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THE ROLE OF THE HORSE IN THE SPILLOVER EVENTS AND ITS IMPACT ON ANIMAL AND HUMAN HEALTH

Il ruolo del cavallo nel fenomeno dello spillover ed il suo impatto sulla salute animale e dell'uomo

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Abstract

New emerging diseases are often about a consequence of the spillover event. Spillover or cross-species transmissions, interspecies transmissions or host jumps, are the transmissions of a pathogen between hosts belonging different species. For this event to occur, a break in the balance between the protagonists of this phenomenon (the reservoir, the host and the pathogen), is necessary. This, occasionally, may also involve horses, endangering the health of other animal species but also of humans. Horses are among the most important animals in human history, and therefore, in relation to this, the detection of infectious diseases that affect both humans and horses is crucial, especially in cases of highly transmissible diseases. There are many examples in history that are related to the spillover of different diseases from horses. Among them, there are two of particular importance: the cross-species transmission of the equine influenza virus to dogs, and the spillover of the Hendra virus to humans. The influenza virus affects many different species, including equines. In fact, the equine influenza virus is one of the most frequent and important diseases concerning the equine industry all over the world. In January 2004, one of the two strains of the virus, the H3N8, was firstly isolated in racing greyhounds, becoming, from that moment on, one of the main strains of the canine influenza. The Hendra virus, instead, is an emerging virus jumping from flying foxes to horses and from horses to humans. This virus is confined to Australia only, but it is characterized by a high mortality range, becoming not only a major concern for the Australian National public health ministry, but also for the other health authorities around the world. The Hendra virus is significant because it demonstrates the epidemic potential of an emerging virus.

Introduction

The COVID-19 emergency has attracted international attention on the crucial issue of the spillover event in causing emerging diseases and pandemics. This emergency has highlighted the lack of attention towards this problem both by the scientific community, and the public health authorities from all over the world. Yet, it seems that this event was inevitable. The SARS-CoV-2 pandemic clearly shows how the animal and human world are deeply connected, and often, this connection is made precisely through pathogens that have the ability to adapt to both realities. This problem is occurring with increasing frequency, and this is because of the overcoming of barriers that separate the different ecosystems, and because of the changes affecting the pathogens and the animal species involved. The phenomenon of the cross-species transmission affects a wide range of animals, and among them, the horse. Through time, this animal has become progressively popular and has been used for a variety of activities. Despite this, horses are rarely considered as a source of spillover, capable of causing dangerous epidemics, even if there are numerous episodes of this type that have affected them. Some questions arise from this first analysis. For example, notwithstanding this concrete risk, are owners, the whole equine industry, and the public health authorities aware of this threat? Also, is this risk limited to humans or also to other animal species?

To answer these questions, we can start by describing the spillover process in depth, defining what it is about, and tracing the most important examples of host species jumps. Then, we must outline and analyze all the dynamics that allow the jump to be realized. Firstly, we can look at the dynamics that allow the reservoir, the pathogen and the host to interact and realize a complete spillover. In addition to this, we can observe other fundamental elements that are often overlooked such as the ecological and environmental factors. Moreover, the major concern about the cross-species transmission is its ability to cause pandemics. Therefore, it is important to acknowledge that for a spillover event to be realized, certain conditions are necessary, conditions that, in some way, make the event predictable. This should be the focus of public health authorities. In fact, many different prevention strategies exist, like databases, mathematical models, but also the control of habitats and of the expansion of human activities. Thus, global health authorities and veterinary services should invest more in prevention mechanisms, and in what is defined as the ‘One Health’ approach, a vision in which animal and

human health are mutually dependent. In this task, the role of veterinarians should be further recognised and emphasized.

After this overview of the spillover process, the role of the horse is going to be looked at in the 'One Health' approach, taking into account its relationship with the environment and with humans, its domestication process, and its impact in the socioeconomic and medical fields. To better understand this role, we can retrace the main historical spillover event with regards to the horse, caused by bacteria, but especially by virus. From this analysis, we move to a more indepth overview of the two spillover events, one that represents the jump from horses to dogs, and the other that represents the jump from horses to humans.

The first spillover event that is described is the one of the H3N8 equine influenza virus to dogs. Firstly, we consider the virus itself and all the different characteristics of the disease, as well as how it affects horses and the whole equine industry. Then we frame it in the context in which the jump occurs and how it has been confirmed by researchers. Finally, we analyze both the new canine influenza virus originated from this spillover event, but also the other strain of disease affecting dogs, the H3N2, that instead arises from another cross-species transmission incident. In describing both of these infections, a certain attention is given to the public health implications.

The second spillover case considered is the Hendra virus from horses to human. This virus originates from flying foxes and jumps to horses, who later can transmit it to humans. In this part we firstly describe the host jump incidents, with a description of the main outbreaks and of the properties of the virus. A special focus is then given to the factors that favored the spillover from flying foxes, recalling what has been said in the first part of this work. After this, a full description of the disease in the horse is presented, followed by a focus on the impact on the human and animal community and of the management of the outbreaks by the Australian public health authorities. Finally, the disease in humans is looked at, with a case by case focus due to the peculiarity and particularity of each instance.

In conclusion, what emerges at the end of this analysis is that a greater importance should be given to the role of the horse in the interspecies transmission of viruses, especially due to the potential pandemic risk they pose to both animals and humans. This attention should be given

not only by the equine industry, but also by the international public health authorities of the world.

1. Spillover

1.1 Description of the spillover process

"Infectious disease is all around us. It is about a kind of natural mortar binding one creature to another, one species to another, within the elaborate biophysical edifices we call ecosystems. Darwin's theory is that humanity is a kind of animal, inextricably connected with other animals: in origin and in descent, in sickness and in health. Although infectious disease can seem grisly and dreadful, under ordinary conditions it's every bit as natural as what lions do to wildebeests and zebras, or what owls do to mice. Just as predators have their accustomed prey, their favored targets, so do pathogens. And just as a lion might occasionally depart from its normal behavior, or kill a cow instead of a wildebeest, a human instead of a zebra, so can a pathogen shift to a new target. Accidents happen. Aberrations occur. Circumstances change and, with them, exigencies and opportunities change too. These pathogens aren't consciously hiding, of course. They reside where they do and transmit as they do because those happenstance options have worked for them in the past, yielding opportunities for survival and reproduction. By the cold Darwinian logic of natural selection, evolution codifies happenstance into strategy" (1). In the aforementioned passages from David Quammen's masterpiece, the concept of spillover is introduced. But what exactly is meant by spillover?

Cross-species transmission (CST), also called interspecies transmission, host jump or spillover, is the transmission of a microorganism (virus, bacteria, protozoa and other type of pathogens) between hosts belonging to different species. Once introduced into an individual of a new host species, the microorganism may cause disease for the new host and/or acquire the ability to infect other individuals of the same species, allowing it to spread through the new host population (2). When the interspecies transmission occurs between animals and humans, we are talking about zoonoses (3).

New pathogens continue to emerge in human, domestic animal, wildlife and plant populations, yet the population dynamics of this kind of biological invasion remain poorly understood (4). The rate of emergence of novel disease appears to be increasing as a result of both increased spillover events and our improved ability in detection. The risk of cross-species transmission of known and unknown pathogens has emerged as a threat to human and animal populations due

to various factors, including industrialization, intensive farming, urbanization, rapid transportation and climate change (5).

An emerging pathogen can be defined as "the causative agent of an infectious disease whose incidence is increasing following its appearance in a new host population or whose incidence is increasing in an existing host population as a result of long-term changes in its underlying epidemiology" (6).

Few infectious diseases are entirely human-specific, in fact most human pathogens also circulate in animals, or else originated in non-human host (6). Over the past 10 000 years the deadliest disease and human pandemics recognized as the primary cause pathogens jumping from animals to humans (4). For this reason, it is generally accepted that approximately 75% of emerging infectious diseases (EID) for humans are zoonoses (5) and are responsible for the death of more than 30 million people from 1981 to the present (1). Understanding zoonotic spillover and stuttering transmission are, therefore, two very important public health challenges (7).

1.2 History and evolution of species jump

Pandemics have been driving human history until today and will continue to do so, as demonstrated by the current pandemic of SARS-COV2.

The three most devastating pandemics in human mankind were caused by zoonoses: the Black Death, the Spanish influenza and the Human Immunodeficiency Virus (HIV) that caused the Acquired Immunodeficiency Syndrome (AIDS) (6).

Human society went through a series of major transitions that affected our pattern or infectious disease acquisition and dissemination. These transitions give us the opportunity to illustrate the connection between environmental, social and behavioral aspects and the influences on the emergence and subsequent spread of infectious disease (8).

The first major transition began within the prehistoric/early historic times with the domestication of livestock creating more opportunities for pathogens to move between species. Thus, measles emerged about 7,000 years ago, in all probability from the cattle (*Bos Taurus*) rinderpest and diverged to become an exclusively human infection when population size and density became sufficient to maintain the virus without an animal reservoir. The second historical transition occurred in Classical times as massive Eurasian civilizations came into

commercial and military contact. They inadvertently exchanged their pools of infections and vectors, like rats and fleas across the Mediterranean basin, the Middle East, India and China (*e.g*., typhus fever frequently accompanies human conflict and deprivation). The third historical transition accompanied the era of worldwide exploration and colonization by Europeans from circa 1500 CE onward. A contemporary account by one of Hernan Cortes' fellow conquistadors, Bernal Diaz, recalls that they might well have failed to overthrow the mighty Aztec empire had they not been aided by a raging epidemic (8).
Then bringing us into the present day, several were the episodes of species jump that led to the development of epidemics and epizootics.

HIV, the virus of AIDS in humans, is one of the most important recent example of a virus emergence by host switching. Following its emergence into humans from primates approximately 70 years ago, HIV has infected hundreds of millions of people. Despite our increased understanding of the virus and the development of effective antiviral therapies, it is estimated that 1.8 to 4.1 million new human HIV infections still occur each year (2). HIV is a virus that belong to the genus *Lentivirus* within the *Retrovirus* family. The ancestral viruses that eventually gave rise to HIV were common and well-established simian immunodeficiency viruses (SIV) in Old World monkeys and hominoids, which have been infecting those animals for millions of years and are now mostly associated with very little or no malady. There have been at least four strains of HIV-1 identified that each representing a single transfer from either chimpanzees (*Pan troglodytes*) (M and N strains from the SIVcpz) or from gorillas (O and P strains from SIVgor); the M (major) strain is the pandemic one, and it is responsible for greater than 98% of human infections. It's clear that all SIVcpz strains were derived from one common ancestor that contained sequences derived from SIVs from different species. This virus eventually gave rise to the M and N strains that infected humans, and infected gorillas in which it gave rise to the HIV-1 O and P strains, which were then transmitted to humans. The HIV-1 M strain is the responsible of the global pandemic, while the O strain has caused infection in hundreds of thousands of humans in Central Africa. Alternative spillovers of lentiviruses from other primates are named HIV-2 and they infect fewer individuals in limited outbreaks (9). Different factors have contributed to the cross-species transmission, such as the rapid transportation of game animals to national and international markets; concentration of diverse

wildlife species, domestic animals and humans in wet markets; and exposure to animal blood and tissues during butchering by humans and during predation by animals (2).

Another important event is the spillover of the influenza A virus (IAV). IAVs are members of the *Orthomyxoviridae* family (10). IAVs are often at the top of most lists of emerging viruses, having jumped into prominence in 1918 with the global pandemic caused by the H1N1 strain in humans and in swines. This was followed by the emergence of the H2N2, H3N2 and H1N1 pandemic strains of IAV in humans in 1957, 1968 and 2009, respectively. Other epidemic strains have arisen in horse (*Equus caballus*) (H7N7 and H3N8), pig (*Sus scrofa domesticus*) (besides the H1N1, there have been two different H3N2 strains and reassortant forms), seal (Fam. *Phocidae*) and dog (*Canis lupus familiaris*) (H3N8 and H3N2). The jump of H3N8 from horses to dogs will be analyzed in detail in the next chapter. Birds in fresh or saltwater environments are the primary reservoirs of IAVs where infections are largely non-pathogenic, replicating in the gastrointestinal tract, and the viruses are shed into the water where they are taken up, likely by oral and respiratory routes, to infect other individuals. With the exception of bat viruses, all IAVs in mammalian species, including humans, ultimately originate from viruses in wild birds, either directly or via intermediate hosts. The H1N1 pandemic virus that emerged in 2009 had a complex history and contained segments from a range of various host sources, including segments with swine (from which probably originated in Mexico), avian and human viral origins. While spillovers of IAV are quite common, epidemics are relatively rare and making the shift from an intestinal infection and fecal-oral transmission in water birds to a respiratory infection and aerosol transmission in mammals would appear to be a significant problem. Genetic changes have been identified as one of the primary factors which allow the virus to cause epidemics, and this is made possible by determinants like the animal environments that differ between the avian gastro-intestinal tract, which is around 41°C, and the upper or lower respiratory tracts of mammals, which are approximately 34 or approximately 37°C. Other differences include the display and binding of specific sialic acid (SA) receptors and linkage forms which influence haemagglutinin (HA) binding and neuraminidase (NA) cleavage, release of the viral ribonucleoproteins from the endosome and transport to the nucleus, RNA replication and/or transcription, antagonizing various interferon-associated innate immune responses, viral budding from the cell and release from the cell surface, shedding and transmission among individuals of that host (9). The medical and veterinary communities are challenged with a virus

that continually changes through different mechanisms, as it adapts to different species and reassorts with other IAVs of avian and mammalian origin (10).

Coronaviruses (CoVs) are a family of viruses that cause severe diseases such as the Middle East Respiratory Syndrome (MERS-CoV) and the Severe Acute Respiratory Syndrome (SARS-CoV). In December 2019, an outbreak of pneumonia with an unknown cause occurred in Wuhan, Hubei province, China, with an epidemiological link to the Huanan Seafood Wholesale Market, a local live animal and seafood market. Considering the clinical signs, this disease greatly resembled viral pneumonia. Through deep sequencing on the lower respiratory tract samples of patients, a novel CoV was identified, the name of which was then determined as SARS-CoV-2. CoV are shown to have a wide range of hosts, and some of them can infect humans. Thus, it is critical to determine the natural reservoir and the host tropisms of them, especially their potential of causing zoonosis. In the last two decades, apart from the recent SARS-CoV-2 pandemic, also SARS and MERS have caused serious outbreaks in humans, leading to thousands of deaths. CoVs usually cause respiratory and gastrointestinal tract infections. This family of virus is genetically classified into four major genera: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus* and *Deltacoronavirus*. The former two genera primarily infect mammals, whereas the latter two predominantly infect birds. In addition to SARS-CoV-2, other members of the *Betacoronavirus* genus caused the 2003 SARS outbreaks and the 2012 MERS outbreaks in humans. Quite often, bats are identified as the major CoV reservoir hosts, while a direct contact between humans and bats seems impossible. It seems to be more likely that the spillover in this CoVdisease emerged thanks to the intervention of intermediate hosts, like the palm civets (*Paradoxurus hermaphroditus*) for SARS-CoV and dromedary camels (*Camelus dromedarius*) for MERS-CoV. A study published on May 14, 2020 on 'Plos Pathogens' has identified the pangolin (*Manis javanica*) as a possible intermediate host for the emergence of SARS-COV-2: pangolin-CoV-2020 is genetically associated with both SARS-CoV-2 and a group of bat CoV. In fact, it seems that there is a high sequence identity between pangolin-CoV-2020 and SARS-CoV-2. A prior study suggested that SARS-CoV-2 and SARS-CoV bind to the same angiotensin-converting enzyme 2 (ACE2) as one of the main receptor. For all of these reasons Pangolin-CoV is very likely to use ACE2 as its receptor as well. A comparative analysis of the interaction of the S proteins of CoV with ACE2 proteins of humans and pangolins showed that the S proteins of SARS-CoV-2 and pangolin-CoV can

potentially recognize ACE2 in both humans and pangolins. The study substantially does not support that pangolins could be intermediate hosts for the emergence of SARS-CoV-2, but the results do not exclude the possibility that other CoVs could be circulating in pangolins (11). According to the World Health Organization (WHO) September 2021 the cases of COVID-19 are of 219 million with 4,55 million deaths, and the number are is still expected to increase (12). Many are the causes that have favored the spillover and must be investigated to prevent next spillover and pandemics event. Severe disease related to CoVs associated with bats are not limited to humans. In 2016-2017, there was a major outbreak of swine acute diarrhea syndrome (SADS) in piglets in multiple southern China farms in a region geographically close to where the SARS outbreak began in 2002. The origin of the causative agent, SADS-CoV, was quickly traced back to a bat colony in the vicinity of the pig farms. Examination of pig farmers with close contact with affected piglets did not yield evidence of human infection, hence it would appear that humans may not be susceptible to SADS- CoV (5).

Cross-species event are not isolated to humans, but may also affect the animal population. This is the case of the canine parvovirus (CPV). CPV is a member of the *Protoparvoviruses*, in the Family *Parvoviridae*. The emergence of the pandemic CPV in dogs, gained the canine host range and spread worldwide in 1978. The viruses that gave rise to CPV have been known to infect many different hosts within the order *Carnivora*, and they were first found to cause disease in cats (*Felis silvestre*) in the 1920s, in raccoons (*Procion lotor*) in the 1930s and in mink (*Neovison vison*) in the 1940s. Those viruses were variously named feline panleukopenia virus (FPV), raccoon parvovirus or mink enteritis virus, although it has long been recognized that those viruses are related and could infect some other carnivore hosts. In 1978, new diseases were identified in dogs by severe gastroenteritis with profuse bloody diarrhea, while neonatal puppies developed a myocardial disease. The cause was recognized as a parvovirus similar to FPV, that was named CPV type-2 (CPV-2) to differentiate it from the distantly related minute virus of canines, also known as canine boca-virus. Sequence analysis showed that CPV was new in dogs, and that all viruses in dogs share a common ancestor present around the mid-1970s, and they were greater than 99% identical to the FPV-like viruses. The causes of the jump can be found in the frequent exposure of dogs to viruses from other hosts, particularly cats. As a result, the CPV-2 develops a close host adaptation. It is also worth nothing that the strain that emerged in 1978 did not infect cats (9).

1.3 The biology of jump

Spillover transmission is promoted by successive processes that enable a pathogen to establish infection in another species, including humans, and when the transmission takes place from non-human species, we are referring to zoonosis. The probability of zoonotic spillover is determined by interactions among several factors, including disease dynamics in the reservoir host, pathogen exposure and the within-human factors that affect susceptibility to infections. Considering the zoonosis model, these factors can be partitioned into three functional phases that describe all major routes of transmission (13): the first stage is when a pathogen exclusively infects animals ('reservoir dynamics') (7). In this first phase, the amount of pathogen available to the human host at a given point in space and time, known as the "pathogen pressure", is determined by interactions among reservoir host distribution, pathogen prevalence and pathogen release from the reservoir host, followed by pathogen survival, development and dissemination outside of the reservoir hosts (13). The second phase is when the pathogen occasionally jumps to the dead-end-host human population ('spillover') (7). In this phase, human and vector behavior determine "pathogen exposure"; specifically, the likelihood, route and dose of exposure (13). This stage can be followed by a third phase, once human-to-human transmission becomes possible but leads only to self-limiting chains of transmission ('stuttering transmission'). The final stage is when a pathogen gains the ability to transmit effectively between humans and no longer requires zoonotic transmission (7): genetic, physiological and immunological attributes of the recipient human host, together with the dose and route of exposure, affect the probability and severity of infection (13). An additional scenario is when the pathogen infects both animals and humans in a sustainable manner (7). Sometimes, even if capable of infecting a different host species, pathogens are usually, although not always, less infectious. This is referred to the "species barrier", and it can be substantial, implying that much higher doses are required to infect the new host (4). Spillover requires the pathogen to pass every barrier and this can only occur when gaps align in each successive barrier within an appropriate window in space and time. Many of the individual determinants of spillover are subjects of intensive study, each of these usually being addressed in a specialized discipline (13). The probability of spillover is determined by the possibility of the pathogens to cross the barriers and this is, in turn, influenced by their changes and by the modification of other determinants involved in the process, that will now be addressed in more detail.

In order to better describe the various factors involved, it is necessary to recall that the spillover of the emerging pathogens requires a series of hierarchical enabling conditions: reservoir hosts must be present; reservoir hosts must be infected; if transmission is indirect, reservoir hosts must be shedding pathogen that must survive outside of its reservoir host with access to the recipient host; recipient hosts must be exposed to the source of the virus in sufficient quantity for an infection to establish; and recipient hosts must be susceptible to the virus (14).

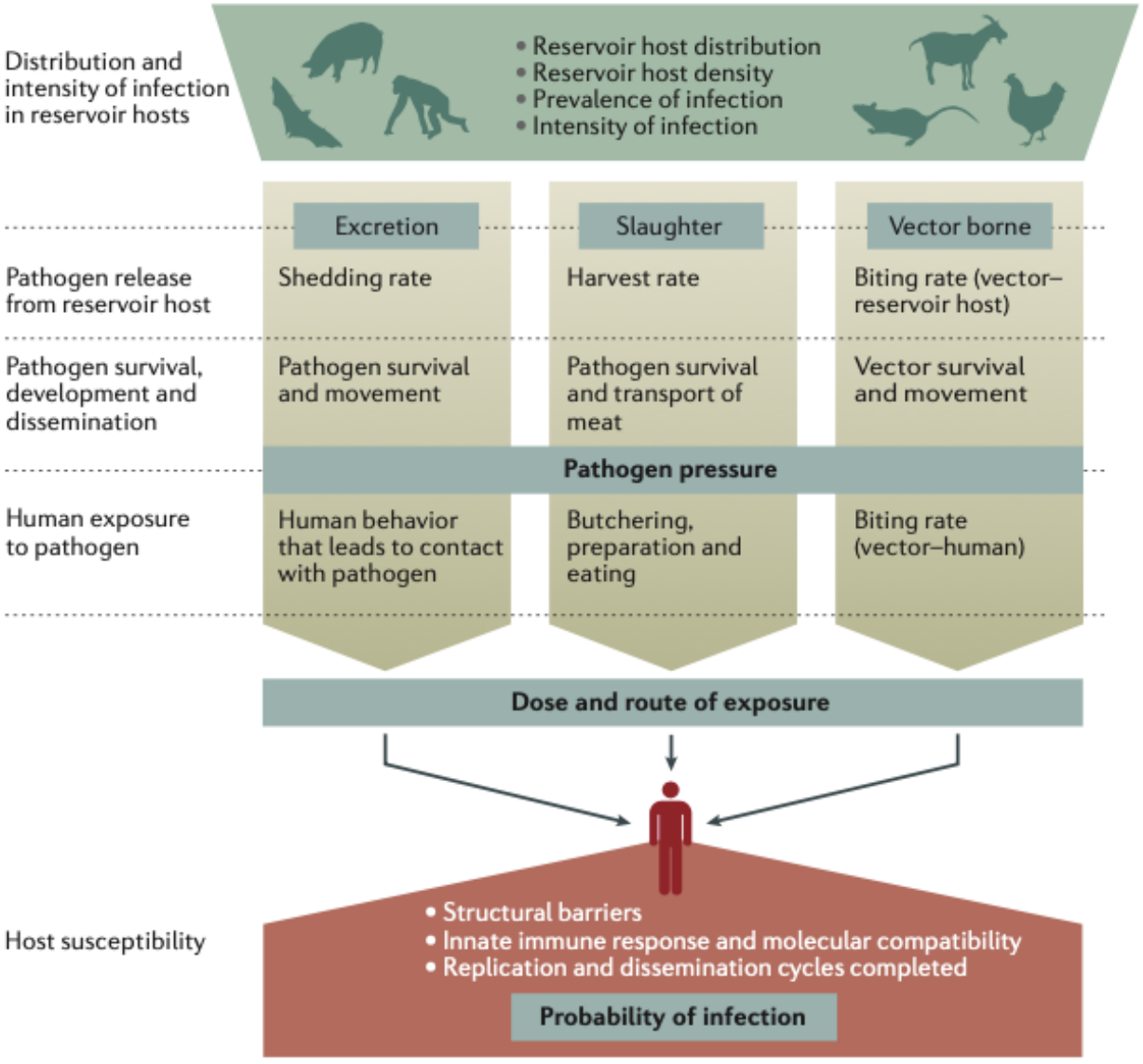

Figure 1: Pathways to zoonotic spillover
('Pathways to zoonotic spillover', Plowright et al. (2017))

1.3.1 The reservoir

According to Haydon et al., a reservoir can be defined as "one or more epidemiologically connected populations or environments in which the pathogen can be permanently maintained and from which infection is transmitted to the defined target population". Previous reservoir definitions often required that the relevant infectious agent be nonpathogenic to the reservoir host species. A more modern definition describes the reservoir as an ecological system within which the infectious agent survives indefinitely. In addition, reservoirs must be defined with reference to the particular target populations (15).

Domesticated species, primates and bats were known as having more zoonotic viruses than other species. Domestication of animals, human encroachment into habitats high in life biodiversity and hunting of wild animals have been proposed as key anthropogenic activities driving infectious disease emergence at the global scale. Johnson et al. combine data on all zoonotic viruses detected in terrestrial mammalian species through the International Union for Conservation of Nature (IUCN) Red List of Threatened Species and systematically evaluate data on both wild and domesticated mammalian species that have viruses in common with humans. It appears that species abundance and specific extinction threats are related to the number of viruses shared with humans across mammalian species, with important implications for understanding virus spillover risk. The number of viruses detected in each mammalian species was summed to estimate zoonotic virus richness for each species. The result of the study is that the highest proportion of zoonotic viruses were reported among species in the orders *Rodentia* (61%), *Chiroptera* (30%), *Primates* (23%), *Artiodactyla* (21%), *Carnivora* (18%) and fewer viruses were detected in other mammalian orders. The mammalian orders with more species are the source of more zoonotic viruses. Three mammalian orders, rodents, bats and primates, have together been implicated as hosts for the majority (75.8%) of zoonotic viruses described to date, and these orders represent 72.7% of all terrestrial mammal species. The study highlights that the domestication of livestock has played a well-recognized role in transmission of zoonotic viruses to humans: domesticated species status had the largest influence on the number of mammalian viruses shared with humans with eight times more zoonotic viruses .The top 10 mammalian species with the highest number of viruses shared with humans included eight domesticated species: pigs (n = 31 zoonotic viruses), cattle (n = 31 zoonotic viruses), horses (n = 31 zoonotic viruses), sheep (*Ovis aries*) (n = 30 zoonotic viruses), dogs (n = 27

zoonotic viruses), goats (Gen. *Capra*) (n = 22 zoonotic viruses), cats (n = 16 zoonotic viruses) and camels (*Camelus bactrianus*) (n = 15 zoonotic viruses). Aside from humans, accurate detection and reporting of zoonotic viruses would be most probable in domesticated species. The only wild animals among the top ten species with detected zoonotic viruses were the house mouse (*Mus musculus*) and the black rat (*Rattus rattus*), with 16 and 14 zoonotic viruses, respectively. Both of these species in the *Rodentia* order are considered invasive in most regions of the world, commonly inhabit domestic and peri-domestic structures, and have dubious non-domestication status given their use in laboratory studies and as pets worldwide. Towards the end, the study postulate that wild mammals were the original host for the majority of viruses, sharing viruses with domesticated species over centuries of co-evolution and domestication.
Primate, rodent and bat species appear to harbor zoonotic viruses that are not well connected to domesticated species and other wild animal species, supporting the premise that these species share zoonotic viruses directly with humans, without domesticated amplifying hosts facilitating viral sharing among species in other orders. It seems that bats hosted significantly more zoonotic viruses than other orders and that primates drove the phylogenetic effect as a determinant of zoonotic spillover, while the close phylogenetic relationship of humans with non-human primates is recognized as a causal factor for spillover.

Bats, and all of species in the order *Chiroptera,* have been frequently implicated as the source of recent emerging disease involving high consequence pathogens, including the severe acute respiratory syndrome SARS-CoV, Nipah virus (NiV) encephalitis and hemorrhagic fevers caused by *Filoviruses*, and have been noted previously to host more zoonotic viruses per species than rodents (16). The importance of bats as a source of emerging viruses has been proven in numerous studies in the last two decades. Bats are the only mammals capable of powered flight and are among the most ancient of mammals. There are currently more than 1000 species of bats, making them the second most diverse mammalian group, after rodents, and representing 20% of extant mammalian species. Although the recent interest in bats is mainly driven by their association with many of the most lethal viruses, bats are known for their exceptionally long life span and for being less prone to cancer. They also have great variation in their geographical locations, dietary preferences, physiological range of body temperatures, social behavior and navigation and vision systems. One of the challenging scientific questions is why many of the

bat-borne zoonotic viruses are so lethal when they spillover into human and/or livestock animal populations (5).

It has been observed that viruses which severely affect other mammals, including humans, are apparently nonpathogenic for bats. This adaptability of bats to harbor many viruses without showing any clinical signs suggests that bats have evolved immune mechanisms that allow for benign virus–host relationships.

