetuses with diagnosed microcephaly (Martines et al. 2016; Calvet et al. 2016), thus suggesting that also this biological fluid could be useful, in particular situations, to interpret the diagnostic data (Nicastri et al. 2016a, b).

10 Biological Basis for Pathogenetic Effects

The majority of ZIKV infection is transmitted when mosquitoes bite the skin of humans. The role of the skin in the pathogenesis of infection carries potential significance as an entry-point into the body. Mosquito-borne viruses have evolved to bypass the physical skin barrier by hitch-hiking on blood-sucking arthropod vectors. As keratinocytes and fibroblasts are the most abundant cell population in the epidermis, viruses acquiring the capacity to replicate in these resident cells represents an attractive strategy for host colonization. Epidermal keratinocytes have been demonstrated to support WNV and DENV replication (Lim et al. 2011; Surasombatpattana et al. 2011), while CHIKV antigens revealed the presence of virus infected cells at the level of deep dermis, suggesting a role of fibroblasts located in the basal skin layers (Couderc et al. 2008).

Skin fibroblasts and epidermal keratinocytes have been demonstrated to be highly susceptible to ZIKV infection with active replication soon after 6 hours post infection, and immature dendritic cells also resulted permissive to ZIKV infection (Hamel et al. 2015) (Fig. 2).

The susceptibility of the host tissue to the virus depends on the abundance and distribution of cell receptors, that promote virus entry into the target cell (Grove and Marsh 2011). A large number of receptors and/or attachment factors seem able to mediate entry of ZIKV into permissive cells, thus providing an evolutionary advantage for the virus that is able to infect a wide range of targets and invade the human host.

ZIKV entry is mediated by adhesion factors including the adhesion molecule of dendritic cells (DC-SIGN) interacting with the viral envelope protein, as already described also for DENV (Cruz-Oliveira 2015). Moreover, transmembrane receptors TIM and TAM (AXL or Tyro3), known to act as DENV promoting viral infecton by attaching and possibly internalizing viral particles in human cell (Meertens et al. 2012; Perera-Lecoin et al. 2014), have been also involved in ZIKV entry. More in detail, TIM-1 has been demonstrated to have additive effect on the efficacy of AXL-mediated viral entry, acting as a possibly cooperator able to bind viral particles and transfers them to AXL (Hamel et al. 2015).

The envelope protein is the primary flavivirus antigenic site and dictates attachment of the virions and penetration into the host cell. Mutations in the envelope protein of other arboviruses have been demonstrated to have an impact on the pathogenesis: for instance the envelope A226V mutation of CHIKV has been associated with enhanced replication and fitness of the virus in *Ae. albopictus* vector (Tsetsarkin et al. 2007) and was the object of studies aimed at investigating its possible involvement in enhancing human pathogenesis (Bordi et al. 2008, 2011). In a recent study aimed at comparing genome of pre-epidemics and epidemic ZIKV strains, a number of amino acid substitution in E protein were found, being V603I and D679E present in all epidemic strains, but in none of the pre-epidemics strains, thus suggesting a possible involvement in the pathogenesis of the severe complications that seem to be associated only with the recent strains (Zhu et al. 2016). Faye and colleagues demonstrated that, among recombination events, ZIKV have experienced the recurrent loss and gain of the N-linked glycosylation site around position 154 of E protein, which could be related to vector competence (Faye et al. 2014; Chang et al. 2016; Lee et al. 2010). Mutations at the NS1 N-glycosylation sites have been demonstrated to significantly affect viral replication and virulence in YFV (Muylaert et al. 1997) and WNV (Hindiyeh et al. 2001; Scherret et al. 2001; Shirato et al. 2004). Moreover, N-linked glycosylation site of the E protein in WNV and other flaviviruses has been linked to alterations in pH sensitivity and virus yield

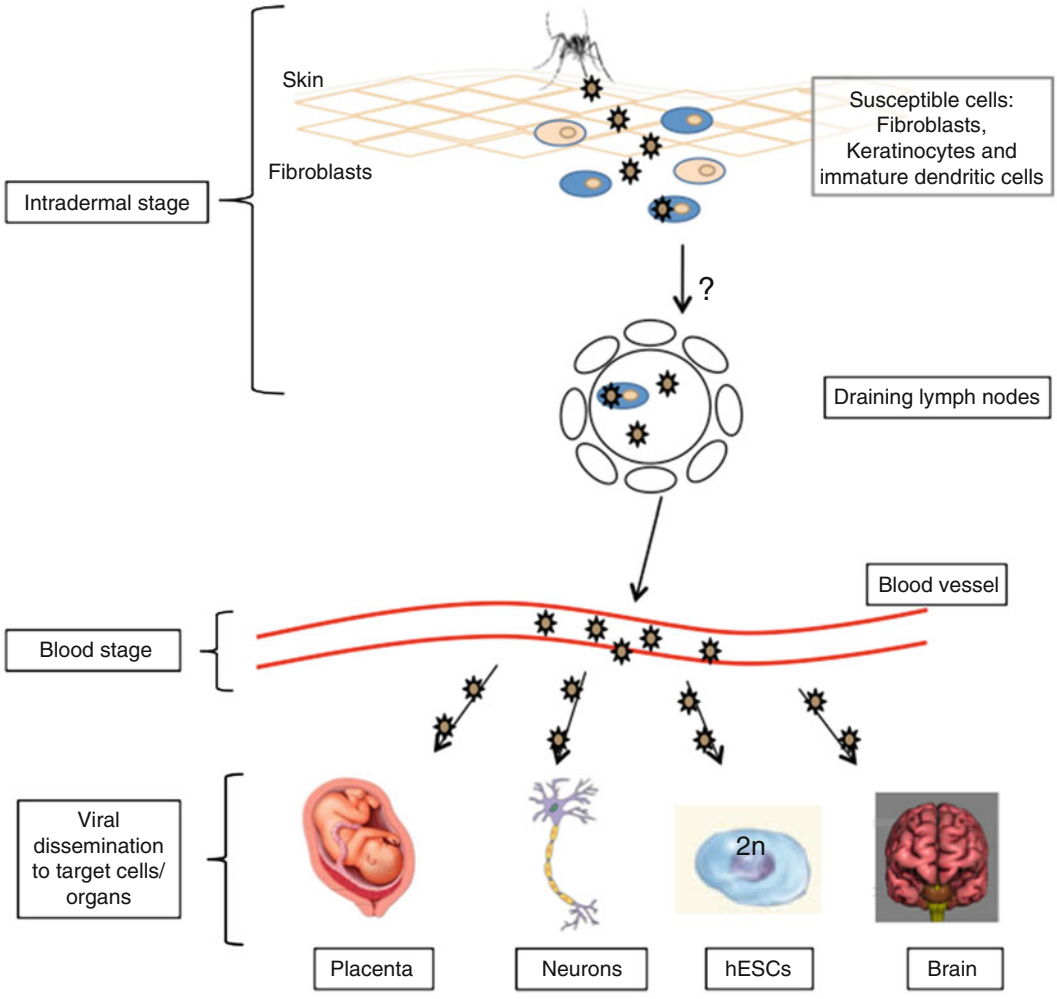

Fig. 2 Schematic representation of ZIKV entry and dissemination to different cells and organs

(Vorndam et al. 1993; Lee et al. 1997) and has been postulated to play a significant role in the interaction between DENV and DC-SIGN on the surface of immature dendritic cells in vitro (Tassaneetrithep et al. 2003). Altogether these evidences support the hypothesis that modulation of N-glycosylation could be associated with a different ability to bind the surface of host cells. Another important protein involved in flavivirus pathogenesis and in immune response evasion is NS1. Dengue NS1 protein has been reported to share common epitopes with human blood-clotting integrin-adhesion proteins and endothelial cell surface protein (Falconar 1997, 2007). Antibodies generated against the NS1 protein have been described to cross-react with host proteins thereby leading to autoimmune damage of the host tissues. A study from de Melo Freire and colleagues showed that ZIKV Asian lineage spread is associated with significant NS1 codon usage adaption to human cells, facilitating viral replication and increasing viral titers, thus suggesting a role of NS1 protein in the higher pathogenicity of the Asian lineages, together with NS4A (de Melo Freire et al. 2015).

NS2A, NS2B, NS4A and NS4B of flaviviruses are small, hydrophobic proteins, playing important roles in the assembly or anchoring of the viral replication complexes on the endoplasmic reticular membrane (Miller

et al. 2006) and exerting important effects on the host immune response (Aguirre et al. 2012). Two amino acid substitutions were recently found in all the ZIKV epidemic strains, including V2449I and L2451S, together with eight amino acid substitutions in the NS3 and NS5 (M1970L, T2630V, A2783V,N2892S, K3046R, P3158S, S3219D and D3383N), whose significance should be further investigated (Zhu et al. 2016).

The innate immune response is the first barrier against viruses, being able to inhibit viral replication through cytolytic and non-cytolytic mechanisms. ZIKV infection has been demonstrated to strongly induce the expression of several antiviral gene, in particular those for pattern recognition receptors (PRRs), able to detect the presence of pathogen-associated molecular patterns (PAMPS). More in detail, a rapid induction of Toll like receptor3 (TLR3), already detectable at 6 hours post infection was observed, whereas that of RIG-1 and MDA-5 was delayed, while no modulation of TLR7 expression was reported.

Following ZIKV infection, enhanced interferon-α (IFN-α) and interferon-β (IFN-β) expression was detected, together with up-regulation of several interferon-stimulated genes (ISGs) such as OAS2, ISG15, Mx1; induced expression was also observed for the two CXCCR3 ligands, CxCL10 and CXCL11, that are chemokines involved in the innate and adaptive immune response (Hamel et al. 2015).

A polyfunctional immune activation was seen during the acute phase of ZIKV infection, as reflected by elevated cytokine profiles associated with Th1 (IL-2, and non-significantly IFN-γ), Th2 (IL-4, IL-13), Th17 (IL-17), and also Th9 (IL-9) responses, followed by a decrease in the reconvalescent phase; IFN-γ and TNF-α seem not to increase significantly, pointing toward a Th2 bias in ZIKV infections (Tappe et al. 2015). A recent issue of Cell Host & Microbe presented two elegant studies: one on ZIKV mouse model (Lazear et al. 2016) and another on placental restriction of ZIKV infection (Bayer et al. 2016), both underscoring the importance of innate immunity in modulating ZIKV infection and disease outcome.

Moreover, ZIKV infection has been shown to induce an autophagy program in cultured skin fibroblast, as demonstrated by the presence of characteristic autophagoseme-like vesicles in association with enhanced viral replication (Hamel et al. 2015).

Usually, ZIKV is responsible for a mild self limiting disease, despite during recent epidemics increasing cases of neurological manifestation and GBS occurred. In early 1952 Dick and colleagues demonstrated ZIKV tropism for the brain in the intraperitoneally infected mice (Dick et al. 1952), and about 20 years later Bell and colleagues showed that the virus was able to infect both neurons and glia, producing a variety of intracytoplasmic inclusions, which they termed, "virus factories" (Bell et al. 1971). A very recent study describing microcephaly on fetus at 32 weeks of gestation revealed an high copy number of ZIKV RNA only in the brain, thus confirming the strong neurotropism of the virus (Mlakar et al. 2016) (Fig. 2). Another important evidence comes from recent publication of Tang and colleagues, who demonstrated that ZIKV is able to infect human cortical neuronal progenitor cells with high efficiency, resulting in stunted growth of this cell population and transcriptional dysregulation (Tang et al. 2016) (Fig. 2). Noteworthy, the AXL tyrosine kinase is expressed by glioma cells, providing a clue for its role in the pathogenesis of brain development dysfunction putatively associated with ZIKV infection (Hamel et al. 2015). In a recent study has been further demonstrated that AXL is highly expressed by human radial glial cells, astrocytes, endothelial cells, and microglia in developing human cortex and by progenitor cells in developing retina and that AXL expression in radial glia is conserved in developing mouse and ferret cortex and in human stem cell-derived cerebral organoids, thus highlighting multiple experimental systems that could be applied to study mechanisms of ZIKV infectivity and effects on brain development (Nowakowsk et al. 2016).

A study by Anaya and colleague suggested that, beside the direct toxicity of ZIKV on neuronal cells, also indirect effects induced through

an autoimmune response similar to those observed in GBS should be considered (Anaya et al. 2016). Gangliosides are crucial in brain development and their expression correlates with neuronogenesis, synaptogenesis, synaptic transmission, cell proliferation (Ghiulai et al. 2014) and antibodies that recognize gangliosides play a crucial role in the pathogenesis of GBS. Authors speculated that gangliosides could be the link between ZIKV, GBS and microcephaly and targeting the autoimmune response to gangliosides could represent an opportunity to examine the increased incidence of neurological complications (Anaya et al. 2016). Li and colleagues showed that peripheral ZIKV exposure in a mouse model could infect adult neural stem cells in the brain, leading to cell death and reduced proliferation. Thus, in addition to impacting fetal development, ZIKV infection may also have negative effects on the adult brain (Li et al. 2016).

Moreover, histopatological findings indicated the presence of ZIKV in fetal and placenta tissues, thus suggesting that ZIKV can cross the placenta barrier (Fig. 2) (Martines et al. 2016; Calvet et al. 2016). It has been demonstrated that primary human placental macrophages and placental villous fibroblasts are permissive for ZIKV replication in isolated cultures in vitro and that, in addition to amplifying infectious virus within a usually inaccessible area, the putative migratory activities of Hofbauer cells may aid in dissemination of ZIKV to the fetal brain (Jurado et al. 2016). Understanding the susceptibility of placenta-specific cell types will aid future work around and understanding of ZIKV-associated pregnancy complications.

Presently, comprehension of ZIKV immunobiology is still in its early moments and there is a long way to go before answers related to the direct and indirect interactions between virus and host homeostasis and immunity will be obtained. These will certainly be important in designing novel antiviral control strategies against ZIKV infection.

11 Perspective for ZIKV Control and Treatment

11.1 Vector Control

The rapid spread of ZIKV in Latin America is believed to be the result of high *Aedes* mosquito densities, their adaptation to urban environment and the lack of immunity (Chang et al. 2016). An important strategy to mitigate the spreading of the virus is therefore source control of the mosquito vectors. In the immediate future the best prospects for controlling ZIKV rely on reducing contact between the vector and susceptible humans, especially pregnant women who represent the highest risk of severe disease. The most effective approach to reducing such contact would be to eliminate or reduce these mosquito populations by: (1) Mechanical approaches, such as ovitraps; (2) Chemical approaches such as outdoor residual spray, insecticides with both adulticidal and larvicidal properties (despite considered environmentally unacceptable today), the replacement of chemical compound with plants extract, the use of insect growth regulator; (3) Biological approaches such as the use of bacterial (*Bacillus thuringiensis israelensis* or *Wolbachia*) and fungal control measures which can spread through natural populations and suppress viral transmission by interfering with replication in the mosquito; the use of Copepods as predators; (4) Genetic approaches such as the release of genetically modified mosquitoes that express a dominant, lethal gene at the larval stage, resulting in the death of all offspring from mating with wild females, leaving no risk to persistence of the transgene in nature; (5) RNAi-boosted insect immune responses and homing endonuclease genes.

11.2 Vaccine Development

Clinically approved vaccines are available for four flaviviruses: (1) Vaccine for YFV (17D

strain) is a live attenuated virus successfully used in humans since 1937; (2) Both inactivated-virus vaccine are available for JEV (3) A chimeric virus, representing the 17D vaccine strain of YFV that contains the structural membrane and envelope genes from DENV, was recently approved for clinical use for dengue virus. It is assumed that ZIKV vaccine can be developed adopting the above approaches. The landscape analysis revealed around 18 active programmes exploring different vaccine approaches including purified inactivated virus, nucleic acid based vaccines, live vectored vaccines, Virus like particles (VLPs) technologies, subunit vaccines and live recombinant approach. Each vaccine approach has its pros and cons. For instance, subunit vaccines could have better safety and shorter development time as compared with a live attenuated virus vaccine; on the other hand, a live attenuated virus vaccine may trigger more robust humoral and cellular immune response for better protection.

11.3 Therapeutics

No clinically approved therapy is currently available for treatment of any flavivirus infections (Lim et al. 2013; Shan et al. 2016). Over the past decade, significant effort has been made towards dengue drug discovery. Due to the structural similarity between ZIKV and DGV, knowledge derived from dengue drug discovery could be applied to ZIKV, taking into account that the biology of the two flaviviruses could be very different. Among the therapeutic substances under evaluation there are Amadiaquine, Cloroquine, Ribavirin, Interferon α, BCX4430, NITD008, whose activity has already been tested for some other flaviviruses in hamsters or mice or in vitro. GS-5734 is a new substance under investigation never tested before. Concerning monoclonal antibodies, passive immunization is expected to provide short-term protection. Animal models that recapture human diseases of ZIKV infection, including MC and GBS, are urgently needed for vaccine and therapeutic development.