As is well known the immune response has two primary components, innate and adaptive. As shown in *Figure 2* genes such as those for sensing and repairing DNA damage and the inflammatory process are under positive selection in black flying foxes (*Pteropus alecto*) and David's myotis bats (*Myotis davidii*). For instance, there are mutations in the coding sequence of p53 functional domains that are unique to these bats. Interferons are the primary innate effector molecules that control viral replication and infection. Several types of interferon have been identified in bats, especially Type I and Type III. The number of variants of interferon present in these bats is lower compared to those in the gene loci of other vertebrate species. Despite this decrease in interferon gene diversity, black flying foxes express these variants at higher basal levels than other mammalian species. This suggests that the interferon and interferon-stimulated genes (ISGs) are constitutively expressed in these bats (17). A study carried out by La Cruz-Rivera et al., suggest that in bats of several species, higher level of ISGs are always present in their cells, which makes them better prepared to control viruses. Overall, bats seem to possess either an "always ON" interferon strategy plus or a better antiviral ISG defense strategy (18). Bats also have mechanisms to avoid over-induction of inflammatory genes. It is probable that many bats have a mechanism to suppress the expression of TNFα, a key inflammatory cytokine, and thereby maintaining a balanced response to viral infection. Several species of bats control inflammation thanks to a mutation at a highly conserved serine residue in one of the key adaptor molecules for sensing damaged DNA, *i.e.,* stimulator of interferon genes (STING), which reduces its functionality.

Mechanisms to control inflammation may have evolved to mitigate the detrimental effects of flight. Excessive exposure to cytosolic DNA in bat cells during flight might have posed a strong natural selection pressure to reduce the activation of bat DNA sensors. Thus, it appears that bats have developed a unique immune features related with the evolution of flight. The increased rate of metabolism accompanying flight would lead to higher levels of oxygen-free radicals.

This makes bats more prone to generating damaged DNA. As mounting an immune response is energetically expensive and would be detrimental, bats probably evolved mechanisms to suppress activation of immune response due to damaged DNA generated via flight, thereby leading to reduced inflammation. In these animals the evolutionary suppression of inflammation and consequent susceptibility to pathogens infection is counteracted by constitutive expression of innate immune genes or novel genes to target viruses as described earlier.

Another issue is the viral persistence in bats. For an animal species to be a viral reservoir, the virus needs to persist in the population. Two probable ways in which this can happen are: (a) virus infection and clearing from infected individuals is an ongoing process and introduction of naïve individuals maintains the virus in the population and (b) individuals infected with the virus are able to maintain the virus in the form of a persistence infection (17). Plowright et al. proposed a hypothesis called "The Parsimonius theory" according to which bats are persistently infected with some virus but shed these virus only when they are immunocompromised. Virus infect naïve susceptible bats leading to acute infection, which subsequently progresses to a chronic or latent infection. Then, it reactivates from time to time in response to a variety of physiological and environmental triggers. This chain of events is called the "SILI hypothesis": Susceptible–Infectious–Latent–Infectious (19). Among other factors, arousal from hibernation features as one of the responsible stressors. Gerow et al. demonstrated by isolating and characterizing a *Gammaherpervirus* (*Eptesicus fuscus herpesvirus*, *Ef*HV) autochthonous to North America big brown bats to better understand the Henipavirus (HNV) emergency, that the virus reactivates latency when big brown bats arouse from hibernation, leading to the detection of the virus in blood. This reactivation was also associated with a low level of antibodies against the virus. Following hibernation, the antibody levels increase, which subsequently drives the virus into latency (20). An additional factor is the presence of secondary infections: little brown bats are particularly susceptible to a frequently lethal fungal infection known as the "white nose syndrome" caused by *Pseudogymnoascus destructans.* A study looking at the effects of white-nose fungus on a persistently infecting CoV showed that bats having the fungal infection and showing signs of the infection on their wings had 60 times more CoV in their intestines as compared to fungal uninfected bats. The gastroenteric system of the fungus-infected bats exhibited a gene expression profile suggesting suppression of the innate antiviral response, which may have contributed to unrestrained viral replication. This suggests that secondary

infections in bats persistently infected with viruses could increase the potential of viral shedding. These studies indicate that waning antibody levels and suppression of innate immune responses due to stress might be some of the factors leading to an increase in viral levels in persistently infected (21). Last, but not least, it is also important to look into various factors that might stress bats such as habitat destruction (deforestation), pregnancy, change in seasons, nutritional factors and climate change (17).

Bats represent an important but largely uncharacterized source of known human pathogens. A major problem facing all studies of bat-derived virus cell biology is the lack of available reagents and animal models, compounded by the enormous taxonomic diversity of these animals. Most mammalian cell lines currently available do not support replication of the majority of viruses being discovered. There is an urgent need for more cell culture reagents that can better facilitate virus isolation, either new cell lines capable of supporting replication of bat viruses or genetically modified versions of existing cells, such as Vero cells, to increase their susceptibility to viral infection with bat viruses. Lastly, live animal models will also be crucial for understanding the implications of molecular findings in bat cells for the course of infection in the natural host. Therefore, efforts to mitigate the public health impacts of bat-borne viruses must integrate research across these disciplines, applying a 'One Health' approach, from field to lab, to address the problem. The future of bat virus research lies in a combined and concerted effort to evaluate the molecular and macro-ecological risk factors of transmission, shine light on which viruses carry the potential to spillover and conduct large-scale, longitudinal surveillance studies that will support the deployment and evaluation of next-generation interventions (22).

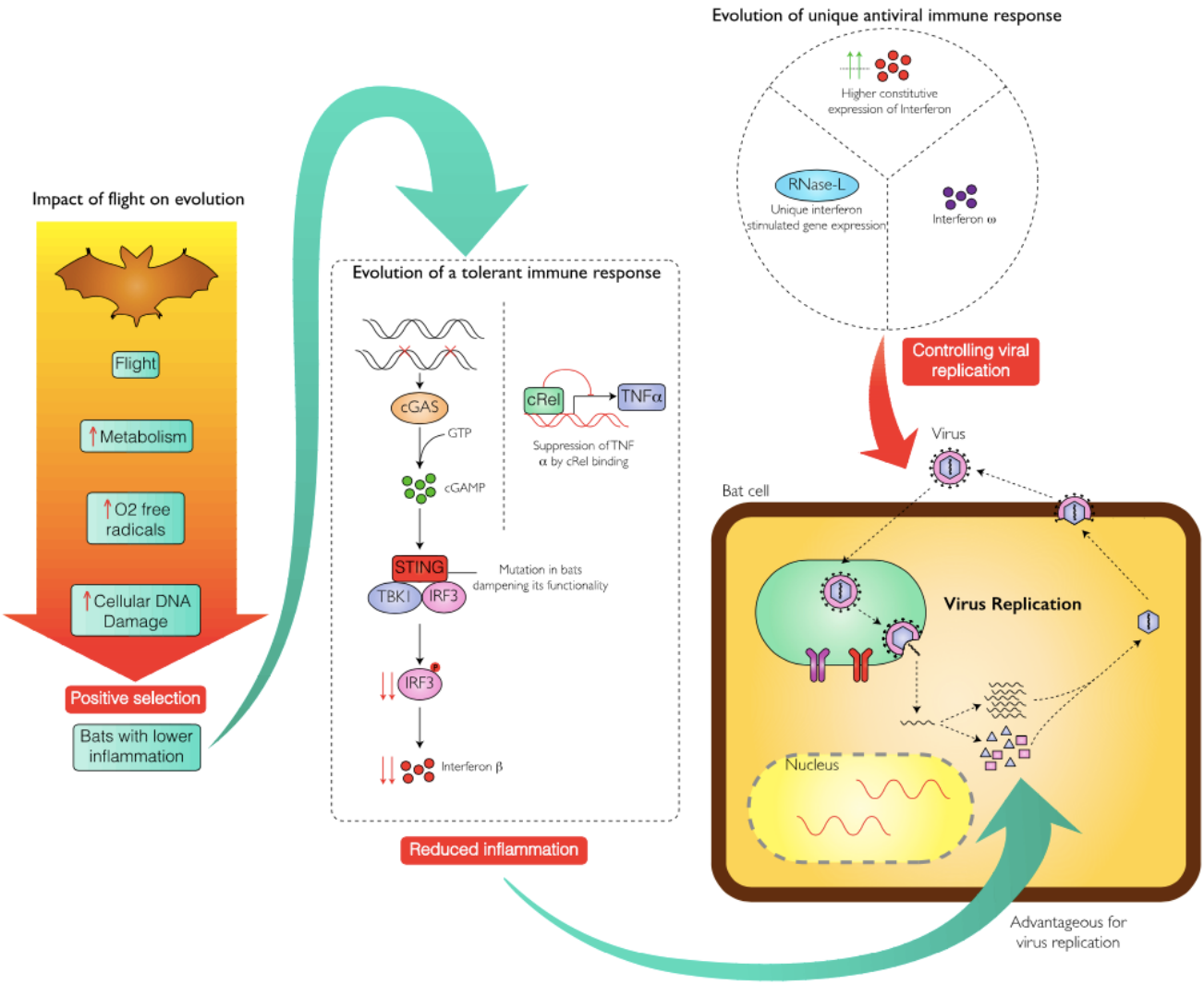

Figure 2: Tolerance to DNA damage and unique antiviral immune response in bats
('Immune System Modulation and Viral Persistence in Bats: Understanding Viral Spillover', Subudhi et al. (2019))

1.3.2 The host

A crucial role is played by the changes in the various factors that concern the host. Many are the barriers that a virus need to overcome in the new host, including receptor binding, entry or fusion, trafficking within the cell, genome replication and gene expression. The production and shedding of infectious virus may also be host specific. As a matter of fact, multiple host barriers to infection would each require one or more corresponding changes in the virus, making the host range barrier more difficult to cross. Other significant impediments to infection can include

innate antiviral responses (such as interferon and cytokine-induced responses) or other cellular barriers or responses that restrict infection by particular viruses.

The genetic host separation is important to consider, as spillover or epidemic infections have occurred between hosts that are closely or distantly related, and no rule appears to predict the susceptibility of a new host. Repeated virus transfers between chimpanzees and humans, who are closely related, resulted in HIV establishment, while the transfer of a feline panleukopenia virus (FPV) to dogs reflected adaptation between hosts from different families in the order *Carnivora*. While the evolutionary relatedness of the hosts may be a factor in host switching, the rate and intensity of contact may be even more critical.

An initial level of protection of the host against viruses occurs at the level of viral entry into the skin or mucosal surfaces or within the blood or lymphatic circulation or tissues. Defenses should include mechanical barriers to entry as well as host factors that bind to virion components to prevent infection. For example, glycans or lectins (often called serum or tissue inhibitors) may bind and eliminate incoming viruses (23). Mucus is composed of a variety of different glycans and proteins, which differ widely between various hosts and their tissues. Mucus composition and susceptibility to viral infection also vary depending on the health of the individual, the presence of resident commensal microflora and coinfections, and physiological stressors. Many different viruses carry glycosidases, sialidases, or esterases that can act as countermeasures against mucin glycans upon entry as well as during egress and shedding of viral particles. Mixed infections of newly transferred viruses with other viruses or bacteria may alter the host mucus and facilitate infection, as it happens in respiratory diseases. Also, many viruses have glycosylated proteins on their surfaces which are host-specific. For that, viruses produced in one host will carry an imprinted source glycosylation that is determined by the specific cell or tissue they are produced in. Upon exposure to a new host, these variant glycans (*e.g.*, N-glycolyl neuraminic acid (Neu5GC) or a1,3-galactose (a-gal)) may be targeted by preexisting host antibodies to these glycan forms and block the viral infection (9).

Another crucial element is represented by the host receptors. A key determinants of host and tissues tropism and therefore of host range, are often viral receptors and those that may be expressed at the cell surface or within the entry pathway. The specificity of virus-receptor interactions may involve host-specific structural interactions that control the affinity of binding, progression to internalization, triggering cell infection, along with key roles for co-receptors

where those are present. Changes in receptor binding often play a role in host transfer. For example, the SARS-CoV was derived from viruses circulating enzootically in a number of bat reservoirs, and the bat-derived viruses interact differently with the angiotensin-converting enzyme 2 (ACE2) receptors of humans and carnivore hosts such as Himalayan palm civets (*Paguma larvata*), which harbor viruses that are closely related to the human viruses (23). In this sense, the SAs receptor may play a key role in solving the enigma of interspecies transmissions. Receptors are major determinants of host susceptibility to viruses. The role of the SAs receptors is better explained in the article of Kuchipudi et al. According to this, animal species sharing host cell receptors that support the binding of multiple viruses that can play a key role in virus spillover and the emergence of novel viruses and their variants. SAs, which are linked to glycoproteins and ganglioside serve as receptors for several human and animal viruses. In particular, influenza and CoVs, which represent two of the most important zoonotic threats, use SAs as cellular entry receptors. Glycans are structural polysaccharides found ubiquitously on eukaryotic and prokaryotic cells and contribute to the cellular protective, stabilizing, organizational, and barrier functions. SAs are a striking exception to the family of sugar units with a nine-carbon backbone and typically found attached to the terminal position of N- and O-linked glycans. The unique location of these nine-carbon alpha-keto acids helps them play a critical role in several intrinsic and extrinsic interactions of a cell. Currently, in nature, more than 50 structural variations for SAs have been detected. Many viruses often target particular classes of receptors, such as sialylated glycans, cell adhesion molecules, to mediate cellular entry. The redundancy in receptor usage indicates evolutionary conservation in the way viruses target particular receptors to take advantage of their cellular function. Sialo-glycoconjugates expressed on cell surfaces serve as ligands or receptors for intrinsic or extrinsic SA specific lectins. Many RNA viruses and DNA viruses exploit these glycans as initial anchors to gain access to the host cells. Host cell receptors undergo evolutionary alterations to avoid rapidly emerging pathogens while maintaining the critical endogenous function. Consequently, many of the microbial interactions with host cells expressing cognate SA are detrimental. Furthermore, most viruses have evolved to express enzymes that can cleave the interactions with these SA receptors, aiding their release from infected host cells. These sialidases act as decoy receptors, which bind to virions, preventing their access to host epithelial cells. The presence or absence of an appropriate host SA receptor is a major determinant of the host tropism of a virus,

including the tissue and the specific cell types within the host that the virus can infect. Host receptor distribution is therefore a key to understanding their susceptibility to a particular virus and to determine the body systems that will likely be infected and the type of clinical symptoms it may generate. The distribution of SA variants in various tissues and hosts may contribute to the evolution of specific viral glycoproteins, which can interact with SAs. The expression and distribution of these SA receptors differ depending on the location within the body, type of the cell, and their intended functional role. Just to give some examples the susceptibility of a host to IAV infection is determined by the type of SA receptor present on the host cell surface along with other host factors. Here, increasing evidence shows that sialylated compounds of cellular glycocalyx can serve as an important factor in the mechanism of CoV infection (24).
In conclusion from a receptor standpoint, widespread presence of SA receptors in domestic, wild animals and humans that a particular virus can bind provides an opportunity to jump species and adapt to the human host (4).

Other than receptor binding, restriction may also occur at other levels in viral infection cycles. For example, for *Retroviruses*, several intracellular mechanisms restrict cell infection. The TRIM5α protein binds the incoming capsid protein in the cytoplasm and restricts infection in a host-specific process that depends on the capsid protein structure. The adaptation of HIV-1 to humans from chimpanzees for instance, was associated with a change in the p17 Gag protein, which may be involved in the specific targeting of the protein within the host cell cytoplasm (23).

Certainly, the immune response of the host is an essential weapon, which consists of innate and adaptative response. Protease and protein modification has been found to control infection and activation of some viruses, and can differ between different hosts and their tissues. These may include the activation of viral glycoproteins by specific cleavage of the protein, as well as recruitment of post-translation modifying pathways such as ubiquitination or phosphorylation, which may be specific for various hosts and tissues and therefore control host susceptibility. Additionally, the innate immune response driven by IFN and innate intracellular blocks is important. The innate immune system vary significantly between different animal hosts and often control infection or replication. Viral-encoded countermeasures to IFN or ISGs or their products are often host-specific, and can influence the success of viral transfers (9).

Adjustments in hosts may occur at different level and facilitate pathogen spillover.

Among these changes, we can list the variations in host genetics. As an example, the loss of major histocompatibility complex haplotypes or other genetic diversity in inbred livestock or small populations might increase susceptibility to infection. This shifting may involve also the host phenotype. For example, immunosuppression during hospital treatments or due to the effects of HIV/AIDS has been cited as contributing to the spread of numerous infections. The loss of cross-immunity might also increase the potential for invasions by new pathogen as has been suggested for several pairings: yaws and syphilis, leprosy and tuberculosis, yellow fever and dengue fever, smallpox and monkeypox and vivax malaria and falciparum malaria.

Changes in host behavior and movements must also be considered. For example, patterns of sexual behavior directly affect the potential spread of sexually transmitted diseases, and global travel exacerbated the spread of SARS.

Lastly, important changes in host ecology and environment and the impact of humans in the natural habitats of the reservoir species need to be accounted for (23).

1.3.3 Ecological, environmental and anthropological determinants

"Perhaps a tiny, invisible virus will be what actually, hopefully tips the scale toward a critical mass of global understanding of the fact that our own health is intimately tied to how we trat the natural world" said Steve Osofsky, professor of Wildlife Health & Health policy at the Cornell University. This quote is the basis for the comprehension of the multitude of events that govern the cross-species events, especially considering the role of wildlife health and the way in which humans trespass and damage habitats.

In the same vein, David Quammen wrote "These disease outbreaks coming one after another, and they are not simply happening to us, they represent the unintended results of things we are doing. They reflect the convergence of two forms of crisis on our planet. The first crisis is ecological, the second is medical. As the two intersect, their joint consequences appear as a pattern of weird and terrible new diseases, emerging from unexpected sources and raising deep concern, deep foreboding, among the scientists who study them. There are three elements to the situation: a) mankind's activities are causing the disintegration (a word chosen carefully) of natural ecosystems at a cataclysmic rate. We all know the rough outlines of that problem. By

way of logging, road building, slash-and-burn agriculture, hunting and eating of wild animals (when Africans do that we call it "bushmeat" and impute a negative onus, though in America it's merely "game"), clearing forest to create cattle pasture, mineral extraction, urban settlement, suburban sprawl, chemical pollution, nutrient runoff to the oceans, mining the oceans unsustainably for seafood, climate change, international marketing of the exported goods whose production requires any of the above, and other "civilizing" incursions upon natural landscape by all such means, we are tearing ecosystems apart. This much isn't new. Humans have been practicing most of those activities, using simple tools, for a very long time; b) millions of unknown creatures include viruses, bacteria, fungi, protists, and other organisms, many of which are parasitic. They don't live independently. They don't cause commotion. They might kill some monkeys or birds once in a while, but those carcasses are quickly absorbed by the forest. We humans seldom have occasion to notice; c) now the disruption of natural ecosystems seems more and more to be unloosing such microbes into a wider world. When the trees fall and the native animals are slaughtered, the native germs fly like dust from a demolished warehouse"(1). Zoonotic disease outbreaks in humans are triggered by the outcome of pathogens from animals and locations where humans and animals meet frequently are potential spillover hotspots. Alongside factors such as human population density, living conditions and environment characteristics, proximity to ecosystem boundaries is suspected to mediate rates and risks of infectious disease spillover events (25). As humans encroach further into previously uncultivated environment, new contacts between wild fauna and humans and their livestock increases the risk of cross-species infection. An example of such contact is the destruction of natural forest that encouraged fruit bats to relocate nearer human habitation, like the large colony in the botanic gardens in the heart of Sydney. Indeed, in 1997, Hendra virus (HeV), a related *Paramyxovirus* of Australian fruit bats, fatally infected a veterinarian examining a sick horse (8).

The ecological events that drive interactions between source and recipient species are seldom understood, probably as a result of the enabling conditions and drivers of cross-species transmission occur over many scales of time, space and ecological organization, from within-host pathogen evolution to spatially extensive processes such as land-use and climate change. As said before, the spillover of the emerging viruses requires a series of hierarchical enabling conditions: reservoir hosts must be present; reservoir hosts must be infected; if transmission is

indirect, reservoir hosts must be shedding pathogen and virus must survive outside of its reservoir host with access to the recipient host; recipient hosts must be exposed to the source of the virus in sufficient quantity for an infection to establish; and recipient hosts must be susceptible to the virus (14).

It is necessary to introduce a distinction between *spillover rate* (the number of spillover events for a single host-parasite system) and *spillover diversity* (the number of parasite species spilling over). The rate of spillover across ecosystem boundaries depends on the likelihood that source and recipient hosts, as well as the parasite, are present in or near a boundary region. This likelihood can be represented by a boundary's permeability, a concept used in landscape and movement ecology to describe an organism's ability or willingness to move through a certain habitat. Applied to spillover, this idea can be used to characterize how likely a parasite is to spillover across ecosystem boundaries. Spillover of a parasite across an ecosystem boundary requires boundary permeability for at least one of three actors involved in spillover: source host(s), recipient host(s) or parasite. The interactions between the levels of boundary permeability for each of these components will determine the spillover rate for a given system. Permeability for hosts will depend on host traits, and all factors that influence behavior and abundance near the boundary. As an example reported by authors Borremas et al., boundaries will have high permeability for species whose home ranges extend into both ecosystems. For many host and parasite species, permeability will relate to the contrast between adjacent ecosystems. Ecosystems that share many characteristics are more likely to facilitate cross-boundary movement, while boundaries dividing distinct ecosystems sharing few characteristics will more likely have low permeability for most species. An important question that is relevant for the risk of spillover to humans is whether anthropogenic boundaries are less permeable to host and parasite movement than natural boundaries. Host traits that increase the probability of occupying or crossing ecosystem boundaries may lead to such host species functioning as bridge hosts that link different host species occupying distinct ecosystems. Bridge host traits can include being a generalist consumer, having high tolerance to different habitats, or being an edge-habitat specialist. The presence of this kind of hosts can be particularly important for spillover between two other host species for which the boundary has low permeability. In turn, arthropod vectors themselves can often act as crucial bridge species. Hosts with broad

environmental tolerance and generalist resource use are more likely to be able to cross ecosystem boundaries than specialists. Examples of generalists host are small mammals.

The route of transmission of a parasite is likely to affect which host traits and ecosystem conditions will be important for boundary permeability. Directly transmitted parasites require individuals of two different host species to come into close contact, which means that the conditions determining host movement and presence in the boundary will drive permeability for the parasite. Parasites with a free- living stage or ectothermic host will be more sensitive to abiotic conditions, and spillover risk in the boundary will depend on conditions affecting parasite survival as well as those affecting host presence. Furthermore, passive transport in the environment can lead to spillover even between host species that have no overlap in habitat use. Generalist parasites are the ones that are able to infect a wider range of host species, thereby increasing the chances of infecting a host that is able to enter or cross the ecosystem boundary. Similarly, broad tolerance to environmental conditions will allow a parasite to survive in a wider range of ecosystems, which can increase the opportunities for encountering new host species in adjacent ecosystems or boundaries. The presence of parasites near ecosystem boundaries is not static, and should be expected to vary over time owing to source host dynamics impacting pathogen release: temporal variation in parasite pressure near ecosystem boundaries depends on host movement near or across boundaries, that can vary in short or long time intervals. Seasonal change, environmental conditions near boundaries, seasonal rainfall are all determinants in time exposure variability.

In summary spillover near ecosystem boundaries is expected to increase relative to ecosystem interiors when bridge hosts/vectors and edge specialists are present or abundant, when the proportion of generalist hosts and parasites is high, or when there are high levels of biodiversity, host density, and species interactions (25).

Another point of view that should be considered is the theory of “land-use spillover”. When land use changes, which we regard as anthropogenically-induced ecosystem change, it drives this infect-shed-spill-spread cascade, and we refer to this process as land use-induced spillover. Considering bats as major reservoir host: firstly, bat distribution, abundance, and density are determined by resource availability, mainly food, and the availability of mates and roosting sites. In fact, a key resources can be the destruction and fragmentation of bat habitat reduces. Thus, bats might be forced to change behavioral norms, for example shifting from feeding in

native forests to feeding in human-dominated landscapes and roosting in urban parks or anthropogenic structures. Accordingly, the likelihood and intensity of bat infection changes with the host population distribution, as bats that are nutritionally or physiologically stressed are more likely to become infected. Secondly, bats are more likely to shed pathogens into the environment during periods of stress as we have already analyzed above talking about the reservoir host. Thirdly, wildlife-human contact is a key determinant of spillover: if that same bat sheds virus while foraging on fruit trees in a village or being slaughtered for human consumption, human exposure is more likely.

Multiple factors affect the likelihood of onward transmission, including pathogen biology, human population size, and human population connectivity. Over the past three decades, viruses such as Ebola virus, influenza A (pandemic H1N1, H7N9) virus, CoV, HeV, and NiV have aptly showed the interdependence of human, animal, and ecosystem health and that local land use decisions can have large scale socioeconomic consequences (26).

Social and economic conditions, behavioral changes and geopolitical instability have also influenced the cross-species transmission. Biologically weakened and vulnerable populations, especially if also living in circumstances of privation, unhygienic conditions and close contact, are susceptible to microbial colonization. The severity of the bubonic plague in mid-fourteenth-century Europe seems to have reflected the nutritional and impoverishment consequences of several preceding decades of unusually cold and wet weather with crop failures compounding the incipient destabilization of the hierarchical feudal system. Many of the rapid and marked changes in human social ecology in recent decades have altered the probabilities of infectious disease emergence and transmission. Among these changes we can include increases in population size and density, urbanization, persistent poverty, the increased number and movement of political, economic and environmental refugees, conflict and warfare. Political ignorance, denial and obduracy often compound the risk of infectious disease transmission, as has been tragically observed with HIV/AIDS in parts of Africa, where widespread poverty, a culture of female disempowerment and political instability further exacerbate the problem.

The urban environment has only recently become the dominant human habitat. Urbanism typically leads to a breakdown in traditional family and social structures, and entails greater personal mobility and extended and changeable social networks. These features, along with access to modern contraception, have facilitated a diversity of sexual contact and, hence, the

spread of sexually transmitted diseases. More generally, cities often function as highways for "microbial traffic". Rapid urbanization boosts certain well established diseases, such as childhood pneumonia, diarrhea, tuberculosis and dengue, and facilitates dissemination of various emerging diseases .

Furthermore, there is also the possibility that technological advances in medicine and public health that may inadvertently promote the emergence and spread of infectious disease, having developed problems such us multidrug-resistant pathogens, medical vectors such as reused syringes and needles, biological medicines produced from animal-cell substrates that present an inherent potential hazard for introducing new infections and at least xenotransplations, practice that seems to increase the risk of transmission of emerging pathogens such us the porcine retroviruses (8).

1.3.4 The pathogen

Wolfe et al. proposed a useful classification scheme for pathogens, delineating five stages spanning the range from those exclusively infecting animals (stage I) to those exclusively infecting humans (stage V). There is no inevitable progression of microbes from Stage I to Stage V.

- Stage I: Microbe that is present in animals but that has not been detected in humans under natural conditions.
- Stage II: An animal pathogen that, under natural conditions, has been transmitted from animals to humans ('primary infection') but has not been transmitted between humans ('secondary infection'). Examples: the West Nile virus (WNV) or *Brucella abortus*, that can transmit from animals to humans to cause 'primary' infections but do not exhibit human-to-human ('secondary') transmission.
- Stage III: Animal pathogens that can undergo only a few cycles of secondary transmission between humans, so that occasional human outbreaks triggered by a primary infection soon die out. Examples: the monkeypox virus and *Leishmania infantum*, spill over into human populations from animal reservoirs and can cause limited cycles of human-to-human transmission that stutter to extinction.
- Stage IV: A disease that exists in animals, and that has a natural (sylvatic) cycle of infecting humans by primary transmission from the animal host, but that also undergoes

long sequences of secondary transmission between humans without the involvement of animal hosts. Such pathogens persist in animal reservoirs but can cause self-sustaining chains of transmission in human populations; examples include *Yersinia pestis* (plague) and pandemic influenza. This stage can be further categorized into:

  - Stage IV a: where the sylvatic cycle is much more important than direct human-to-human spread
  - Stage IV b: where both sylvatic and direct transmission are important
  - Stage IV c: where the greatest spread is between humans
- Stage V: the pathogen becomes exclusive to humans (27).

The most credited theory is that each epidemic starts from a single ancestral virus that made the transition to successful replication and spread in the new host by overcoming a number of barriers, in the form of host-specific proteins or processes that differed between the reservoir and the new outbreak host. Viruses may encounter one or more barriers in alternative hosts and will require a number of adaptations in order to achieve sustained transmission in those hosts. Between these barriers, as highlighted before, there is the block created by the mucosal surfaces, the bind with host-specific receptors and the activation of the host immune system. It is clear that efficient transmissibility between individuals of the new host is a critical hurdle that must be cleared for an emerging virus to create an epidemic (9).

In order to increase the success rate of jump and establish an infection in the new host, the pathogen is subject to many different mutations and acquires different strategies to infect.
"Generalist" viruses, which infect many different hosts, might be expected to show an increased likelihood of shifting to additional hosts, as they can already use the host cell mechanisms of many hosts to infect and replicate. In contrast, specialist viruses, which naturally infect only one or a few closely related hosts, appear likely to be more strongly restricted by the different receptors and replication mechanisms in newly encountered hosts. However, both generalist and specialist viruses are known to have become established successfully in new hosts, suggesting that there is no generalization that can be made about the likelihood of either type of virus infecting a previously resistant host to create a new epidemic pathogen (23).
Evolutionary changes are not always necessary for viruses to emerge in new hosts. For example, the canine distemper virus has a very wide host range in mammals, naturally infecting marine mammals, lions, black-footed ferrets, and other hosts, and its emergence in these species appears

to be limited primarily to contact. However, in other cases, emergence requires the evolution of the virus to allow efficient infection and transmission within the new host. The level of genetic variation is important, and most viruses transferred to new hosts are poorly adapted, replicate poorly, and are inefficiently transmitted, so that the greater the rate of variation the more likely a virus is to adapt to the new host. This indicates that cross-species transmission should be more common in rapidly evolving viruses (23). RNA viruses are the most abundant molecular pathogens infecting humans, animals and plants. A comparative analysis of the structures, genetic organization, and replication pathways of RNA viruses indicates that RNA viruses use disparate strategies to ensure their multiplication in cells and their stability as free particles. According to Domingo et al., the emergence of new viral pathogens is favored by the genetic plasticity of RNA viruses and by alterations in the environment that have an effect on viral traffic. As a result, viruses may come in contact with potential new hosts, thus facilitating host jumping. However, current evidence suggests that the origins of such an ability include: the frequent generation of mutations by RNA viruses, the continuous competition among variant genomes, and the selection of those variants which are best adapted to each particular environment. This is made possible by the limited complexity of genomes (in terms of the number of encoded proteins), their high mutability as a consequence of a lack of a proofreading mechanism, and rapid replication rate (28). In contrast, most DNA viruses are less variable and more often associated with virus- host co-speciation.

For many viruses, recombination allow the acquisition of multiple genetic changes in a single step and can combine genetic information to produce advantageous genotypes or remove deleterious mutations. For instance, retroviruses such as HIV have high rates of recombination, while the SARS-CoV appears to have arisen from a recombinant between a bat CoV and another virus (most likely also a bat virus) before infecting humans and carnivore hosts. Recombination and reassortment may also be important for incremental host adaptation after the switch to the new host has occurred.

A fundamental challenge for host-switching viruses that require adaptation to their new hosts is the viral fitness: mutations that optimize the ability of a virus to infect a new host will likely reduce its fitness in the donor host. Interactions between the virus and hosts determine the fitness land-scape for the virus and, after a host-switching event, combinations of genetic drift and

selection will determine the viral genetic variation that remains in the long term. However, only a small proportion of the viral mutational spectrum will exhibit increased fitness.

An important constraint influencing the emergence and successful host transfer is the mode of virus transmission. For instance, arthropod vectors that feed on a range of mammalian hosts can facilitate cross-species viral exposures. Adaptation to interhost transmission by droplet spread, by sexual inoculation, and by fecal-oral transmission each represent different adaptational challenges due to host differences and variation in environmental exposure. In fact, it is not clear why IAVs are enteric viruses in their natural avian hosts but mainly infect the respiratory tract in mammals, but this likely influences the host adaptation of the virus and its ability to spread efficiently. In addition to optimizing the replicative efficiency in cells and tissues, a new virus may have to intensify the viral shedding from appropriate sites for transmission (*e.g.,* mucosa, respiratory tract, skin, feces, urine, blood, and other tissues): for instance, they may have to induce sneezing to achieve respiratory shedding, or, for arthropod-transmitted viruses, they may have to establish high levels of viremia or replication in vectors.

The process of virus transfer to a new host is rarely observed directly but can be inferred by comparing viral ancestors in donor hosts with emergent viruses from recipient hosts. Thus, if several changes are required to allow host switching, then intermediate viruses would likely be less fit in either the donor or recipient hosts than the parental or descendant viruses. Crossing any evolutionary "low-fitness valley" for partially adapted viruses may be a key step for virus host switching and may explain the rarity of such transfers: partially adapted viruses would quickly go extinct, as they would be unfit in the donor host and also insufficiently adapted to allow efficient replication and spread in the recipient host. Early detection of inefficiently spreading viruses in a new host would provide opportunities for epidemic control (23).

Another aspect to be considered is the survival of a virus outside the reservoir as a determinants in the success of spillover. The stability of free viruses in the environment determines the time frame during which indirect cross-species transmission can occur. Considering bats as the reservoir host, we need to take into account the fact that they are volant, spending most of their time in trees in which they roost or feed, in caves or in transit. Bats spend little time on the ground. Therefore, virus transmission from bats to non-volant species is most likely to occur indirectly via free virus particles shed from bats onto fomites or surfaces, or through virus-laden

aerosolized urine or feces. HNV, *Filoviruses* and CoV are enveloped RNA viruses that are sensitive to increases in temperature, changes in pH, ultraviolet light and desiccation. Environmental conditions in nature may be less optimal for viral survival; temperature, humidity and microclimate under trees and in caves may influence viral decay rates and ultimately the likelihood of spillover (14).

1.4 Spillover in emerging infectious diseases (EIDs) and its pandemic potential

"An emerging disease is 'an infectious disease whose incidence is increasing following its first introduction into a new host population'. The key words, of course, are 'infectious', 'increasing', and 'new host'. A re-emerging disease is one whose incidence is increasing in an existing host population as a result of long-term changes in its underlying epidemiology. Emergence and spillover are distinct concepts but interconnected. Spillover leads to emergence when an alien bug, having infected some members of a new host species, thrives in that species and spreads among it" (1).

Emerging infectious diseases (EIDs) are a significant treats on global economies and public health. Their emergence is thought to be driven largely by socio-economic, environmental and ecological factors (29).

The key event in a successful species jump is for the pathogen to be sufficiently transmissible between individuals within the new host population, and this is the occurrence that led into EIDs (2). This phenomenon can be described by the basic reproduction number (R_0), also called the basic reproduction ratio or rate or the basic reproductive rate. This is an epidemiologic metric used to describe the contagiousness or transmissibility of infectious agents. R_0 is also the number of secondary cases one case would produce in a completely susceptible population. Fine supplements this definition with the description of "average number of secondary cases"(30). In the article of Woolhouse et al., this concept is well analyzed. The relative fitness defined by R_0 is a composite of three terms: c - the contact rate or number of contacts per unit time, p - the transmission probability per contact, and d - the duration of infectiousness.

The expected size of an outbreak depends on the number of introductions, so-called 'primary' cases of infection and the potential for transmission of the pathogen from one new host to another. Pathogens that enter a new host population via a species jump can be placed in two categories depending on the value of R_0: if R_0 is <1 in the new host population, even if the new

host repeatedly acquires the pathogen, each primary case will, on average, fail to replace itself (although short chains of transmission are still possible) and each single introduction will lead to no more than a minor outbreak. This category of emerging pathogen is unlikely to constitute the greatest disease threat; examples in humans include the Ebola, monkeypox and avian influenza viruses (IVs) and the Variant Creutzfeldt-Jakob disease (vCJD) agent. Conversely, if R_0 is >1 in the new host population, each primary infection will, on average, generate more than one secondary infection and the pathogen is capable of invading the host population; following this, there is a finite chance that a major epidemic will occur. This category is likely to constitute the greatest disease threat; examples in humans include HIV, influenza type A virus and SARS-CoV.

The difference between the two categories lies in the origin of infections within the new host population: if R_0 is <1, then a large proportion of infections will be acquired directly from the original source host population; if R_0 is >1 (and the outbreak takes off), then most infections will be acquired from within the new host population, the resulting positive feedback potentially fueling a major epidemic. There is a transition between these behaviors in the region R_0=1, where the size of an epidemic is highly sensitive to small changes in the transmission potential. This is especially relevant to pathogen emergence because it implies that relatively small changes in R_0 can have large impacts on the incidence of infection. As has been widely discussed above, there are many reasons why R_0 might change.

If R_0 is >1, then there is a sense in which there is 'an epidemic waiting to happen'; numerous recent examples include the introductions of the WNV into North American birds or the phocine distemper virus into North Sea seals. Indeed, emerging pathogens are often of special concern because the absence of shared evolutionary history with the new host implies an absence of evolved constraints on susceptibility and pathogenicity, which might, at least in some instances, enable disease outbreaks of large magnitude and unusual severity. Conversely, if R_0 is <1, this implies that each primary case will result in a chain of transmission in the new host population that will stutter to extinction. This, however, might be overly optimistic because of the possibility that the pathogen evolves so that R_0 becomes >1 and, as a result, could go on to generate a major epidemic. This evolution, or 'adaptation', of the pathogen can involve a series of mutations that we have discussed above. Adaptation might be so rapid that pathogen lineages adapt to different host tissues or vector cells versus host cells. The probability of successful

adaptation occurring depends on several factors such as: (i) the number of primary infections, I_0; (ii) the initial R_0 of the infection in the new host population; (iii) the number of mutations or other genetic changes required; and (iv) the likelihood of these changes occurring and how R_0 changes at each step. It is relatively simple to see that the probability of emergence increases linearly with I_0, but is much more sensitive to the evolution of R_0, particularly when this is close to 1. This is because the probability of each (rare) evolutionary step is proportional to the expected size of the initial outbreak and, hence, the number of opportunities for the required genetic changes to occur. Even though it is sometimes possible to state the genetic differences between pathogens in the original and new host, it is often difficult to ascribe these change because of: (i) events in the original host population before the jump (*i.e.*, predisposition of novel genotypes to jump species); (ii) events during the 'adaptation' phase where there was a shift from $R_0<1$ to $R_0>1$ in the new host; or (iii) subsequent divergence once the pathogen is established in the new host (4).

As previously described, it has been hypothesized that socioeconomic factors (such as human population density, antibiotic drug use and agricultural practices) are major determinants of the spatial distribution of EID events, in addition to the ecological or environmental conditions that may affect overall (emerging and non-emerging) human pathogen distribution. Furthermore, EID events caused by zoonotic pathogens from wildlife are significantly correlated with wildlife biodiversity, and those caused by drug-resistant pathogens are more correlated with socio-economic conditions than those caused by zoonotic pathogens (29).

1.5 Prevention and control programmes: predicting and preventing the next pandemic

"Some knowledgeable and gloomy prognosticators even speak of the *Next Big One* as an inevitability"(1).

Novel infectious diseases can emerge in any part of the world at any time, and it is difficult to predict when a new disease will come next or where it will appear. New viruses will continue to emerge unexpectedly, but there is a lot we can and must do to be better prepared. If researchers can identify the next pandemic pathogen before the first case appears, communities could drastically improve strategies for control, and even stop it from taking hold (31). An effective public health and/or veterinary response then requires prompt, coordinated action by multi-

disciplinary teams, as exemplified by the WHO (12). ‘One Health’ approaches should be used, addressing zoonosis at the human, animal and environmental levels (22).

Prevention strategies can be applied at many different levels.

An ambitious biodiversity-based approach to outbreak prediction was announced in February 2018, the Global Virome Project (GPV), which is a ten year collaborative initiative to discover zoonotic viral threats and stop further pandemics. GVP is a strategic response to the growing need to better predict, prevent, and respond to future viral pandemic threats and to protect us all from their worst consequences with a multidisciplinary unit. The mission of the project is to develop an innovative partnership network among public, private, philanthropic and civil organization to discover the majority of our planet’s unknown virus to improve human health and food security. The project estimates that other mammals and birds contain 1.67 million unknown viruses from the families of viruses that are most likely to jump to humans, and will use the funding to conduct a genomic survey of these unknown viruses, with the aim of predicting which of these might eventually infect people. This will certainly advance our understanding of virus diversity and evolution (32). According to the report of Holmes et al*.,* this approach has a little practical value, and it is necessary to focus funds and efforts on a simpler and cost-effective way to mitigate outbreaks, namely the proactive, real-time surveillance of human populations. The public has increasingly questioned the scientific credibility of researchers working on outbreaks. In the 2013-16 Ebola epidemic, for example, the international response was repeatedly criticized for being too slow. During the 2009 H1N1 influenza epidemic, people asked whether the severity of the virus had been overblown, and if the stockpiling of pharmaceuticals was even necessary. Supporters of outbreak prediction maintain that if biologists genetically characterize all of the viruses circulating in animal populations (especially in groups such as bats and rodents that have previously acted as reservoirs for emerging viruses), they can determine which ones are likely to emerge next and, ultimately, prevent them from doing so. Determining which of more than 1.6 million animal viruses are capable of replicating in humans and transmitting between them would require many decades’ worth of laboratory work in cell cultures and animals. Even if researchers managed to link each virus genome sequence to substantial experimental data, all sorts of other factors determine whether a virus jumps species and emerges in a human population, such as the

distribution and density of animal hosts, as well as accounting for the fact that viruses are not fixed entities.

The author's conclusions are that currently the most effective and realistic way to fight outbreaks is to monitor human populations in the countries and locations that are most vulnerable to infectious disease. In practice, at least four kind of analysis are required: genomic, virological, epidemiological and clinical, and in the end link this data within days of an outbreak being detected, including information about how people in an affected community are interacting (31).

1.5.1 Mathematical and data modelling

To predict transmission between species many different strategies has been proposed, and among which the use of mathematical models for the analysis of cross-species events. The practical use of epidemic models depends heavily on the degree of realism of these models. Mathematical models can be defined as a method of emulating real life situations with mathematical equations to predict their future behavior. In epidemiology, mathematical models play a role as a tool in analyzing the spread and control of infectious diseases (33). This doesn't mean that a reasonable model can include all possible effects but that can rather incorporate the mechanisms in the simplest possible fashion so as to maintain major components that influence disease propagation. Great care should be taken before epidemic models are used for prediction of real phenomena. However, even simple models should pose important questions about the underlying mechanisms of infection spread and possible means of control of the disease or epidemic (34).

We can say that an epidemiological model uses a microscopic description (the role of an infectious individual) to predict the macroscopic behavior of disease spread through a population. Mathematical models have both limitations and capabilities that must be recognized. Sometimes, questions cannot be answered by using epidemiological models, but often the modeler is able to find the right combination of available data, an interesting question and a mathematical model which can lead to the answer. Models can often be used to compare different diseases in the same population, the same disease in different populations, or the same disease at different times. Epidemiological models are useful in comparing the effects of

prevention or control procedures, for example to compare gonorrhea control procedures such as screening, rescreening, tracing infectors, post-treatment vaccination and general vaccination. Communicable disease models are often the only practical approach to answering questions regarding which prevention or control procedure is most effective. Quantitative prediction of epidemiological models are always subject to some uncertainty since the models are idealized and the parameter values can only be estimated. An underrecognized value of epidemiological modeling is that it leads to a clear statement of the assumptions about the biological and sociological mechanisms which influence disease spread. The parameters used in an epidemiological model must have a clear interpretation such as a contact rate or a duration of infection. Models can be used to assess many quantitative conjectures. Epidemiological models can sometimes be used to predict the spread or incidence of a disease. An epidemiological model can also be used to determine the sensitivity of predictions to changes in parameter values. Once the parameters are identified which have the greatest influence on the predictions are identified, it may be possible to design studies to obtain better estimates of these parameters (35).

Firstly, we have to differentiate between two types of epidemic model: the stochastic and the deterministic one. “Stochastic” means being or having a random variable. This kind of model is a tool for estimating probability distributions of potential outcomes by allowing for random variation in one or more inputs over time. It depend on the chance variation in risk of exposure, disease and other illness dynamics. The “Deterministic model” is otherwise use in case of large population. In this case individuals in the population are assigned to different subgroups or compartments, each representing a specific stage of the epidemic (36).

Other category of models include the most famous among them, the ‘SIR model’ (Susceptible-Infectious-Recovered): this model starts with the assumption that all members of the community are initially equally susceptible to the disease, and that a complete immunity is conferred after the infection. The population is divided into three distinct classes: the susceptible, ‘S’, healthy individuals who can catch the disease; the infected, ‘I’, those who have the disease and can transmit it; and the removed, ‘R’, individuals who have had the disease and are now immune to the infection (or removed from further propagation of the disease by some other means) (34). The standard SIR model is often not a realistic representation of the human behavior driving an epidemic, however. Even in very large populations, individuals do not mix randomly with one another. This issue becomes especially important when considering the spread of infectious

diseases across a geographic area, because geographic separation inherently results in nonrandom interactions, with more frequent contact between individuals who are located near each other than between those who are further apart. It is important to realize, however, that there are many other dimensions besides geographic space that lead to nonrandom interactions among individuals (37). The standard SIR epidemic model can be reconsidered into the 'SIR model without vital dynamics' and the 'SIR model with vital dynamics'. The first one accounts for infection that confers permanent immunity. When such a SIR disease goes through a population in a relatively short time (less than one year), then this disease outbreak is called an epidemic. Since an epidemic occurs relatively quickly, the model does not include births and deaths (vital dynamics). Epidemics are common for diseases such as influenza, measles, rubella and chickenpox. With this model if an epidemic occurs in a homogeneous population and there is no vaccination during the epidemic, then it is possible to estimate the contact number for the disease in that population from epidemic data. The second one, the 'SIR model with vital dynamics', consider the behavior population over a long time period, includes births as a source of new susceptible population and natural deaths in each class. In addition, another variation of the standard SIR model is the 'SIS model' (Susceptible-Infectious-Susceptible) that is used for diseases or which infection that does not confer immunity. It is called an 'SIS model' since individuals return to the susceptible class when they recover from the infection. This model is appropriate for some bacterial agent diseases such as gonorrhea, meningitis and streptococcal sore throat (35).

Models can also evaluate the potential influence of unknown information, helping to set priorities for data collection and define the uncertainty associated with model outcomes (6).

1.5.2 Surveillance and prevention strategy

Environmental changes due to human activity, increased international mobility, poor public health systems, and microbial adaptations are some of the main drivers of spillover events and pandemics. To efficiently combat EIDs, scientific and governmental communities use different approaches focused on the prediction, rapid detection, and surveillance of pathogens with the potential to cause outbreaks. However, the high pathogen diversity in nature makes the prediction of which pathogens have a real potential of causing diseases in humans a significant challenge. The discovery of new potential human pathogens is a useful strategy for EID

prevention. However, considering the high cost and uncertainty about its effectiveness, this strategy has been highly criticized. Considering that testing animal populations for previously unrecognized pathogens is expensive, testing for pathogens that have already crossed the barrier between animals and humans could be a more cost-efficient use of limited resources. Coordinated strategic planning is certainly critical for the rapid responses required to confront new viruses early after emergence. 'One Health' surveillance approaches are needed, integrating animal and human health in monitoring for emerging infectious diseases and consider environmental change that is likely to intensify close proximity animal–human interactions in the near future. Against this backdrop, the following question emerges: What are the target for investment in emerging infectious disease prevention? (38)

Target 1: Animals

The investigation of already known or even unknown pathogens hosted in animals would help the identification and the tracking of pathogens that, at some point, may cause human maladies. However, this strategy can be very costly and has little immediate practical applicability, as we have seen with the 'Global Virome Project'. In this regard, focusing investigations on key animals (*e.g.,* companion animals, livestock, and select wild animals, such as bats) may be more advantageous since they are in close contact with humans and can act as spillover intermediates (38). Among these animals, the role of bats is well recognized, as one of the primarily reservoir species involved. In this regard, efforts should be taken to reduce all the stressors that facilitate bat virus shedding, such as habitat destruction, which results in increased contact between bats and humans and is considered a cause of viral spillover (22). For example, at the level of virus shedding, conservation and restoration of critical bat feeding habitats should reduce the risk of nutritional stress and reduce urban colonization by bats, while at the level of virus survival, delaying recipient hosts' interaction with bat excreta to allow viral decay should reduce exposure (14).

Target 2: Human sentinels for spillover events

Zoonotic pathogens found in human biological samples represent a small number of pathogen that have successfully moved from animals to humans. Individuals in close and frequent contact with wild animals and livestock (*e.g.*, hunters, farmers, and veterinarians) can act as human sentinels of recent spillover events. Once a new human pathogen is detected, response measures

such as the elucidation of its medical importance and surveillance intensification can be taken. However, if the host jump is not rapidly identified or if adequate control measures are not taken, the pathogen will have the opportunity to spread among the human population (38).

Target 3: The general human population

Screenings performed in blood donor samples or samples from other specific groups may be useful to detect the circulation of emerging pathogens at a population level. This action requires an adequate laboratory and technical infrastructure. In this context, it is important to emphasize that low-income countries will require substantial efforts concerning investments to build laboratories and train staff to address the diagnosis of infectious diseases. Microbial screening in the general population can be very useful for EID prevention but can sometimes trigger false alarms (38).

Target 4: Environment and wildlife habitat

Curbing disease emergence will prove challenging until we have a more deep knowledge of the epidemiologic circumstances that facilitate pathogen spillover, particularly from wild animals, which are the source of the majority of recently EIDs and continue to constitute a substantial gap in disease detection efforts worldwide.

We mentioned that species that have increased in abundance and even expanded their range despite large-scale anthropogenically driven landscape change and urbanization are more likely to be generalist species that have adapted to human-dominated landscapes. Approximately one quarter of mammalian species have stable or increasing trends in abundance, half of which are rodents. Large-scale surveillance efforts are necessary to accurately identify epidemiologically relevant animal reservoirs for zoonotic viruses, as well as the periods of heightened shedding that might be related to specific host traits and environmental factors measured at the species level. Informed mitigation efforts aimed at ensuring biosafety in livestock production, minimizing interactions between wildlife and domesticated animals and limiting close contact with wildlife are especially needed given global trends in urbanization and food production (16).

Broader prevention is possible by addressing the upstream stressors resulting from ecological disruption that set the wildlife disease process in motion. Fragmented landscapes and fragmented solutions increase this vulnerability as shown by the COVID-19 pandemic. Fostering landscape immunity should be regarded as a biosecurity imperative and actions need

to be taken to maintain and enhance landscape immunity as part of the national and global security plans. Investments in landscape conservation provide returns for human health, climate change, international trade, sustainable development, environmental justice, and other policy issues associated with human wellbeing. Landscape immunity (that arises from the ecological conditions that, in combination, maintain and strengthen the immune function of wild species within a particular ecosystem while preventing the conditions that lead to high pathogen prevalence and shedding) corresponds to ecological integrity. Any land use practice that reduces ecological integrity and resilience erodes the barriers to zoonotic spillover. Minimizing anthropogenic habitat fragmentation and penetration, and the perimeter of habitat edges, should be one of the first principles in landscape management to reduce wildlife zoonoses risk. Because interaction and connectivity among species and the environment define the essence of all life, promoting ecological connectivity should be a conservation priority at the local and global scale (26).

Target 5: Vaccination and progress in therapies

Vaccines represent some of the most effective interventions available against infectious diseases.

Vaccine strategies could be used in some control programs, but the current rate of development and approval of human vaccines is too low to allow the control of most newly emerging virus diseases. Existing vaccines can be used to control the emergence of known viruses when sufficient lead-time is available, as might veterinary vaccines which can be developed relatively quickly and used to combat outbreaks, along with the culling or quarantine measures that are now often used. New and improved vaccine technologies include molecularly cloned attenuated viruses that can be rapidly changed into the appropriate antigenic forms with sufficient efficacy and a level of risk low enough for use in the face of some outbreaks (23).

With the absence of immunization, it is projected that the world would experience as many as 5 million deaths a year from smallpox, 2.7 million deaths from measles, 2.0 million deaths from neonatal tetanus, 1.0 million deaths from pertussis and 600000 deaths or paralytic cases of polio. Since smallpox has been eradicated, all of those deaths are being prevented; at least 60% of expected deaths due to the other diseases are also being prevented. The eradication of various infectious disease was possible thanks to various vaccination campaigns (39).

Next-generation vaccine technologies are platforms that are rapidly adaptable for different types of viral pathogens. Importantly, several of these technologies use genetically modified viruses, such as the vesicular stomatitis virus (VSV) and ChadOx1 platforms, that can induce protective immunity in humans, mice, guinea pigs (*Cavia porcellus*), non-human primates and livestock to a number of pathogens, including bat-borne Ebola virus and NiV. Vaccine efficacy in animals, for example horse vaccination for HeV, including live-stock and other peri-domestic animals, may even enable proactive measures to reduce cross-species transmission of bat viruses to humans. Novel platforms such as DNA-based and mRNA vaccines offer the potential for an incredibly rapid response time from pathogen discovery to therapeutic intervention. Using these technologies, researchers were able to test the first Zika vaccine in mice and non-human primates within 3.5 months of the initial outbreak in 2015 and, more recently, a similar RNA-based vaccine was designed for SARS-CoV-2 and entered human clinical trials only 2 months after the virus sequence was published.

One of the biggest hurdles to preventing zoonosis at the animal level is the limited feasibility of wildlife vaccination. Given that *Filoviruses* and CoVs are likely hosted in a variety of different animal populations, including multiple bat species and other mammals, covering large geographic regions, current vaccination delivery methods are impracticable and likely insufficient to induce effective herd immunity. Some progress has been made on this front, for example, in the form of oral vaccines against rabies in dogs and bats (22).

The ultimate weapon that we have against emerging infectious disease are drugs. Specific therapies notably include antibiotics, antiviral and anti-parasitic drugs. These have primarily had impact in reducing the mortality from infectious diseases but also have affected the incidence of disease by shortening the period that an infected individual remains infectious to others (39). Antiviral drugs may be used where available, although cost, logistic problems, and side effects may make those more difficult to use in a large-scale outbreak, and they would likely work only in the context of other control measures (23). Finally, there is the problem of antimicrobial resistance (AMR), that is becoming day by day more important. Major causes of antibiotic resistance are the indiscriminate, inappropriate, and incomplete use of antibiotics. Whatever the cause, the result is the selection of strains of micro-organisms which are resistant to available anti-microbial agents. If this resistance is transferred from one type of organism to another (as may occur with plasmid-mediated resistance) the result can be the rapid development

of multi-drug resistant organisms which might make the management of once-simple infections a real challenge. This has occurred with tuberculosis, pneumococcal disease, and enterococcal disease, among others (39).

1.5.3 Global health authorities and veterinary services in managing disease outbreaks

Veterinary professionals constitute an invaluable workforce capable of delivering public services essential to weathering the current pandemic and preventing future pandemics (40). Veterinary services are well-organized all around the world. Animals, and the Veterinary Services which ensure their protection, are a global public good playing a vital role in the security and the economic and social wellbeing of humanity.

For global and national health security, prevention is better than cure, and there has been a steady and growing realization targeting "risk at source" in animal populations is a vital strategy in safeguarding the planet from risks posed by emerging zoonoses, neglected zoonoses and AMR (41). With regard to this, in 2018 the WHO (12) reviewed its "Blueprint" list of diseases to prioritize in public health emergency contexts due to their epidemic potential. It is notable that all 7 identified pathogens are zoonoses. The increased interest in preventing zoonosis disease led authorities like the WHO (12), the OIE (Office International des Epizooties or World Animal Health Organization) (42) and the FAO (Food and Agriculture Organization of the United Nation) develop the "Tripartite Guide to Zoonosis (TGZ)", a multidisciplinary and multisectoral guide addressed to all the health care institutions around the world. The aim of this guide is to establish a 'One Health' approach to building an international mechanism for coordination, communication and collaboration in the fight against zoonoses (43).

The OIE is the reference authority for all the veterinarians. The OIE is a unique intergovernmental organization to work with in order to achieve a healthier and safer planet. It has a strong technical and governance reputation, harnesses the best international experts, and provides value for money and efficiency, with a relative lack of bureaucracy. As seen with the TGZ, the OIE also has established a powerful 'One Health' partnership with the WHO integrating the OIE strategies with the WHO International Health Regulations (IHR) Monitoring and Evaluation Framework in addressing global health security (41). The primary function of the OIE is to inform governmental Veterinary Services of the occurrence and evolution of animal epidemics which could endanger animal and human health.

The OIE has been developing for a long time now an integrated management system for emergency animal outbreaks: the organization drew up a list (List A) (44) of the most important animal disease that need to be monitored. This notification system is useful also for new emerging diseases. The OIE provides for transparency, continuous update and open communication with all the national health departments (45). In Italy the management of animal disease has been regulated since 1954 with the "Regolamento di polizia veterinaria" (46), a national regulation for notification systems and outbreak management similar to those provided for by the OIE. For the OIE, preparedness for epizootics or epidemics is fundamental. To this end, they organize simulation exercises, which are controlled activities where a hypothetical situation, that could exist in reality, is imitated for training or assessment of capabilities and testing of plans purposes (47).

As we have seen Veterinary services are well prepared to handle with epidemics, but can we say the same for human public health system? Undoubtedly, pandemics and their management raise major concerns and challenges among governments and authorities all over the world. Particularly in the case of the current COVID-19 pandemic, questions are being raised by the public concerning the efficiency and efficacy of the responses so far. The interest in emerging outbreaks and how to best manage them has been reflected in many different publications which aimed to provide unified guidelines at a global level. Examples include the "Managing epidemics" handbook (48) proposed by WHO and the recent report of the European Commission "Improving pandemic preparedness and management"(49). These manuals have the purpose to create a global unified "modus operandi" and across the different sectors involved into outbreaks emergency response.