12 Conclusions

Given the rapid geographic spread of ZIKV in recent years and the severe ZIKV-associated neurological manifestation, a coordinated and global effort is needed to contain further expansion of the current outbreak. The key public health research priorities emerged from PAHO Public Health Research Meeting included:

- to ensure the availability of validated diagnostic kits, guidelines for laboratories to construct sample panels for validation and use as reference material, purchase and distributing of basic reagents for laboratory detection and diagnostics, harmonizing protocols and data collection tools
- to determine the absolute risk of microcephaly and other birth defects by gestational age, mother's clinical course and viraemia, and other co-factors with the objective of defining evidence based protective measures for pregnant women.
- to describe the characteristics, neurological impairment, evolution, complications, and mortality of newborns with microcephaly, in order to design interventions for improving outcomes.
- to assess countries' capacity to support intensive care beds to manage patients with GBS.
- to define best practices and accelerate the assessment of new tools for vector control.
- to characterize the dynamics of the epidemic, and that of the vector, including better defining the species of vector mosquitoes most involved in transmission, and other potential routes of transmission.

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Syrian Hamsters as a Small Animal Model for Emerging Infectious Diseases: Advances in Immunologic Methods

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Abstract

The use of small animal models for the study of infectious disease is critical for understanding disease progression and for developing prophylactic and therapeutic treatment options. For many diseases, Syrian golden hamsters have emerged as an ideal animal model due to their low cost, small size, ease of handling, and ability to accurately reflect disease progression in humans. Despite the increasing use and popularity of hamsters, there remains a lack of available reagents for studying hamster immune responses. Without suitable reagents for assessing immune responses, researchers are left to examine clinical signs and disease pathology. This becomes an issue for the development of vaccine and treatment options where characterizing the type of immune response generated is critical for understanding protection from disease. Despite the relative lack of reagents for use in hamsters, significant advances have been made recently with several hamster specific immunologic methods being developed. Here we discuss the progress of this development, with focus on classical methods used as well as more recent molecular methods. We outline what methods are currently available for use in

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hamsters and what is readily used as well as what limitations still exist and future perspectives of reagent and assay development for hamsters. This will provide valuable information to researchers who are deciding whether to use hamsters as an animal model.

Keywords
Syrian hamster • Golden hamster • Syrian golden hamster • Immunological tools • Molecular methods • Mesocricetus auratus • Animal models

1 Introduction

The need for a reliable and representative animal model for the study of a particular disease is critical for expanding our understanding of the disease and for developing therapeutics and ultimately a cure. The first steps in developing intervention strategies involve establishing a small animal model for study of the disease, typically in rodents where mice are the most common model due to their low cost, ease of handling, availability of reagents, and potential for genetic manipulation. Despite their prominence and widespread use, there are many instances where mice are not suitable candidates for a disease model and alternative model is necessary. The guidelines for selecting an appropriate animal model have been outlined by the Canadian Council for Animal Care (CCAC) (Animal Care 1997) and the United States Food and Drug Administration (FDA) (FDA 2015). A model should be chosen such that the disease is pathophysiologically similar to the human condition in terms of onset, progression, symptoms, pathology, and disease outcome (FDA 2015). Additionally, the challenge agent should reliably cause disease in the model animal consistent with the parameters above and the host should be susceptible to a realistic challenge of the disease agent (FDA 2015).

Due to the ability of Syrian golden hamsters (*Mesocricetus auratus*; hereafter referred to as hamsters) to satisfy many of the conditions outlined by the CCAC and the FDA, they have been used as an alternative to mice in many disease models. There are many advantages to using hamsters as a disease model. They are outbred animals, allowing for disease modelling with more genetic diversity than inbred mice. Also, the requirements for housing of hamsters is similar to that of mice and rats, and facilities designed for housing rodents can typically accommodate hamsters without the need for additional equipment. Hamsters can be cohoused in small cages, which is a significant advantage over other alternative disease models such as guinea pigs and ferrets. These advantages are why many consider hamsters a superior alternative to other small animals for use in research. For the development of vaccines and therapeutic approaches, some consider hamsters a higher standard as small animal model than mice and as such hamsters have been utilized in a wide range of models, from those examining diabetes, atherosclerosis, neural plasticity, to cancer (Table 1) (Bhathena et al. 2011; Dillard et al. 2010; Jové et al. 2013; Staffend and Meisel 2012; Woods et al. 2015; Vijayalingam et al. 2014). However, the use of hamsters for models of pathogenic human diseases may be the most valuable due to comparable disease progression seen in hamsters to that of humans for many infectious diseases including bacteria, viruses, and parasites (Dondji et al. 2008; da Silva-Couto et al. 2015; Kuehne et al. 2014; Safronetz et al. 2012). Specifically, hamsters are used as a disease model for many high consequence

pathogens such as bunyaviruses, arenaviruses, henipaviruses, flaviviruses, alphaviruses, filoviruses, and SARS-corona virus (Table 1) (Safronetz et al. 2009, 2012, 2013; Brown et al. 2011; Schountz et al. 2015; DeBuysscher et al. 2013; Gowen and Holbrook 2008; Steele and Twenhafel 2010; Ebihara et al. 2012; Gowen et al. 2010; Roberts et al. 2010). Additionally, hamsters can be used for the evaluation of vaccines and therapeutic treatments against these viruses. In some cases, as with Andes virus, hamsters are the only lethal model of the disease (Safronetz et al. 2012). The value of hamsters as an animal model is research is only recently being realized. The popularity of hamsters used for infectious disease research has increased significantly the last several years (Fig. 1). This growing use of hamsters highlights the need for the development of hamster-specific reagents for a wide range of applications including immunological assays.

Table 1 Infectious disease models utilizing Hamsters

Infectious disease models	References
Hantavirus pulmonary syndrome	Safronetz et al. (2012)
Eastern equine encephalitis	Steele and Twenhafel (2010)
Leishmaniasis	da Silva-Couto et al. (2015)
Leptospirosis	Silva et al. (2007)
Nipah virus encephalitis	DeBuysscher et al. (2013)
Scrapie	Sokolowski et al. (2003)
Ebola hemorrhagic fever	Ebihara et al. (2012)
Rift Valley virus	Scharton et al. (2014)
SARS-corona virus	Roberts et al. (2010)
Yellow fever virus	Gowen and Holbrook (2008)
Clostridium difficile	Kuehne et al. (2014)
Helicobacter spp.	Woods et al. (2015)

In spite of the growing use of hamsters as disease models, there remains a lack of available immunological reagents developed for assessing immune responses in these animals. Often, researchers are left with examining clinical signs of disease progression and pathology (Zivcec et al. 2011). For the study of disease progression and pathophysiology, this is of little concern, but for the development of vaccines, therapeutic drugs, and determining correlates of immune protection for infectious diseases, evaluating the immune response is critical. Fundamental tools for the study of both innate and adaptive host immune responses commonly used in other models such as mouse and non-human primates have not yet been developed. The result

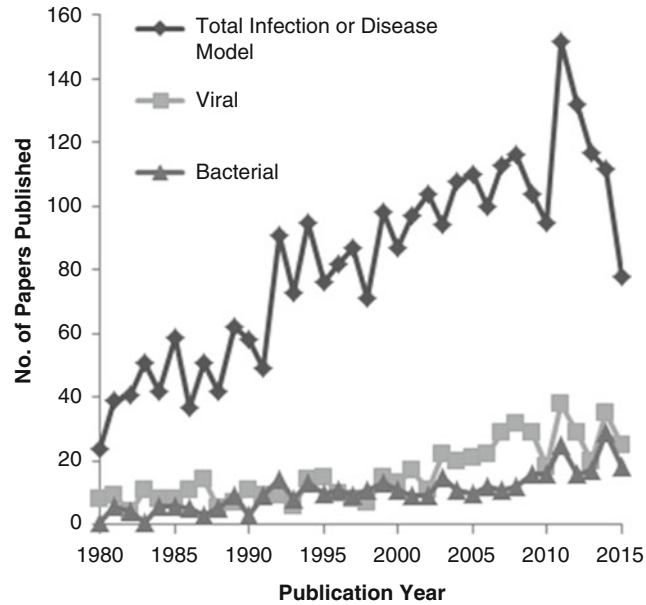

Fig. 1 Number of publications using Hamsters as a disease model. The publications using hamsters as an animal model from 1980 through 2015 are shown. For each criterion, the number of publications was determined via a search using the Scopus abstraction and citation database. Searches were performed with the keywords "Syrian-golden-hamster", "Mesocricetus", or "Syrian-hamster" and the keyword "model", as well as either (a) "viral" or "virus", (b) "bacteria", (c) "infection" or "disease"

is that researchers who are using or plan to use a hamster model of disease need to use alternative methods for evaluating the immune response.

The widespread use of laboratory mice over the course of the last century has led to great advances in many fields. The combination of whole genome sequencing for *Mus musculus* being complete and the nearly universal use of mice for many decades have led to the development of countless mouse-specific reagents used in many disciplines (Chinwalla et al. 2002). The complete sequencing of the hamster genome has been performed at the BROAD Institute (NCBI BioProject 77669) and assembly of the hamster genome only recently completed (http://www.genome.gov/27557963). This recent completion will hopefully lead to a surge in the development of hamster specific research tools. Currently, there are 874 cDNA sequences or expressed sequence tags (ESTs) available from the hamster genome in the NCBI-dbEST database (Boguski et al. 1993). The lack of available sequence data and resulting insufficient tools for molecular biology in hamsters has hamstrung scientists who are looking to use them as a disease model. In addition to the insufficient sequence data for hamsters until very recently, the characterization of many immune-specific markers in the hamster remains to be done. The number of these markers that have been described in the mouse, including cell surface markers, transcription factors, signaling proteins, cytokines, chemokines, and even secreted effectors molecules dwarfs the work that has been done in almost every other species, including hamsters. Additionally, monoclonal antibodies against nearly all of the described immunological markers in mice can be readily found commercially, whereas monoclonal antibodies against hamster specific immune markers are almost completely non-existent. The considerable lag in the time taken to sequence the hamster genome coupled with an almost non-existent commercial collection of monoclonal antibodies developed against hamster specific proteins and immunological markers has limited the advancement of hamster models from an immunological perspective.

Despite the scarcity of available reagents for immunological assays in hamster models, there have been significant advances in the methods used to characterize the immune response in hamsters and there are still many reasons why hamsters are a good choice as an animal model of disease. The development of assays to evaluate immune responses in hamsters has been critical in their use as a model for infectious disease and vaccine development in addition to the aforementioned ability of hamster models to closely mimic the human condition of many diseases and satisfy requirements of a suitable animal model. In this review, we focus on how the progress of immunological assay development in hamsters, from determination of cross-reacting antibodies against hamster markers, hamster specific ELISAs and qRT-PCR, to transcriptome analysis and microarrays. We discuss how the assays that have been developed to this point are being utilized in current hamster models to assess immune responses as well as advantages and disadvantages of these currently available assays in the context of particular models. Finally, we address how recent advances in developing immunological tools for use in hamsters can potentially influence future progress along with what remains to be resolved in this area, providing ideas of what we think would be valuable additions to a growing resource for researchers who plan to use hamsters as an animal model.

2 Immunological Methods Currently Used in Hamster Models

2.1 qRT-PCR

While quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) has been a technique of choice in diagnostics, detection of pathogens, and determination of viral loads, the potential for utilizing qRT-PCR for determination of immune responses in tissues has been realized in many models. For example, in human models qRT-PCR has been employed

for the detection of innate cytokines and transcription factors involved in immune responses in cells infected with SARS-coronavirus, the detection of upregulated genes implicated in immune escape in circulating tumor cells, and evaluating immune responses generated in patients given the live attenuated yellow fever vaccine (Zielecki et al. 2013; Steinert et al. 2014; Gaucher et al. 2008). Similarly, in mice, qRT-PCR has been used to describe the mechanisms of immune activation in certain vaccine models, to determine the transcription factors involved in dendritic cell mediated presentation of antigen and the subsequent activation of T cells, to investigate the mechanisms behind macrophage polarization, and to evaluate the role of certain subsets of T cells in infectious disease models (Pollard et al. 2013; Seillet et al. 2013; Davis et al. 2013; Stross et al. 2012). Despite the fact that in many models, qRT-PCR is used to determine the relative gene expression of both innate and adaptive cytokines and chemokines (Zivcec et al. 2011; Safronetz et al. 2011a; Prescott et al. 2013; Overbergh et al. 2003), the examples above illustrate that the ability to detect mRNA of other non-cytokine and non-chemokine genes such as transcription factors and cell surface markers can play a valuable role in evaluating immunity generated in certain instances (Gaucher et al. 2008; Seillet et al. 2013). The fact that qRT-PCR is used in models such as human and mouse models, whereby immunological reagents are readily available and many aspects specific immunity can be analyzed with relatively ease using other methods shows the value and relevance of qRT-PCR among today's available assays.

In hamsters, qRT-PCR is currently the method of choice for many in evaluating immune responses in infectious disease models. Recently, a panel of TaqMan® prime/probe assays for 51 specifically targeted genes in the hamster that were chosen out of a set of more than 800 reference mRNA sequences in GenBank was described (Zivcec et al. 2011). Each are involved in either pro-inflammatory, anti-inflammatory, innate immune responses, T cell responses, as well as non-immune pathways such as apoptosis, cell junction, or coagulation responses in the hamster (Zivcec et al. 2011). Additionally, the validation of an appropriate housekeeping gene for use in qRT-PCR assays in the hamster was simultaneously performed, with ribosomal protein L18 (RPL18) identified as the most stable of the housekeeping genes tested among β-actin, β-2-microglobulin, and Hypoxanthine phosphoribosyltransferase (Zivcec et al. 2011). Consequently, the use of qRT-PCR in hamster models as an immunological tool has increased greatly. qRT-PCR has been utilized in hamster models studying disease caused by Andes virus (Safronetz et al. 2011a, b; Prescott et al. 2013), ebola virus hemorrhagic fever (Ebihara et al. 2012), Nipah virus (DeBuysscher et al. 2013), and Leishmania spp. (da Silva-Couto et al. 2015) among others.

The development of specific primer/probe sets for use in qRT-PCR in hamsters has improved upon the limited options that are available for researchers who are looking to use hamsters as a small animal model. This advancement allows for detection of a broad range of immune and cellular factors and has been crucial to expanding the use of hamsters as an animal model. That said, while qRT-PCR is in all likelihood the best immunological tool available in hamsters currently, it is still an assay that has its considerable disadvantages. First, the number of immune-related genes that have been sequenced and their mRNA sequences entered into GenBank is still relatively low. The panel of primers that was validated contained only 51 genes that played a role in host immune responses (Table 2) (Zivcec et al. 2011). While many of the genes reported by play an important role in host immunity, the number of immune factors that can be assays pales in comparison to what is available in mouse and human assays. Second, while the study of the immune-related genes involved in disease systems can provide valuable insight into the class of immunity generated or what type of immunity is needed for protection against certain pathogens, the relative amount of mRNA present does not always correlate directly with the amount of expressed protein (Overbergh et al. 2003). In many instances, the presence of

Table 2 Genes validated for qRT-PCR in Hamsters

Genes validated in Hamsters for qRT-PCR	
IL-1β	Complement component 5
IL-2	Complement component C1qBP
IL-2Rα	Chemokine ligand 17
IL-6	Chemokine ligand 22
IL-6 transducer	Muc1
IL-12p35	IL-4
IL-12p40	IL-10
IL-21	FoxP3
CXCL10	IRF2
ICAM-1	TGF-β
STAT1	TGF-β2
STAT1β	TGF-β3
STAT2	TGFβ type I receptor
IFNγ	MHC II α chain
Interferon regulatory factor (IRF) 1	PECAM
TNFα	Bcl-2
P75 TNF membrane receptor	Bcl-2 associated protein
Myxovirus resistance protein 2	Ecadherin
Protein kinase R	Tight junction protein
IFNα inducible protein p27	Junction adhesion molecule
CD83	Claudulin-1
CCL20/MIP3-α	Occludin
NOS2	Matrix metallinoproteinase-2
Inducible NOS	Tissue inhibitor of matrix metalloproteinase-2
Complement C3d region	Fibrinogen A α chain
Vascular endothelial growth factor	

Reference: Zivcec et al. (2011)

expressed protein will be very small, whereby qRT-PCR and relative gene expression must be used instead. In these instances, and particularly in using qRT-PCR for analyzing the expression of immune-related genes in hamsters, a discrepancy between mRNA and protein levels should be considered (Overbergh et al. 2003). In spite of this, there is evidence that there is a good correlation between mRNA and protein levels in some instances (Hein et al. 2001; Blaschke et al. 2000). Finally, the methods used for collection of tissues in hamsters for analysis by qRT-PCR in most models do not allow for the evaluation of specific gene expression in individual cell types. Perhaps the most important flaw to consider when using qRT-PCR for analyzing the expression of immune-related genes is that hamster tissues are often harvested and total RNA is extracted from the tissues for analysis by qRT-PCR (Safronetz et al. 2011a; Prescott et al. 2013; Chattopadhyay et al. 2014). A lack of hamster specific antibodies does not allow for the isolation of individual cell types for analysis of gene expression. Therefore, the detection of antigen-specific immune responses at the individual cell level is nearly impossible, leaving only systemic responses and total cytokine, chemokine, and other markers to be detected. This can cause issues when attempting to detect primary and secondary immune responses, what types of innate cells play a role in protection against pathogens, and determining what the correlates of protection are against certain diseases.