What the COVID-19 pandemic clearly highlights is the fact that the potential role of the veterinarian sector is not sufficiently taken into account. The pandemic is an opportunity and obligation for change. Veterinarians are uniquely poised to help safeguard global food security and stability through contributing their expertise in food animal production, food safety, epidemiology and biosecurity. This expertise will prevent future pandemics and maintain public health infrastructure including the safety of people engaged in animal origin food production. Veterinarians need a seat at the table of discussions on strategies and models to improve national and global food security plans and emergency operation efforts. The 'One Health' concept highlights connections between human, animal, and environmental health and promotes

collaboration between diverse sectors. Veterinarians often drive ‘One Health’ initiatives and attempt to gain a seat at the table of collaborative efforts. Despite their best efforts and attempts at reinforcing the importance of collaboration between sectors, veterinarians’ voices often remain unheard. The American Veterinary Medical Association (AVMA) states that veterinarians play an integral role in ‘One Health’ because animals both impact and are impacted by people and the environment. However, global health leaders like the WHO have been slow to integrate animal and environmental health into strategies to combat human diseases. The COVID-19 pandemic is an opportunity for veterinarians to demonstrate their utility in ‘One Health’ efforts. Veterinarians have an opportunity to assert themselves as key players in transforming the ‘One Health’ theory into collaborative action in preventing future pandemics (40).

2. Horses as a crucial part of public health

2.1 Horses in the One Health approach

'One Health' is a holistic approach which defines the health of humans, animals and the environment as a coherent system. 'One Health', as defined by the WHO, includes the design and deployment of policies, legislation and research at multidisciplinary level to assure better public health. Horses are among the most important animals in human history. They have been used in wars, as a means of transport, and even facilitated work in mines. In the late 19th century, horses played a crucial part in developing the first antidote to cure diphtheria. Since then, the rate of contact between domesticated horses and humans steadily increased. Furthermore, the detection of infectious diseases that affect both humans and horses are crucial, especially in cases of highly transmissible diseases. Beside infectious diseases, non-communicable diseases (NCDs) such as skeletal and joint diseases or metabolic disorders are of concern to both. Several risk factors concerning the health of humans and horses exist (50).

The horse-environment relationship

Horses have a a very important impact on soil and vegetation, the biodiversity of plants, and several animal species, such as reptiles and small mammals, ants, herbivores and grassland birds. Nevertheless, among the genus *Equus* itself, great diversity can be observed. In fact, from a 'One Health' perspective, horses' influence on the environment depends strongly on several factors such as plant-animal coevolutionary history, soil development, climate, frequency of grazing, and animal density (50).

The domestic horse-human relationship

In contrast to wild horses, domesticated horses live in close contact with humans. Some analysis discovered that the domestication of horses started about 6000 years ago in the Ukraine, southwest Russia and west Kazakhstan. The utilization of horses depends on the cultural background. In some countries, horses are needed for work, and thus have an impact on the economic status of the owner. In high-income countries, horses are primarily used for sport, breeding, animal assisted therapy, or as companions for leisure. Besides that, horse meat is a

common food source, especially in France, Mexico and Argentina. Nevertheless, horses have always been deployed as a sign of power (50).

Socioeconomic impact of working horses

The working horse can have a drastic impact on the socioeconomic status of the owner and consequently on mental health particularly in case of sickness or death. In low-income communities, the owner's livelihood is limited by factors such as poverty, low status and restricted access to resources. Therefore, working horses enhance capital and secure sustainable livelihoods. Besides the economic factors, owning working horses can benefit status, leading to stronger social relations. Illness and injuries of working horses are often the result of a lack of knowledge of wound and disease management. Other reasons that influence the performance of working horses are overloading the horse, insufficient access to water and food as well as veterinary care and inadequate recovery phases (50).

Horses in medical field

In 1890 horses played a crucial part in developing the first antidote to cure diphtheria (*Corynebacterium diphteriae*) in humans. Horse serum is also used as an anti-venom, for instance, when humans are bitten by snakes. As an animal model, horses were used in the research of *Hepatitis C*, since the virus displays great similarities to the *Equine hepacivirus*. Furthermore, horses were employed as a model for human respiratory diseases (human allergic neutrophilic asthma), orthopedic problems (focal articular cartilage injuries), and in defining the causes of depression as an ethological animal mode (50).

Antimicrobial resistance (AMR)

AMR in horses was first documented in 1970, almost twenty years after the first discovery in animals. Several studies report about methicillin-resistant staphylococcus aureus (MRSA) and extended spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae*, which also risks human's health. Recently, some cases of *Salmonella typhimurium* have raised the awareness of multidrug resistance (MDR) in horses (50).

2.2 Horses and main spillover events

There are relatively few diseases that are transmitted directly from horses to people (51) , but many viruses affecting equines are also important human pathogens (52). Horses are not immune to EID, and many of them are zoonotic. Recently identified emerging diseases in horses include the equine protozoal myeloencephalitis, clostridial enterocolitis, ehrlichiosis, Japanese encephalitis, vesicular stomatitis virus infection, Venezuelan equine encephalomyelitis (VEE), HeV infections, WNV encephalitis, Eastern equine encephalitis (EEE), Western equine encephalitis (WEE).

Some diseases have long been forgotten in developed countries because of improved management and the advent of antimicrobials. However, some of these diseases may still pose significant threats. Among them, for example, the *Burkholderia mallei,* the glanders agent. In case of this pathogen, the last reported case of naturally acquired glanders in the United States was in 1945, and the most recent case demonstrated the difficulty of recognizing ‘‘nearly forgotten’’ diseases. Nowadays, glanders is an example of a disease that can have significant human health implications if used as a bioterrorism agent, and, as a result, it is important for equine practitioners to be aware of this danger and to recognize that horses may serve as sentinels for a potential intentional biologic release.

Another forgotten pathogen that can be transmitted from horses to humans is rabies. With the widespread use of rabies vaccine since the 1950s, human cases have virtually disappeared. Rabies is occasionally identified in horses, and cases have been also reported in mules and donkeys, and it still represents a public health concern. In existing literature, no documented human cases of rabies have been attributed to equine exposure, yet diligence is necessary because of the severity of human disease. Furthermore, there has been documented evidence of illness even in vaccinated horses.

Horses’ pathogen can also be involved in nosocomial infections: until recently, *Rhodococcus equi* has been considered strictly an equine pathogen, nonetheless the incidence of the agent infecting people has increased markedly with the emergence of HIV (51).

Among the zoonoses diseases that involved horses and humans, for sure the viral ones have a greater impact. These include virus such as the WNV, which is a virus transmitted by mosquitoes and causes fatal encephalitis in human and equines. The WNV belongs to the Japanese encephalitis virus family. The mortality rate in humans varies from 3-15% and can

reach up to 50% in clinically affected horse. Horses and humans are the main hosts and are thusly referred to as ‘‘dead-end’’ hosts, as their infection does not contribute to additional cases either directly or indirectly through infection of an arthropod vector due to the low levels of viremia. Animals other than horses may be susceptible to the WNV, but rarely become ill (53). The WNV is amplified by continuous transmission cycles between mosquitoes and birds. Generally, *Culex* mosquitoes are the vectors and passerine birds are the reservoirs in enzootic transmission cycles. The virus is carried in the salivary glands of infected mosquitoes and transmitted to susceptible birds during blood-sucking. Competent bird reservoirs sustain an infectious viraemia for 1 to 4 days subsequent to exposure, and then develop life-long immunity. Few cases in humans have been spread through blood transfusions, organ transplants, breast feeding and during pregnancy. Vaccination of horses protects valuable animals from a potentially fatal disease, but trade and competition practices make this undesirable as some countries use positive antibody tests and impose import restrictions (52). In Italy we have an *integrated surveillance system* which provides for monitoring equine, birds and insects and the results are reflected on monitoring the blood transfusion center all over the nation. Data tell us that in Italy, from 2008 to 2018, there have been 475 autochthonous confirmed cases of WN human encephalitis (54) and 7 imported, while the equines surveillance reported 108 cases in the 2020 autumn-summer period (55).

Another important virus, that we are going to examine in detail in the next chapter, is the HeV. This virus causes respiratory and neurological disease and death in human and horses. The virus was first identified in 1994 during the first recorded outbreak of the disease in Brisbane, Australia. Fortunately, HeV caused limited outbreaks that are confined especially to Australia. Despite the less number of cases, HeV infection continues to threaten equine and human health in Australia (56).

Next, we have the vesicular stomatitis. This is a viral disease which primarily affects cattle, horses, and swine. The vesicular stomatitis virus (VSV) is the prototype of the genus *Vesiculovirus* in the family *Rhabdoviridae.* The virus is endemic in South and Central America. Outbreaks traditionally occurred in all regions of the USA but have been limited to western states. VSV firstly appeared in Europe during the First World War and periodically appears in South Africa. The virus is zoonotic and causes flu-like symptoms. Horses of all ages appear equally susceptible, but lesions do not appear in all susceptible horses. The lesions of the disease

resemble foot-and-mouth disease in cattle and the other viral vesicular diseases in pigs. Horses are resistant to foot-and-mouth disease and susceptible to VS. VSV is the only viral vesicular disease of livestock that infects horses and it is also the most important zoonotic agent for humans among the vesicular viruses (52).

Equine encephalitis viruses are certainly the most worrying zoonoses transmitted from horses to humans and they all belong to the genus *Alphavirus*, family *Togaviridae*. Among them we consider Western- (WEEV), Eastern- (EEEV), Venezuelan equine encephalitis virus (VEEV).

The WEEV is maintained in an enzootic cycle between its natural vertebrate hosts, passerine birds, and its most common mosquito vector, *Culex tarsalis*, a species associated with irrigated agriculture and stream drainages in the western USA. Transmission to horses and humans is mediated by so- called bridging mosquito vector (57). Changes in irrigation practices turned out to be a successful mosquito control programs and can also control the number of animals and humans cases. Horses and humans are often referred to as "dead-end" hosts as the virus does not build to high enough levels in the blood to infect other mosquitoes (52). WEEV infections in humans tend to be asymptomatic or cause mild disease after a short incubation period of 2–7 days with non-specific symptoms, while in a minority infected individuals, encephalitis or encephalomyelitis occur (57). In horses, infections with WEEV begin with fever, inappetence and lethargy, progressing to various degrees of excitability and then drowsiness, ultimately leading to paresis, seizures and coma during the 5-10 days course of the disease. The WEEV mortality rate in horses is higher than in humans. The mortality rate in horses showing clinical signs of WEE is 20–50% (52).

The EEEV was first isolated in 1933 from infected horses in Virginia and New Jersey. This virus has North American and South American variants. Infection of birds and mosquitoes maintains these viruses in nature. *Culiseta melanura* and *morsitans* species are primarily involved. Transmission of EEEV to mammals occurs via other mosquitoes which are primarily mammalian feeders and act as bridge vectors. Most people bitten by an infected mosquito do not develop any symptoms, with symptoms generally appearing 3 to 10 days after the bite. Clinically affected patients may exhibit pyrexia, muscle pains, headache, photophobia, and seizure. The disease in horses is characterized by fever, anorexia, and severe depression. In severe cases, the disease in horses progresses to hyper-excitability, blindness, ataxia, severe mental depression, recumbency, convulsions, and death. The nervous system symptoms may

appear due to brain lesions. This may be followed by paralysis, leading to difficulty in the causing the horse raising its head. Vaccines containing dead virus are used for prevention of the disease. These vaccinations are usually administered as combination vaccines, most commonly with WEE, VEE, and tetanus (57).

Lastly, we have the VEEV that was first isolated in 1938. The VEEV complex is composed of six subtypes (I-VI) that are differently distributed in all South America countries. Like the WEEV and EEEV, the VEEV is a zoonotic pathogen, transmitted between vector mosquitoes and vertebrate hosts, namely rodents and humans in enzootic cycles, and horses and humans in epidemic or epizootic cycles. In recent years, spillover to humans during equine epizootics has resulted in epidemics of VEEV (52). The primary vectors for the bird or rodent-mosquito life cycle are members of the *Melanoconion* subgenus (*Culex cedecci*). Infections with the VEEV may present, in both humans and horses, as either encephalitic disease or as simply a febrile disease without profound neurologic signs. Horses could die after a very acute course, even without any neurologic signs, but mortality in humans is generally low. Horses are not dead-end hosts for VEEV epizootic strains as they are for EEEV and WEEV. Horses, in fact, are the key reservoir species for the epizootic strains of VEEV that cause clinical disease in both horses and humans. Epizootic subtypes highly pathogenic to equines can spread rapidly through large populations. Equines are the primary animal species and serve as amplifying hosts for epizootic VEEV strains. Blood-sucking insects feed on infected horses, pick up this virus and transmit it to other animals or humans. The two VEE vaccines, a modified-live vaccine and an inactivated adjuvant vaccine, have been used in field (57).

In concluding this brief review of the main spillover events that involve horses, we talk about the equine influenza virus (EIV). It is acknowledged that the H3N8 jumped into dogs becoming today one of the main strains of canine influenza. In contrast, historically it hasn't been known for the virus to affect humans, although many scientists have mixed opinions. Based on different publications, one may find considerable experimental and observational evidence that the H3N8 EIV occasionally infected humans. Morens and Taubenberger found out that from 1658 to the early 20th century, EIV outbreaks in horses often preceded 3 weeks or so human influenza-like-illnesses. The likely cause of human pandemic in 1889 has also been considered to be a H3N8 EIV. Serological studies of the people who lived in the 1892 times have also shown elevated antibodies against H3N8 EIV (58).

Lately, for updating this review, it has been discovered that around 10% of horses in the USA were positive for β-CoV, which is the cause of the COVID-19 (50).

3. The equine influenza virus (EIV) H3N8 and its spillover to dogs

Part 1: The equine influenza virus (EIV)

Influenza is a well-known and ancient disease. In fact, the earliest evidence of a disease resembling influenza date back to Hippocrates in 412 BC. Although millions of dollars have been spent on research, the influenza virus (IV) continues to challenge our understanding of its ecology and our ability to control its spread. Two key reasons why IV has remained one of the most important causes of viral respiratory disease are its potential for establishing genetic and antigenic diversity and its ability to occasionally transmit between different host species (59).
Among the emerging and re-emerging animal diseases, the influenza group is the prototype member associated with severe respiratory infections in a wide variety of host species. Within this group, we find also the equine influenza (EI), an influenza A virus (IAV), OIE listed, which is the main cause of respiratory illness in equines across the globe, including horses, mules, donkeys and zebras (60). In addition, the EI is one of the most economically important horse diseases (61).

3.1 Etiology

3.1.1 Taxonomy

The EIV is part of the Domain *Riboviria*, Kingdom *Orthornavirae,* Phylum *Negarnaviricota*, Subphylum *Polyploviricotina*, Class *Insthoviricetes,* Order *Articulavirales*, Family *Orthomyxoviridae.* The family includes seven genera and nine species. The genus are: *Alphainfluenzavirus, Betainfluenzavirus, Deltainfluenzavirus, Gammainfluenzavirus, Isavirus, Quaranjavirus* and *Thogotovirus*. The EIV is part of Genus *Alphainfluenzavirus* or *Influenzavirus A*, Species *Influenza A virus* (62).
Type A is differentiated from type B and C on the basis of the identity of the major internal proteins antigens, the nucleoprotein (NP) and matrix (M1), which we are going to better describe later (63). In contrast to influenza A viruses (IAVs), which can be isolated from a wide variety of species (including horses), influenza B viruses (IBVs) appear to infect primarily humans.

Influenza C (IC) has been isolated mostly from humans, although they have also been shown to infect pigs and dogs (64).

3.1.2 Virion properties

Morphology

The IV virion has 1% of negative single chain RNA, with a molecular mass (Mr) of $4x10^6$ Daltons. It has a helicoidal nucleocapsid of the scale of 6-9 nm, probably made by eight different subunits. The particles, equipped with 80-120 nm peplos, are shaped of spherical or elongated with numerous cavities and cylindrical structure, 9 nm long and 1,5-2 nm wide (65). The virion envelope is derived from the cell membrane, incorporating virus glycoproteins (one to three in number) and non-glycosylated proteins (one or two in number). Virion surface glycoprotein projections are 10-14 nm in length and 4-6 nm in diameter. The virus genome is segmented, has helical symmetry, and consists of different size ribonucleoproteins (RNP), 50–150 nm in length (66).

Physiochemical and physical properties

Virions are sensitive to heat, lipid solvents, non-ionic detergents, formaldehyde, irradiation and oxidizing agents (66).

Nucleic acid

Depending on the genus, virions contain different numbers of segments of linear, negative sense ssRNA: eight segments: IAV, influenza B virus (IBV) and infectious salmon anemia virus (ISAV); seven segments: influenza C virus (ICV) and Dhori virus (DHOV); six segments: Thogoto virus (THOV). Segment lengths range from 736 to 2396 nt. Genome size ranges from 10.0 to 14.6 kb. RNA segments possess conserved and partially complementary 5′- and 3′-end sequences with promoter activity. Shorter viral RNA segments may occur in defective particles (66).

Proteins, lipids and carbohydrates

Structural proteins common to all genera include: structural proteins which are termed as hemagglutinin (HA), neuraminidase (NA), NP, matrix proteins (M1 and M2), three polymerase proteins (PB1, PB2, and PA), one nuclear export protein (NEP) and a non-structural protein named as NS1. Each protein is determined by a different genome segment, which in the case of IAV are eight (60).

- PB2 polymerase: PB2 polymerase is encoded by RNA segment 1. It is a member of the protein complex providing viral RNA-dependent RNA polymerase activity. It is known to function during initiation of viral mRNA transcription as the protein which recognizes and binds the 5' cap structures of host cell mRNAs for use as viral mRNA transcription primers. Endonucleolytic cleavage of these cap structures from host mRNAs is also at least in part a function of PB2. The role of PB2 in the other virus-directed RNA synthetic processes, *i.e.,* synthesis of full-length template cRNA and new negative-sense viral RNA (vRNA), is not known since these processes do not require host cap priming. Newly synthesized PB2 proteins migrate to the nucleus of infected cells (60).

- PB1 polymerase: PB1 polymerase is encoded by RNA segment 2; it functions in the RNA polymerase complex as the protein responsible for elongation of the primed nascent viral mRNA and also as elongation protein for template RNA and vRNA synthesis. PB1 proteins localizes in the nucleus of infected cells (63). Also, PB1 subunit can give rise to three proteins namely, PB1, PB1-F2, and PB1-N40. +1 reading frame of PB1 segment codes for the PB1-F2 (on average 90 amino acid length) which has apoptosis induction function. N40 is another version of PB1 where there is truncation in the N terminal region of PB1 (60).

- PA polymerase: PA polymerase is encoded by RNA segment 3. It also localizes in the infected cell nucleus and is a member of the RNA-dependent RNA polymerase complex along with PB1 and PB2, but its role in viral RNA synthesis is unknown. There is evidence for possible roles as a protein kinase or as a helix-unwinding protein (63).

- HA: The HA protein is an integral membrane protein and the major surface antigen of the IV virion. It is responsible for binding of virions to host cell receptors and for the fusion between the virion envelope and the host cell. HA is encoded by RNA segment 4. There are three kinds of post-translational processing involving the protein: proteolytic cleavage,

glycosylation, and fatty acid acylation. Newly synthesized HA is cleaved to remove the amino-terminal hydrophobic sequence of 14 to 18 amino acids, which are the signal sequence for transport to the cell membrane. Carbohydrate side chains are added, whose number and position vary with the virus strain. Palmitic acid is added to cysteine residues near the HA carboxy terminus. The final processing step is the cleavage of the HA into two subunits, HA_1 and HA_2 (uncleaved HA is called HA_0), connected by disulfide linkages. This process is accomplished by host-produced trypsin-like proteases and is required for infectivity because virus-cell fusion is mediated by the free amino terminus of HA_2. The three-dimensional structure of a complete HA trimer has been determined. In essence, each HA molecule consists of a globular head on a stalk. The head is made up of HA_1 and contains the receptor-binding cavity as well as most of the antigenic sites of the molecule. The stalk consists of all of HA_2 and part of HA_1. The carboxy-terminal region of HA_2 contains the hydrophobic transmembrane sequence and a terminal cytoplasmic anchor sequence where palmitate is attached. Owing to error-prone viral RNA polymerase activity, IV HA is subject to a very high rate of mutation. Selection for amino acid substitutions is driven at least in part by immune pressure, as the HA is the major target of the host immune response. Although the amino acids making up the receptor-binding site, as well as cysteine and most proline residues, are highly conserved, the remainder of the HA molecule is highly mutable. In nature, there are presently 14 recognized subtypes of HA (called H_1, H_2, ect.), which differ by at least 30% in the amino acid sequence of HA_1 and which are serologically not cross-reactive. Subtypes may include several variant strains which are partially serologically cross-reactive (63).

- Nucleoprotein: NP is encoded by RNA segment 5. It is transported into the infected cell nucleus, where it binds to and encapsidates viral RNA. It has a structural rose, but also, NP is believed to play a role in the switching of viral RNA polymerase activity from mRNA synthesis to cRNA and vRNA synthesis. NP is abundantly synthesized in infected cells and is the second most abundant protein in the IV virion. NP is also a major target of the host cytotoxic T-cell immune response (63).
- Neuraminidase: NA, encoded by RNA segment 6, is also an integral membrane glycoprotein and a second major surface antigen of the virion. NA cleaves terminal SA from glycoproteins or glycolipids. Thus, it functions to free virus particles from host cell

receptors, to permit progeny virions to escape from the cell in which they arose, and so facilitate virus spread. NA is glycosylated and possesses an amino-terminal hydrophobic sequence which functions both as signal for transport to the cell membrane and as transmembrane domain; it is not cleaved away. The distribution of NA has not been conclusively understood; immunogold-labeling experiments suggest that the NA tetramers are not evenly distributed over the virion envelope, as is HA, but aggregate into patches or caps. The complete three-dimensional structure of an NA tetramer, bound to antibody, has been determined. Like HA, NA is highly mutable with variant selection partly in response to host immune pressure. Nine subtypes of NA (called Ni, N_2, etc.) have been identified in nature; they are not serologically cross-reactive. Different variants of several subtypes are known (63).

- Ml protein: IV RNA segment 7 is bicistronic, encoding both Ml and M2 proteins. Colinear transcription of segment 7 yields mRNA for the matrix protein. This is the most abundant protein in the IV virion. Matrix protein forms a shell surrounding the virion nucleocapsids, underneath the virion envelope. In the infected cell, it is present in both cytoplasm and nucleus. It has no known enzymatic activity, although it has been speculated to play an important role in initiating progeny virus assembly (63).

- M2 protein: The mRNA for M2 is also transcribed from RNA segment 7. It is derived from the colinear (Ml) transcript by splicing. M2 is an integral membrane protein, whose membrane-spanning domain acts as a signal for transport to the cell surface. It is present as a tetramer in large amounts on the infected cell surface, and a small amount is found in the virion. It is believed to act as a proton channel to control the pH of the Golgi during HA synthesis and to allow acidification of the interior of the virion during virus uncoating (63).

- Nonstructural NS1 and NS2 proteins: in IAVs, RNA segment 8 encodes the two nonstructural proteins NS1 and NS2. These proteins, particularly NS1, are abundant in the infected cell (NS1 primarily in the nucleus, NS2 primarily in the cytoplasm) but are not incorporated into progeny virions. Both proteins play roles in virus replication, but those roles have not been fully defined (63). There are two functional domains in case of the NS1 protein named as RNA binding domain (N terminal end) and effector domain (C terminal end). NS1 has different epitopes, hence having multifunctional activities. NS1 protein plays

a crucial role in influenza infection by antagonizing type I interferon of the host and reducing IFN β production (60). NS2 appears to modulate the synthesis of NS (63). Complete transcription of segment eight leads to the expression of NS1, while pre-mature splicing leads to expression of NEP. Previously, NEP was thought to be a non-structural protein and termed as NS2, while later studies indicated that this protein was found within the virion and has interaction with the M protein. NEP has an essential role in the release of viral ribonucleoprotein from the host nucleus. On the basis of nucleotide homology, NS segments of IAVs are divided into A and B allele. All mammalian influenza isolates, except equine origin H3N8, belong to allele A (60).

Lipids in the virion envelope constitute about 18–37% of the particle weight. They resemble lipids of the host cell plasma membrane.

Carbohydrates in the form of glycoproteins and glycolipids constitute about 5% of the particle weight. They are present as N-glycosidic side chains of glycoproteins, as glycolipids, and as mucopolysaccharides. Their molecular composition is host- and virus-dependent (66).

Infection and replication

Virus entry involves the HA and occurs by receptor-mediated endocytosis. The receptor determinant of IV consists of SA bound to glycoproteins or glycolipids. In endosomes, low pH-dependent fusion occurs between viral and cell membranes. For IV, infectivity and fusion depend on the post-translational cleavage of the virion HA protein (IAV into HA_1 and HA_2) to result in the production of a hydrophobic group of amino acids at the amino terminal of the HA_2 molecule. Among other factors, cleavability depends on the number of basic amino acids at the cleavage site. Integral membrane proteins migrate through the Golgi apparatus to localized regions of the plasma membrane. New virions form by budding, thereby incorporating matrix protein and the viral RNPs, which align below regions of the plasma membrane containing viral envelope proteins. Then budding occurs from the apical surface in polarized cells. Viral RNPs are transported to the nucleus where the virion transcriptase complex synthesizes mRNA species. For IV, mRNA synthesis is primed by capped RNA fragments 10–13 nm in length that are generated from host heterogeneous nuclear RNA by viral endonuclease activity that is associated with the PB1 and PA proteins, after cap recognition by PB2.

Protein synthesis occurs in the cytoplasm. However, NP, M1 and NS1 proteins accumulate in the cell nucleus during the first few hours of replication, then migrate to the cytoplasm. Cytoplasmic inclusions of NS1 may take place. Complementary RNA molecules, which act as templates for new viral RNA synthesis, are full-length transcripts and are neither capped nor polyadenylated. These RNAs exist as RNPs in infected cells (66).

Antigenic properties

The best studied antigens are the NP, HA, NA, M1 and NS1 proteins of IAV and IBV. Considerable variation occurs among the IAV HA and NA antigens. Antibodies to HA, NA, or GP neutralize virus infectivity.

IVs agglutinate erythrocytes of many species. Serotype-specific antibodies may block agglutination. The NA of attached influenza virions may destroy SA on the erythrocyte surface and the virus receptors, resulting in the elution of virus. Hemolysis of erythrocytes may be produced by HA at acid pH (66).

Receptor binding characteristics and distribution

The receptor-binding site of the HA glycoprotein recognizes the SA bond attached to galactose (Gal) in either α2-3 or α2-6 linkage. IAVs recognize mainly two species of SAs, NeuAc (N-acetylneuraminic acid), and NeuGc (N-glycolyl- neuraminic acid), which are attached to galactose in SAα2-3Gal or SAα2-6Gal linkages. For instance, avian viruses preferably recognize SAα2-3Gal linkages, which are mainly found in the intestine and respiratory epithelia of birds, whereas human IVs recognize SAα2-6Gal linkages, which mainly populate the human upper respiratory tract (URT) epithelia. Pigs are known to exhibit dual expression of both SA linkages in the respiratory tract, similar to the receptor distribution in human URT, which is why pigs and humans may have similar susceptibility to IAVs infection.

Additional factors may play a role in species differences, including the relative abundance of the preferred glycan topology, which might influence the viral binding kinetics and/or equilibrium shift.

Taking a look at other species, the presence of SAα2-6Gal in the alveoli of dogs, cats, tigers, pigs, and ferrets and in the trachea of chickens and ducks has been reported.

The amino acid residues in the receptor-binding site of HA cells also affect the virus host range. IV receptor interactions are more complex than the simple α2- 3 versus α2- 6 dichotomy on the host range restriction, suggesting that glycan species (linked to SA) and their topology could also play an important role. On the other hand, SA glycans are classified as having umbrella-like (long α2- 6) and cone-like (α2- 3 or short α2- 6) structural topology, which in turn may also influence virus-receptor affinity (10).

Biological properties

As previously announced certain IAV naturally infect humans and cause respiratory disease. Particular IAV infect other mammalian species and a variety of avian species. Interspecies transmission, though rare, is well documented, and this theme will be better dealt with later. IBV strains appear to naturally infect mainly humans and cause epidemics every few years. ICV cause more limited outbreaks in humans and may also infect pigs.

Human IAV and IBV replicate in the amniotic cavity of embryonated hen eggs, and after adaptation they can also be propagated in the allantoid cavity. ICV replicate only in the amniotic cavity. Primary kidney cells from monkeys, humans, calves, pigs and chickens support replication of many IAV and IBV strains. Most of these viruses require the addition of trypsin to the growth medium, so that proteolytic HA activation and multiple cycles of replication can occur in some continuous cell lines. IV are also characterized by hemagglutination activity, that is the ability of the virus to induce the agglutination of blood red cells, and this property is widely exploited in different diagnostic methods (66).

Mutations

Mutations, including substitutions, deletions, and insertions, are one of the most important mechanisms for producing variation in IV. The lack of proofreading among RNA polymerases contributes to replication errors in the order of 1 in 10^4 bases. This contrasts with the much higher replication fidelity found among DNA polymerases, *i.e.,* errors in the order of 1 in 10^9 bases per replication cycle. Each round of RNA virus replication results in a mixed population with many variants, most of which are not viable, but some of which have potentially advantageous mutations that can become dominant under the right selective conditions (63).