Until more hamster specific monoclonal antibodies become commercially available, qRT-PCR for determining the relative expression levels of immune response genes is one of the best tools at the researcher's disposal today. Despite certain flaws such as the number of existing analytes to test, and inherent issues with the assay like discrepancies between mRNA and protein levels, and an inability to distinguish between certain cell types, qRT-PCR has become a standard immunological method for use in hamster models. It allows for the simultaneous detection of a large number of immune markers (Table 2), and is a reliable and sensitive assay, with a typical limit of detection between 10^{-6} and 10^{-8} ng of gene-specific RNA, corresponding to approximately 9–900 RNA copies (Chattopadhyay et al. 2014). qRT-PCR is one of the most valuable methods we have for immunological analysis in hamster models.

2.2 ELISA

Enzyme-linked immunosorbent assays (ELISA) have become standard practice for the detection of cytokines, antibodies, and other proteins since being described in 1971 by Engvall and

Perlmann (Engvall and Perlmann 1971). Although originally described as a method for the detection of IgG antibodies, ELISAs have subsequently been developed and optimized for detection of a wide variety of proteins, the most common being cytokines that can be detected in biological samples (Hornbeck 1991). For species like mice, non-human primates, and humans a large number of commercially available kits can be obtained for a reasonable price for the detection of nearly every important cytokine or chemokine. Alternatively, antibodies against cytokines from these species are readily available as well, including antibodies with enzymatic conjugates, allowing for the optimization of individual protocols to each researcher's liking. In hamsters however, these antibodies for the detection of cytokines are not available. A study examining cross-reactivity of hamster proteins reported that out of 64 antibody-based assays including luminex and ELISA for the detection of cytokines and chemokines in various species, 14 showed significant cross-reactivity with hamster proteins (Zivcec et al. 2011). Out of eight ELISA kits that were tested, only three showed an acceptable level of cross-reactivity with hamster proteins (Zivcec et al. 2011). Due to the high level of sequence homology of the genes for these proteins in the species tested compared with hamsters, this low percentage of cross-reactivity is surprising. The authors concluded from these experiments that ELISA kits and kits for the detection of cytokines and chemokines from other species such as mice and rats were of little value for use in hamsters (Zivcec et al. 2011).

Because of the lack of antibodies for the detection of hamster cytokines, it follows that another valuable and related immune assay, the enzyme-linked immunospot (ELISPOT) assay, is also of little value when using a hamster model. This assay detects individual cytokine producing cells in culture rather than the presence of cytokines in a culture supernatant or serum, but similarly utilizes anti-cytokine antibodies to detect cytokine secreting cells. The lack of antibodies that can detect hamster specific cytokines limits the use of both ELISA and ELISPOT for cytokine detection in hamster models. Development of monoclonal antibodies against various cytokines from hamsters would greatly improve the number of assays that could be utilized for examining immune responses in hamsters.

Despite the inability to use ELISAs for detection of cytokines in hamsters, ELISAs can still be a valuable tool for the detection of antibodies in hamster models. True to its original use, ELISAs can be used to quantify the presence of hamster IgM and IgG. Out of the limited number of commercially available anti-hamster antibodies, anti-hamster IgM and IgG are available and have been used to show the presence of antigen specific antibodies in various hamster models (Safronetz et al. 2009; Prescott et al. 2015; de Wit et al. 2013). In general, the use of a direct ELISA assay is ideal for detection of antibodies in the serum of hamsters. Therefore a recombinant or purified antigen is necessary to coat ELISA plates before the detection of specific antibodies in serum can be achieved. This has been performed in models for hantavirus cardiopulmonary syndrome caused by Andes virus (Safronetz et al. 2009), Ebola hemorrhagic fever (Prescott et al. 2015), and MERS-CoV (de Wit et al. 2013) among others. This has provided a valuable tool for the evaluation of humoral immunity in hamsters in response to infectious agents.

Despite the value of being able to detect IgM and IgG in hamsters, these are the only two isotypes for which there are currently available antibodies against. The detection of different isotypes such as IgG1 and IgG2 would be invaluable to researchers in evaluating the immune response given the other limited options available in hamsters. Additionally, being able to detect IgE, IgA, and IgD, also important players in many immune models, would increase the value of using hamsters as an animal model. Once again, the lack of hamster specific reagents limits the ability to examine the full scope of the immune response. Currently, we are left determining titers of hamster specific IgM and total IgG until further detection antibodies are developed.

2.3 Flowcytometry

Using flowcytometry for the detection of specific cell types has become standard immunological practice. Flowcytometry can identify the presence of specific markers on cells, the activation status of lymphocytes, whether specific subsets of cells are present in tissues, and what cell subsets are producing certain cytokines. In addition, fluorescence activated cell sorting (FACS) can sort cell types upon recognition of fluorescent markers bound to cells via specific antibodies. The specificity and sensitivity of flowcytometry, along with the advent of large panels capable of recognizing over a dozen fluorescent markers on cells make it one of the most valuable tools for immunological analyses. For hamsters, there are currently no monoclonal antibodies against cell surface markers specific to hamster cells for use in flowcytometry. However, many studies have used cross-reactive antibodies against cell surface markers of mice and rats for use in flowcytometry (Prescott et al. 2013; Hammerbeck and Hooper 2012). Hammerbeck and Hooper reported that out of a panel consisting of 52 commercially available antibodies for use in flowcytometry, four were able to identify hamster cells (Hammerbeck and Hooper 2012). The four cross-reactive antibodies included anti-mouse/rat MHC II (I-E^k), anti-mouse CD4, anti-rat CD8β, and anti-mouse Thy1.2 (Hammerbeck and Hooper 2012), confirming some which had been reported to cross-react with hamster cells previously (Dondji et al. 2008; Liu et al. 1990). Antibodies against hamster IgM and IgG can also be used to detect hamster B cells in flowcytometry as well (Hammerbeck and Hooper 2012).

The limitations regarding flowcytometry use with hamster cells is that there are only these very few antibodies available, with many antibodies developed for use with mouse and rat cells non cross-reactive. Another issue is that each specific clone should be tested to confirm cross-reactivity with hamster cells, as the clones generated by different companies may not react with the same specificity with hamster cells in every case. Additionally, the few antibodies that are available that cross-react are limited to cell surface proteins on T and B cells. This results in an inability to determine activation status, cell subset, or identify other non-T and B cells like macrophages, dendritic cells, neutrophils, and NK cells. As with ELISA and ELISPOT assays in hamsters, the lack of antibodies against hamster-specific cytokines prevents using flowcytometry to detect cytokine producing cells by intracellular staining for phenotyping of the immune response. This is a major limitation on the use of flowcytometry in hamster models. Finally, the use of anti-mouse CD4 and anti-rat CD8β antibodies for use in hamsters must be done in conjunction with anti-mouse/rat MHC II to allow for the exclusion of myeloid lineage cells expressing either CD4 or CD8β, which has shown to be the case in mice (Hammerbeck and Hooper 2012). Fortunately the combination of these antibodies has been shown to be effective in identifying CD4 and CD8 T cells in hamsters due to the lack of binding of cells by both anti-mouse CD4 or anti-rat CD8β and anti-mouse/rat MHC II (Hammerbeck and Hooper 2012). Overall, the use of flowcytometry in hamsters in severely limited due to the lack hamster-specific antibodies. The identification and possible sorting of B cells and CD4 and CD8 T cells is possible due to the cross-reactivity of anti-mouse and anti-rat antibodies, but should be done with caution and optimization of protocols by individual researchers to determine cross-reactivity levels of specific clones.

Related to the use of monoclonal antibodies against mouse and rat cell surface markers that are able to cross-react with hamster proteins for use in flowcytometry, these antibodies have been shown to be effective at depletion of CD4 and CD8 T cells *in vivo* in hamsters (Prescott et al. 2013, 2015; Hammerbeck and Hooper 2012). The ability of these antibodies to recognize hamster T cells and mediate depletion *in vivo* allows for depletion studies and for determining the importance of CD4 or CD8 T cells in infectious disease models. The ability of cross-

reactive antibodies to deplete T cell *in vivo* is a considerable advantage in examining host immune responses in disease models. With the limited ability to assess certain immune parameters *ex vivo* in hamsters, this provides a critical tool for immunological studies in hamsters. The same caveats exist for the use of these antibodies for depletion of cells *in vivo* as for their use in flowcytometry, but this provides an interesting avenue for researchers to pursue when evaluating the immune response against certain pathogens or testing drug and vaccine efficacy.

2.4 Immunohistochemistry

Immunohistochemistry has been a powerful method for the detection of specific antigens within formalin-fixed tissues for decades (Schacht and Kern 2015). Since the advent of hybridomas for the production of monoclonal antibodies in 1975, immunohistochemistry has adopted the use of monoclonal antibodies for the detection of specific antigens with great specificity (Schacht and Kern 2015; Köhler and Milstein 1975). The use of specific antibodies allows for the detection within fixed tissues of bacteria, viruses, certain cell types such as lymphocytes, and cellular markers of disease pathology. While immunohistochemistry has important applications in diagnostics, in animal models of disease, it is particularly useful for assessing pathology in affected tissues, and can detect the present of tissue infiltrating immune cells that may be causing immunopathogenesis during the course of disease.

Immunohistochemistry in hamsters for assessing disease pathology has been a popular technique due to the lack of reagents available for classical immunological methods. It has been used to examine the course of disease in many infectious disease models including Andes virus, Sin Nombre virus, Chikungunya virus, Nipah virus, and Ebola virus (Safronetz et al. 2013; DeBuysscher et al. 2013; Ebihara et al. 2012; Safronetz et al. 2011b; Bosco-Lauth et al. 2015). It is also useful in non-infectious disease models in hamsters such as cancer and encephalopathic diseases (Woods et al. 2015; Clouse et al. 2015; Elder et al. 2013). While the use of this technique in hamsters is typically limited to examining preserved tissues that display disease pathology, the advantages are that cross sections of entire tissues can be visualized to give a more representative image of disease tropism and the presence of immune cells or of particular pathogens can easily be detected (Schacht and Kern 2015). As with other immunological methods in hamsters, the detection of most immune cells is limited to few cross-reactive antibodies available, and antibodies against common cell surface markers found in many tissues are not available or their cross-reactivity with antibodies against these markers in other species has not been assessed. Other inherent limitations of immunohistochemistry include the alteration of antigens during the fixation process, the relative insensitivity of the assay as compared to other techniques like ELISA and PCR, and the technical demands involved in the procedure (Schacht and Kern 2015). Particularly in the case of pathogens that require high containment facilities, the procedure for fixation of tissues can be up to several weeks long, increasing the possibility of altered tissues when best results are obtained as soon after euthanization as possible. Despite these issues, the value of using immunohistochemistry in hamster models is the ability to examine multiple tissues that impacted during the course of disease, determining specific tissue tropism of pathogens by visualizing pathogens in infected tissues, and studying possible immune cells infiltration into tissues that could be contributing to immunopathogenesis during certain diseases.

2.5 Transcriptome/Microarray Analysis

In recent years, transcriptome sequencing and analysis has been utilized to provide sets of mRNA that are expressed in a given species, help provide insights into the expression profiles of these species, and for the development of

microarrays for the detection of gene expression (Tchitchek et al. 2014; Ying et al. 2015). The description of the transcriptome of mice, rats, and humans has been critical for the increasing use of microarray for examining expression profiles in tissues in many models (Yu et al. 2009; Okazaki et al. 2002; Yang et al. 2010; Maywood et al. 2009). Recently, hamster transcriptome sequencing and analysis has been performed in several models (Ying et al. 2015; Yu et al. 2009; Yang et al. 2010; Maywood et al. 2009; Hohlweg et al. 2003; Schmucki et al. 2013). In recent years, the use of microarray analysis in hamsters has been limited to cross-reactive hybridization of hamster RNA to cDNA from other species such as rats, mice, and humans (Yu et al. 2009; Wahl-Jensen et al. 2012). cDNAs comprising the hamster transcriptome have been sequence aligned to the transcriptome of species that have been described previously (Tchitchek et al. 2014). This transcriptome sequencing has allowed for the identification of a large set of genes that play a role in a number of biological processes. Subsequently, Ying et al were able to sequence and annotate over 34,000 sequences comprising the hamster transcriptome for the development of a custom hamster microarray (Ying et al. 2015). The microarray that was developed from the hamster transcriptome was validated by comparing gene expression profiles in mice infected with Adenovirus using the custom microarray and qRT-PCR (Ying et al. 2015). This was one of the first descriptions of a hamster specific microarray capable of detecting changes in hamster gene expression. This newly developed microarray following the sequencing and annotation of the hamster transcriptome will hopefully lead to the production of more hamster specific microarrays. While RNA-seq has been recently used to examine the regulation of genes in hamster in a model for Arenavirus infection (Schountz et al. 2015), the use of RNA-seq has not yet become common in hamster models of infectious disease. It is likely that the use of RNA-seq will become more popular in coming years as the genome and transcriptome of hamsters becomes fully characterized and publicly available, the description of the hamster transcriptome and its value in the development of hamster specific microarrays should not be overlooked. This recent work on the hamster transcriptome will hopefully lead to beneficial tools like microarrays for researchers looking to use hamsters as an animal model of disease.

2.6 Kinome Analysis

The ability to evaluate host responses to pathogens has historically relied upon examining gene expression or protein synthesis in the form of antibodies or cytokines. Since many of the intracellular pathways involved in immune cell signalling are well known, and many proteins have been characterized, the cell signalling proteins within host cells have become recently become a target for therapeutics. The ability of kinases to phosphorylate proteins is critical in cellular signalling, and allows for rapid responses to environmental stimuli such as stress or infection (Arsenault et al. 2011; Falcinelli et al. 2015). Only recently has the potential of examining the presence of kinases involved in cell signalling, collectively called the kinome, been realized. The study of the kinases involved in immune signalling can give important indicators of the outcome of disease in certain models (Falcinelli et al. 2015). Kinome analysis involves synthesizing peptides representing phosphorylation sites on hundreds of proteins that are immobilized onto an array surface (Arsenault et al. 2011). Samples containing cellular kinases phosphorylate the immobilized peptides, which can then be visualized to determine the level of relative phosphorylation and the activity of the kinases in the sample (Arsenault et al. 2011). The examination of the kinome during the host response to pathogens has been used as a tool to define cellular responses and evaluate host immune responses in different disease models (Kindrachuk et al. 2012, 2014; Arsenault et al. 2013; Kindrachuk and Napper 2013).

In hamsters, a kinome peptide array was recently synthesized by Falcinelli et al. in a model for Arenavirus infection (Falcinelli et al. 2015). This hamster specific kinome was developed with peptides focused primarily on immune pathways, and showed that Arenavirus

infection in hamsters is characterized by lung vascular endothelial growth factor and interleukin responses as well as NF-kB and TLR signalling (Falcinelli et al. 2015). This presents a novel assay for assessing hamster-specific immune responses to infection by examining the activity level of host cell kinases.

2.7 Genetic Manipulations

The ability to genetically manipulate animals for use in research has been invaluable for decades in countless disease models. Since the first description of genetically manipulated mice in the late 1980s by Martin Evans, Oliver Smithies, and Mario Capecchi, which led to the Nobel Prize in Physiology or Medicine, the use of genetically modified animals has revolutionized biomedical research (Manis 2007; Thomas and Capecchi 1987; Capecchi 2005). For immunologists, the use of knockout and transgenic mice has been essential for determining the roles of cell types, cytokines, and transcription factors as well as providing valuable insights into things like immune memory and regulation (Manis 2007). Genetically modified mice have become so common, that commercially available transgenic and knockout mice for dozens of genes are readily available.

Until recently, gene targeting in hamsters has been limited due to the lack of a completely sequenced genome. However, Fan et al. have reported a CRISPR/Cas9 system for the targeting of hamster specific genes (Fan et al. 2014). They were able to successfully target the STAT2 gene of hamsters with reliable efficiency to produce STAT2 knockout hamsters (Fan et al. 2014). These hamsters have been subsequently used by others in an Adenovirus model to show that type I IFN responses are critical in controlling Adenovirus infection (Toth et al. 2015). These are the first studies to report on and employ genetically modified hamsters. These results show the potential for not only using STAT2 knockout hamsters in studying disease models, but also the potential to knockout other genes that play important roles in immune pathways. These studies will hopefully serve as the first step in developing many more knockout and possible transgenic hamsters for use as models for infectious disease.