IAVs evolve using different mechanisms. The most frequent one is the antigenic drift, as a result of mutations introduced during replication of the viral genome by viral RNA polymerase, which lacks proofreading activity. The rate of mutation during replication of the IVs genome is about 1 nucleotide change for every copied genome. Antigenic shift occurs through viral reassortment, which can result in the shuffling of entire gene segments. For example, the transmission of H5N1 HPAI from poultry to humans was first reported in Hong Kong in 1997. Even if human-to-human transmission has been limited, H5N1 is believed to be a significant health threat due to "spillover" infections in humans associated with widespread infection in poultry populations. The single mutation HA-Q192H in some H5N1 strains isolated from humans increased viral binding to SAα2-6Glu, correlating as well with an increased virulence. However, mutations enhancing the binding to SAα2-6Glu are not in themselves sufficient for host switching and transmission, meaning that other virus factors may be involved. In this regard, the adaptation of the IAV polymerase to host factors is an important mechanism underlying interspecies transmission (10).

Reassortments

Since IVs have segmented genomes, reassortment is an important mechanism for producing diversity very rapidly; it occurs among IAVs in nature and is important in the emergence, of pandemics in human populations. Reassortment has been demonstrated between IBV strains in the laboratory but is probably not important for producing novel gene combinations, for there is no known IBV gene pool except in humans (63).

In the case of IAVs, the genome consists of eight separate RNA segments and the coinfection of one host cell with two different strains can result in progeny viruses containing gene segments of both parental viruses. Theoretically, there are 256 possible combinations of the eight gene segments between two viruses. Swine are considered as the main candidates for generating reassortant viruses between human and avian IAVs. Available reports have demonstrated the isolation of whole avian IAVs in pigs, while complete genomic analyses have confirmed the reassortment of swine, avian, and/or human viruses in pigs worldwide, as reported in China. Swine are also capable of spreading reassortant viruses to humans, as demonstrated during the last 2009 "Swine flu" pandemic.

The continuous circulation among different hosts has provided the conditions for the evolution and generation of multiple novel genotypes through reassortment events. It has been reported that significant inter- and intra-subtype reassortments associated with specific amino acid substitutions may result in increased transmissibility in mammals (10).

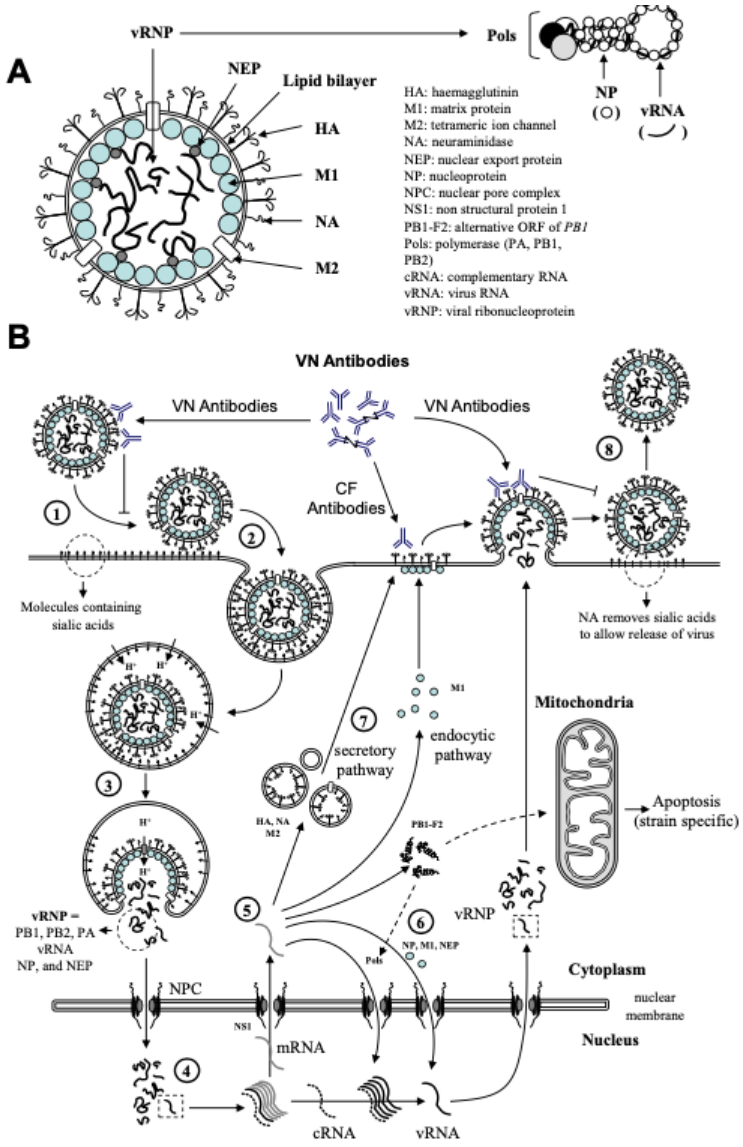

Figure 3: Structure and replication of influenza viruses
('A Systematic Review of Recent Advances in Equine Influenza Vaccination', R. Paillot (2014))

Other mechanisms for producing genetic variation include: defective-interfering particle-mediated interference and intramolecular recombination. Although defective-interfering particles can influence evolution by reducing the yields of non-defective particles and modifying

pathogenicity, their role in IV evolution has not received much attention. While intramolecular recombination in negative-stranded viruses is rare, recent studies have shown one instance of insertion of cellular mRNA sequences into the HA gene with acquisition of virulence. This provides another mechanism that enables rapid evolutionary changes (63).

3.1.3 Influenza A viruses

As mentioned above, EIV is part of IAV genus. Only a single species is currently recognized in this genus, that is comprised of a cluster of strains that replicate as a continuous lineage and can genetically reassort with each other.

One distinguishing feature of IAV is that member viruses of the genus all have eight genome segments. The HA and NA receptor-destroying enzyme are different glycoproteins. Based on antigenicity, 18 subtypes of HA and 9 subtypes of NA are identified for IAV, a criteria used for the classification of subtypes.

By convention, species names are in italic script: names of isolates are in roman script. Sequence accession numbers [ ] and assigned abbreviations ( ) are also listed. New isolates are designated by their antigenic type/host species/geographical site of origin/strain designation number/year of origin and (HA and NA subtype): *e.g.* A/chicken/Novosibirsk/65/2005 (H5N1) (66).

IAV main pandemics throughout history

Influenza is an ancient disease which has affected all countries in the world and caused millions of deaths and continues to represent a major treat for all international and national authorities in modern times. There have been many pandemics in the history of animal and human health, and we will proceed to retrace some of the most important ones.

Reports of possible human influenza can be found as early as the Greek writings of 412 BC. The outbreak of 1510 was probably a pandemic, reported as spreading from Africa to engulf Europe. The outbreak of 1557 may have also been a pandemic; but the first influenza pandemic agreed on by all authors occurred in 1580. The first influenza pandemic of the 18th century began in 1729: the outbreak started in Russia in the spring months, spread westwards in expanding waves to embrace all Europe within a 6-months period. The next pandemic occurred after a gap of some 40 years between 1781-2 and began in China in the autumn, spread to Russia and from there westwards in widening circles to encompass the whole of Europe in a period of 8 months.

The pandemic of 1830-3 ranks in terms of severity the pandemic of 1918-20. In the 20th century, a pandemic was recorded in 1898-1900, in 1918-1920 and other different times. The pandemic "Spanish influenza" from 1918-1920 is regarded as one of the greatest pandemic in history, both in terms the number of death, estimates more than 50 million people, and considering the particular historical framework context in which it occurred, the First World War. This pandemic has been describe as "the greatest medical holocaust in history", an event similar in scope to the Black Death. The pandemic was caused by the IV H1N1 subtypes, derived from the avian virus but, in reality, the origin of the virus is not entirely clear. Another major pandemic was the one between 1957 and 1958, the "Asian flu", which originated in the Yunan Province of China in February 1957 and evolved to affect all of Europe and the rest of the world, with estimated deaths worldwide ranging from 1 to 4 million. It was caused by the H2N2, a human and avian resultant virus (67). Another event in the IV history is the 1968 flu pandemic, also called "Hong Kong flu pandemic", a global outbreak of influenza that originated in China in July 1968 and lasted until 1969–70. The outbreak was the third influenza pandemic to occur in the 20th century. The pandemic resulted in an estimated 1 million to 4 million deaths, and was initiated by the emergence of a virus known as IAV subtype H3N2, which was also a resultant from avian and human viruses (68). The 1977 pandemic, the "Russian Flu", was a relatively benign flu pandemic, mostly affecting population younger than the age of 25-26. These characteristics turned out to have a simple scientific explanation: the virus was not novel. The 1977 strain was virtually identical to an H1N1 influenza strain that was prevalent in the 1950s but had since dropped out of circulation, and that happened because older people had already gained immunity against it, since it was an older virus. It is estimated that 700.000 people died (69). The last significant pandemic event occurred in 2009 and was called the "swine flu". In the spring of 2009, in Mexico, a novel IAV H1N1 emerged. It was first detected in the United States and spread quickly across the United States and the world. The WHO emitted a global warming in the following June. This new H1N1 virus contained a unique combination of influenza genes not previously identified in any animal species. Although the 2009 flu pandemic primarily affected children and young and middle-aged adults, the impact of the H1N1 virus on the global population during the first year was less severe than that of previous pandemics, leading to 7000 deaths (70). Swine influenza (SI) is a common respiratory disease of pigs caused by IAV viruses that regularly cause outbreaks of influenza in pigs. IVs that commonly circulate

in swine are called “swine influenza viruses” or “swine flu viruses.” Like human IVs, there are different subtypes and strains of SI viruses. The main SI viruses circulating in U.S. pigs in recent years have been H1N1, H3N2, and H1N2 virus. In this case, the virus was a human-avian-swine resultant, but the pre-existence of antibodies against this subtype allowed the limiting of the pandemic extension (71).

Influenza pandemics do not affect only humans and swine, but also the avian population. The most widely quoted date for the beginning of the first ever recorded incident of avian influenza (AI) is 1878, when researchers first differentiated a disease of poultry (initially known as fowl plague, but later renamed highly pathogenic avian influenza) from other diseases with high mortality rates. Current evidence indicates that highly pathogenic AI (HPAI) viruses arise through mutation when low pathogenicity AI viruses of H5 or H7 subtype are introduced into poultry. Between 1877 and 1958, a number of epizootics of HPAI occurred in most parts of the world. From 1959 to 1995, the emergence of HPAI viruses was recorded on 15 occasions, but losses were minimal. In contrast, between 1996 and 2008, HPAI viruses caused emergency at least 11 times and four of these outbreaks involved many millions of birds. Events during this recent period are overshadowed by the epizootic of HPAI due to an H5N1 virus that spread throughout Asia, Europe and Africa, affecting over 60 countries and causing the loss of hundreds of millions of birds. All sectors of the poultry population were affected, but free-range commercial ducks, village poultry, live bird markets and fighting cocks seem to be especially significant in the spread of the virus. The role of wild birds has been extensively debated over the years, but it is likely that both wild birds and domestic poultry are responsible for its spread. Even without these H5N1 outbreaks, the period 1995 to 2008 is considered significant in the history of HPAI because of the vast numbers of birds that died or were culled in three of the ten epizootics during this time (72).

In the next chapter we will explore the pandemic potential of other mammalian IVs, like the EIV and the canine influenza virus (CIV).

Ecology of IAV: The Webster Theory

IAVs infect a variety of animals, including humans, pigs, horses, sea mammals, and birds. Recent phylogenetic studies of IAVs have revealed species-specific lineages of viral genes and

have demonstrated that the prevalence of interspecies transmission depends on the animal species. They have also revealed that aquatic birds are the source of all IVs in other species. According to Webster et al., IVs have a well-defined biological cycle:

- Reservoir host: There is convincing evidence that all the subtypes of IAVs are perpetuated in the aquatic bird populations of the world, especially in ducks, shorebirds, and gulls. There is no evidence to show that IVs persist for extended periods in individual animals. This indicates that some mechanism has evolved for maintaining IVs in aquatic avian species. The infected birds are presumably immune to reinfection with the predominant influenza subtype. For this reason probably this influences the changes in the subtype predominating in a particular flyway from year to year. Several possibilities have been suggested for the perpetuation of IV in the aquatic bird populations of the world:

    a) Continuous circulation in aquatic bird species: it is suggested that each of the IV subtypes could be maintained in the wild duck population. The detection of low levels of IVs throughout the winter months and the detection thereof in ducks as they arrive back from migration at the beginning of the breeding season support this notion.

    b) Circulation between different avian species: since IVs are prevalent in shorebirds in the spring and in wild ducks in the fall, there may be interspecies transmission. About half of the IV isolated from gulls and shorebirds may experimentally infect ducks; however, sampling of shorebirds during August and September failed to reveal any IVs in shorebirds and gulls when they were prevalent in ducks.

    c) Persistence in water or ice: when wild ducks are present in August and September, IV can be isolated from lake water without concentration. It is possible that IVs are preserved frozen in ice or in lake water and reinfect ducks in the spring. Tests of lake water in the winter and spring have so far failed to detect IVs. The infectivity of IVs in water is dependent on the virus strain tested and the salinity, pH, and temperature of the water. At 17°C, some strains remain infectious for up to 207 days, and at 4°C they remain infectious for longer times, raising the possibility of persistence of IV in water when the ducks are absent.

    d) Persistence in individual animals: although virus shedding from the intestinal tract in some ducks can continue for 2 to 4 weeks, there is no evidence that continued

shedding occurs. The possibility has been raised that IV persists in an integrated or episomal form in the genetic material of humans or lower animals. To determine whether IV can persist in the tissues of ducks, by some as yet unexplained mechanism, the polymerase chain reaction (PCR) method of gene amplification was used to detect IV in experimentally infected ducks. Infectious virus and RNA cleared concurrently after oral infection of ducks with IV. There was no evidence from PCR analysis of the HA gene for persistence of viral genetic information. There is ample evidence from several laboratories that IVs can produce persistent infections in cell culture. The conditions that lead to this state are still not understood. There is much less evidence for persistent infections of animals, although there are reports of extending shedding of IVs from immunocompromised animals, including nude mice. There is evidence that H3N2 IVs can persist in pigs after disappearing from the human population, but some of these H3N2 strains were most probably derived from recent infection with H3N2 avian IAV.

e) Continuous circulation in subtropical and tropical regions: there is increasing evidence that, in the tropical and subtropical regions of the world, IVs of humans are isolated year-round, whereas in temperate climates, influenza is a winter disease and the virus is infrequently isolated in the summer months. IVs have been isolated year-round from domestic ducks in Hong Kong. Although surveillance studies for IVs in wild ducks and shorebirds have not been carried out in tropical regions, the possibility has to be considered that the epicenter of IV perpetuation is in the tropical regions of the world and that ducks, shorebirds, and gulls transport viruses from the tropical regions to the temperate regions during spring migration. The argument against a tropical epicenter is that high-density congregations of wild birds are not found in these regions of the world.

At this time, the most convincing data supports the first alternative, by which there is a continuous circulation of IVs in wild ducks with very low levels of detectable virus while the birds are in their overwintering sites in the subtropics (63).

- Melting pot: Current human IVs are believed to have arisen by genetic reassortment between previous human IVs and non-human viruses. Where did the reassortment between genes of

human and avian IVs occur? Reassortment requires simultaneous infection of a host animal with both avian and human IV. The pig has been the leading contender for the role of intermediate host for reassortment: swine are the only mammalian species which are domesticated, are reared in abundance, and are common hosts for human IV. The evidence supporting the role of the pig is as follows:

a) Pigs are susceptible to infection by subtype HlN1 and H3N2 IV of both human and avian origin, although the mechanism by which pigs contract avian viruses is undetermined. Pigs probably acquire human viruses by inhalation of aerosols during period of close contact.

b) Humans occasionally contract IV from pigs, such as in 1976 or 1988, when isolated cases of SI in humans caused deaths. About 10% of persons with occupational exposure to swine develop antibody to swine influenza in humans.

c) Genetic analyses indicate that genes for most internal proteins of human IVs share a common ancestor with the equivalent genes of most swine IVs, but not with the equivalent genes of other mammalian IVs, after diverging from the avian virus lineage.

d) There is no evidence that humans are susceptible to natural infection with true avian IVs.

It is also worth noting here that there has not been any "smoking-gun" IV that appeared in swine before starting a human pandemic (63).

- Humans: the emergence of new human pandemic viruses is rare, occurring at unpredictable intervals in the order of decades. But is there an epicenter for IVs? Historical records and the appearance of the Asian, Hong Kong, and Russian pandemic strains of IV in China suggest that the majority of pandemics of human influenza since about 1850 have originated in China. The exception seems to be the Spanish influenza, which may have been brought over to Europe by U.S troops in 1918. The possibility has been raised that southern China is an influenza epicenter. Unlike the temperate or subarctic regions of the world, where influenza in humans is a winter disease, in the tropical and subtropical regions of China influenza occurs year-round. In China, IAV of all subtypes are prevalent in ducks and in water frequented by ducks and the different subtypes are present year-round with peak incidence in summer months. While IVs occur in humans, pigs, and aquatic birds in China, the question

arises regarding the way in which this differs from other tropical and subtropical regions of the world. Ecological studies show that IVs occur in each of these species in all countries where tests have been conducted; these countries include temperate as well as tropical climates. In temperate climates, influenza in people and pigs is a winter disease and usually occurs when free-flying aquatic birds are absent. Tropical and subtropical regions of the world include Southeast Asia, China, India, Central Africa, and Central America. If we examine the distribution of people, pigs, and ducks in these countries, we find that the human population is largest in India and in China, smaller in Central Africa, and smallest in Central America. The pig population is largest in China, small in India and Central America, and moderate in Central Africa. The distribution of wild and domestic ducks in the world is influenced largely by the availability of surface water, and they are prevalent in all regions except the Antarctic. The domestic duck population is largest in China and smaller in the other regions; however, aquatic birds migrate through or overwinter in subtropical and tropical regions.

Religious customs may influence the regions where influenza may originate. Pigs are a common domestic animal throughout the globe, but their prevalnce is often influenced by religion, social customs, and climate. Pigs are not an approved source of protein in the Muslim and Jewish religions and are considered a dirty animal in India, where up to 80% of the population are vegetarians. Since pigs may play an important role in the inter-species transmission of influenza, they might be the limiting factor in some countries and may explain why IVs do not occur in tropical regions of the world. These considerations leave southern China as a possible region where IVs can circulate in people, pigs, and ducks, thus providing the opportunity for inter-species transmission and genetic exchange among IVs. All these considerations are useful to frame the ecological contest of regions like Southern China, a place where rural zootechnics and the commingling between animals and humas promotes the creation of new strains.

All the above considerations about the regions of the world where influenza pandemics originate are interesting, but they are still merely speculation. In fact, only circumstantial evidence exists for the appearance of pandemic IVs in Southern China. Further studies on influenza in Southeast Asia are necessary to determine whether an epicenter does exist in this region and whether it is an important source of strains for inclusion in vaccines (63).

In accordance with the aforementioned theory, the primary natural reservoir of IAVs are aquatic birds and EIVs H7N7 and H3N8 are considered to be of avian origin too (73). During bird-to-horse IAV transmission, and subsequent adaptation to equine host, viruses undergo changes in the genome and consequently in the encoded proteins, resulting in the ability to replicate and spread in equine hosts. This is corroborated analyzing conserved equine IAV marker amino acids, incidence in individual proteins, the type of amino acid substitution and the possible influence on the function of a particular protein (74).

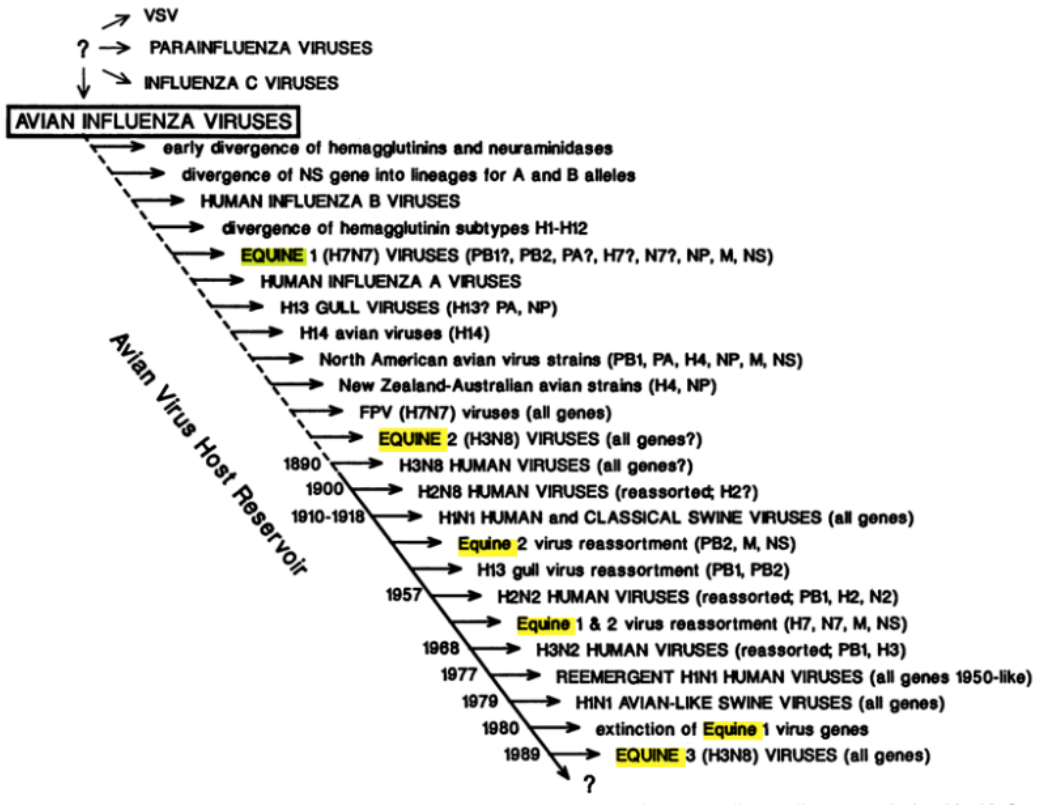

Figure 4: Evolution of IAV
('Evolution and ecology of Influenza A viruses', Webster et al. (1992))

3.1.4 Subtypes, Lineages and Sublineages of EIV explained by the historical events

EIV has two recognized subtypes namely H7N7 and H3N8, of which H3N8 predominantly circulates in equines. In 1956, the first reported outbreak of EIV occurred in Eastern Europe and was caused by a H7N7 strain (A/equine/1/ Prague/56 H7N7), firstly classified as "subtypes 1" or "equi 1". Since then, the H7N7 virus was isolated from horses in many countries all over the

world. In fact, the last epidemic was reported in Italy in 1979, and some sporadic case were reported in India and Egypt around the end of ‘80s and the beginning of ‘90s. Despite this, the presence of antibodies against this subtypes is a prove of its circulation. Even if specific antibodies have been detected in unvaccinated horses in Maghreb and Sahel, no virus was isolated. In 1963, the H3N8 subtype (A/equine/2/Miami/63), firstly named “subtypes 2” or “equi 2” caused an epidemic of EIV in Miami (USA) (75) and subsequently spread throughout North and South America and Europe, leading to massive outbreaks during 1964 and 1965. Between 1978 and 1981, there were widespread epidemics of the A/equine/2 strain throughout the USA and Europe, despite the development of vaccines. In 1986, the H3N8 virus was introduced to the naïve horse population of South Africa by a sub-clinically infected vaccinated horse. A similar scenario occurred in India in 1987. Outbreak in Europe and Ireland during 1989 happened due to a genetic drift away from the current vaccine strains. Also in 1989, a severe epidemic with unprecedented high morbidity and mortality occurred in China. This outbreak was caused by a unique H3N8 strain that was thought to have a link with a reassortant virus originated in the avian gene pool. Thankfully, this highly virulent strain (A/Equine/Jilin/1/89) did not spread beyond Northern China and died out quickly. Since the late 1980s, the evolution of the H3N8 virus has diverged into two families: an “American-like” lineage and a “European-like” lineage. The American lineage has further diverged into three lineages, designated the South American/Argentinian, Kentucky and Florida lineages (76). The Florida lineage is further divided into Clade 1 and Clade 2, that are the main responsible lineages for epidemics nowadays (75). Some analysis showed that Clade 1 viruses have been circulating more on the American continent, while Clade 2 viruses have been incriminated for most of the outbreaks in Europe and Asia. Both Clades have been reported in major outbreaks throughout the world (60). An EI outbreak that included vaccinated horses occurred in Sweden in 1991/1992. Another epidemic occurred in China between 1993 and 1994 in an unvaccinated rural horse population and was caused by a strain related to the H3N8 virus circulating in Europe. Two H3N8 Europe-like strains were isolated from an outbreak in the Netherlands in 1995. Many outbreaks have occurred in recent years, including in Tunisia in 1998, Egypt in 2000, the UK and South Africa in 2003, and Argentina, Canada, Croatia, Denmark, France, Germany, Greece, Hungary, Ireland, Italy, Sweden, the UK, and the USA during 2004. The American lineage of the virus has been responsible for all of the 2004 outbreaks (76). However, outbreaks due to both of the

Clades keep on occurring across the geographic barriers. Florida Clade 1 viruses have been responsible for major outbreaks also in Japan and Australia in 2007-08, while Clade 2 viruses caused huge outbreaks in China, India, and Mongolia. A/eq/South Africa/04/2003-like or A/eq/Ohio/2003-like viruses are representative of Clade 1, A/eq/Richmond/1/2007- like viruses represents Clade 2 and Newmarket/2/93 represents the Eurasian lineage. Since 2013, some of the isolates from Europe have been consistently showing two amino acid changes (A144V and I179V) in the antigenic region and have been referred to as subgroups of the Clade 2 lineage (60).

By virtue of the capacities of the IV in mutations and reassortments, we can expect the creation of new subtypes/lineage/clade, which may have an important epidemic potential among equine population in the future.

3.2 Epidemiology and evolution

3.2.1 World Distribution of EIVs

Influenza is the most frequently diagnosed and economically important cause of viral respiratory disease of the horse (64). Since first diagnosed during an epidemic of respiratory disease in 1956, when marked widespread respiratory epidemic disease occurred in equines due to EIV, Europe and North America are the most endemic regions for EI, and almost all nations in the world have witnessed outbreaks caused by EIV except a few small island countries like New Zealand and Iceland. Currently, EI is prevalent worldwide *viz*. Europe, Canada, USA, Turkey, Scandinavia, and South America. Increase in equine traffic has favored the spread of EIV to other countries, including South Africa in 2003. Australia has reported the disease for the first time in 2007, when the disease was also re-introduced in Japan and South Africa. Improper quarantine of sub-clinically affected animals, which were not sufficiently vaccinated, led to the spread of this virus to Australia, but also to China and Japan, as seen in this important outbreak, when about 76000 horses were found infected (60). In 2008, Australia, along with New Zealand and Israel, was declared free of the virus (77).

A more detailed description of the epidemiology situation is as follows:

- North America: A/equine/Montana/9564-1/2015 (H3N8) was isolated from an outbreak in USA during 2015 from the unvaccinated equines and sequence analysis showed that

the virus was identical to A/equine/Tennessee/29A/2014 (H3N8) based on its polymerase acidic (PA), polymerase basic protein 1 (PB1), hemagglutinin (HA), matrix (M) and nucleoprotein (NP), while the analysis of non-structural proteins (NS), neuraminidase (NA), and PB2 showed maximum identity with A/equine/Malaysia/M201/2015 (H3N8). A Canadian study at a racetrack showed a 76% prevalence of EIV. A recent analysis conducted in West Indies among 140 horses and 40 donkey serum samples revealed 49 samples positive for EI antibodies. This was the first report of EIV infection from the Leeward Islands of West Indies.

- South America: seropositivity of EIV is observed in 92% of the equines amongst Brazil's equine establishments. The high prevalence of EV antibodies suggests that the virus circulated extensively among the animals, and statistical analysis indicated that the movement and high aggregation of animals are associated with virus transmission. In 2015, an EIV outbreak was reported among both the vaccinated and unvaccinated equines in Brazil. Notably, all the 12 isolates recovered were classified as Florida Clade 1 EIV.

- Europe: The EIVs evolved in France during 2005–2010 in a similar manner as in other parts of world. The genetic evolution of all the EIV isolated in France from 1967 to 2015 was studied and it was found that, until 2003, the American and Eurasian lineages were predominating, while the Florida sublineage Clade 2 predominated after 2005. The genetic characterization of Italian isolates revealed a close relatedness to American, European, and also the prototype vaccine lineage A/eq/South Africa/4/2003 isolate. The first incidence of the Florida clade 1 virus in Nordic countries was reported in 2011 in Sweden, which supports the use of both Clade 1 and 2 Florida sublineage viruses in the vaccine. In 2014, EIV outbreaks were reported in 19 premises in Ireland. Although there was clear vaccination history against EIV, the outbreak may be due to the non-updating of vaccines with Clade 2 of the Florida sublineage. Phylogenetic analysis of the HA and NA gene of the Greek EIV isolates recovered during 2003–2007 showed that they are related to the Eurasian lineage and Florida sublineage Clade 2, respectively. This study suggests that there may be the possibility of reassortment. Recently, in February 2018, there was a report of EI from Scotland and the virus belonged to Florida clade 1 sublineage and this sublineage has not been reported in UK after 2009.

- Africa: African countries also reported this virus. A study in Nigerian horses showed the presence of H3 and H7 subtypes in their sera by ELISA. Later, in a serosurvey conducted in Nigeria employing nucleoprotein-based ELISA, 173 out of 284 animals screened were found to be positive for the presence of EIV antibodies.
- Asia: During 2007–2008, China and its neighboring countries, like Mongolia, India and Japan, were invaded by various EIV strains. Further, phylogenetic analysis revealed that the Chinese strains, the Indian strain (Jammu-Katra/6/08) and the Mongolian strain (Mongolia/1/08) were of Florida sublineage Clade 2 type. All strains were derived from European strains of this Clade as the Newmarket/1/07 and Cheshire/1/07 strains, but were unrelated to the Japanese strains isolated around the same time (Florida sublineage Clade 1) or to the Chinese strains isolated in the 1990s (European lineage). Since 2007, several outbreaks of EI have occurred in Kazakhstan, western Mongolia, India, and western China and they all have similarities with EIVs circulating around the same period in neighboring countries. The genetic characterization of the viruses revealed the formation of an EIV cluster and the continued evolution of this lineage in central Asia between 2007 and 2012. In India, influenza-like symptoms in equines were first reported in 1964 from the Bombay Turf Club, Mumbai, where around 400 horses showed symptoms of coughing. Since then, India has experienced two major epizootics, the first of which was recorded from January to August 1987, involving over 83,000 equines in north and central India. The second epizootic was reported in 2008–2009 after a gap of 20 years, which initially started in Jammu and Kashmir and covered almost 14 states in the country. Further phylogenetic analysis shows that the HA gene of the isolates were related to the Florida sublineage Clade 2 in the American lineage (H3N8) and also very similar to Chinese isolates of 2007–2008. The Indian isolates were clustered in the Yokohama/10 isolate subgroup together with Chinese, Mongolian, and Kazakhstan isolates. In 2007, an outbreak had been reported in China among Asian wild horses (*Equus przewalskii*). The virus had been isolated and completely sequenced and then designated as the strain A/equine/Xinjiang/4/2007 which showed 99% homology with the Florida-2 sublineage rather than with the A/equine/Qinghai/1/1994 (European lineage) strain responsible for previous outbreaks in China. In March 2017, an EIV outbreak in donkeys from the Shandong province of China was reported, where the virus

was found to be A/donkey/Shandong/1/2017 (H3N8) belonging to the Florida sublineage Clade 2. The report suggested the circulation of newly emerging EIV in donkeys in China. The Japanese EIV isolate Kanazawa/07 phylogenetically relates to American sublineage Florida virus Clade. During the period 2015–2016, an outbreak of EI among equines in several districts of Khyber Pakhtunkhwa Province of Pakistan was noticed. Turkey reported their EIV first outbreak in the year 2013 and the virus was found to be a Florida Clade 2 sublineage H3N8 which was similar to the one circulating in Europe. IAV has been isolated from camels in Mongolia, which is more evidence of the expansion in the host spectrum of this virus.

- Australia: In Australia, the first outbreak of EIV was reported in 2007. The major outbreak appeared in New South Wales and Queensland, affecting more than 1,400 equines within a month (60).

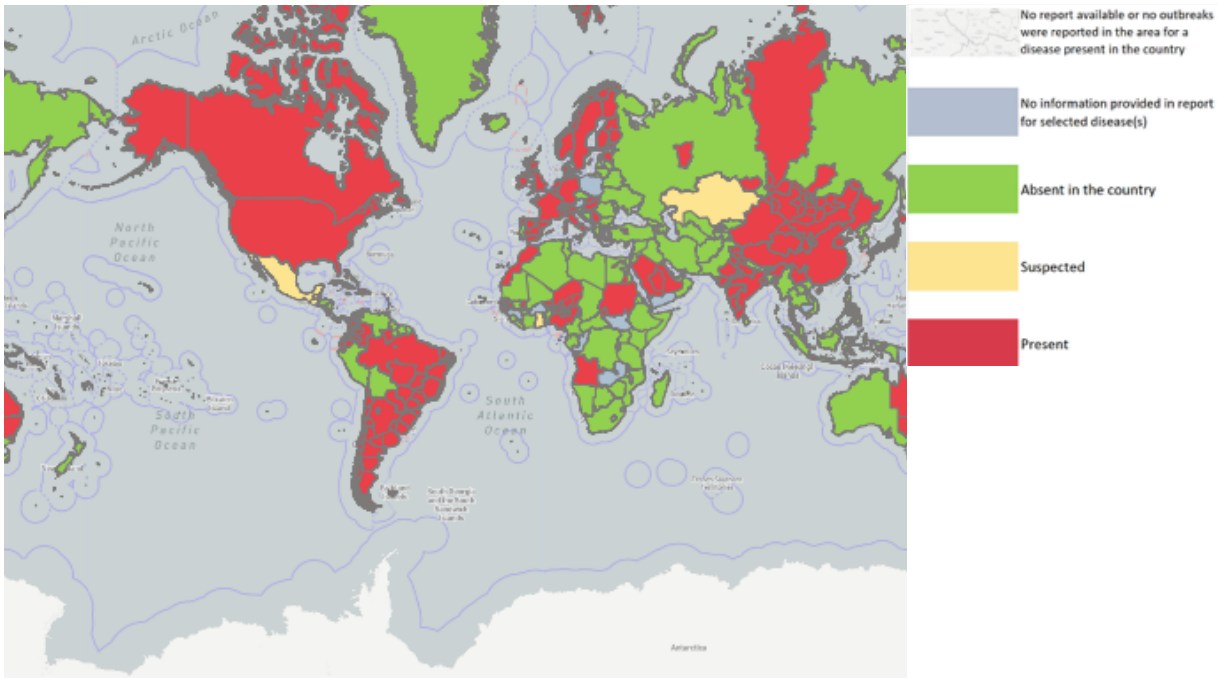

Figure 5: Epidemiological distribution of EIV
(OIE 2021)

3.2.2 Epidemiological characteristics of the virus

As noted before, EIV is manifested both with and epidemic both with and endemic trend. Endemicity is a term used to describe a disease that is constantly present or very frequent in a population, is maintained by sporadic clinical cases and by subclinical infection in susceptible horses introduced into the population by birth, through waning immunity, or after movement from other areas or countries. A carrier state is not recognized for EI. The clinical outcome after viral exposure largely depends on the immune status of the animal in question; clinical disease varies from a mild, inapparent infection to severe disease in susceptible animals. Epidemics, instead, is a term used to describe an increase in cases compared to the condition of endemia, arise when one or more acutely infected horses are introduced into a susceptible group. The epidemiologic outcome depends on the antigenic characteristics of the circulating virus and the immune status of a given population of horses at the time of exposure. Frequent natural exposure or regular vaccination may contribute to the degree of antigenic drift seen with specific strains of A/equine-2 virus in some parts of the world (78). In humans, due to the frequent antigenic drift, we talk about “seasonal flu”, because the virus appears during the winter months and the strain involved is different from the ones of the previous year. In the case of EIV, there is no currently evidence of seasonal incidences, thus it can occur at any time of the year (60).

3.2.3 Economic importance

EI is not serious in itself in terms of health impact, but it causes much inconvenience in racing stables because it occurs in explosive outbreaks and affected horses have to break training. Such outbreaks have capacity to close down the racing industry in a country for several months. An additional cost is incurred because of the restriction on movement of horses and associated quarantine periods. Recent outbreaks of EI in India suspended the racing activity for a prolonged period of time, resulting in marked economic losses of equine the industry (79). For example, in the case of the Australian outbreak of 2007, 76000 horses were infected, and the estimated economic losses amounted to 1 billion dollars (75).

3.3 Transmission and risk factor

The virus is transmitted by aerosol, wind, nose-to-nose contact, and fomites such as tack, grooming equipment, machinery, water, feed, and human contact (76). The virus is extremely contagious, but it can be easily fought with acid substances (pH=3) and warmth (56°C for 30 minutes), lipidic solvents and detergents (75). Aerosol spread occurs over distances of 35 meters and is enhanced by the frequent coughing characteristic of the disease. EIV in aerosols survives longer (24-36 hours) than human or porcine strain (79).

The virus transmission by inhalation happens due to aerosol particles that can spread effectively through air up to 1–2 km of distance. Droplet infection also plays a major role in the transmission, as nasal discharge/fomites aid in animal-to-animal transfer. Horse-to-horse spread is fairly rapid and faster than other respiratory infections in the equine species.

The worldwide distribution is certainly enhanced as a result of international trade and traffic, also leading to the spread of the disease to previously disease-free regions of the world (60).

The incubation period lasts between 1 and 3 days, while the shedding of the virus in nasal secretions begins as soon as 24 hours after infection. In addition, shedding can continue for 7 to 10 days in horses that have been exposed to the virus for the first time. It is important to recognize that vaccinated and previously exposed horses may be sub-clinically infected and shed virus transiently, thereby constituting a difficult-to-detect source of infection for naïve horses. The introduction of sub-clinically infected horses into a susceptible population is the largest risk factor for the initiation of an outbreak.

Infection may occur within any age group, breed, sex, and season. The disease tends to occur more frequently during cooler months due to indoor housing, which places the horses in close contact with one another and sharing a common air space. Stable that are poorly ventilated are a well-documented risk factor for spread. Young immunologically inexperienced horses (age groups 1-5 years old) and unvaccinated horses are particularly susceptible to infection (76). Partially immune horsess tend to become infected sub-clinically. Further, the spread of the virus in partially immune animals is slower than in naïve animals. In contrast, due to the presence of maternally derived antibodies, the incidence of the disease is quite low in foals (60).

Morbidity can be as high as 100% in susceptible populations, while mortality is generally low but may vary depending on the virus strain and the health of the horse (76).

3.4 Clinical signs

The clinical signs can be distinguished in:

- The minor form: it is the most common clinical form among vaccinated horses. The animals show moderate and fleeting hyperthermia (75), weakness, and poor performance (60). The cough is rare and there is the presence of abundant nasal discharge associated with transitorial tumefaction of pharyngeals lymphonodes.

- The major form: it is the most commonly observed form in unvaccinated horses. The animals present a febrile syndrome with high hyperthermia (from 39,1 to 41,7°C), associated with lethargy and anorexia. These signs are complemented by a characteristic repetitive, harsh, dry explosive nonproductive cough for the duration of 1 to 5 days, if there are no complications (75). The cough is a characteristic clinical sign that can last from 1 to 3 weeks and the frequency decreases with the clinical improvement of the animal (60). Inflammation and irritation of the airways may cause affected horses to cough or gag while eating, particularly if the feed is dusty. Paroxysms of coughing can often be elicited by pinching the larynx or trachea. Coughing is a predominant clinical feature but is not always present. When present, the cough may persist for 1 to 3 weeks with diminishing frequency as the horse recovers (76). An increase in the intensity of inspiratory and expiratory bronchovesicular sounds are often heard on auscultation of the chest (76). The horse manifests great fatigue, nasal and ocular discharge and hyperemia of the nasal and ocular mucosae. Other clinical signs observed are edema of the limb hinds, stiffness, epiphora, tachypnea and dyspnea, nasal and ocular mucosal congestion and muscle and joint pains. Without major complications, there is an improvement of the situation in 5-7 days, with a recovery of the respiratory epithelium in 3 weeks. The mortality is very low among adult horses. On the contrary, foals and young naïve horses can develop a severe pneumonia with a very quickly evolutions that can lead to death (75). Hematological changes are non-specific but may include anemia, leukopenia and lymphopenia (73).

- The major complicated form: Horses with severe viral infection or the onset of a secondary bacterial infection may show nostril flare, increased respiratory effort, crackles and wheezes on auscultation of the thorax, anxiety, and reluctance to move. In

particular, foals and young horses may develop a severe, rapidly progressive pneumonia that may prove fatal. Fever prolonged beyond 5 days and/or the development of a mucopurulent nasal discharge is suggestive of a secondary bacterial infection. The severity of the clinical signs is likely related to the exposure dose, virulence of the infecting strain, and the specific immune status of the host as a result of previous exposure or vaccination, including the type of vaccine used and the similarity between the vaccine strain and the infective strain (76).

- Other less common clinical signs: Influenza-associated-encephalitis/encephalopathy (IAE) in horses and rapid fatal pneumonia in foals and donkeys have also been recorded but their pathogenesis is not clear (60). In vaccinated racehorses, poor performance, with or without a cough or nasal discharge was the common clinical factor. In pregnant mares abort or resorb of the fetus may occurr, probably in response to severe illness and fever rather than viral invasion of the fetus. Rarely, viral myocarditis is observed and may result in tachycardia, electrocardiographical abnormalities, and arrhythmias such as atrial fibrillation. Horses with myocarditis are febrile, depressed, and intolerant of exercise and, in severe cases, may develop valvular insufficiency and congestive heart failure. The 1989 Jilin-strain outbreak in China involved the unique clinical feature of enteritis, in addition to pneumonia. Partially immune horses may demonstrate mild or no clinical signs, and the respiratory component may be indistinguishable from other forms of respiratory disease (76).

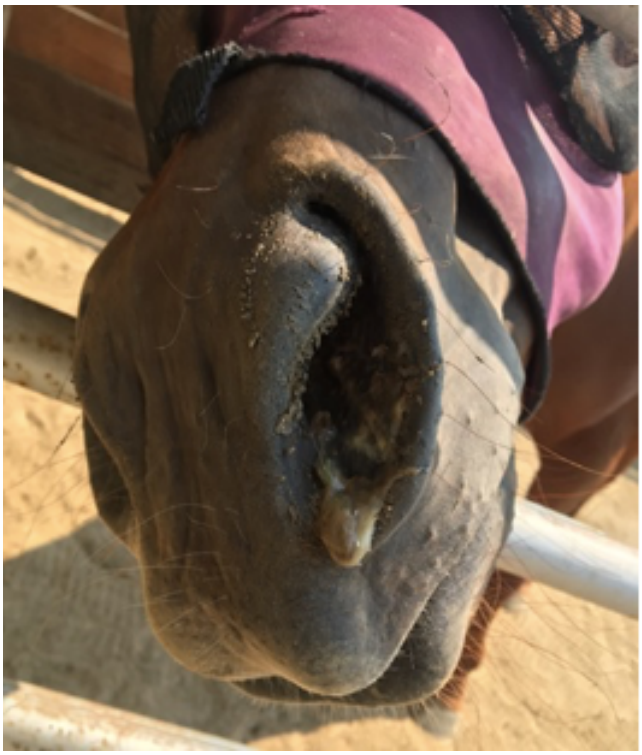

Figure 6: Nasal discharge is one of the EIV clinical signs
(UC Davis Veterinary medicine, centre for Equine Health)

3.5 Pathogenesis and pathology

IAVs replicate and induce pathologic changes throughout the entire respiratory tract, with the most significant pathology present in the lower respiratory tract. The primary targets of IAVs in mammalian species are the airway epithelial cells. After inhalation of the aerosolized virus, infection is initiated by binding of the IV HA to SA residues (*N*-acetylneuraminic or *N*-glycolneuraminic sialyloligosaccharides) on target cells located in the upper respiratory tract. However, for the virus to access to the cellular receptors, the particle fist needs to penetrate the mucus layer that forms a protective barrier over the cell surface to gain access to cellular receptors. In this regard, it is thought that the viral NA promotes virus access to respiratory epithelial cells by destroying mucous glycoproteins and removing decoy receptors present on mucins, cilia, and cellular glycocalyx. After viral attachment, the virion is internalized into the cell through receptor-mediated endocytosis. After internalization and acidification of the endosomal compartment, the HA undergoes an irreversible conformational change, which leads to the insertion of the hydrophobic fusion peptide into the endosomal membrane and then to the

fusion of the viral and cellular membranes. After membrane fusion and release of the RNPs into the cell's cytoplasm, the RNPs are actively transported into the nucleus through nuclear pores, where messenger RNA (mRNA) synthesis is initiated. During virus replication, the virus-encoded NS1 shuts off cellular protein synthesis by inhibiting the maturation of cellular mRNAs. NS1, that is a multifunctional protein, apart from inhibiting both polyadenylation and splicing of cellular pre-mRNAs, it also counteracts IFN-dependent and IFN-independent antiviral responses. This occurs by binding to the dsRNA-binding region of protein kinase, RNA activated (PKR), thus blocking its activation, and by inhibiting the activation of IFN regulatory factor 3106 and nuclear factor kappa B (NF-κB). The switch from mRNA synthesis to complementary RNA (cRNA) and viral RNA (vRNA) synthesis is believed to be triggered by an increased concentration of free NP. At a later infection stage, the main translation products are M1, HA, and NA. These are synthesized on membrane-bound proteins and transported across the membrane of the endoplasmic reticulum (ER) by means of a signal recognition particle (SRP). During transport through the ER, the proteins undergo a stepwise conformational maturation and folding, and additional processing may occur in the Golgi complex. In polarized epithelial cells, IVs assemble and bud from the apical surface of the cells. Interaction between the cytoplasmic tails of the HA and NA proteins and the internal proteins (most likely M1) are thought to be the principal driving force on the formation of the budding particles. By removing carbohydrate chains from the virion and the cell surface, NA enzymatic activity is thought to be required to release the newly formed IV virions completely from the cell. The virus spreads quickly throughout the respiratory tract, damaging the respiratory epithelial cells, particularly in the trachea and bronchial tree. Virus replication leads to cell death, largely through virus-induced apoptosis, and subsequent desquamation and denudation of respiratory epithelial cells. Histological evaluation of infected respiratory epithelium in fact reveals vacuolization and swelling of the columnar ciliated cells, accompanied by clumping and subsequent loss of cilia. In addition, tracheal mucociliary clearance is impaired, predisposing to the development of secondary bacterial infections. Within 1 day after the onset of clinical signs, focal erosions of the respiratory cells down to the basal layer are evident. Viral antigen can be demonstrated predominantly in the respiratory epithelial cells and mononuclear cells and only rarely in the basal cell layer. Bacteria have the opportunity to invade the respiratory epithelium thanks to the disruption of the superficial cell layers of both the upper and the lower respiratory tract, leading

to bacterial bronchopneumonia and other complications. Submucosal edema and hyperemia occur with peribronchial and peribronchiolar infiltration by neutrophils and mononuclear cells. About 3 to 5 days after onset of illness, regeneration of the epithelium begins, characterized by the appearance of mitotic figures in the basal cell layer. In uncomplicated cases, complete resolution of the epithelial damage takes a minimum of 3 weeks (64).

Due to the accumulation of fluid in the respiratory tract, there is the likelihood of secondary bacterial infection. The presence of organisms like *Streptococcus equi var. zooepidemicus, Pastorella spp and Actinobacillus spp* increases the inflammation leading to bronchopneumonia, bronchitis, bronchiolitis and leads to a risk of developing transient airway hyperactivity, allergic bronchitis and bronchiolitis, resulting in compromised pulmonary function that can lead to recurrent airway obstruction (RAO). Other long-term complications can include myocardium degeneration and exercise induced pulmonary hemorrhage (EIPH) (75).

The determinants of organ tropism and virulence of IAVs are polygenic, although the HA plays a pivotal role. The final processing step in HA maturation is the proteolytic cleavage of the HA precursor form (HA_0) into two disulfide-linked subunits (HA_1 and HA_2). This cleavage step is accomplished by host proteases and, because uncleaved HA cannot undergo the low-pH–induced conformational change necessary for membrane fusion, it is a prerequisite for the virus to be infectious. The HA of most IAVs (including EIV) are usually cleaved only by a limited number of organ-specific trypsin-like proteases (*e.g.*, tryptase Clara in the respiratory tract), so that the viruses cause only localized infections. In contrast, HAs of highly virulent influenza strains (*e.g.*, HPAI) are cleaved in a broad range of different host cells and are therefore capable of causing severe systemic infections. Thus, HA cleavability is one of the major determinants of IAV tissue tropism. In horses, however, attempts at virus recovery from the heart or brains of affected animals have been unsuccessful, while myocardial dysfunction may be caused by other mechanisms, such as an increase in the expression of inflammatory mediators and cytokines (*e.g.*, nitric oxide). Alternatively, myocardial dysfunction may be caused by viral products, such as the lipid envelope (64).

Macroscopic lesions include hyperemia, edema, necrosis, desquamation, and focal erosion of trachea and bronchi (76), heavy and dark red lungs, with signs of emphysema, congestion and

edema hyperinflated with dark red coalescing on the cut surface (80). Viremia rarely occurred, but is possible if the virus crosses the basement membrane and enters the blood vessels, potentially causing inflammation of skeletal and cardiac muscle (myositis and myocarditis), encephalitic signs, and limb edema (76).

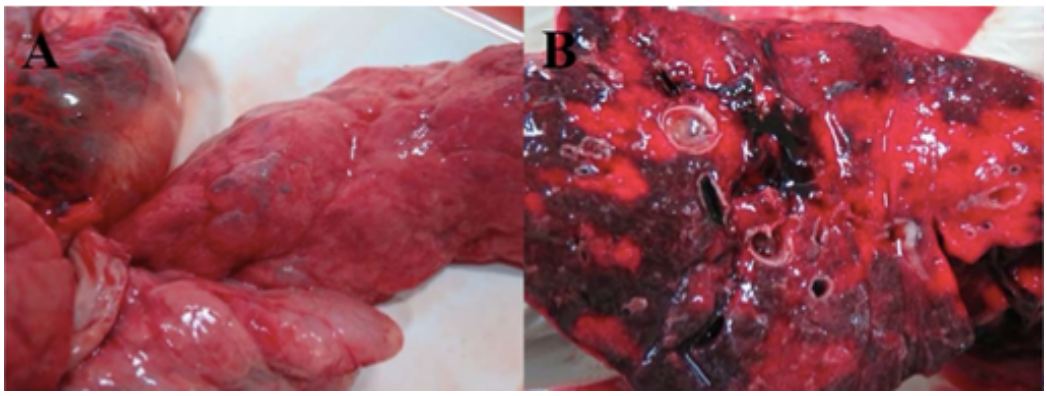

Figure 7: Macroscopic lungs lesions of EIV in donkeys

(A) Emphysema in the lungs; (B) Congestion and edema hyperinflated with dark red coalescing on the cut surface of the lung

('Emergence of H3N8 equine influenza virus in donkeys in China in 2017', Yang et al. (2018))

Microscopic lesions also include histological changes like necrosis of bronchioli and alveoli, infiltration of neutrophils, formation of hyaline membranes and airway epithelium undergoing hyperplasia and squamous metaplasia (60), formation of hyaline membranes in alveoli, hyperplasia of type II pneumonocytes, macrophages, and lymphocytes, necrosis of neutrophils and necrotizing bronchiolitis and hemorrhage (80). A study conducted by Pavulraj et al. on the pathology of EIV H3N8 in murine model has outlined the pattern of disease progression, lesions and virus recovery from nasal washings and lungs in mice were found comparable to natural and experimental EIV infection in equines. These findings establish BALB/c mice as an attractive small animal model for studying EIV (H3N8) infection (81).

With rest, the regeneration of the respiratory tract takes at least 3 weeks. Without rest and rehabilitation, the respiratory tract is vulnerable to invasion by opportunistic bacteria leading to a bacterial bronchopneumonia (76).

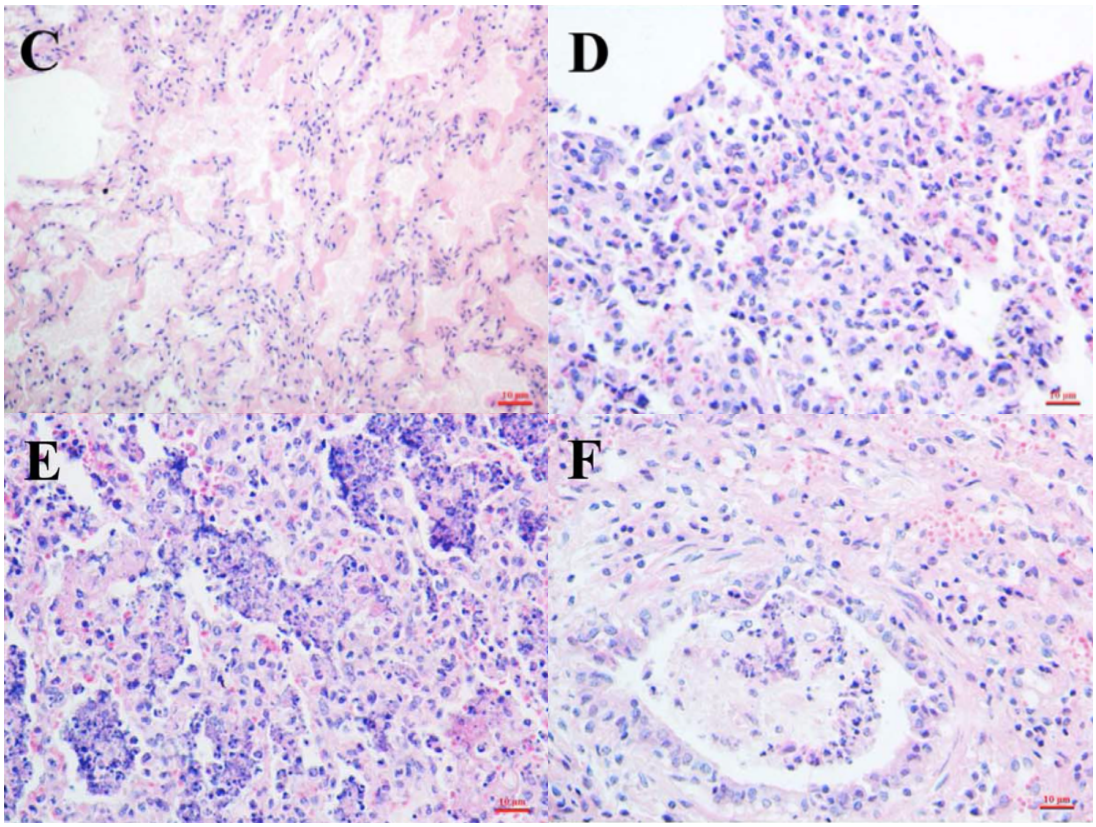

Figure 8: Microscopic lesions of EIV in donkeys

(C) Formation of hyaline membranes in alveoli; Hematoxylin and eosin (HE) staining. (D) Hyperplasia of type II pneumonocytes, macrophages, and lymphocytes; HE staining. (E) Necrosis of neutrophils; HE staining. (F) Necrotizing bronchiolitis and hemorrhage; HE staining.

('Emergence of H3N8 equine influenza virus in donkeys in China in 2017', Yang et al. (2018))

3.6 Immunity

EIV infection generates a broad vary of adaptive immune responses in systemic and mucosal compartments, also stimulating important innate immune system responses. In horses, many resarchers have demonstrated that antibody responses can be strongly associated with protection. A protective immune response, such as that following infection, is characterized by the induction of IV–specific immunoglobulin G isotype a and b (IgGa, IgGb) and immunoglobulin A (IgA) antibodies in both the blood circulation and the nasopharyngeal secretions, with the IgG isotype responses predominating in the circulation and IgA in the respiratory tract. Nasal IgA is an important mediator of protective immunity to IV infection in other species, through neutralization of viral particles at the respiratory epithelium and in the intracellular compartment. Nasal IgA responses are a characteristic of the protective immunity

that follows EI infection, and IV–specific IgA-producing B lymphocytes have been detected in mucosal lamina propria and lymphonodes draining the nasopharynx of the horse. Virus-specific IgG antibodies can also contribute to immune exclusion at the respiratory epithelium in a mouse model, although the lack of specialized mechanisms for transporting IgG to the respiratory surface means that its role is less important. In horses, there is evidence for local production of IV–specific IgGa and IgGb at respiratory mucosal surfaces after infection, and indirect evidence that virus-specific nasal IgGb antibody responses can contribute to a reduction in nasal shedding of IV. However, IgG antibody responses tend to be more short-lived in equine respiratory secretions than IgA responses.

In the circulation, IgGa and IgGb are thought to be the principal protective IgG sub-isotype responses to IV, whereas IgG(T) responses are not associated with protection. Circulating antibody has been measured in a number of ways in horses, including conventional hemagglutination inhibition (HI) assays, single radial haemolysis (SRH) assays, virus neutralization assays, and enzyme-linked immunosorbent assay (ELISA). Of these techniques, SRH and ELISA may have the greatest sensitivity and utility, and it appears that SRH results correlate closely with IgGb ELISA results.

Much attention has targeted on the correlation between levels of circulating antibody measured by SRH tests and protection from IV infection. This tool has proved very useful, and SRH responses are often used to measure vaccination effect and predict protection. However, after circulating antibody responses to a prior IV infection have waned, horses can remain protected against a further challenge. Furthermore, circulating antibody responses measured by SRH to a cold-adapted, modified live influenza vaccine are almost undetectable, although this vaccine provides long-lasting protection against challenge infection. Taken together, these observations illustrate that although circulating antibody responses are an important predictor of protection against IV protection, a lack of antibody does not invariably predict susceptibility.