3 Future Directions

The growing use of hamsters in small animal models of disease has brought to light a glaring need for the development of hamster specific reagents for use in immunological assays. The study of the immune response in nearly any disease model is critical for developing therapeutic options and an understanding of the disease course. The number of available reagents for use in hamster models right now is not where it needs to be for sufficient insight into how these animals are protected from or develop disease (Table 3). As of now, the best methods for immunological assays in hamsters are qRT-PCR for detection of expression of immune-related genes and ELISA for the detection of humoral immune responses. Despite being used as an animal model for decades, immunological tools for hamsters remains years behind other animal models such as mice, rats, and non-human primates. For the full potential of hamsters as an appropriate disease model to be realized, developing of many different hamster-specific tools for assays that are specific to hamsters need to be created.

Table 3 Immunological methods used in Hamster models

Immunological methods used in Hamsters	References
qRT-PCR	Zivcec et al. (2011) and Safronetz et al. (2011b)
ELISA	Safronetz et al. (2009) and Brown et al. (2011)
Flowcytometry	Prescott et al. (2013) and Hammerbeck and Hooper (2012)
Immunohistochemistry	DeBuysscher et al. (2013) and Ebihara et al. (2012)
Transcriptome analysis	Tchitchek et al. (2014) and Ying et al. (2015)
Microarray	Ying et al. (2015)
Kinome analysis	Falcinelli et al. (2015)

The use of cross-reactive monoclonal antibodies against immune markers and cytokines from other species has given researchers using hamsters a viable option for certain assays, but the development of monoclonal antibodies that are hamster-specific for a variety of immune cell surface markers and cytokines would greatly improve upon the current state of immunological assays done in hamster models. The production of hamster-specific monoclonal antibodies against common immune markers and cytokines should be number one on the wish list of anyone doing immunological assays with hamsters. The ability to perform a plethora of techniques from ELISA, ELISPOT, flowcytometry, and microscopy would increase greatly with the production of only a few dozen hamster-specific monoclonal antibodies.

In the coming years, with the ability to produce monoclonal antibodies with increased efficiency and the increase in the use of hamsters as an animal model of diseases where understanding the immune response is critical, antibodies for use in hamsters will hopefully see an increase in demand and production. With the sequencing of the hamster genome and transcriptome, we have seen the development of novel assays for use in hamster models like microarrays and kinome analysis via peptide arrays. The first use of gene knockout hamsters has been reported. These advances will hopefully open the flood gates in terms of what becomes available for researchers in the near future. The recent sequencing projects that have gone on have given us the ability to uncover the gene sequences of many immune-related genes and the proteins that are encoded by them. The demand for the reagents available for use in other species like mice and rats should increase by a large amount as hamsters become more and more popular as animal models of disease. We now have the ability to develop the repertoire of reagents that is available in commonly used species. The recent advances in hamster-specific immunological tools gives us reason to hope that soon researchers will have a number of assays at their disposal when conducting experiments in hamsters.

4 Concluding Remarks

The use of hamsters as an animal model has increased greatly in recent years due to their ability to recapitulate human disease in models for diseases like Hantavirus, Ebola virus, Nipah virus, C difficile, Leishmania spp., as well as cancers, and atherosclerosis (Dillard et al. 2010; Jové et al. 2013; Woods et al. 2015; da Silva-Couto et al. 2015; Kuehne et al. 2014; Safronetz et al. 2009, 2012; DeBuysscher et al. 2013; Ebihara et al. 2012). The realization of hamsters as valuable animal models has led to their use in studying disease course for many pathogens. However, the lack of immunological reagents available for use in hamsters limits their value when it comes to developing therapeutic options, vaccines, and determining correlates of protection or immunopathogenesis of disease. The methods available currently pale in comparison to those available for use in other species like mice, rats, and non-human primates and the ones that are available have many inherent disadvantages. For hamsters to continue to grow into a common animal model of disease, and one that can hopefully lead to important therapeutic and vaccine developments in the coming years as the number of available immunological reagents in this species needs to improve greatly.

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Enabling Rapid Response to the 2014–2016 Ebola Epidemic: The Experience and the Results of the National Institute for Infectious Diseases Lazzaro Spallanzani

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Abstract

The unprecedented epidemic of Ebola virus disease (EVD) in West Africa highlighted the need for stronger systems for disease surveillance, response, and prevention worldwide. Tackling an epidemic event today requires a broader view, not only limited to medical management of the patients, but which also includes heroic efforts by clinicians and public health personnel.

Since its foundation in 1936, INMI has been devoted to the prevention, diagnosis and care for infectious diseases. In 2009, INMI became a WHO collaborative center for clinical care, diagnosis, response and training on Highly Infectious Diseases. This paper is aimed to present the activities and the challenging issues encountered by INMI during the 2014–2015 EVD outbreak in terms of preparedness and response to the epidemiological, clinical, diagnostic and research controversial aspects of EVD, both in Italy and in the field.

Keywords

Ebola virus • Clinical management • Preparedness • High containment laboratory • Mobile laboratory • High isolation unit

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1 Introduction

On March, 19 2014 the Guinea Conakry health authorities reported at least 35 cases of an unknown disease characterized by diarrhoea, vomiting, high fever and in some cases bleeding.

Most of the victims had been in contact with the deceased persons or had handled their bodies. The patients were put in isolation and samples were shipped to Senegal for testing. Samples were also delivered to Biosafety Level 4 (BSL4) laboratories in Lyon and Hamburg, where the diagnosis of Ebola virus disease (EVD) was confirmed (Baize et al. 2014).

This was the beginning of an unprecedented EVD epidemic in West Africa, with 28,610 confirmed/suspect cases reported in Guinea, Liberia, and Sierra Leone, and 11,308 deaths (as of 30 March 2016) (WHO Sitrep 30 March 2016); this epidemic highlighted the need for a new model of preparedness in emerging infections, based on stronger and more integrated systems, high patient care capabilities, diagnosis, disease surveillance, rapid response, research and prevention (Di Caro et al. 2015).

The management of EVD patients is a severe commitment for all involved players. Firstly, well-trained staff, logistics and infrastructures for adequate isolation, updated infection control procedures, along with high-level diagnostic capabilities, are needed. Secondly, preparedness and response activities, such as translational research, established international links and collaborations, and capability to deploy resources on-the-field in affected areas, are also crucial in EVD management. Finally, a certain amount of enthusiasm and courage is the necessary complement of a successful response.

The challenge arising from the unprecedented spread of EVD promoted every healthcare system in the world to assess its preparedness and capability to safely manage suspected and confirmed patients, not only in respect of EVD, but also from other highly threatening contagious diseases.

Since the initial phases of the epidemic, the National Institute for Infectious Diseases "L. Spallanzani" (INMI) has been committed to cope with EVD, through a multidisciplinary approach based on a common strategic path: to make available to public health authorities and the community a long lasting preparedness model, skills and capabilities; to concur to the international response against EVD outbreak; to advance the level of treatment of patients and protection of health care workers (HCWs); to contribute to the advancement of knowledge on Ebola virus (EBOV) and EVD.

The aim of this review is to summarize INMI activities in response to the West African EVD outbreak, in order to describe the leading role of a single institution in clinical, epidemiological, diagnostic, and research activities, and to illustrate the challenges and the results of this integrated approach.

2 The National Institute for Infectious Diseases "Lazzaro Spallanzani": A Bit of History

Since its foundation in 1936, INMI is devoted to infectious diseases (IDs), and at present it is recognized as a leader institution in care, diagnosis, prevention and research in this field. Over the years, INMI followed a pathway to adapt to the evolving patterns of IDs threats. This pathway is illustrated in Fig. 1. In late '70, a high isolation unit was built by the Italian Ministry of Health (MoH), and INMI was identified as national reference center for the management of patients affected by Highly Infectious Diseases (HIDs). Since then, INMI started a continuous program based on long-term investment in preparedness and response against emerging IDs, on improving infrastructure and increasing integrated capabilities for research, diagnosis, patient's management, infection control, risk assessment and communication to respond to infectious health threats, whether naturally occurring, newly emerging, or deliberately released (Armignacco et al. 2001; Ippolito et al. 2005). In 1995, MoH identified INMI as national reference center for viral hemorrhagic fevers and in 2001, for national reference center for IDs and bioterrorism. In 2014, INMI was confirmed by the MoH as the national reference center for patients with EVD during the current West African Outbreak.

Across the years, INMI established long-term connections and agreements with internationally recognized institutions in the field of research, public health, diagnosis and management of HIDs. Some relevant milestones of this

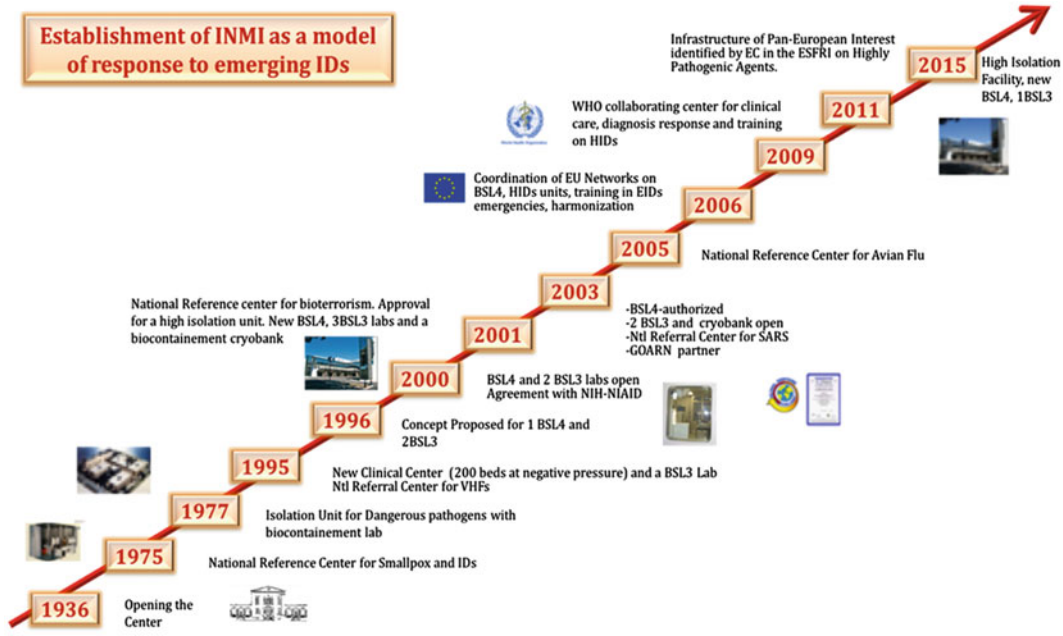

Fig. 1 Timeline of INMI pathway towards the establishment of a model of preparedness and response to IDs

pathway are the following. In 2000, INMI signed an agreement with US-NIH-NIAID; in 2003, INMI became member of GOARN; in 2009, the Institute was appointed as WHO Collaborating Center for clinical care, diagnosis, response and training on HIDs. Furthermore, INMI plays a role as coordinator or participant in several public health and research projects on hospital and laboratory preparedness for emerging pathogens, working in close collaboration with the European Commission, ECDC, WHO and other International and Public Health bodies.

Since March 2014, immediately after the identification of the EVD outbreak, INMI contributed to the international efforts to control the epidemic with: (a) deployment of scientists, laboratorians, clinicians and epidemiologists in affected countries; (b) establishment and field management of mobile laboratories; (c) support to national and international public health authorities in the outbreak response; (d) coordination of the high bio-containment laboratory European network; (e) development of a local clinical and preclinical research plan; (f) participation to international consortia for research; (g) management of international health cooperation programmes.

This paper is aimed to summarize INMI's activities during the 2014–2015 EVD outbreak.

3 The Added Value of Long-Term Preparedness: The INMI Model

3.1 Preparedness: How to Respond to an EVD Outbreak

The INMI preparedness model is founded on a 20-year long inter-sector pathway, based on the following cornerstones:

- *Prevention, infection control and bio-containment:* implementation of a uniformed protocol for a safe transport system in collaboration with the Italian Air Force, presence of areas specifically designed for high-level isolation and advanced diagnostic infrastructures for

the handling of risk group 3 and 4 agents (Fusco et al. 2015);

- *Advanced diagnostic capabilities*: state-of-the-art methods for the detection of risk group 3 and 4 agents, as well as availability of high profile diagnostic methods and competence to perform differential diagnosis. The virology team coordinates an international network of BSL-4 facilities to exchange data, specimens, reagents and provide validation of new diagnostic tests and research results (Brouqui et al. 2009);
- *High level infection prevention and control (IPC) expertise:* IPC procedures are revised and updated in real time, according to the evolving HIDs scenario, to guarantee a high level of safety in the management of highly infectious diseases (Schilling et al. 2014; Bannister et al. 2009; Puro et al. 2015; Fusco et al. 2016);
- *Well-trained and skilled clinical staff*: a long-standing multidisciplinary task-force of infectious diseases, epidemiology, laboratory and intensive care experts;
- *Well-established international links:* INMI is involved in several preparedness and research projects and networks, and works in close collaboration with the European Commission, ECDC, WHO and other International Public Health bodies;
- *Field experience in developing countries:* INMI contribution to the field activity is longstanding and involves a team of enthusiastic health care workers, ranging from physicians, virologists, microbiologists and immunologists, who not only provided support and training at INMI, but have also been deployed in developing countries, during outbreaks of emerging HIDs.

3.2 Laboratory Challenges: Etiological Testing

The diagnosis of EVD is mainly based on molecular testing; however all the steps involving the manipulation of infectious samples must be performed under the highest level of biosafety. At the beginning of the outbreak, no commercial tests were available, the diagnosis was performed using in house tests developed by few groups worldwide with expertise in risk group 4 viruses; these tests had been shared and validated in the pre-pandemic phases, through the networks established in this field, such as EuroNetP4/Quandhip, coordinated by INMI. Soon after the identification of EBOV as the causative agent of the 2014 outbreak, the first commercial tests became available and were widely distributed to laboratories with diagnostic capability for HIDs.

Following the internationally agreed policy, EVD testing outside the epidemic area is based on two steps (screening and confirmation, based on different RT-PCR target regions), in order to overcome the low predictive value of molecular testing in low incidence settings. The most widely used screening test was the RealStart Filovirus RT-PCR kit 1.0 (altona Diagnostic GmbH, Hamburg, Germany), while as confirmatory testing was the RealStar Filovirus Type. RT-PCR Kit 1.0, from the same company; these tests were adopted by the INMI laboratory team, after the first phases of the outbreak, when only in house tests were available.

The diagnostic algorithm developed at INMI for the EVD diagnosis is illustrated in Fig. 2.

A plethora of in house accessory tests have been developed and widely used at INMI during the outbreak, to perform sequence analysis and to measure the specific and non-specific immune response, as detailed below (INMI experience with two Italian EVD patients).

3.3 Basic Laboratory Testing Capability Within High Containment Laboratory

Since 2001, in parallel with the establishment of the bio-containment unit, INMI developed a plan to establish laboratory capability for the diagnosis of HIDs. The strategy included the establishment of a biosafety level 3 and 4 Point Of Care (POC) laboratory that could support all the necessary testing at the required biosafely level, close to the isolation unit, to perform a core set

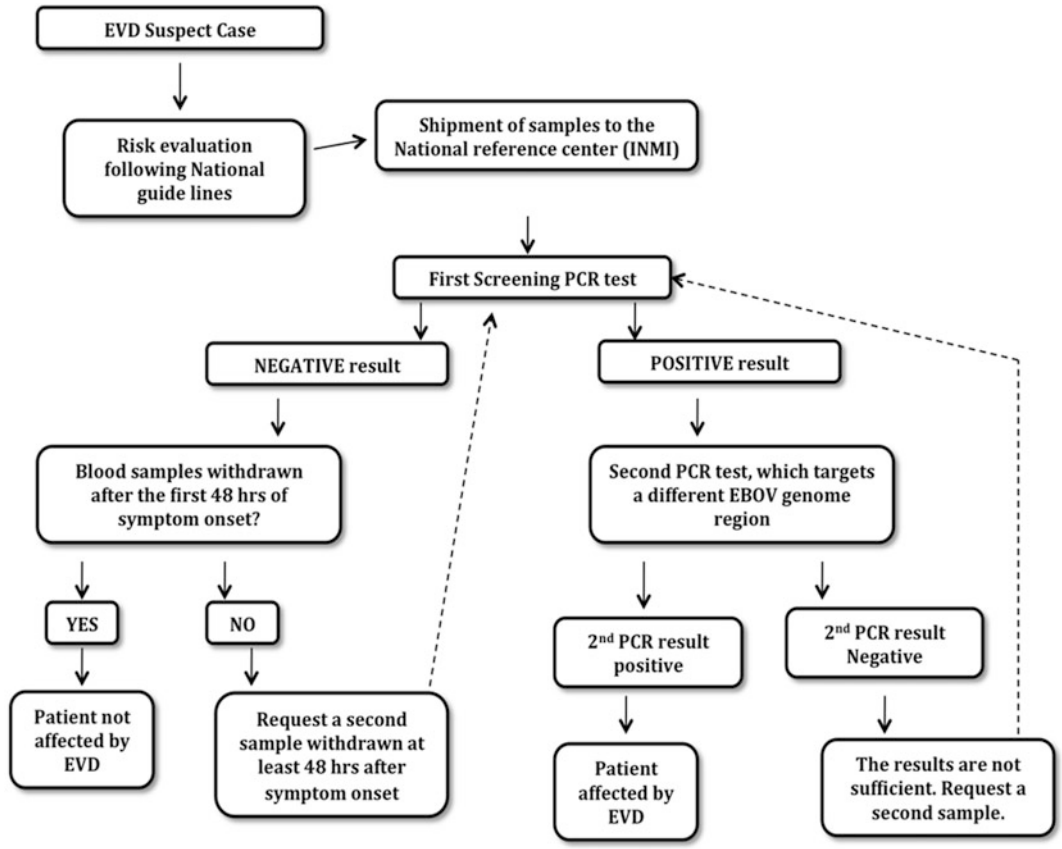

Fig. 2 INMI algorithm for the laboratory diagnosis of EVD

of metabolic, coagulation and other basic blood assays. The platforms included: chemistry analyzer (Reflotron Plus, Roche Diagnostics GmbH, Germany); arterial blood-gas analyzer (GEM Premier 3000, Werfen, Spain); coagulation analyzer (Hemochron Signature Elite, Accriva Diagnostics, USA); hematology analyzer (ABX Micros ES60, HORIBA ABX SAS, France). In addition to biochemistry tests, microbiological diagnosis was implemented within the BSL3/4 laboratory, using a minimal set of instruments, including a dry incubator for microbial cultures and antimicrobial susceptibility testing and a small automated blood culture incubator. The laboratory computer was linked to the hospital network for data transmission.