The role of cellular effectors in ensuring resistance to EIV infection is less well investigated. Virus-specific cytotoxic T lymphocytes (CTLs) are important for protection from IV infection, and there is a single description of the measurement of MHC-restricted CTL responses to EIV. The lack of other CTL studies reflects the difficulty in detecting this equine immune response to IV using available methods. Currently, IV–specific lymphoproliferative responses and IFN-γ gene expression may be the best available measures of virus-specific cellular immune

responses in the horse. Production of IFN-γ is an indicator of T-helper 1 (Th1) cell–mediated immunity and can contribute to immunologic protection of humans from IV infection and disease. Also, several researches indicate an association between IFN-γ production and the concomitant generation of antigen-specific CTL responses. A number of studies have demonstrated the development of equine IFN-γ responses to IV consequent to either infection or vaccination. Similarly, IV–specific lymphoproliferative responses have also been associated with protective immunity. The importance of HA–specific immune responses in protection from IV infection is well known, and vaccination studies in horses using deoxyribonucleic acid (DNA) vaccination and recombinant vaccines expressing the HA gene all confirm the importance of the HA antigen for protection. Recent studies using modified Ankara vector vaccines have demonstrated that NP–specific equine immune responses can also result in reduced clinical disease after challenge infection, although the degree of protection was inferior to that induced by HA vaccination IV NP is an internal viral structural protein, and therefore NP-specific antibodies are not capable of virus neutralization and do not control virus shedding in the horse or other species. NP typically serves as an important target antigen for cellular immune responses and can elicit cross-protective immunity to heterologous strains of IV. In the equine vaccination studies conducted to date, NP-specific immune responses included both lymphoproliferative and IFN-γ responses, and it is possible that NP-specific immune responses could make a significant contribution to equine immunity to IV infection (64).

3.7 Diagnosis

Veterinarians must act quickly, as soon as infection is suspected, to prevent an outbreak from occurring because IV spread very quickly. Rapid diagnosis and isolation of affected horses are the front lines of defense against outbreaks (76). There is a vast availability of diagnostic techniques for EIV infection, and laboratory diagnosis is fundamental to differentiate the disease from other affections.

The methods presently used for diagnosis of IV infections include virus isolation in embryonated chicken eggs and cell culture, antigen detection by fluorescent antibody and ELISA testing, reverse transcriptase–polymerase chain reaction (RT-PCR) assays, and serologic analyses. Unfortunately, many of these methods have one or more serious disadvantages, such

as lack of sensitivity, long turnaround time, prohibitive costs, or the need for a high degree of technical expertise in the laboratory. Thus, to institute optimal control measures, it may be necessary to combine several of these diagnostic tools to identify the etiologic agent accurately and rapidly (64). The OIE published the "Manual of Diagnostic Tests and Vaccines for Terrestrial Animals" which provides an extremely useful resource for current testing methodology (82).

We shall now illustrate and differentiate the various methods.

- Anamnesis and clinical signs: Recognition of clinical signs (fever, depression, dry and hacking cough, and nasal discharge) in conjunction with a history that lends itself to the potential for infection are vital. Important features of the history include vaccination status, age of horse/s affected, travel, stabling conditions, and recent exposure (*e.g.,* new horses, race meet, or show) (75).

- Blood count and clinical biochemical determinations: In the early stages, these may reveal a mild to moderate normocytic normochromic anemia and leucopenia with a moderate to marked lymphopenia which persists for 1 to 5 days. Monocytosis is occasionally present in the early stages. A variable leukocytosis with a modest neutrophilia may occur between days 3 and 7, becoming more pronounced and possibly accompanied by a left shift in the event of secondary bacterial infection. Fibrinogen levels are generally within the normal range, except for severe cases and those with secondary bacterial infection. Elevated fibrinogen concentration is an indication that the patient may require more aggressive diagnostics and therapeutics. Protein serum amyloid is an inflammatory marker that increases during the first 48 hours of infection and returns to baseline over 11 to 22 days. The level of protein serum amyloid closely corresponds to the severity of infection. Blood chemistry should not be abnormal unless the animal has suffered complications such as dehydration and inappetence that result in electrolyte derangements and increased indirect bilirubin concentration. Moderate to marked increases in concentrations of the muscle enzymes, creatine kinase, aspartate aminotransferase, and lactate dehydrogenase are manifested in those horses that develop myositis. Affected horses often show muscle stiffness or soreness and are at risk of

developing severe rhabdomyolysis, myoglobinuria, and myocarditis if forced to exercise (64).

- Direct diagnosis - antigen detection:
    - Sample: To perform direct diagnosis, the preferred type of sample is the nasopharyngeal swab. It should be collected form any horse showing clinical signs. An immunologically naïve horse will shed large amounts of virus in respiratory secretions for 4 to 10 days post-infection, whereas a horse that has previously been exposed to viral antigens through vaccination or natural infection will shed less virus over a shorter period of time. Nasopharyngeal swabbing of the most immunologically naive horses is, therefore, most likely to enable the veterinarian to confirm a diagnosis of EI. Theoretically, the swab should be taken within 24 hours after the onset of fever and preferably includes mucosal cells as well as nasopharyngeal secretions. Higher concentrations of virus are found within the mucosal cells. The swab should then be placed directly into virus transport media containing calf serum and antimicrobials. If the sample cannot be tested immediately, the sample should be either cooled if being submitted to the laboratory within 24 hours, or frozen if submission will take longer than 24 hours. Bronchioalveolar lavage (BAL) and transtracheal aspirate may detect virus by virus isolation and antigen detection. However, these tests are typically not indicated because less expensive and less invasive procedures are available. Cytology and bacteria culture may be of interest, if a secondary bacterial infection or small airway inflammatory disease is suspected (76).

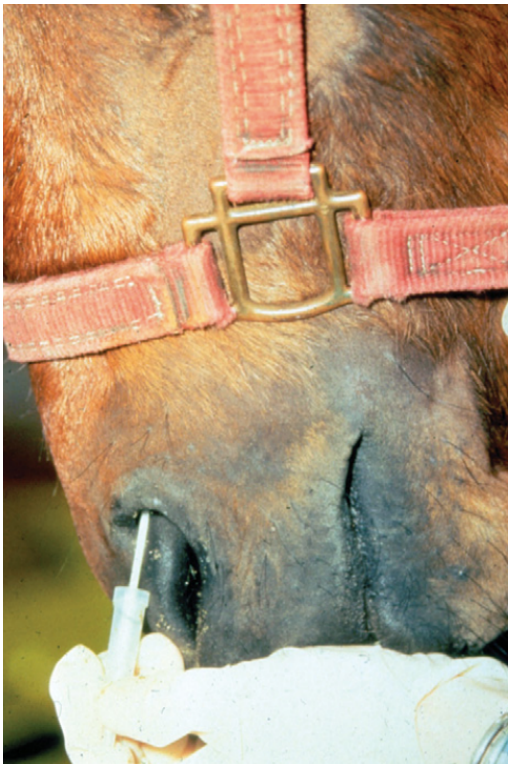

Figure 9: Technique to obtain nasal mucosal swab for antigen detection or virus isolation

Short, polyester-tipped (non cotton-tipped) swab is most appropriate for sample collection. Virus isolation from a nasal mucosal swab is most sensitive for detection of EIV if obtained during the first 24 to 48 hours of fever

('Equine infectious disease', Sellon,& Long (2007))

- Virus isolation: from clinical samples is critical for epidemiologic investigation and for vaccine production and is generally carried out in embryonated chicken eggs or cell culture. In the naïve horse, by the third or fourth day after infection, large amounts of virus are shed into the secretions of the respiratory tract. Therefore, the best results for virus isolation can often be achieved by collecting nasopharyngeal or nasal passage swabs within the first 24 to 48 hours after the onset of clinical illness. Historically, nasopharyngeal swabs obtained using a mare uterine culture swab or similar instruments have been recommended as the optimal sample type for IV isolation, although a swab of the nasal mucosa provides equivalent sensitivity. In partially immune animals, the length of virus shedding is

often shorter, decreasing the diagnostic sensitivity of virus isolation. Therefore, it might be useful to sample more immunologically naïve horses in a group to increase the likelihood of demonstrating the presence of infectious virus. Nasopharyngeal or nasal passage samples are best collected using polyester-tipped swabs. Cotton swabs should be avoided because IV can adhere to the cotton fibers, decreasing the likelihood of isolating virus from the sample. The swabs need be placed in sterile viral transport medium and kept on ice until further analysis. Traditionally, embryonated chicken eggs have been the biologic system of choice for isolating IVs. At 10 to 12 days after the fertilization of the egg, the sample is injected by either the amnionic or the allantoic route. After 24 to 72 hours of incubation, the allantoic or amnionic fluid is checked for hemagglutinating activity. In practice, twofold serial dilutions of the sample are prepared and mixed with a defined quantity of erythrocytes. If IV is present in the sample, the HA protein binds to SA–containing glycoproteins on erythrocytes. This hemagglutination activity results in the formation of a lattice. The robust yield of virus from eggs has led to their widespread use in research laboratories and for vaccine production. However, their use may be of limited value for diagnostic laboratories. Depending on the virus strain, the amount of virus present in the sample, sample quality, and handling, the detection of the presence of infectious virus by egg inoculation can take a minimum of 2 or 3 days. Horses with mild or subclinical infections, viral titers in nasal secretions are often low, sometimes requiring several egg passages before sufficiently high viral titers are produced to allow detection using conventional hemagglutination assays. In addition, there is overwhelming evidence that the growth of IAVs in eggs can lead to the selection of HA variants. Finally, the lack of reliable high-quality chicken eggs is a serious limitation in their use. Alternatively, EIV can be propagated in cell culture. Although the virus can infect a variety of primary and continuous cell lines, most cells do not support productive viral replication. Thus, the most globally used cells are Madin-Darby canine

kidney (MDCK) epithelial cells. The use of cell culture for IV isolation also has several limitations. For example, depending on the protocol and cell lines used, substantial differences in influenza recovery rates from clinical samples can occur. In addition, MDCK cells are generally considered less permissive than embryonated chicken eggs for EIVs. Other, less frequently used cell lines include mink lung epithelial cells (Mv1Lu) and chick embryo fibroblasts (64).

- Immunoassays: A number of ELISAs using monoclonal antibodies to detect the viral NP in nasal swab samples have been developed as a more rapid alternative to virus isolation. Although IAVs can differ substantially in their HA and NA genes, the sequences of the internal genes, such as the M, NP, and the NS genes, are highly conserved among all subtypes and strains of IAVs. As such, a diagnostic test aimed at the detection of NP is likely to be capable of identifying a wide variety of IAVs from different host species. An additional advantage of ELISA-based assays over virus isolation is that these tests are able to detect virions that have lost their infectivity during sample handling, storage, and transport to the laboratory. Originally intended for the diagnosis of human influenza infections, an antigen-capture ELISA has been adapted for the detection of equine H3N8 viral antigen. Basically, to detect viral antigen, a "capture" antibody, directed against the influenza NP, is linked to a 96-well plastic plate. The clinical sample is added to the wells, and if viral antigens are present, they will be bound to the immobilized antibody. Then, bound viral antigen is detected by use of a second enzyme-linked antibody. Commercial development of optical immunoassay (OIA)-based test kits, designed to detect the NP of human IAVs, has facilitated the widespread use of this procedure. Two of these test kits have been evaluated for use in the diagnosis of EIV infection; the Flu OIA assay and the Directigen Flu-A assay. Many investigators found these commercial diagnostic kits to be useful, and frequently these assays were considerably more sensitive than traditional virus isolation. In addition, these OIAs proved to be highly specific and rapid, whereas virus isolation

in embryonated chicken eggs required up to three passages before hemagglutination became evident. Although these test kits were able to identify infected horses consistently at the peak of virus shedding, they may not be sensitive enough to detect low levels of virus shedding reliably. When possible, horses with severe clinical signs during suspected IV outbreaks are preferable for testing to increase the likelihood of obtaining positive results (64).

- Immunofluorescence: Employing IV-specific fluorochrome-labeled antibodies, immunofluorescence (IF) is based on the immuno-detection of virus-infected cells obtained from nasal scrapings or tracheal washes. Although the assay was reported to be highly sensitive and rapid, IF requires substantial sample preparation and handling. Substantially the samples are centrifuged to separate the cells from respiratory mucous. The cells are washed, spotted onto glass slides, acetone fixed, and incubated with influenza-specific antibodies. Antigen-positive cells are detected by use of a secondary fluorochrome-labeled antibody. A recent study compared the detection of IVs by IF to a commercially available OIA for diagnosis of IV infections in humans and found that the IF had a significantly higher sensitivity than the OIA (64).
- PCR techniques: During the past decade, advances in PCR technology and other DNA amplification techniques have resulted in these methods becoming key tools in diagnostic laboratories. This techniques works by choosing appropriate oligonucleotides, a selected region of the viral genome can be amplified. The oligonucleotides acts as primers for in vitro DNA synthesis, which is catalyzed by a special DNA polymerase isolated from a thermophilic bacterium that is stable at high temperatures. PCR is extremely sensitive and can theoretically detect a single-copy DNA in a sample. Trace amounts of RNA can be detected in the same way by first transcribing them into DNA with reverse transcriptase (RT). RT-PCR–based assays have been used successfully for the detection of a broad range of IAV subtypes from clinical samples. By selecting primers specific for a region of the highly

conserved M gene, such assays are capable of detecting minuscule amounts of a wide range of IV strains and subtypes. In a recent study, RT-PCR was found to be a highly sensitive and specific method for the detection of IAVs of human, swine, avian, and equine lineage. In contrast to virus isolation, RT-PCR–based antigen detection techniques do not require the presence of viable virus, and therefore the sensitivity of RT-PCR methods is often substantially higher than for virus isolation. Although RT-PCR–based techniques have proved to be powerful tools for investigating respiratory disease caused by a variety of pathogens, the technique can also have a number of shortcomings. Because of the assay's high sensitivity, the greatest problem facing the diagnostic application of PCR is the generation of false-positive results. These are often attributable to contamination by nucleic acids, particularly from previously amplified material (carryover). Any contaminant, even the smallest airborne remnant carried over from the previous PCR procedure or from a strong positive sample, may be multiplied and produce a false-positive result. Furthermore, statistical analysis that take into consideration the sensitivity of RT-PCR–based assays on nasal or nasopharyngeal swabs have encountered false-negative results. Such false-negative results may result from the nature of the sample tested. Nevertheless, RT-PCR–based assays offer a more sensitive tool for the diagnosis of IAV infection than conventional techniques such as culture or immunoassays. In diagnostic laboratory settings, the use of RT-PCR can be limited by cost and sometimes the availability of adequate test sample volume. To overcome these shortcomings and also to increase the diagnostic capacity of PCR, multiplex PCR assays have been developed. In multiplex PCR techniques, more than one target sequence can be amplified by including more than one pair of primers in the reaction. Recently, multiplex PCR has been shown to be a valuable and cost-effective tool for monitoring the emergence of new variants and subtypes of IAV. Given the advantages already demonstrated by the use of multiplex PCR assays in human and veterinary medicine, this procedure will undoubtedly prove

beneficial in the diagnosis and differentiation of pathogens that commonly cause respiratory infections in horses (64).

- Indirect diagnosis - antibody detection: As horses will shed virus before showing clinical signs and may shed for only a short period of time, it is important to also collect a blood sample when taking a nasopharyngeal swab sample during the acute phase of infection. If the shedding window has been missed, then the swab sample may test negative; however, virus infection can still be confirmed by serology. The test is comparative, so the acute sample needs to be taken as early as possible, while a convalescent sample needs to be taken any time starting from two weeks later. In the absence of recent vaccination, a four-fold rise (seroconversion) in antibody titre between the acute and convalescent samples will confirm recent virus infection. Even if is no longer required, some vaccines still contain an H7N7 antigen. This can be useful when interpreting serological test results by differentiating infection from vaccination (DIVA). In this scenario (where a vaccine containing H3N8 and H7N7 antigens had been used), a seroconversion on paired blood samples to both H7N7 and H3N8 antigens would suggest recent vaccination, whereas seroconversion to just H3N8 antigens would suggest recent infection (Table 1). However, to enable DIVA, the full vaccination history of the horse, including the vaccines used, is essential (83).

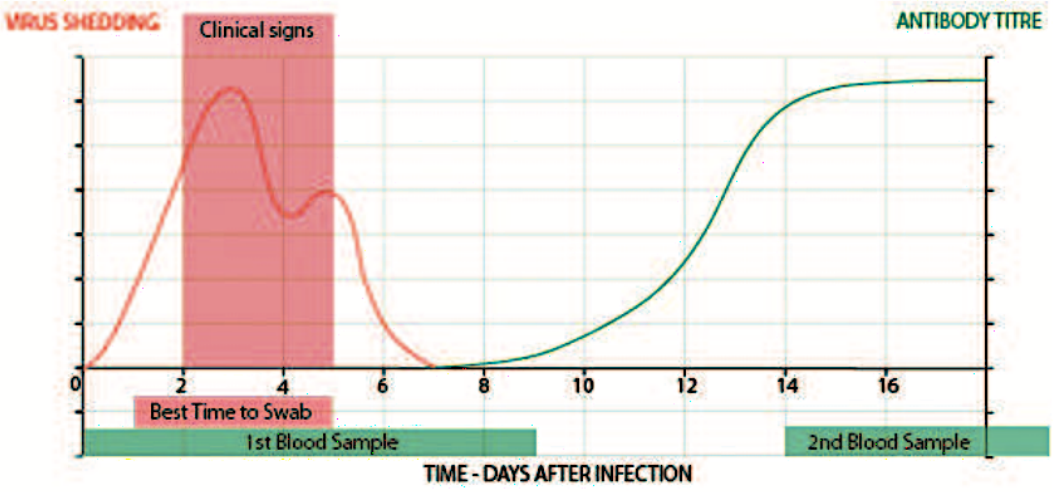

Figure 10: Distribution of viral shedding and antibody titres post infection indicating when to sample
('Diagnosis of Equine Influenza', Adam Rash, Veterinary records; Surveillance focus (July2017))

	Day 1, fist sample	Day 14, second sample
EI H7N7 – Prague'56 antibody	0	0
EI H3N8 – Miami '63 antibody	0	256
EI H3N8 – Nmkt '93 antibody	16	256
Test reading titres: 0, 16, 32, 64, 128, 256, 512, 1024, >1024 (maximum dilution at which antibody is detected)		

Table 1: Example of a seroconversion indicating recent infection with H3N8 viruses

- HI: HI tests are simple, sensitive, inexpensive, and rapid and therefore are often the method of choice for assaying antibodies to IAV. The test relies upon the hemagglutination activity of the influenza HA and the ability of HA-specific antibodies to inhibit the virus from agglutinating erythrocytes. Basically, dilutions of serum are incubated with virus, and erythrocytes are added. After incubation, the HI titer is read as the highest dilution of serum that inhibits hemagglutination. A fourfold or greater increase in HI antibody titer is regarded as evidence of infection. HI antibodies define subtype-specific antigens on the virus particle, thus allowing the differentiation of EI H3N8 and H7N7 subtypes. One of the main shortcomings of the HI test is the wide inter-laboratory variation, with results for the same serum sample varying as much as 100-fold. A study comparing the sensitivity of HI and SRH tests on human sera found that both tests had similar sensitivity, but the inter-laboratory reproducibility of HI was significantly lower (64).
- SRH: For SRH tests, sheep erythrocytes, which have previously been incubated with IV, are mixed with guinea pig complement and incorporated in agarose gels. Then, heat-inactivated serum samples are added to wells cut into the gel, and the antibody titer is determined based on the zone of hemolysis induced by diffusion of the antibody-positive sample from the well. An increase of 50% or 25 mm^2 is considered evidence of recent infection. Since it has been found that the level of antibody measured by SRH after

vaccination correlates well with the level of protection, SRH could be used to predict the level of antibody-mediated immunity and determine the need for revaccination (64).

- ELISA-Based Assay: ELISAs detecting antibodies to EI HA (H3N8) have been developed as an alternative method to traditional HI tests and were found to be sensitive, rapid, and reproducible. Because horses are often vaccinated, and conventional serologic tests do not provide information as to whether antibodies were produced in response to infection or vaccination, an ELISA aimed at the detection of antibodies to the NS1 protein has been developed. Because antibodies to NS1 could be demonstrated only in IV–infected horses, and NS1 is antigenically and genetically highly conserved across IAVs, NS1 is a good candidate for a differential diagnostic marker, capable of distinguishing infected from vaccinated horses. Thus, the ELISA is considered a useful tool to distinguish post-vaccination antibody titers from those generated by recent infection (64).

Differential Diagnosis

In a group of susceptible horses, a presumptive diagnosis of IV infection can be made based on the rapid spread of an acute, febrile respiratory disease characterized by a dry, hacking cough. However, laboratory diagnosis is required to confirm and differentiate influenza from *Equine herpesvirus types 1 and 4* (EHV-1, EHV-4), *Equine viral arteritis* (EVA), and other respiratory pathogens like the *Rhinovirus A and B*, the *Equine adenovirus 1 and 2*, the *Reovirus* and also the CoV. Some bacterial infections, like the one from *Streptococcus equi*, can also exhibit similar symptoms (75).

3.8 Treatment

Once a horse is demonstrating clinical signs of EI (cough, fever, serous nasal discharge), treatment is largely symptomatic. Strict stall rest and adequate nutrition cannot be emphasized enough, to allow the horse a complete and uncomplicated recovery (76). As a general protocol, horses should rest for the number of days/weeks equal to the number of days/weeks they had suffered fever, to allow for the recovery of the respiratory epithelium. (60). Returning to work before this time may lead to the establishment of secondary bacterial infections and long-term

complications such as myocarditis and recurrent airway obstruction RAO (76). Free airflow should be present in the stall during the resting period so that good quality oxygen supply will be available. Good hygienic food, water and dust free bedding materials should be provided at the stall (60). Electrolyte solutions may be provided in addition to clean plain water. These hydrating fluids should be placed so that they are easily accessible to the febrile and lethargic horse. Rest of greater than 4 weeks is recommended for horses showing elevations in muscle enzymes. Nonsteroidal anti-inflammatory drugs (NSAIDs) like phenylbutazone, flunixin meglumine or dipyrone may be administered to counteract fever and subsequent inappetence, but should be used with caution in dehydrated horses. The veterinarian should also be cognizant that NSAIDs may mask some clinical signs. Antimicrobials sre indicated if the horse is at risk of secondary bacterial infection or is demonstrating clinical signs (fever of greater than 5 days duration, mucopurulent nasal discharge, and adventitial lung sounds). Veterinarians should consider using penicillin G or trimethoprim/sulfonamide over a period of 7 to 10 days that extends at least 3 days past the resolution of clinical signs, as *Streptococcus* spp. and *Actinobacillus* spp. are commonly involved. Antitussives are contraindicated, as the cough reflex enhances clearance of virus. Bronchodilators, such as clenbuterol hydrochloride, are of limited value in the case of uncomplicated infection, as the pathophysiology of the disease does not include significant bronchoconstriction.

There are no specific antiviral drugs available on the market for the treatment of EIV (60): antiviral agents such as amantadine and rimantadine are of little benefit to an already infected horse; however, these agents may be useful to protect susceptible an valuable horses during an outbreak. Ideally, antivirals should be administered before exposure to the virus. Horses that have been in contact with infected horses may be treated prophylactically or should have their temperature monitored two to three times daily and therapy commenced if the rectal temperature rises above 38.5°C. Amantadine has been shown to prevent disease in 90% of experimentally infected horses. However, it has also been reported to induce seizures that can be fatal. In acute situations, intravenous administration at a dose of 5 mg/kg bwt every 4 hours has been reported to allow for effective plasma and respiratory concentrations. Rimantadine is a safer and more effective derivative of amantadine. Oral administration has been shown to be effective at a dose of 30 mg/kg bwt twice daily for 7 days, beginning 12 hours before exposure. This regime experimentally decreased rectal temperatures and lung sounds (76). Among other medications

that can be used, we find the neuraminidase inhibitors such as peramivir, recommended at the early stage of infection. This drug was found to reduce virus shedding and, as such, limits the spread of infection from one horse to another. Peramivir is a selective NA inhibitor, having a strong affinity for NA and with a slow off-rate of NA from the NA-peramivir complex, providing a prolonged inhibitory effect and subsequent lower dose requirement. Single intravenous dose of peramivir treatments (7.8–9.3 mg/kg of body weight) exhibited significant reduction in pyrexia, nasal discharge and cough, as well as the duration of viral shedding. This indicates the great potential of peramivir as treatment of EI and as a means to contain the spread of the disease. Some other promising therapies includes the use of cytokines, microRNA, si-RNA, TLRs, potent immunomodulators, nanotechnology-based therapeutics, herbal and plant metabolites, and others which may counter EIV and need to be exploited optimally (60).

3.9 Prevention and prophylaxis

3.9.1 Biosecurity

The control of EIV infection can be substantially addressed by adequate husbandry procedures (64). Biosecurity measures and appropriate management practices are essential for the prevention and control of EI. Strategies that contribute toward the control of EI include following strict biosecurity measures, restricted movement and traffic, proper quarantine practices, and post-vaccination surveillance programs. In the event of an outbreak, the adoption of biosecurity measures can provide protection to horses. Simple preventive measures like personal hygiene, decontamination, and other biosecurity practices prevented the spread of infection in 2007 in Queensland, Australia (60). An hygiene protocol practices devised by a practice in which all veterinarians were involved on a daily basis in visiting infected premises, including sampling, handling of equines, treatment of clinical cases, and other common veterinary work has been described, which should be strictly followed in the face of an EI outbreak (84). The implementation of strategies like the *high health, high performance* (HHP) by OIE outlines the mitigation measures for limiting the spread of equine diseases including EI. There are various factors and many different players responsible for the prevention and control of EI in equine sports arenas: vaccine producers, vaccine regulators, OIE, various government bodies in different countries, veterinary practitioners, owners, riders, trainers, etc. Each has a

unique and important role to play in the proper management of equestrian sports. A cross sectional study was conducted on 759 Australian horse owners to determine their biosecurity practices and perceptions. The results demonstrated that young people or people with no commercial involvement with horses or likely to suffer no business impact as a result of an EI outbreak, were more likely to have poor biosecurity compliance (60).

3.9.2 Vaccination

The prevention and control of EI depends on vaccination and the application of management rules designed to reduce the exposure of susceptible horses to the virus and the generation and aerosolization of large amounts of virus. It has been suggested that 70% of a given population of horses needs to be fully vaccinated to prevent epidemics of influenza (61). The main cause of EIV infection is the H3N8 subtype and vaccine virus different from prevailing subtypes leads to subclinical infection, which is followed by viral shedding from vaccinated animals as well. IAVs are able to evade host immunity even in vaccinated horses. The study of intra- and inter-host evolution of EIV in vaccinated horses revealed a similar level and structure of genetic diversity to those in naïve horses. However, intra-host bottlenecks were more stringent in vaccinated animals and mutations were present near putative antigenic sites (60). EI vaccines have been available since the 1960s and vaccination is mandatory in countries like the UK for racing Thoroughbreds since 1981. The following elements are taken into account for the commercialization of an EI vaccine: the safety of the product, a demonstrated efficacy and protection against at least one of the strains contained in the vaccine, with a significant reduction in clinical signs of disease and virus shedding (85).

The role of antigenic drift

It has been shown that animals immunized with heterologous strains are not fully protected and have been shown to excrete significantly more virus than animals vaccinated with homologous strains, despite having comparable antibody titres (61). Antigenic drift at the HA gene level led to vaccine failure in various parts of the world. Genetic reassortment taking place during a mixed infection can lead to the development of new strains and ultimately vaccine failure. To deal with this problem, continuous checks and monitoring through surveillance programs and updating of vaccines with recent strains remains the best and most effective way of preventing and

controlling this disease. Some study revealed that the EIV strain changed from a prevalence of the Eurasian lineage to clade 1 and clade 2 of Florida lineage. Accordingly, changes made in vaccine strains lead to scientific or effective control of the disease. A study was conducted in the UK to understand the antigenic changes occurred in the EIV isolates during 2013–2015 to get a clear picture on the efficacy of the vaccine. The results showed that the Florida sublineage clade 2 was diverging. The study also suggested the inclusion of the Florida sublineage clade 1 and 2 in the vaccine for EIV. Thus, an epidemiological surveillance of IV along with monitoring the impact of immunization is extremely important. The disease control is influenced by the antigenic variations of the virus, target group, goal of immunization, rate of antigenic variation, and vaccine composition (60). For this reason, the OIE issued its recommendation on EIV vaccination in the "Terrestrial Animal Code". A formal EI global surveillance program has been in place since 1995. The OIE Reference Laboratories and other collaborating laboratories collect data on outbreaks of EI and strain variation, reviewed annually by an Expert Surveillance Panel (ESP) including representatives from OIE and WHO. This panel makes recommendations on the need to update vaccines, published annually in the "OIE Bulletin" and on the OIE website. The criteria for updating EI vaccines are similar to those for human influenza vaccines and based on the analysis of antigenic changes, genetic changes and, when possible, supporting experimental challenge data. Many vaccines still contain an H7N7 strain. However, the ESP has recommended that the H7N7 component should be omitted as reports of infections with this subtype have not been substantiated during the past 30 years. On the contrary, for H3N8 antigenic and genetic variants of the viruses co-circulate, which points to the need to include a strain or strains that are epidemiologically relevant. For this reason, all vaccines should contain the contemporary clades 1 and 2 of the Florida sublineage H3N8 EIV strains (86).