3.4 European Mobile Laboratory (EMLab)

One study estimated that if 60 % of patients with Ebola virus disease are diagnosed within 1 day of symptom onset, instead of the current average of 5 days, the virus attack rate drops from 80 % to nearly 0 % (Dhillon et al. 2015). This finding emphasizes the substantial effect of a rapid and accurate clinical diagnosis of EVD supported by laboratory tests. According to this concept, mobile laboratories for highly dangerous pathogens were deployed in West Africa during the 2014–2016 outbreak of EVD, to provide on-site diagnostics (Mansuy 2015).

Since March 2014, i.e. immediately after the identification of the EVD outbreak, INMI has contributed to the deployment and field

management of the "European Mobile Laboratory for BSL4 agents", in the context of the Consortium (EM-Lab) funded by the European Commission (DEVCO) and coordinated by the Institute of Tropical Medicine Bernhard-Nocht in Hamburg. For the Ebola outbreak, three European Mobile laboratory units have been activated by the Consortium, and deployed in Guinea, Nigeria, Liberia and Sierra Leone to continuously provide fast molecular diagnostics of EVD; all the EM-Lab units were connected with treatment centers managed by Medicines Sans Frontiers (MSF). INMI has contributed with the deployment of 8 experts, 1 physician and 7 virologists, in Guéckédou and Coyah (Guinea), Foya (Liberia) and Hastings (Sierra Leone) for a total of 10 shifts of 1 month each (Wölfel et al. 2015; Reusken et al. 2015; Strecker et al. 2015).

3.5 The Italian Diagnostic Laboratory in Goderich, Sierra Leone

On 4 December 2014, INMI started a new advanced lab, located in the premises of the Ebola Treatment Center (ETC) managed by the Italian NGO "Emergency" in Goderich, Freetown (Sierra Leone). From December 2014 to June 2015, 16 virologists and 1 epidemiologist from INMI have been deployed in Sierra Leone for 5 weeks shifts, under the umbrella of the agreement between INMI and the Italian Ministry of Foreign Affairs. The main task of the team was to perform the diagnosis of EVD by Real Time PCR and of malaria by rapid test; to establish a biobank of samples stored at −20 °C; to define a database with clinical and laboratory annotation, including quantitative data (i.e. viral load). At present, INMI is involved in the EVD surveillance program, which started during the decaying phases of the epidemic and is still ongoing, under the direction of the MoH of Sierra Leone, and consists of molecular testing for EVD on all the samples collected from the community deaths.

4 Research on Diagnostics

4.1 Setting Up of New Protocols: Differential and Immunological Characterization on EBOV Positive Samples

A significant laboratory issue is the inactivation of EBOV-positive samples in order to safely run routine and research activity at lower biosafety level. A considerable effort was devoted at INMI to establish the optimal procedures to perform experiments with samples from EBOV patients minimizing the steps conducted under BSL4 conditions.

Based on experimental and observational data, the CDC recommends the blood-borne pathogen standards laboratory procedures, and use Triton X-100 alone or in combination with heat treatment to inactivate viral infectivity. We confirmed that 1 h exposure to 0.1 % TritonX-100 at room temperature substantially reduces the infectious titer of EBOV preparations, while not affecting the performance of the most common laboratory tests, nor disturbing rapid and molecular malaria test results as well as parasite recognition in blood smears (Tempestilli et al. 2015). Therefore this protocol was adopted to perform all laboratory tests that could not be performed in the BSL4.

However, TritonX-100 causes damage of cell membrane, therefore it is not suitable for the immunological characterization of immune cells, that is usually performed by flow cytometry and Elispot assay on living blood cells. These protocols require several manipulation steps, including centrifugation and processing of open tubes. To optimize a rapid, easy and safe procedure to handle EBOV-positive samples for flow cytometry, we modified the standard protocol, in order to minimize the experimental steps to be conducted in the BSL-4 laboratory. Thus, the separation of peripheral blood cell (PBMC) through Ficoll gradient centrifugation was replaced by an easy red cell lysing step. The blood cells were then washed and stored at −80 °C in living conditions (i.e. with 10 % DMSO), in BSL-4. After thawing, leucocytes

were fixed by using 10 % paraformaldehyde (a procedure that inactivates EBOV infectivity), then transferred to BSL-2, where staining with cocktails of monoclonal antibodies and flow cytometry analysis were performed. By contrast, the Elispot assay requires BSL-4 conditions throughout the entire procedure, since it is based on a stimulation of viable leucocytes. Therefore all the ELISPOT experiments were conducted in BSL-4.

4.2 Validation of New Diagnostic Methods

To date, diagnosis of EVD, due to the scarcity of reference materials, has relied primarily on real-time reverse transcription PCR (RT-PCR) performed in field bio-containment laboratories on submitted venous whole blood or buccal swab specimens. Different assays are being currently evaluated using residual samples available in West Africa, after approval of the Ethical Committee and Pharmacy Board of the involved countries. With the European Funded Projects IF-Ebola, EbolaMoDRAD, FiloDiag, and Mofina, INMI is currently performing validation assays on the rapid detection of Ebola Virus antigens and the detection and quantification of viral RNA through new qRT-PCR methods. Furthermore, the WHO recently launched the initiative to establish the first International Standards for molecular and serology assays. Besides participating to the evaluation of the candidate molecular and serological standard preparations, INMI contributed to this initiative by making available one unit of convalescent plasma collected during the management of the first Ebola case treated at INMI.

INMI is also involved in a collaboration project funded by the Food and Drug Administration (FDA) for the validation of serological tests developed by Gerardo Kaplan in the FDA laboratories, based on a molecular construct of EBOV that can be handled under BSL-2 conditions. This test addresses a compelling need to perform on site sero-prevalence and vaccine efficacy studies with simple methods not requiring BSL-4 conditions. The main goal of the collaboration is to perform the field validation of the tests established at FDA, and will be conducted at the INMI Laboratory in Freetown, Sierra Leone, with the support of Emergency.

5 Research on Clinical Management

5.1 Clinical Management in High Isolation Setting

INMI was directly involved in the treatment and care of two Ebola cases: a 50-year-old Italian male Emergency NGO physician, who contracted EVD in Sierra Leone while working at the Ebola Treatment Center (ETC) in Lakka, Freetown, Sierra Leone, and a 37-year-old Italian male nurse, who contracted EVD while working at the Emergency NGO Intensive Care Unit of the ETC of Goderich, Freetown, and developed symptoms after his return home in Sassari, Sardinia, Italy (Petrosillo et al. 2015). Both patients presented challenging issues in the EVD clinical course. The clinical course of patient 1 (outlined in Fig. 3) was characterized by severe gastro-intestinal symptoms, profound asthenia and lethargy, followed by respiratory failure, accompanied by *Plasmodium vivax* co-infection. He received experimental antiviral therapy including favipiravir, EBOV monoclonal antibodies and EBOV convalescent plasma. Patient 1 underwent intensive care support, including mechanically ventilation, central line introduction, lung

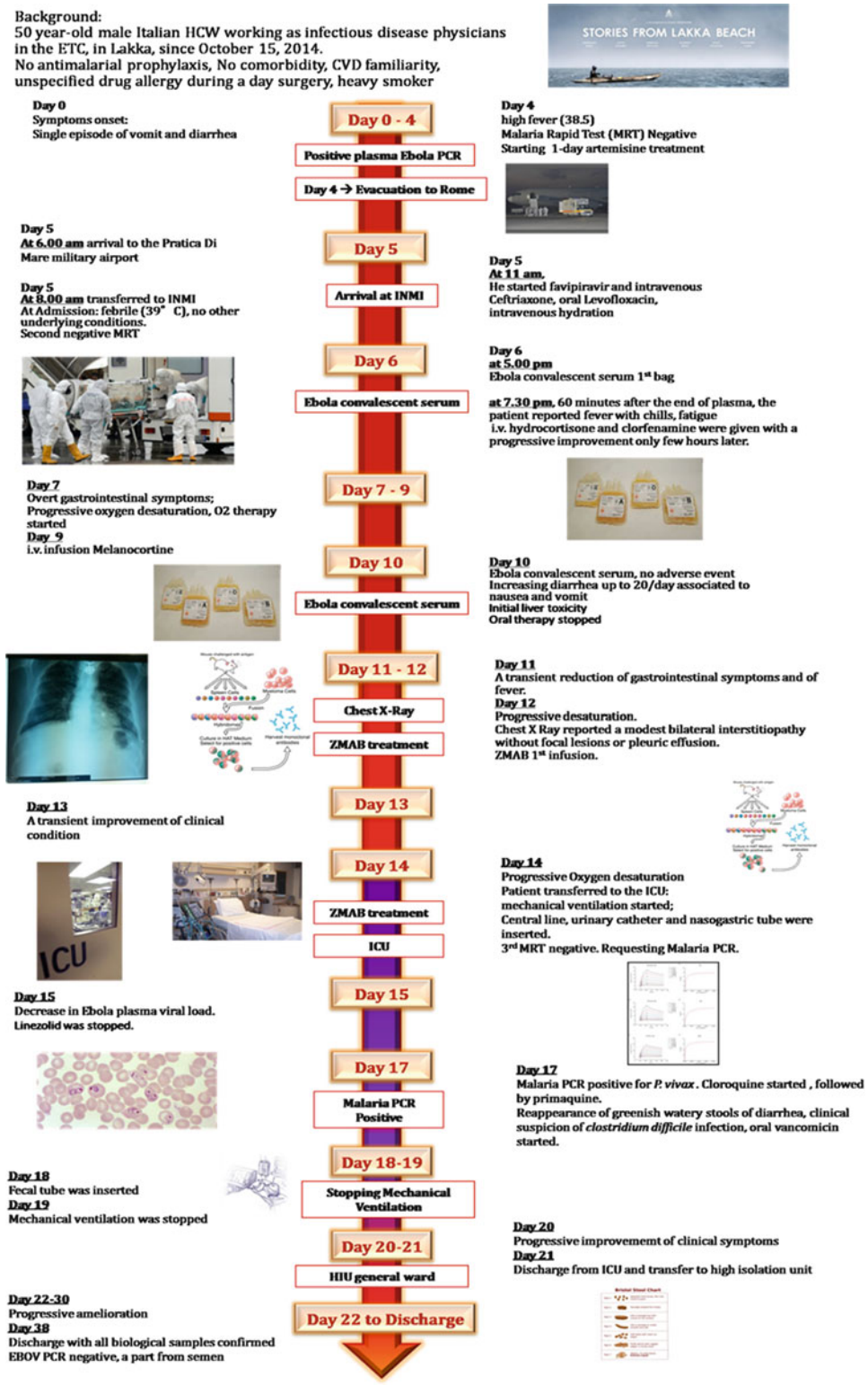

Fig. 3 Workflow of events surrounding the management of the first Italian EVD case

ultrasound monitoring and parenteral nutrition for 7 days. Specific procedures for infection control during imaging testing were drafted and rapidly implemented (Busi Rizzi et al. 2015). EBOV genomes were detected at high concentration in deep respiratory secretions, at a time when EBOV

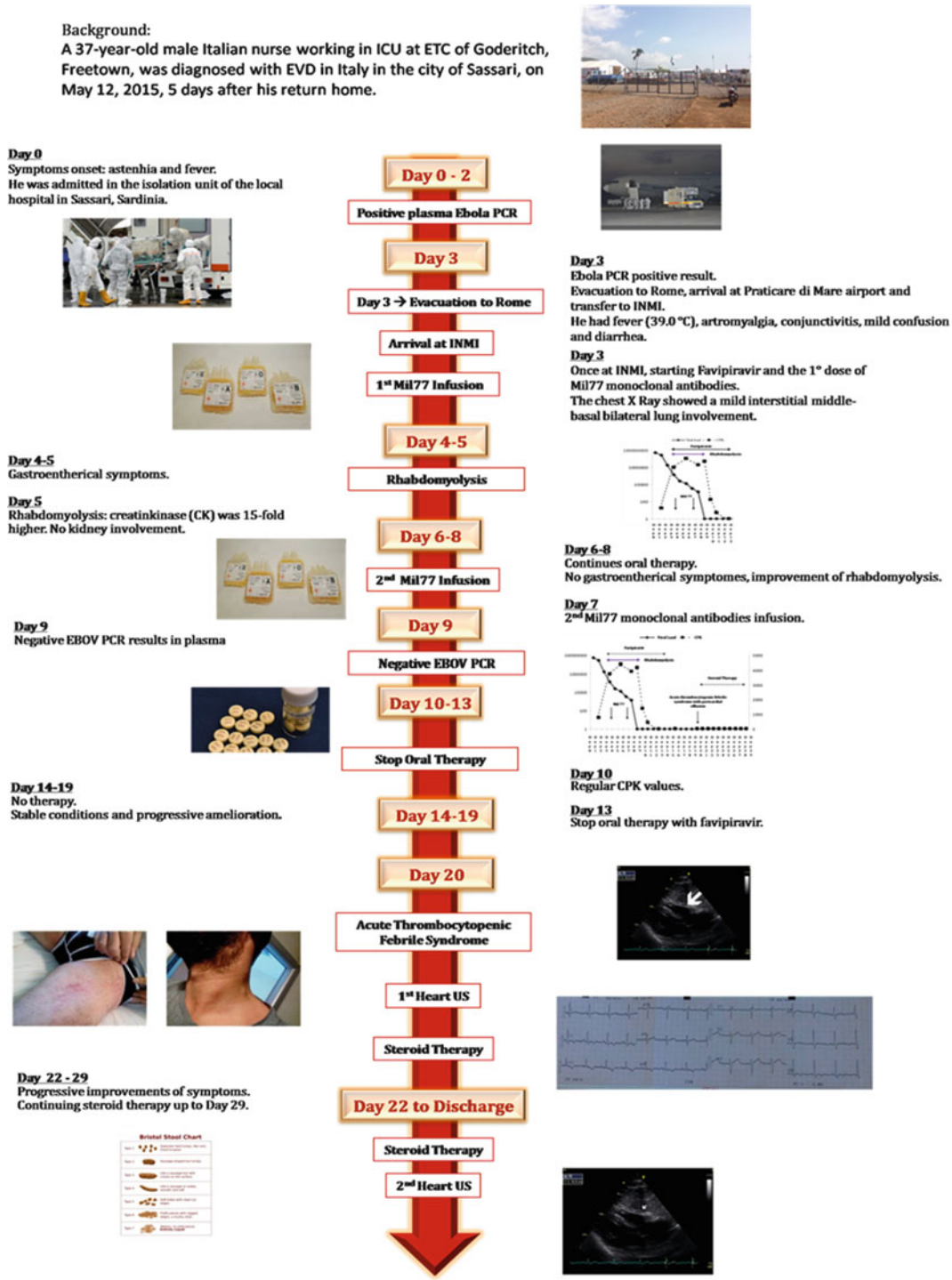

Fig. 4 Workflow of events surrounding the second Italian EVD case

viraemia was already halted, suggested the lung localization of virus replication, raising concerns on the adopted infection control policies and on the accepted mode of EBOV transmission. However, the patient fully recovered and was discharged on day 39, and no at-risk events occurred among HCWs. There is no evidence of sequels at month 18 follow-up visit.

The second Italian EVD case developed symptoms 5 days after his return home in Italy. The clinical course of patient 2 is outlined in Fig. 4. At admission, he presented with fever, artromyalgia, conjunctivitis, mild confusion and diarrhea. Upon arrival, he received experimental antiviral therapy and EBOV viraemia became undetectable since 2 weeks after admission. On illness day 19, a febrile syndrome with diffuse adenopathy, confluent skin rash and marked thrombocytopenia occurred. Pericardial effusion was suspected on electrocardiogram and confirmed by echocardiography. High-dose corticosteroid therapy was initiated with immediate clinical improvement. At discharge on day 29, a minimal pericardial effusion was still present. Corticosteroid treatment was stopped and oral indomethacin was prescribed. At day 60, after discharge, indomethacin therapy was stopped (Nicastri et al. 2016a, b). There is no evidence of relapse at month 10 follow-up visit.

Six further patients have been admitted in the INMI high isolation unit as EVD suspected but unconfirmed cases: 4 of them had *P. falciparum* malaria and two cases had a high respiratory tract infection.

5.1.1 Neuropsychological Tests

Considering that both EVD patients reported an altered state of consciousness, neuropsychological examinations were performed twice during the hospital stay and at follow-up. The first evaluation showed normal results in tasks involving mental flexibility, concentration and speed of mental processing. Conversely, at baseline memory tasks showed mild deficits during learning and delayed recall in both cases. During follow-up visits, a better performance in all cognitive domains was reported and no permanent cognitive disorder is currently present. An early access to fluid resuscitation, antivirals and convalescent plasma could be likely to have driven a less pronounced neuropsychological impairment as compared to other described cases (Mohammed et al. 2015; Nicastri et al. 2016a, b; Uyeki et al. 2016).

5.1.2 Discharge Criteria

No established, widely accepted discharge criteria (DC) are available, and different policies had been adopted in different settings and countries.

The policy adopted by INMI was to discharge the patient after at least two consecutive PCR-negative samples from all biological samples (apart from semen). However, the convalescent patient could still be infectious, harbouring compartmentalized long-standing viable virus in other body fluids/compartments, and he/she could be at risk of clinical relapse. However, the discharge policy requires an organized and evidence-based approach to ensure that patients, HCWs, family and community members are protected at all times. The main issues on the discharge policy were addressed at INMI through the DC revision of 14 cases of EVD released from hospitals in high-resource countries and reviewing the international literature. International organization as WHO, US CDC and ECDC did not issued specific recommendations for the discharge procedure of EVD patients out of Africa. In the resource-limited setting with intense transmission, DC included 3 days without gastrointestinal symptoms and, if available, a negative blood RT-PCR. Considering the risk of EBOV transmission in the community through unprotected sexual intercourse with EVD convalescent patients, public health policies should include a precautionary principle, which could drive the affected countries out of the epidemic setting (Bevilacqua et al. 2015).

5.1.3 Follow Up

The two EVD patients were monitored at day 15 from discharge and then monthly, during the first 6 months. The follow up was performed on standard clinical samples from blood and semen for the presence of the viral RNA in seminal fluid, and for both humoral and cell-mediated specific response. EBOV RT-PCR on semen was carried out until two consecutive negative results were obtained. In the second case, the pericardial effusion was monitored using heart ultrasound and standard immune profile pattern. Ebola convalescent plasma has been collected

Table 1 VHF treatment options

Virus, Family	Incubation	Virulence	Human-to-human transmissibility	Isolation required	Treatment option(s)
Ebola, *Filoviridae*	2–21 days	High	Yes	Yes	Favipiravir, monoclonal Ab[a], small interfering RNA or antisense oligonucleotides passive immune therapy; post exposure vaccination & antivirals
Marburg, *Filoviridae*	2–21 days	High	Yes	Yes	Monoclonal Ab, small interfering RNA or antisense oligonucleotides
Lassa, *Arenaviridae*	6–21 days	Moderate	Yes	Yes	Ribavirin
Lujo, *Arenaviridae*	6–21 days	High	Yes	Yes	Ribavirin
Junin, *Arenaviridae*	6–21 days	Moderate	Yes	Yes	Ribavirin & passive immune therapy
Machupo, *Arenaviridae*	6–21 days	Moderate	Yes	Yes	Ribavirin
Sabia, *Arenaviridae*	6–21 days	Moderate	Unknown	Yes	Ribavirin
Guanarito, *Arenaviridae*	6–21 days	Moderate	Unknown	Yes	Ribavirin
Chapare, *Arenaviridae*	6–21 days	Unknown	Unknown	Yes	Ribavirin
Nipah, *Paramyxoviridae*	4–18 days	High	Potential	Yes	Ribavirin & passive immune therapy
Hendra, *Paramyxoviridae*	4–18 days	High	Unknown	Yes	Ribavirin & passive immune therapy
Crimean Congo Hemorrhagic Fever, *Bunyaviridae*	3–7 days	Moderate	Yes	Yes	Ribavirin
Rift Valley fever, *Bunyaviridae*	2–14 days	Low to moderate	No	No	
Hantan, Seoul, *Bunyaviridae*	Days to months	Low to moderate	No	No	Ribavirin, haemodialysis, ECMO
SinNombre, Andes, *Bunyaviridae*	Days to months	Moderate	No	Yes	
Yellow fever, *Flaviviridae*	3–6 days	Moderate	No	No	
Dengue, *Flaviviridae*	3–14 days	Low	No	No	
Russian spring summer encephalitis, *Flaviviridae*	7–14 days	Moderate	No	No	
Omsk/Kyasanur Forest Disease/Alkhurma Hemorrhagic Fever, *Flaviviridae*	3–8 days	Moderate	Unknown	Yes	

[a]Ab = antibodies

monthly in only one case, due to the detection of anti HLA antibodies in the first survivor.

5.1.4 Treatment Options, Advancing the Level of Treatment

Table 1 summarizes the main clinical and epidemiological features of different VHFs agents with specific recommended interventions to undertake (Qiu et al. 2014; Ippolito et al. 2012).

6 Research on Infection Control

Since the beginning of the epidemic, the pre-established INMI Task Force for Highly Infectious Diseases was activated. This task force consists of a multidisciplinary team including members from all relevant fields of the Institute, such as infectious diseases specialists, intensive care specialists, nurses, virologists and microbiologists, epidemiologists and clerical staff. A written protocol for the management of EVD cases was soon developed, and was put in force when the first patient was admitted at INMI. The protocol has been periodically reviewed and updated, covering all aspects of infection prevention and control (IPC, e.g. PPE policies, cleaning and disinfection, waste management, handling of dead bodies and policies for staff safety and surveillance) according to the evolving situation and knowledge. On voluntary basis, medical doctors and professional nurses had weekly or bimonthly training sessions on donning and doffing PPE, supervised by peers and by infection control specialists. On-going training curriculum of all HCWs potentially involved was implemented, consisting of seminars and epidemiological updates, and practical sessions and simulations at laboratory and clinical settings.

Written records of entry and exit times were taken for all HCWs involved in direct caring of the patients. There have been a minimum of two nurses and one physician per shift; the day shift lasted 6 h, while the night shift lasted 12. At least two HCWs were always present at the bedside, performing regular buddy check of the procedures. All involved HCWs were instructed to monitor actively and record in sign-in sheets their body temperature twice daily from the first up to 21 days after the last exposure, or after the return from work in the epidemic area. A Post-Exposure Prophylaxis (PPE) was developed as well.

7 Research on Epidemiology

7.1 Innovative Approach for Interventional Study Design

Several experts argued that randomized controlled trials to assess clinical interventions could have been both unethical and infeasible during large and deadly epidemics such as the Ebola outbreak in West Africa. However, implementing a framework of field research, which only relies on observational studies, can be dangerous. In fact, non-randomized studies can hardly assess safety and efficacy of novel intervention for new emerging pathogens. Through a simulation approach, adaptive clinical trial to assess the efficacy of novel treatment for patients infected with Ebola were demonstrated to be feasible and safer than non-randomized studies. The investigational approach proposed was represented by a sequential group design with "Early stopping" for either efficacy or toxicity. The simulation included two interim analyses (i.e. a three stage design) and three different scenarios (i.e. mortality in non treated at either 50 %, 60 % or 70 %). Such a design could have assessed the efficacy of an experimental compound to reduce mortality 20 % in the interventional arm with a power of 80 % using a sample size between 70 and 210 participants and an overall time to carry out the study between 2 and 6 months (Lanini et al. 2015b).

7.2 Innovative Approach in Field Epidemiology

The amount of information gathered during the management of individual patients represents a unique opportunity for improving the general knowledge about the natural history of Ebola infection. The main hindrance for exploiting this large resource of information is that it is made of sparse and not homogeneously collected longitudinal sets of data. To overcome these issues, an analysis based on "*latent trajectory modeling*" with a multilevel regression model framework was implemented. *Latent trajectory models* explore the variation of dependent variables (represented by a repeated measure) as a function of time and, possibly, other measures. Such longitudinal data share the features that the same subjects are observed repeatedly over known time points. In this way the different behaviors of EBOV viral load between days 2 and 13 after symptoms onsets were explored and the EBOV viral kinetics either in survivor and non-survivors were defined. This approach has been shown to produce reliable estimates with minimal ethical and economical impacts, and, in principle, can be used for any future outbreak of emerging pathogens (Lanini et al. 2015a).

7.3 Ethical Clearance

By introducing the effort made by INMI in European and International research Projects, aimed at better understanding the nature of the virus, its way of transmission and its interaction with the host immune response, it has to be taken under consideration that the collection of samples during the outbreak was done without the acquisition of the informed consent, as part of the response outbreak. However, ethical clearances from the Ethical Boards and the Pharmacy Boards of each country from which the samples originated from and INMI Ethical Board have been obtained for the exploitation of the residual samples available in the field laboratories, allowing the conduction of several studies.

8 Research on Virology

Rapidly obtaining genome sequences during disease outbreaks is crucial for clarifying patterns of virus evolution, monitoring the validity of diagnostic assays, and investigating transmission chains (Carroll et al. 2015; Mate et al. 2015). Three EBOV isolates were obtained at INMI: two from the two Italian patients attending the INMI clinical facility, the third one from a HCW whose samples were submitted for diagnostic reasons from Freetown. Sequencing was based on Sanger techniques. Complete EBOV genome sequences were obtained from the three isolates and from clinical sample of another HCW whose samples were submitted for diagnostic reasons from Freetown (Zaire ebolavirus isolate Ebola virus/H.sapiens-tc/SLE/2014/Makona-Italy-INMI1, accession number: KP701371; Zaire ebolavirus isolate Ebola virus/H.sapiens-tc/ITA/2015/Makona-INMI2, accession number: KT961624; Zaire ebolavirus isolate Ebola virus/H.sapiens-wt/SLE/2014/Makona-Goderich2, accession number: KT946869; and Zaire ebolavirus isolate Ebola virus H.sapiens-wt/SLE/2015/Makona-Goderich1, accession number: KT345616).

The first sequence originates from the first Italian confirmed EVD case. The INMI1 isolate sequence shows high similarity with the previously published sequences from the Western African outbreak. Single nucleotide variants (SNV) of the INMI1 isolate are located both in coding (mostly synonymous) and in noncoding regions (Castilletti et al. 2015). The whole genome sequence of the second isolate, originating from the second Italian case attending INMI, showed high similarity with the previously published sequences from the Western African outbreak.

The third sequence was obtained from a HCW who was infected while attending patients from the Aberdeen fishermen community. The viral sequence from this case presents three

uncommon nonsynonymous substitutions, one in NP (c1958t:P497S) and two in GP (a7267t:R410S and a7352g:K439E). Among the Sierra Leone EBOV sequences available so far, these mutations are represented only in the Aberdeen fishermen community phylogenetic cluster (Capobianchi et al. 2015).

Overall, obtaining a broader view of viral evolution during the outbreak has been difficult, because of political and logistical obstacles that limited the export of samples to laboratories capable of performing these analyses. Furthermore, establishing conventional Sanger or next-generation sequencing technologies in affected countries is logistically challenging because of the size and weight (≈ 40 to ≈ 100 kg) of the necessary equipment, the high potential for transport damage related to the sensitive optics many of these machines incorporate, limitations on supportive infrastructure, and complex sample processing procedures. An additional challenge is the required installation or calibration of sequencing machines, which often has to be done by field engineers employed by the manufacturers, who may be reluctant to send their employees into outbreak areas. Many of these problems can be resolved by adding a genome sequencing capability to an EVD diagnostic laboratory. Recently, a new genome sequencer has become available, with dimensions and operability consistent with the usage in the field (Hoenen et al. 2016). The MinION (Oxford Nanopore Technologies, Oxford, UK) is highly portable, being the size of a chocolate bar and weighing <100 g, is powered by the controlling laptop battery, and streams real-time data via a Universal Serial Bus (USB) port. The MinION has been used for sequencing activities in the field by the EMLab teams (Quick et al. 2016). In addition, thanks to the MinION, sequencing activities have been implemented in the permanent laboratory in the premises of the Princess Christian Maternity Hospital (PCMH), Freetown, Sierra Leone, run by INMI and Emergency NGO, and the residual EBOV positive samples collected during the outbreak are currently under investigation. Combining these genome sequences with epidemiological investigations will help confirm or confute transmission chains and will inform outbreak control efforts and resource allocation.

9 Research on Immunology

Defining human immune responses to EBOV infection, pathogenesis and correlates of protection are important for designing effective therapeutic and vaccination interventions. Nevertheless, data on human immune responses to EBOV virus are scanty due to limitations imposed by biosafety requirements and logistics of performing studies on sequential samples during the course of disease. The admission of two EVD patients at INMI provided the opportunity to collect sequential samples soon after symptom onset until recovery to study the kinetic of the immune response, by evaluating T-cell subset dynamics, T-cell activation, expression of exhaustion marker and T cell functions (Table 2).

Table 2 Immunological analysis performed on samples from the two Italian EVD cases

	Innate immune cells		Adaptive immune cells		
	NK	γδ T cells	CD3	CD4	CD8
Frequency	CD56 + CD16+	Vδ2 + CD3+	CD3+	CD3 + CD4+	CD3 + CD8+
Activation	CD69+	CD69	CD69/HLA-DR	CD69/HLA-DR	CD69/HLA-DR
Differentiation	n.t.	CD45RA/CD27	CD45RA/CD27	CD45RA/CD27	CD45RA/CD27
Apoptosis	n.t.	CD95+	CD95+	CD95+	CD95+
Autophagy	n.t.	n.t.	AMBRA-1+	n.t.	n.t.
Exhaustion	n.t.	PD-1+	PD-1+	PD-1+	PD-1+
KIR expression	p44/CD161	n.t.	n.t.	n.t.	n.t.
Cytotoxicity	n.t.	n.t.	Granzyme B+	n.t.	Granzyme B+
Proliferation	n.t.	n.t.	Ki-67+	Ki-67+	Ki-67+

A modified flow cytometry protocol was used to minimize the experimental steps in BSL-4.

The results showed an early and sustained decrease of CD4 T-cells in both patients, with an inversion of the CD4/CD8 ratio that was reverted during the recovery period. The marked reduction of the CD4 T-cell population is likely an important driver in the overall loss of lymphocytes, and may limit the initiation and maintenance of effective humoral and cytotoxic T-cell immunity. In parallel with the CD4 T-cell depletion, a massive T-cell activation was observed, mainly in CD8 T cells, and was associated with autophagic/apoptotic phenotype. Moreover, an enhanced expression of the exhaustion marker PD-1 on CD4 T cells was associated to impaired IFN-gamma production (Falasca et al. 2015; Agrati et al. 2016). The immunological impairment was accompanied by EBV reactivation, suggesting a general immunosuppression during EVD. The association of an early and sustained dysfunctional T-cell activation in parallel to an overall CD4 T-cell decline may represent a previously unknown critical point of EBOV-induced immune subversion.

Within the context of the EU-funded project Evident, the clinical samples collected and stored by the EMLab during the mission in Guinea represent an invaluable source of information to conduct a large study on innate immune cells (γδ T cells and NK cells) and analyzed their phenotype, activation and exhaustion markers expression during EVD. Due to the current complexities of preparing and shipping samples outside of Africa, the INMI staff has been deployed in the field to perform the flow cytometry analysis in the field laboratory. At present samples collected at Donka Hospital during the EMLab mission have been characterized (Ruibal et al. 2016).

10 Research on Communication

The 2014–2015 EVD outbreak in Western Africa received unprecedented media attention. During the management of the two Italian patients at INMI, one of the encountered challenges was the establishment of an effective communication strategy. The Institutional Press Office working together with the press offices of the Ministry of Health was able to provide the high level of expertise necessary within both medical and communication contexts. Upon the patient's admission to INMI, a Crisis Communication Management Group (CCMG) was established. Its primary objective was to produce and disseminate frequent, timely, and uniform communication. A stringent communication approach, with a pre-established communication plan, was adopted based on a daily press-conference delivered by a unique authorized spokesperson and a CCMG-driven control of type and quality of the information provided to journalists, to grant accuracy and appropriateness of the information open to the public. Multiple communication tools (including social media) were used to reach as many people as possible. A large press-conference involving the patients their self was organized at discharge (Salce et al. 2015).

11 Conclusions

A remarkable time-lapse occurred between the recognition of the EVD outbreak and the implementation of an effective response and of the related translational clinical research model. The opportunity for timely implementation of translational research, including diagnostic, clinical and infection control issues, has been hampered by several gaps, including the lack of a specific preparedness strategy, the lack of a sufficiently strong research coordination policy and of research capacity mobilization, and the lack of specific, flexible, and easy-to-access and easy-to-use fundings (Ippolito et al. 2015a, b, c). Despite the fact that many interventional studies and research projects have been planned, the majority of them have been implemented in the late phase of the outbreak, i.e. too late for the exploitation of their achievements during this outbreak. However, the experience and knowledge gained, although late for this outbreak, will be of great value in facing future events, which we do not

know when and where will happen, but are most likely to occur.

The experience of INMI suggests a model for an effective and integrated response of a single institution to an outbreak due to a highly infectious pathogen. This experience includes the successful management of two patients, the support to preparedness and response in Italy and Europe, the active contribution to the containment of the outbreak in the affected countries, and the leading of, or participation to, many research projects on EVD. The pillars of this response are based on the strong preparedness activities, lasting about 20 years, and on the availability of INMI staff to face this great challenge with courage and enthusiastic personal involvement.

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Appendix 1

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Prioritization of High Consequence Viruses to Improve European Laboratory Preparedness for Cross-Border Health Threats

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Abstract

Highly infectious diseases can spread rapidly across borders through travel or trade, and international coordination is essential to a prompt and efficient response by public health laboratories. Therefore, developing strategies to identify priorities for a rational allocation of resources for research and surveillance has been the focus of a large body of research in recent years. This paper describes the activities and the strategy used by a European-wide consortium funded by the European Commission, named EMERGE (Efficient response to highly dangerous and emerging pathogens at EU level), for the selection of high-threat pathogens with cross-border potential that will become the focus of its preparedness activities. The approach used is based on an objective scoring system, a close collaboration with other networks dealing with highly infection diseases, and a diagnostic gaps analysis. The result is a tool that is simple, objective and adaptable, which will be used periodically to re-evaluate activities and priorities, representing a step forward towards a better response to infectious disease emergencies.

In recent years, public health systems worldwide have been challenged by epidemics of emerging infectious diseases, the most notorious of which is the 2014–2016 Ebola Virus Disease (EVD) emergency (Ebola Situation Report 30 March 2016), as well as the ongoing Zika outbreak in Central and South America (Zika situation report 11 August 2016). In an effort to improve the global capacity to respond rapidly and

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effectively to such threats, the World Health Organization (WHO) and the European Commission (EC) have set up strategies to identify research and development (RD) needs to rapidly fill gaps and improve collaboration and coordination of response activities (WHO 'Blueprint for R&D preparedness ad response to public health emergencies due to highly infectious pathogens' 2015, Kieny 2016).

The EC has been active for years in the fight against emerging infectious diseases, through the funding of networks of European high containment laboratories involved in public health response: the European Network of Biosafety Level-4 laboratories (BSL-4) (www.euronetp4.eu) (Ippolito et al. 2008, 2009, Nisii et al. 2009, 2013, Senior 2008, Thibervilla et al. 2012), and QUANDHIP (Quality Assurance exercises and Networking on the detection of Highly Infectious Pathogens), which resulted from the combined forces of European BSL-4 and -3 laboratories [Nisii et al. 2016]. In the framework of its Public Health Programme, and in compliance with Decision 1082/2013/EU on serious cross-border threats to health (Official Journal of the European Union, 2013] the EC renewed its support of the activities of European high containment laboratories by launching a Joint Action named EMERGE (Efficient response to highly dangerous and emerging pathogens at EU level) (www.emerge.rki.eu/Emerge/EN/Home/Homepage_node.html). The overarching goal of EMERGE is to improve the coordination and preparedness of the public health stakeholders involved in the response to cross-border emerging health threats in Europe through the organization of Quality Assurance Exercises (EQAEs) and training, as well as to promote integration with other key European networks and research projects. EMERGE was launched in June 2015, and in the first months it concentrated on developing a strategy to determine which pathogens should be the focus of the EQAEs and other preparedness activities, using a simple, yet objective and methodical assignment of priority.

1 Methodology for Pathogen Selection

The Steering Committee (SC) of EMERGE, made up of representatives of the coordinators (the Robert Koch Institute in Berlin and the 'L. Spallanzani' National Institute for Infectious Diseases in Rome), and other partner institutions in the United Kingdom, Sweden, France, the Netherlands, and Germany, worked together to develop an assessment method for prioritising agents of highly infectious diseases, which resulted in a list of pathogens with the potential to cause cross-border outbreaks in Europe. What was needed was a set of objective criteria that would function as a tool consistent enough to ensure objectivity, but also flexible enough to be adapted to future, unforeseen scenarios. The potential to cause harm and to spread across borders was further broken down into several variables: severity of disease, risk of introduction into European countries, presence of reservoirs or vectors, size of the susceptible population, existence of prophylaxis or treatment options. A four-tiered scoring system was proposed whereby every single aspect was rated (from 0 to 3) to obtain a number resulting from the sum of all the individual scores. A survey was produced and disseminated to the BSL-4 laboratories that form the SC to collect their individual evaluations, which were summed up to obtain a final score and weighted average representing the consensus of the EMERGE SC (Table 1).

The form used in December 2015 for Crimean Congo Haemorrhagic Fever virus (CCHF) is shown as an example; basic information, useful links, and the number of ProMed posts are also reported on the form (Fig. 1). The use of ProMed as an indicator of severity or threat posed by an outbreak has been a matter of discussion because of the difficulty in setting objective and consistent thresholds in a scoring system. The final decision was to provide the total number of events together with those published in the previous year, asking questionnaire respondents to provide a score from 0 to 3. The EMERGE survey was also used to collect information on the

Table 1 Individual scores attributed to viral agents with cross-border threat by the EMERGE consortium, and their weighted average

Agent	INMI Italy	Marburg Germany	FHOM Sweden	PHE UK	INSERM France	Weighted average[a]
Filoviruses						
Ebola Zaire	9	8	8	10	8	**8.6**
Ebola Sudan	9	8	8	10	7	**8.4**
Ebola Cote d'Ivoire	9	3	8	10	7	**7.4**
Ebola Bundibugyo	9	8	8	10	7	**8.4**
Marburg	9	9	7	10	7	**8.4**
Arenaviruses						
Lassa	8	10	7	10	8	**8.6**
Junin	8	10	8	13	7	**9.2**
Machupo	8	10	7	13	6	**8.8**
Guanarito	8	9	7	13	6	**8.6**
Sabia	8	7	4	13	6	**7.6**
Lujo	8	7	4	13	3	**7.0**
Bunyaviruses						
CCHF	12	11	11	12	13	**11.8**
Coronaviruses						
MERS	8	7	7	10	Not scored	**8.0**
Orthomyxoviruses						
HPI	14	11	12	11	16	**12.8**
Paramyxoviruses						
Nipah	7	8	8	9	7	**7.8**
Hendra	7	8	7	9	6	**7.4**
Orthopoxviruses						
Monkeypox	7	9	8	7	Not scored	**7.7**
Cowpox	8	10	10	7	Not scored	**8.7**

CCHF crimean-congo haemorrhagic fever, *MERS* middle east respiratory syndrome, *HPI* highly pathogenic influenza
[a]To calculate the weighted average, the sum of individual scores was divided by the number of respondents, as not all pathogens were scored by all laboratories

diagnostic methods available in every laboratory, as this information is necessary to complete the decision making algorithm: once the SC has identified a pathogen with a score indicating significant dangerousness and cross-border potential, the evaluation process will enter the next phases: (i) verifying that there are no other networks dealing with the agent in question, and (ii) identifying any gaps in laboratory diagnostics (Fig. 2). These two steps in particular are necessary to optimize resources and avoid duplication of activities: for example, Highly Pathogenic Influenza (HPI) and Ebola viruses were not included in the 2016 activity planning on the grounds that very large networks on Influenza are in existence [Surveillance and laboratory Networks on Influenza (website)], and that no considerable gaps in diagnostics exist for EVD owing to the extensive effort made to tackle the recent emergency.

2 Results and Discussion

Highly infectious diseases can spread rapidly across borders through travel or trade, and

BUNYA VIRIDAE	Diagnostic tests available						Rationale 0=None/Not known; 1=Small/Low; 2=Moderate; 3=Large/High			
	Yes	No	Ag detection	Molecular detection	Ab detection	Other, specify:		0	1	2	3
CCHF[1]	☐	☐	☐	☐	☐		Reservoir, presence of the vector	☐	☐	☐	☐
							Risk of introduction/spread into EU member States	☐	☐	☐	☐
							Treatment and prophylaxis	☐	☐	☐	☐
							Size of susceptible population	☐	☐	☐	☐
							Severity of disease	☐	☐	☐	☐
							Promed Hits	☐	☐	☐	☐
							other networks involved	Yes ☐		No ☐	

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1 – CCHF

Pro-Med alerts in the last 12 months (08/12/14 – 08/12/15): 38
Tot. Pro-Med alerts: 377
Latest ProMed bulletin (2015) available from: http://www.promedmail.org/post/3784725
The Crimean-Congo haemorrhagic fever virus is a tick-borne virus and is found in Eastern Europe, particularly the former Soviet Union, the Mediterranean, in Northwestern China, central Asia, southern Europe, Africa, the Middle East, and the Indian subcontinent. Person-to-person transmission occurs through contact with infectious blood or body fluids; human infections also occurred in hospitals due to improper or lacking infection control precautions.
Useful Links:
http://www.cdc.gov/vhf/crimean-congo/
http://www.who.int/csr/disease/crimean_congoHF/en/

Fig. 1 Example of the form sent in December 2015 to BSL-4 laboratories forming the Sterring Committee of EMERGE. It contains general information and number of ProMed posts (*bottom*), and was used to collect data on diagnostic tests available at each laboratory (*top left*), and the rationale for including each virus, based on a four-tiered scoring system. Respondents were also asked if they were aware of the existence of other networks dealing with the pathogen (*top right*)

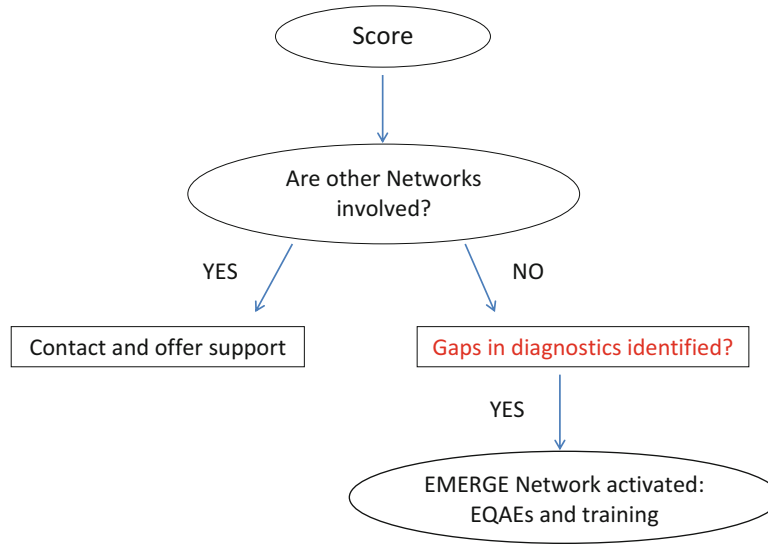

Fig. 2 Algorithm used by the EMERGE consortium for the selection of pathogens to include in the annual work plan, taking into account the score attributed by the Steering Committee members, the lack of other networks and the existence of diagnostic gaps

international coordination is essential to a prompt and efficient response. Public health systems must be on the alert and ready to deal with new emergencies that may arise anywhere in the world, therefore developing strategies to identify priorities for intervention measures, rational allocation of resources for research and surveillance, and preparedness planning has been the focus of a large body of research in recent years (Balabanova et al. 2011, Witt et al. 2011, Brookes et al. 2015, Dahl et al. 2015, Kulkarni et al. 2015, Krause et al. 2008, Matthiessen et al. 2016, Ng and Sargeant 2013, Xia et al. 2013, Wallinga et al. 2010).

At the start of its activity, the EMERGE consortium set out to develop its own strategy to prioritize pathogens for its 3-year EQAEs planning, in order to improve diagnostic capabilities. The approach used is based on an objective scoring system, a close collaboration with other networks dealing with highly infection diseases, and a diagnostic gaps analysis. The results were discussed at length by SC members, representatives of the EC and the European Centre for Disease Prevention and Control (ECDC), in teleconferences and face-to-face meetings. The pathogens chosen for the first year of activities were CCHF, Lassa Haemorrhagic Fever virus, and Orthopoxviruses. As mentioned previously, Ebola was not considered an immediate urgency after the gaps analysis (many commercial kits are available or under development today), but will be re-evaluated annually during the course of the project. CCHF was the virus with the highest score (Table 1) and included as a priority for the 2016 activity planning (having excluded HPI for the reasons explained above); therefore the recent occurrence of an autochthonous infection in Europe is proof of the validity of our work (ProMed 2016). In a 'One Health' approach, Orthopoxviruses (Cowpox and Monkeypox) were also chosen (regardless of their relatively lower score) because of their presence in Europe and cross-border potential, and their relationship to the Smallpox virus, in order to improve the ability of European laboratories to deal with a possible bioterrorism event.

Compared to other more complex prioritization strategies (Balabanova et al. 2011, ECDC technical report on best practice for ranking emerging diseases 2015, Dahl et al. 2015, Krause et al. 2008, Ng and Sargeant 2013), the EMERGE consortium used a pragmatic approach to produce a tool that is simple, objective and adaptable to changing circumstances. This paper describes the results obtained for viruses only, but the same approach was used to produce the annual work plan also for highly pathogenic bacteria.

EMERGE is a large EC Health Programme-funded joint action that brings together about 40 nationally appointed BSL-3 and BSL-4 laboratories; the fact that the assessment and selection of pathogens will be repeated at least annually, together with the flexibility of the project (activities can be focused and funds shifted to accommodate changing demands), represents a step forward in the direction of a better response to infectious disease emergencies.

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The Potential of Social Media and Internet-Based Data in Preventing and Fighting Infectious Diseases: From Internet to Twitter

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Abstract

Health threats due to infectious diseases used to be a major public health concerns around the globe till mid of twentieth century when effective public health interventions helped in eradicating a number of infectious diseases around the world. Over the past 15 years, there has been a rise in the number of emerging and reemerging infectious diseases being reported such as the Acute Respiratory Syndrome (SARS) in 2002, HINI in 2009, Middle East Respiratory Syndrome (MERS) in 2012, Ebola in 2014, and Zika in 2016. These emerging viral infectious diseases have led to serious public health concerns leading to death and causing fear and anxiety among the public. More importantly, at the moment, the prevention and control of viral infectious diseases is difficult due to a lack of effective vaccines. Thus having real-time reporting tools are paramount to alert relevant public health surveillance systems and authorities about taking the right and necessary actions to control and minimize the potential harmful effects of viral infectious diseases. Social media and Internet-based data can play a major role in real-time reporting to empower active public health surveillance systems for controlling and fighting infectious diseases.

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Keywords
Social media • Internet • Infectious diseases • Public health surveillance

Overview
This chapter highlights and discusses the potential role of social media in preventing and fighting infectious diseases and summarizes the advantages and limitations of social media and Internet based data for public health surveillance in general and infectious diseases in particular, leading towards the identification of gaps that still require further research and improvement.

1 Introduction

Major public health threats have been on the rise over the past 50 years. Specifically, infectious diseases such as the AIDS epidemic in the late 1970s, followed by the Severe Acute Respiratory Syndrome (SARS-COV) in Asia (2002–2003), pandemic H1N1 worldwide in 2009, Middle East Respiratory Syndrome (MERS-COV) in Saudi Arabia (2012), the re-emergence of Ebola virus in Africa (2014), and the Zika virus in 2016. All these are examples of infectious diseases which are difficult to control, as they require real-time reporting to alert relevant surveillance systems. Social media and internet based data has shown to play a role in sending early warning signals to public health authorities to take an informed course of action to prevent and control the spread of such diseases.

This noticeable increase in new emerging infectious diseases has led to several challenges facing public health officials operating at different levels, locally, nationally and globally, to take the right action at the right time. Addressing such challenges in the era of Information Technology (IT) has called for effective use of new emerging technology development and approaches towards detecting, tracking, reporting, forecasting, and improving early warning systems and proper response (Milinovich et al. 2014c). Nevertheless, most infectious diseases are still being traditionally monitored by passive and reactive public health surveillance systems, which are officially maintained by national public health authorities. These traditional surveillance systems depend, to a large degree, upon data submitted to public health authorities by health professionals such as hospital physicians, laboratories, public health practitioners, and other health-care providers.

Due to the common problems of time and resource constraints and the lack of operational knowledge of traditional surveillance reporting systems, this negatively affects the timeliness of event reporting. Incomplete reporting leads to no detection of public health threats inclusive of missed on-time detection of infectious diseases. Moreover, the substantial lags between the occurrence of an event and its official notification is common among public surveillance systems due to a number of reasons that govern traditional surveillance systems performance, including the rigid hierarchical structure and verification process dealing with receiving and notification of infectious and communicable diseases. These reasons, among others, led to late or failed reporting of the event to those in charge to take the right action for containment and control of the spread of infectious diseases.

Social media and Internet-based data seem to play a pivotal role in improving real-time reporting in informing both the public and governments about the possible public health threats of infectious diseases (Velasco et al. 2014; Milinovich et al. 2014a, c, 2015). It has been reported that there is, on average, a lag time of at least 2 weeks from receipt of the infectious disease event to dissemination of the data by traditional surveillance systems (Cheng et al. 2009) while the availability of data on the internet and social media has been viewed as playing a better role in accelerating the process of informing both the public and

governments about any looming public health threat, especially relating to infectious diseases. We anticipate that these new technological improvements could provide a new platform to help improving the quality of detection and reporting of infectious diseases threats, utilizing the sensitivity and timeliness capabilities of digital-based surveillance systems.

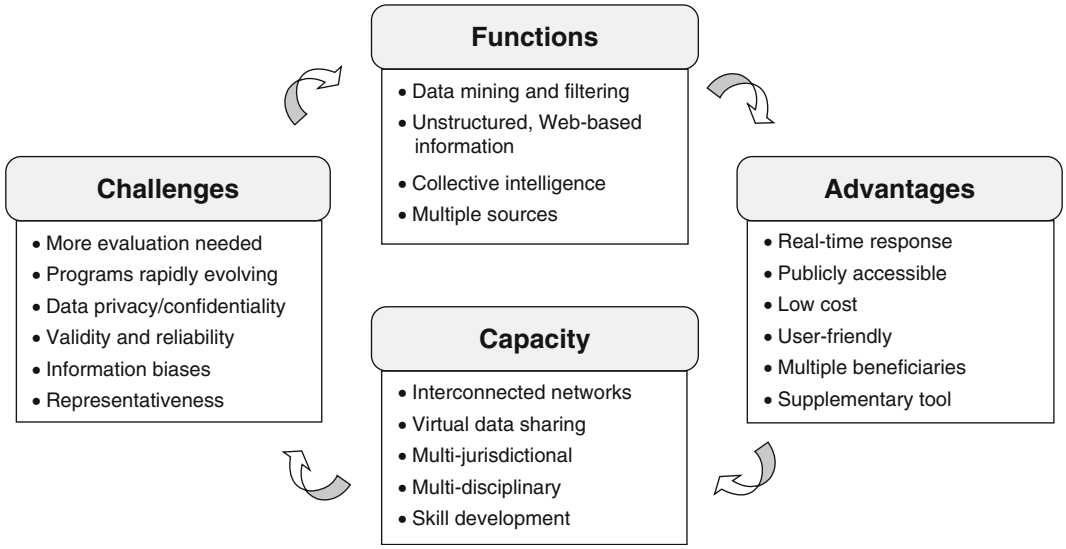
2 Basic Terms and Concepts

Social Media has been defined by Kaplan and Haenlein in 2010 as "a group of Internet-based applications that build on the ideological and technological foundations of Web 2.0 (e.g., Twitter, Facebook, YouTube) that allow the creation and exchange of User Generated Content" (Kaplan and Haenlein 2010). That is, social media is comprised of computer-mediated platforms depending on mobile and web-based technologies which allow people to create, share or exchange information, ideas, and pictures/videos in virtual communities and networks. **Infectious diseases** are the communicable diseases caused by different types of pathogenic microorganisms, such as bacteria, viruses, parasites or fungi (WHO 2015). These types of diseases can spread, directly or indirectly, from one person to another. One of the current emerging sources of infectious diseases is the zoonosis of animals that can cause disease when transmitted to humans. **Disease surveillance systems** have been defined as the ongoing systematic collection and analysis of data and the provision of information which leads to measures being taken in order to prevent and control the spread of the disease (MedicineNet 2015). The disease surveillance systems primarily aim to detect, prevent, control and eradicate sporadic cases and outbreaks, including endemic, epidemic and pandemic, and other public health threats related to biological (viruses, bacteria, parasites, and their toxins) and chemical agents as well (Bernardo et al. 2013). **Digital surveillance** is an internet-based surveillance system which attempts to provide real-time knowledge of public health issues by analyzing information stored digitally (Milinovich et al. 2014b, c). There are now several digital systems being used consisting of non-structured, event-based, and digital data to enhance disease detection and public health responses (Anema et al. 2014; Brownstein and Freifeld 2007; Brownstein et al. 2009; Freifeld et al. 2008; Olson et al. 2015; Sturtevant et al. 2007).

2.1 Social Media-Based Surveillance and Infectious Diseases

Infectious disease surveillance is an epidemiological practice by which the incidence, prevalence and spread of infectious diseases are monitored in order to establish patterns of progression and activate measures of management and control. The main role of infectious disease surveillance is to predict, observe, and minimize the harm caused by outbreaks, epidemics, and pandemic situations, as well as increase knowledge of both practitioners and the public about which factors contribute to such circumstances (Choffnes et al. 2007). Reporting incidences of disease outbreaks has been transformed from manual record keeping to instant worldwide internet communication (Brownstein et al. 2009).

Timely identification of infectious disease outbreaks is critical, both for effective initiation of public health interventions and control measures, as well as the timely alerting of government agencies and the general public at large. Surveillance capacity for such detection can be expensive, and many countries lack the public health infrastructure to identify outbreaks at their earliest stages. The Internet is revolutionizing how epidemic intelligence is activated, and it offers solutions to some of these challenges. Social media and freely available web-based sources of information may allow us to detect disease outbreaks earlier with reduced cost and increased reporting transparency (Wilson and Brownstein 2009). Furthermore, the search and exchange of health information on the Internet and social media has been viewed as an opportunity to improve public health surveillance (Velasco et al. 2014; Kass-Hout and Alhinnawi 2013), and to monitor and predict emerging



Sources: Adapted from Bernardo et al. (2013)

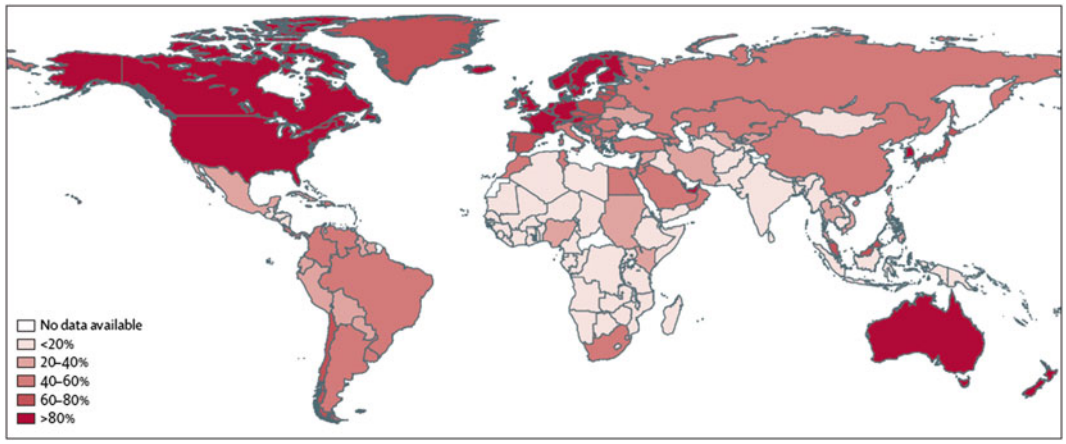
infectious diseases (Milinovich et al. 2014a, c). The diagram above summarizes the key advantages and characteristics of social media-based surveillance and infectious diseases.

3 Social Media and Internet-Based Data

Over the past two decades, the Internet has become an integral component of traditional public health surveillance. Systems using informal electronic information have been credited with reducing the time to recognition of an outbreak, preventing governments from suppressing outbreak information, and facilitating public health responses to outbreaks and emerging diseases. A huge amount of real-time data and information about infectious disease outbreaks is found in various forms of web-based data streams (Brownstein et al. 2008; Freifeld et al. 2008; Brownstein and Freifeld 2007). The web-based data sources exist outside traditional reporting channels; therefore, they are invaluable to public health agencies that depend on timely information flow across national and subnational borders. These information sources, which can be identified through Internet-based tools and social media, are often capable of detecting the first evidence of an outbreak, especially in areas with a limited capacity for public health surveillance (Yang et al. 2013). For example, the World Health Organization's Global Outbreak Alert and Response Network rely on reporting data for day-to-day surveillance activities (M'Ikanatha et al. 2004, 2006). The figure above shows the percentage of population who use the Internet by country.

The Internet and the use of social media are becoming a critical medium for clinicians, public health practitioners, and people seeking health information (Bhatti 2015; Eke 2011; Hartley 2014). Data regarding diseases and outbreaks are disseminated not only through online announcements issued by government agencies, but also through informal channels, including press reports, blogs, chat rooms and analyses of web searches. Collectively, these sources provide a view of global health that is fundamentally different from that yielded by disease reporting done via the traditional public health infrastructure (Brownstein et al. 2008).

The Internet provides a platform to develop efficient, sustainable online resources for patients to research their medical questions, communicate with one another, and support each other,



Source: Lancet Infect Dis 2014; 14: Page 156

such that patients assume more responsibility for their care and decrease the burden on the health care system. A number of online communities have been developed by patient organizations, providers, and nonprofit organizations. Such online communities are virtual forums where patients can discuss their health concerns and exchange information. Participation in online communities heightens levels of emotional well-being, perceived control over disease, overall personal empowerment, and level of public awareness and medical knowledge (Wicks et al. 2010).

The large online population creates a vast network composed of individuals reporting on their activities, their social interactions, and the events around them. These colossal data chunks stream in real time, and are often annotated with context including GPS location, relationships, and images. Extensive data analytics and data mining of social media have been suggested for many applications, such as marketing and financial prediction (Kautz 2013). Recently, researchers have begun to leverage sensor network for public health: preventing, detecting and fighting infectious diseases. Researchers have shown, for example, that Twitter postings can be used to track and predict influenza (Krieck et al. 2011; Sadilek et al. 2012). Such work provides evidence that social media can provide data that helps identify early warning for public health threats.

Some researchers have explored augmenting the traditional notification channels about a disease outbreak with data extracted from Twitter. By manually examining a large number of tweets, they showed that self-reported symptoms are the most reliable signal in detecting if a tweet is relevant to an outbreak or not. Researchers have also tried capturing the overall trend of a particular disease outbreak, typically influenza, by monitoring social media (Culotta 2010). Other researchers focus on more detailed modeling of the language of tweets and their relevance to public health in general (Paul and Dredze 2011) and to influenza forecasting in particular (Paul et al. 2014; Broniatowski et al. 2013).

Extensive data analytics and data mining are concerned with finding models and patterns from the available data. They include predictive algorithms, which result in models that can be used for prediction and classification, and descriptive algorithms for finding interesting patterns in the data, like associations, clusters and subgroups (Lavrac et al. 2007; Corley et al. 2010; Yang et al. 2013). The results of extensive data analytics and data mining need data visualization to enhance the presentation and communicate information clearly and efficiently to users via statistical graphics, plots, information graphics and charts. Effective visualization helps users in analyzing and reasoning about data and evidence. It makes complex data more

accessible, understandable and usable. Data visualization is both an art and a science. Processing, analyzing and communicating the vast amounts of social media data for public health applications present a variety of ethical and analytical challenges for data visualization (Friendly & Denis 2001).

4 Examples of Using Social Media and Internet-Based Data on Infection Diseases

4.1 Google Trends

With the emerging trends of infectious diseases worldwide along with the emerging trend of the Internet being used in a growing number of ways, a number of tools have been developed for the surveillance and discovery of new diseases. Moreover, traditional surveillance systems are not growing proportionately, thus with the rise in the usage of social media, efficient methods are being built for the purpose of internet based monitoring of diseases like Dengue, Influenza, Ebola and others.

Google Trends (GT) is one of the tools, which aims to provide updated recent time data by analyzing search engines with news, websites, images, twitter and YouTube, etc. Google Trend estimates the proportion of keywords from different search engines to estimate the search performed by using Google and relates those keywords and Google results (Nitu et al. 2014). It provides relative search volume (RVS) which is defined as "the query share of a particular term for a given location and time period, normalized by the highest query share of that term over the time-series" (Nuti et al. 2014). Studies have also shown in the past, that GT highly correlates with the incidence of particular infectious diseases, thus the reliability of GT is sound. (Althouse et al. 2011; Ginsberg et al. 2009).

The primary advantage of online surveillance systems are their speed in identifying early warning signals. (Althouse et al. 2011; Chan et al. 2010; 2011; Chunara et al. 2012).

The idea on which Google Trends was built is that during an epidemic of any infectious disease, many people search for that particular disease on the Internet. Google Trends shows that there is a relationship between how many people search for a disease related activity and how many people are suffering from that disease. It has been reported that searched diseases by internet users appeared because of the pattern between online searchs and people suffering from that disease. When compared with the traditional surveillance systems, they found that people search more about a particular disease during an epidemic of the said disease. Thus, this pattern causes the emergence of Google Trends that counts how often people search for particular infectious disease related activity. Through this they estimate the burden of that particular disease in different countries.

5 Advantages of Social Media and Internet-Based Data

Utilizing social media in the prevention and control of infectious diseases can be cost-effective. Social media has the unique advantage of being rapid and can be updated in a timely manner. If properly and scientifically utilized, social media can provide a sensitive and user friendly tool to monitor the distribution and determinants of epidemics both locally and at a global level. For example, systems like GT, present a novel and free tool that allows users to search for information with ease on the internet. This tool can also provide useful clues for understanding characteristics of the disease and the health-related behavior that influences its occurrence (Nuti et al. 2014). The use of social media in infectious disease epidemiology is, however, a new method of surveillance that is emerging. However, with advances in its use, it has the potential of providing an accurate and rapid estimation of the progression of diseases within communities. In addition, social media can be a valuable tool in providing values in distinct climatic and socio-economic context (Gluskin et al. 2014). Most developing countries do not have a

periodically maintained and updated surveillance system for infectious diseases. Countries that own a functioning surveillance system suffer from delay in reporting and many sentinel sites miss out reporting of cases annually or periodically. Lack of resources in most developing countries also hinders communication, training of staff and provision of proper equipment (Madoff et al. 2011). Thus systems like GT, social media and other internet sources can provide a rapid method of surveillance that predicts the real time burden of disease and hence can guide preventive and curative strategies. Systems like GT could also work as a complementary system along with the traditional surveillance system in countries which have already established such systems.

6 Limitations of Social Media and Internet-Based Data

Understanding limitations for using social media and Internet-based data in infectious diseases surveillance is crucial to its proper use. Firstly, most of the information provided is on social media and the internet not moderated by professionals before it gets disseminated online. Reliability of the data is also questionable as the source of information could be from trusted health specialists or from unofficial sources. Lack of standardization for frequency of updates further causes the problem to exacerbate, as too much information becomes available with questionable authenticity. Furthermore, applying algorithms and proper statistical techniques before making it publically available are not usually achieved. Robust monitoring and evaluation of the quality of data needs consideration for its proper usage of information retrieved from social media. (Velasco et al. 2014)

7 Gaps and Future Research

Few challenges and gaps that still exist and need prime attention include collaboration with statisticians, internet and media experts and computer experts to work on different components collectively. Furthermore, training of epidemiologists for monitoring the spread of infectious disease through social media ensures reliability of the evidence extracted. (Velasco et al. 2014) Moreover, strategies and tools should be formulated to compare the traditional system with the online surveillance system, as some diseases can vary drastically. (Keller et al. 2009) Protection and privacy of data should be kept in mind by public health authorities before utilization. This is becoming more important, particularly when the surveillance tools that processes Internet or social media data are within governmental institutions. (Thompson et al. 2011). Future research should focus on factors that hinder the use of social media and internet-based data by health agencies. Social media, weblogs, scientific forums and other electronic communications also have unforeseen social aspects that need to be studied as it can affect human behavior. This in turn has an influence on the information generated by social media and the Internet in general. (Velasco et al. 2014)

8 Chapter Key Points

This chapter reports on the importance of using social media and the Internet in the fight against infectious diseases. Disadvantages and advantages of data gathered from social media and the Internet for public health use are also discussed. Examples and exploration of tools like GT is also given with its own opportunities and challenges. Future challenges and current gaps are also highlighted in this chapter so that future strategies can be formulated in order to improve contemporary surveillance system.

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Emerging Zika Virus Infection: A Rapidly Evolving Situation

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Author's names were initially listed incorrectly which has been corrected in this version as Licia Bordi, Tatjana Avsic-Zupanc, Eleonora Lalle, Francesco Vairo, Maria Rosaria Capobianchi.

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