Type of vaccines

- Killed/Inactivated Vaccine: refers to killed vaccines including whole cells of the H7N7 and H3N8 subtypes. Inactivated vaccines protect horses from disease with no viral shedding. Inactivated vaccines require a booster regimen for better efficacy and are best suited for vaccinating dams so as to protect foals from infection. Some formulations frewuently usded to inactivate viruses in vaccines are formaldehyde, β-propriolactone, ethylene-imine and thimerosal. Killed vaccine adjuvanted with immune stimulating

complexes matrix (ISCOM) has been formulated and found to provide longer duration immunity and be safe to use in pregnant mare and foals. Adjuvants used in vacines are immune stimulating components, which aid in boosting humoral and cell mediated response. They are aimed at presenting antigen to immune cells, targeting toward antigen-presenting cells, and the enhancement of cell-mediated immunity. The administration of combined inactivated EIV vaccine with equine herpesvirus vaccine has shown increased immune response against EIV (60).

- Subunit vaccines: this kind of vaccines encompass purified viral antigens. Among these, two main vaccines are the ISCOM-based vaccines or ISCOMATRIX vaccines. ISCOM based vaccines have ISCOM particles with cage like structures formed spontaneously by viral protein combination with cholesterol, phospholipids and Quillaja saponins. ISCOMATRIX are essentially like vaccines but do not possess cage like structures. ISCOM based EIV vaccine induces strong antibody response along with elevated levels of IFN-γ. The use of ISCOM-based EIV vaccines through an intranasal route in a systemic prime/mucosal boost vaccination program gave transient higher virus-specific IgA in nasal wash. Cell-mediated immune response is also stimulated (60).
- Cold adapted (Ca) vaccines: These vaccines have been developed with the aim of improving both humoral and cellular immunity, therefore mimicking the protective immunity generated by natural infection. The Ca EIV vaccine strain is able to replicate efficiently in the upper respiratory tract to generate local and systemic immune responses. The most advantageous part is that the Ca strain doesn't replicate in the lower respiratory tract the niche of wild type IV and therefore symptoms like bronchitis, pneumonia, and pulmonary edema do not occur (60).
- Modified-Live Cold-Adapted EI A2: These vaccines are administered intra-nasally and have been found to be safe and reduce the onset of EIV outbreaks. Intranasal vaccine confer local protection against EIV, though the circulating level of antibody reduces as time progresses. Although the administration of this vaccine to yearlings was found to be safe, it is not recommended for use in pregnant mares in late gestation (60).
- Canarypox Vector Vaccines: The canarypox-vectored vaccine can evoke antibodies against HA only. As a consequence, in ELISA diagnostics, NP can be detected and

discrimination between infected and vaccinated animals is possible. Since canarypox vectored vaccines provide a longer duration of immunity, they sufficiently protect the equine population during an annual period between booster doses (60). This kind of vaccine is largely used in association with the tetanus (*Clostridium tetani*) toxoid (87).

- Modified vaccine Ankara Vector (MVA): In the case of this vaccine, the vector carries HA and NP gene. It creates good antibody response, as well as IFN-γ and mRNA production (60).
- Modified EHV-1 Vectored Vaccine: EHV-1 vectored vaccines carrying the H3 gene of EIV generated robust protective immune responses against influenza in horses. This vaccine was found to be significantly more effective in terms of reduced viral shedding and mild clinical symptoms during the 1997 EIV outbreak in Australia (60).
- Reverse Genetics-Based Vaccines: The field of reverse genetics (plasmid-based) allows for the simultaneous expression of the components of the virus involved in the replication of the viral genome and gene transcription (60).
- DNA vaccines: DNA vaccines delivered through gene gun have been suggested for EIV. DNA vaccines carrying HA gene elicited good cell-mediated and humoral immunity triggering an IgG response, but it does not provoke IgA responses. These vaccines that are based on the H3N8 virus are quite safe and effective in eliciting both homologous and heterologous immune response . For the purpose of DNA vaccination, intra-lymphatic immunotherapy (ILIT) is the recent strategy, into which HA encoding plasmid is being injected in the sub-mandibular lymph node on days 0, 28, and 98. The EIV specific immune response induced by such vaccination is comparable to the immune response evoked after natural infection, but lower than the conventional canarypox-based EIV vaccine. Intranodal immunization allows vaccine delivery directly at the site of B and T lymphocytes priming, therefore improved immunity is expected. However, the practical feasibility of this technique in the field is questionable due to the skills required for sub-mandibular injection, with the risk of inaccurate injection in the lymphonode (60).

The most common types of vaccines in use today are the one for the intranasal (IN) somministration and the inactivated vaccine for intramuscular (IM) administration route (88).

When to vaccinate

- Adult horses:
    - Adult horses previously vaccinated against influenza should be revaccinated annually. Horses with an increased risk of exposure may be revaccinated every 6 months. Some facilities and competitions may require vaccination within the previous 6 months to enter.
    - Adult horses previously unvaccinated against influenza or of unknown vaccine history:
        - Inactivated vaccine: Dependent upon on the manufacturer's product recommendation, the vaccine may be a two or three dose series with a 3 to 4-week interval between doses (IM injection);
        - Modified live vaccine: Administer a single dose (IN application);
        - Revaccinate annually.
- Pregnant mares: vaccination is important for the production of a colostrum rich in antibodies against EI.
    - Pregnant mares, previously vaccinated against influenza:
        - Inactivated vaccine: Annually with one dose administered 4-6 weeks pre-partum (88);
        - IN administration vaccine: induces good protection, it does not routinely stimulate high levels of circulating antibody (60)
    - Pregnant mares, unvaccinated or having unknown vaccine history:
        - Inactivated vaccine: Dependent upon on the manufacturer's product recommendation, the vaccine may be a two or three dose series with a 3 to 4-week interval between doses (IM), with the last dose administered 4-6 weeks pre-partum (88).
- Foals: The antibody status of a mare at the time of foaling largely determines the post - nursing circulating antibody titer in her foal and, therefore, has a profound impact on the ability of the foal or weanling to respond to influenza vaccines administered during the first year of life. Maternal antibodies have been shown to completely block the serologic response of foals to a primary immunization series comprising two or more doses of inactivated influenza vaccines when the first dose is administered when the foal is

younger than 6 months of age. Maternal antibody interference may persist until 9 months of age or beyond for foals with very high antibody titers post-nursing (76).

  - Foals of mares vaccinated against influenza in the pre-partum period:
    - Inactivated vaccine: best delayed until foals are at least 6 months, and preferably 9 months. Dependent upon on the manufacturer's product recommendation, the vaccine may be a two or three dose series with a 3 to 4-week interval between doses (IM);
    - Modified live vaccine: Administer a single dose (IN) in foals 11 months of age or older
  - Foals of mares previously unvaccinated against influenza or having unknown vaccine history in the pre-partum period:
    - Inactivated vaccine: Dependent upon on the manufacturer's product recommendation, the vaccine may be a two or three dose series with a 3 to 4-week interval between doses (IM) starting at 4-6 months of age.

- Horses having been naturally infected and recovered: Horses with a history of influenza infection and disease are likely to have immunity to the specific strain for more than 1 year, but booster vaccination is recommended 6 months after disease occurrence due to variations in the influenza strain (88).

- Outbreak vaccination: The decision on whether to vaccinate or not in the face of an outbreak is dependent on many factors, the most important of which are the age, vaccination status, and size of the population of horses at risk, the elapsed time since onset of the outbreak, the rapidity with which a diagnosis can be confirmed, the layout of the physical facilities, and availability of personnel. Outbreaks of influenza at racetracks and similar facilities typically take one month or more to spread through the entire population. Therefore, sufficient time exists to enhance the immune protection of many at-risk horses while implementing other management strategies to minimize disease spread. In such situations, it is prudent to booster vaccinate those horses that have been on a regular influenza vaccination program but have not been revaccinated within the previous 3 months. For horses that have not previously been vaccinated or are of unknown vaccination status, it is important to induce protection as quickly as possible (76).

Fédération Equestre Internationale (FEI) and Federazione Italiana Sport Equestri (FISE) recommendation

FEI 2021 veterinary regulations art. 1003 requirements are:

1. All proprietary EI vaccines are accepted by the FEI, provided the route of administration complies with the manufacturer's instructions (*i.e.*IM or IN).
2. An initial primary course of two vaccinations must be given; the second vaccination must be administered within 21-92 days of the first vaccination.
3. The first booster must be administered within 7 calendar months following the date of administration of the second vaccination of the primary course.
4. Booster vaccinations must be administered at a maximum of 12-months intervals. However, horses competing in events must have received a booster within 6 months +21 days (and not within 7 days) before arrival at the event.
5. Horses may compete 7 days after receiving the second vaccination of the primary course.
6. Horses that have received the primary course prior to 1 January 2005 are not required to fulfil the requirement for the first booster, providing there has not been an interval of more than 12 months between each of their subsequent annual booster vaccinations.

If horses do not respect these recommendations, they can't take part in competition (89).

The FISE regulations integrate the FEI regulations (90).

3.10 Disease management and control of outbreaks

In the case of outbreaks, the following recommendations must be followed:

i. Quarantine all horses demonstrating clinical signs, even before a definitive diagnosis is confirmed. Quarantine procedures will depend on whether isolation facilities are available, the nature of the facilities in which the horses are currently housed, and the number of horses potentially exposed.

ii. Isolate horses that have been in contact with affected horses. Prevent contact between these potentially exposed animals and non-exposed horses, particularly those likely to be the most susceptible (*i.e.*, foals, young immunologically naïve horses, unvaccinated or not recently vaccinated horses, stressed or pregnant horses).

iii. Be especially cautious of well-vaccinated horses that have been exposed to infected horses, as they may still act as subclinical virus shedders, yet may show mild or no clinical signs and will not be easy to detect as they shed virus transiently. These horsescould develop clinical signs if the vaccine strain is not closely related to the infective strain.

iv. Carefully monitor potentially exposed horses for early signs of infection and reduce the intensity of exercise if possible.

v. Suspend training of all affected horses as soon as clinical signs are observed.

vi. Avoid intensive housing and crowding.

vii. Stop all horse traffic on and off the premises.

viii. Conduct diagnostic techniques: collect nasopharyngeal swab that will be used for ELISA, PCR and virus isolation. If necessary, collect serum samples for later paired serology.

ix. Keep all suspect horses isolated for 2 weeks.

x. Booster vaccinate non-exposed horses (76).

xi. The IV is fragile and can be quickly inactivated by exposure to ultraviolet light or sunlight and by heating. Phenyl (1%), Chloroxylenol (Dettol) (1 %), Chlorohexidine (1-2 %), formalin (0.2-0.5 %) when applied for 20-30 minutes are effective in inactivating the virus. The exposed parts, such as hands, should be washed with soap and warm water. All surfaces like clothing, equipment and hands should be cleaned and disinfected after the exposure to horses known or suspected to be infected. Inside surfaces of vehicles used for transportation should be cleaned of and sprayed with disinfectant.

xii. Public awareness: Awareness in the horse industry is a must to gain cooperation and build confidence in controlling the disease. All state Animals Husbandry Departments working for the welfare of the equines and organized industry should be fully aware about the disease and measures to be taken to control the disease. This will help in close monitoring and surveillance by seeking active help from individual horse owners and organized industry to identify fresh cases and act suitably and promptly (79).

These are general recommendations also proposed by the OIE. In Italy, in case of an outbreak of EI, one must follow the guidance of the “Regolamento di polizia veterinaria” with reference to art.

1, 10, 16 and 98, with particular attention to the last one that is specific to influenza affections of equids (46).

3.11 Public Health Considerations

In addition to horses, IAVs infect a large variety of species, including humans, pigs, poultry, aquatic birds, sea mammals, and most recently, dogs. While IAVs are occasionally directly transmitted from one host species to another (*e.g.,* the transmission of H5N1 avian IAV to humans in Asia), biologic barriers exist that often limit such spread. This appears to be particularly true for equine-lineage IAVs. Although the susceptibility of human volunteers to infection with H3 equine-lineage viruses has been demonstrated, phylogenetic analyses indicate that exchange of IV genes between horses and other species is limited. These findings suggest that horses may be an isolated or "dead-end" reservoirs for IAVs. The notable exception to this is the recent transmission of an equine-lineage H3N8 virus to dogs (64), which we are about to outline in detail in the following paragraphs.

With regard to human health, historical, observational, and experimental data are supporting the premise that EIV infections occasionally occur in man. While in recent years human infections with EIVs have not often been associated with signs of infection, the propensity for IAVs to change makes these viruses worthy of our attention. In particular, should H7N7 EIV strains again emerge, most humans would have little cross-reacting antibody, and the threat to humans might be quite different from that commonly seen today for H3N8. Studies suggest that these observations support the need for close surveillance for novel IV emergence among equids (91).

Part 2: The spillover of H3N8 EIV to dogs

Prior to 2004, dogs were not considered a reservoir species for IAVs because: (a) they did not appear to maintain their own IV subtype and (b) no sustained transmission of any IV between dogs had ever been recorded (92). Despite this, the interspecies transmission of EIV to dogs represents an unprecedented interspecies transmission event (93).

3.12 Historical aspect

The greyhound racing industry had been struck by significant respiratory problems in the dogs associated with the tracks for several years. Tests for the known pathogens linked to respiratory disease in dogs failed to identify the cause of these recurring problems (94).

H3N8 CIV was first isolated in January 2004 from racing greyhounds affected with respiratory disease in Florida. Thousands of greyhound dogs, at tracks in nine states, were subsequently affected during multiple respiratory disease outbreaks from 2004 to 2006. The virus has been recognized in a majority of states in the contiguous USA, affecting both racing greyhounds and pet dogs. Since the discovery of canine influenza (CI) in the USA, sporadic interspecies transmissions of H3N8 IV from horses to dogs have been reported in other countries. In the UK, a retrospective study revealed that an H3N8 EIV caused a respiratory outbreak among English foxhounds in 2002, which was lately confirmed by many different investigations. During the 2007 epidemic of H3N8 EI in Australia, several dogs in contact with infected horses developed influenza-like illness. Multiple cases were confirmed (95). Before 2004, there was little suggestion that dogs would be a natural host for influenza infection or epidemic spread. However, in 2004, the H3N8 variant of the EIV was isolated and identified as the cause of the epidemics. The H3N8 CIV emerged around 1999 in Florida by transfer of an intact H3N8 EIV virus to dogs, following which the virus circulated continuously among dogs in the United States for more than a decade (96).

Following this first virus isolation in January 2004 in Florida, a second IV was isolated in July 2004 from the lungs of a greyhound that died at a track in Texas (canine/TX/04). Sequence analysis of the virus showed at least 99% nucleotide homology with canine/FL/04 and confirmed the H3N8 equine link to CIV. In April 2005, a respiratory disease outbreak occurred

at an Iowa racetrack, resulting in an essentially 100% morbidity rate, but less than 5% of dogs died with signs similar to the cases in the January 2004 outbreak in Florida. Sequence analysis showed the link to recent H3N8 equine viruses, and subsequent comparisons among the Florida, Texas, and canine/IA/05 isolates showed a common lineage. In aggregate, these data confirmed the widespread CIV infections in greyhounds in the racing industry and made it virtually impossible to deny the existence of an IV in canids capable of horizontal transmission among the dogs. As indicated previously, the problem of respiratory disease and outbreaks in the greyhound racing industry had been evident for several years. In light of this new evidence, archived samples from outbreaks prior to January 2004 were re-evaluated. Examination of tissues from a dog that died in March 2003 yielded another IV isolate (canine/Fl/03) that had high sequence homology to canine/FL/04. Out of a limited number of sera available from previous outbreaks, one of four sera from 2000 was positive for antibodies to CIV. Thus, CIV existed in the greyhound population for at least several years before its initial detection in 2004. Although the finding of CIV in greyhounds was a significant discovery, the focus of the investigation quickly became the non-racing canine population. Serologic data from sera collected from shelters and pet clinics in Florida and New York demonstrated the presence of CIV in the pet population. The isolation of CIV from pet dogs in Florida and New York in 2005 established conclusive proof that CIV infections were not restricted to greyhounds under racing conditions and that all breeds seemed to be fully susceptible to CIV. Data from New York established that the CIV was the cause of a major epizootic in the New York City area in the summer of 2005 and clearly established that CIV had moved from the pet population of Florida to the Northeast by mid-2005. The movement of CIV in the canine pet population has been unpredictable, as is the movement of dogs by owners and the various rescue organizations. The Florida–New York area link is understandable, given the large number of individuals that move between these locations in the spring and fall. Serologic data on CIV began to be collected in the fall of 2005 at the Animal Health Diagnostic Center (AHDC) at Cornell University, Ithaca, New York from submissions throughout the country. Initial results clearly showed the presence of CIV in Florida and the New York City area (New York, New Jersey, and Connecticut). A few animals tested positive in Arizona and California at that time. As there were no isolates of CIV from this region, one could not tell whether this was a CIV infection (greyhound track in Arizona) or simply another type A IV in dogs. Seroconversions to CIV were identified in private

practices in the Washington, DC area and in a shelter in Delaware. Inexplicably, the virus seems to have disappeared from these areas, because no further virus activity has been noted to date (94). In December 2006, started to appear reports of unusual respiratory outbreaks in kennels and shelters in the Denver, Colorado area. In January 2006, serologic data showed the presence of CIV in the Colorado area and subsequent testing detected CIV by PCR assay and by virus isolation. The virus is now enzootic in Colorado, as it is in Florida and New York. The virus was also detected in Wyoming and San Diego, California in May 2005. The San Diego outbreak was linked to the movement of a dog from Colorado to southern California. Strict quarantine of the affected kennel seemed to prevent the spread to other locations in the San Diego area. Seroconversions were also reported in Utah in August 2006, presumably as an offshoot of the Colorado epizootic. Other outbreaks, as defined by isolation of CIV, were noted in Kentucky (September 2006), western Pennsylvania (January 2007), eastern Pennsylvania (July 2007), and Los Angeles, California (July 2007). These were also to the ongoing presence of the virus in Florida, the New York City area, and Colorado. All other areas of the country seem to be unaffected by CIV as of March 2008 based on the lack of viral isolates and the lack of positive sera. The somewhat sporadic movement of the virus is certainly related to the movement of dogs, but also to the exposure and minimal movement controls of susceptible dogs in new locations. As a case in point, a dog was moved from New York City to Ithaca, New York at the end of December 2006. It developed respiratory signs on arrival and was exposed to other dogs in the kennel. CIV was diagnosed based on serology, and a voluntary quarantine was placed on the kennel. Others in the group became infected as determined by serology, but the virus did not spread in the community because of the movement restrictions and lack of contact with other susceptible dogs (94).

The isolation of CIV in 2004 put the basis for further studies in other countries to determine the presence of influenza virus in dogs. The only published reports to date have come from England. In a retrospective study, researchers at the Animal Health Trust detected EIV as the cause of a respiratory outbreak in a quarry hound kennel in 2002, but there was no evidence for ongoing transmission. Limited sequence analysis of nucleic acid recovered from fixed tissues from a death dog with the confirm of the equine origin of the H3 virus linked to the outbreak. No data were presented to show that CIV was involved in the outbreak, however. In a second report, serologic evidence was presented suggesting that foxhounds became infected with a newly

introduced H3N8 virus in the spring of 2003 (97). Also in this case, there was no evidence of horizontal transmission or a link to CIV. The epizootic of H3N8 in equids in Australia in 2007 also resulted in infected dogs. Animals in contact with horses have shown seroconversion and, although clinical signs were noted in a moderate proportion, there was no evidence of horizontal transmission among the dogs. These cases seem to be EIV in dogs and not CIV infections (94). The virus outbreak in Colorado died out around 2012, and had reached very low levels in New York by mid-2016, with very little disease being reported after that time (96).

It is not known when, where, or how the H3N8 EIV was first transmitted to dogs in the USA. Close contact transmission from infected horses is the most accredited transmission route. These transmission routes were also proposed for UK foxhounds that were affected in 2002, in addition to the fact that they were fed with horse meat shortly before the outbreak, leading to speculations that they may have inhaled virus during consumption of raw lung material. Natural infection of dogs during the recent outbreak of H3N8 EI in Australia in an experimental study in Japan have demonstrated the feasibility of close-contact transmission of EIV the dog (92).

3.13 Characterization of the virus

During the 2004 outbreak, Crawford et al. performed a study by analyzing 22 infected dogs. Two clinical syndromes were considered: a milder illness characterized by initial fever and then cough for 10 to 14 days with subsequent recovery (14 dogs) or a peracute death associated with hemorrhage in the respiratory tract (8 dogs for a case-fatality rate of 36%). Postmortem examinations were performed on six of the eight fatal cases. All dogs presented with extensive hemorrhage in the lungs, mediastinum, and pleural cavity. Histological examination of the respiratory tract revealed tracheitis, bronchitis, bronchiolitis, and suppurative bronchopneumonia. The epithelial surface and airway lumens in these tissues were infiltrated by neutrophils and macrophages.

Preliminary evidence of an IAV type was provided by a commercial ELISA kit for detection of the nucleoprotein of influenza A and B viruses, and by PCR analysis using primers specific for the matrix gene of IAVs. In addition, the HA activity was inhibited by reference antisera to the EI A H3 subtype, but not by antisera specific for avian, swine, and human influenza A subtypes H1 to H11 and H13.

To characterize the molecular properties of the virus, they determined the nucleotide sequences of the eight RNA segments of the viral genome. Sequence comparisons with known IV genes and phylogenetic analyses indicated that the eight genes of the canine isolate were most similar to those from contemporary EIA (H3N8) viruses, with which they shared 96% sequence identity. In contrast, representative genes from avian, swine, and human IA isolates had 80 to 94% identity with the canine isolate. These data identified the canine isolate, named A/canine/Florida/43/2004 (canine/FL/ 04), as an influenza A H3N8 virus closely related to contemporary EIV. Because all genes of the canine isolate were of EIV origin, the study concluded that the entire genome of an EIV had been transmitted to the dog (93).

To better understand the role of the canine/FL/04 virus in the clinical and pathological observations in the greyhounds, the researchers performed IHC on lung tissues using a monoclonal antibody to IA H3. To further determine the involvement of a canine/FL/ 04-like virus in the etiology of the respiratory disease outbreak, they analyzed paired acute and convalescent sera from 11 sick dogs and 16 asymptomatic contacts for virus-specific antibodies using HI and microneutralization (MN) assay. Seroconversion, defined as a greater than fourfold rise in antibody titers to canine/FL/ 04 from the acute to convalescent phase, occurred in 8 out of 11 (73%) sick dogs in both assays. Seroconversion occurred in 6 out of 16 (38%) asymptomatic contacts in the HI assay, whereas 8 out of 16 (50%) seroconverted in the MN assay. The seroconversion data demonstrated infection of the dogs with a canine/FL/04-like virus which coincided temporally with the onset of a respiratory disease in most animals. Single serum samples were collected 3 months after the outbreak from an additional 46 dogs with no clinical signs housed with the sick dogs. Of these, 43 (93%) were seropositive in both assays. For the total population of 73 dogs tested, 93% were seropositive in both assays, including 82% (9/11) of the sick dogs and 95% (59/62) of the healthy contacts. The high seroprevalence in dogs with no history of respiratory disease indicates that infections with CIV can be subclinical and suggests efficient spread of the virus among dogs. Post-mortem examination of two dogs on day 5 p.i. revealed necrotizing and hyperplastic tracheitis, bronchitis, and bronchiolitis similar to those found in the greyhounds, but there was no pulmonary hemorrhage or bronchopneumonia. Infectious virus was recovered from the lung tissue of one of the dogs. Postmortem examination of the remaining two dogs on day 14 p.i. showed minimal histological changes in respiratory tissues, no viral H3 antigen by IHC, and no recovery of virus from lung homogenates.

Seroconversion in the last two dogs was detected in MN assays by day 7 p.i., with a further two to threefold increase in antibody titers by day 14. These results finally established the susceptibility of dogs to infection with canine/ FL/04, as evidenced by the febrile response, virus shedding from the upper respiratory tract, presence of viral antigen and infectious virus in the lungs, histopathological findings typical for influenza, and seroconversion (93).

To investigate whether a canine/FL/04-like IV had circulated among greyhound populations in Florida before the January 2004 outbreak, researchers tested archival sera from 65 racing greyhounds for the presence of antibodies to canine/FL/04 using the HI and MN assays. The results suggest that the virus circulated among racing greyhound long before then the 2004 outbreak. This finding was further supported by the isolation of three closely related IVs from fatal canine cases from different geographic locations over a 16-months period, together with the substantial serological evidence of widespread infection.

The high prevalence of CI infection in racing greyhounds suggested that the pet dog population might also be at risk of infection. Serological tests were also performed on 70 dogs with respiratory disease in a shelter facility in northeast Florida, four veterinary clinics located in the northeast, north central, south, and southwest regions of Florida, and one veterinary clinic located in New York. 96% of the shelter and pet dogs were positive for antibody to canine/FL/04. The serologic evidence of IV infection associated with respiratory disease in shelter and pet dogs of various breeds indicated the lack of genetic barriers to infection in the dog population and the spread of the virus to pet populations of regions of the country without greyhound racing (93).

The CI genes were most closely related to the equine H3 “Florida lineage” that emerged in the early 1990s. Phylogenetic analysis and pairwise nucleotide sequence comparisons of the other seven genomic segments supported the segregation of the canine genes as a distinct sublineage most closely related to the equine virus lineage. Together with the identification of infected dogs in widespread geographical locations from 2003 to 2005, these data are most consistent with the possibility of a single virus transmission event from horses to dogs with subsequent horizontal spread of the adapted virus in the canine population (93).

The viral HA is a critical determinant of host species specificity of IV. To identify residues within HA that may be associated with the adaptation of an equine virus to the canine host, researchers compared the amino acid sequence of canine HAs to those of contemporary equine

viruses. Four amino acid changes differentiate the equine and canine HA consensus amino acid sequences: N83S, W222L, I328T, and N483T (93). The W222L at the receptor binding site of HA seemed to be particularly involved (98). Although these amino acid changes in the HA are important for the adaptation of an equine virus to dogs, the receptor binding for the SA is also fundamental, as we underlined before. In fact, both equine and canine IV prefer binding to SA α(2 →3)-gal in N-glycolylneuraminic acid (60).

Yamanaka et al. performed a study to test the possibilities of interspecies transmission of EIV to dogs due to close contact with experimentally EIV infected horses. The infected horses were kept with healthy dogs in three groups in close proximity for 15 days and HI test revealed seroconversion in all dogs of two groups, with viral shedding in dogs from two groups without apparent clinical symptoms (99). The same study was also performed in inverse order by the same researchers with healthy horses and CIV infected dogs kept in close contact to investigate interspecies transmission. Though all the dogs infected with CIV presented clinical signs of lung consolidations after euthanasia, none of the horses showed clinical signs, virus shedding, seroconversion or lesions in the respiratory tract (100).

The emergence of CIV, like the emergence of any IV lineage in a novel mammalian host population, may constitute a direct or indirect pandemic risk. While the lack of transmission of EIV or CIV to humans suggests that long-standing infection of a mammal does not necessarily indicate a threat to humans, a better understanding of the ecological, evolutionary, and molecular mechanisms of influenza emergence is essential to accurately determine which viruses pose a risk to human health (95).

Part 3: The canine influenza virus (CIV)

3.14 Influenza subtype H3N2 in dogs

We have already discussed extensively how the EIV H3N8 jumped to dogs and became one of the principal CIV subtypes, but it is not the only example of such jump. For, in fact, a second CIV is the one of avian origin-H3N2 CIV. The H3N2 CIV was first identified in South Korea and China around 2006 and it seems to have arisen by transfer of a single avian virus into dogs around 2005. The virus circulated widely in dogs in both countries after it was identified and was also found in both Northern and Southern China, with some genetic differences developing between the viruses in the different regions, suggesting that they were circulating locally for a number of years with less long-range movement. In early 2015, an outbreak of respiratory disease in the area of Chicago, Illinois was identified as being caused by the H3N2 virus strain. Soon after, it caused an outbreak of disease in Georgia and nearby states. Even if the outbreak in the Southeastern states died out relatively quickly, there were continuing new cases in Chicago and nearby areas, which seemed not to cause any other major secondary outbreaks. A second wave of infections emerged in 2017, with the virus spreading widely for the next year, being first recognized in Florida and causing outbreaks in many other areas of the country over the next several months. The virus involved in that outbreak was a new introduction from Asia, most likely from South Korea. A third wave of infections occurred in 2018, with an initial outbreak in California near San Francisco, followed by a second outbreak in the New York City area.

It is clear that each of the CIV epidemics ultimately derived from a single cross-species transfer event, with H3N8 subtype transferring from horses, while the H3N2 subtype arose from a virus in an avian reservoir. In dogs, both H3N8 and H3N2 CIV infections are associated with mild upper respiratory tract disease, often including frequent coughing and fever. Where it has been examined, infection of the lungs may occur, and that is rarely associated with more severe disease or death. The more severe disease is likely associated with mixed infections by other viruses or bacteria, or with other health issues for the dogs. The natural risks to other animals, including humans, are largely unknown, but no human infections by either strain of CIV have been reported (96).

3.15 Epidemiology and transmission

In companion animal species, CI typically circulates in shelters and boarding facilities after the introduction of an infected animal due to the number of unexposed dogs in the population, as well as the constant introduction of new, susceptible animals into the population (101). CIV is spread through contact with respiratory secretions or contaminated surfaces and inhalation of airborne particles. The virus can be aerosolized for up to 20 feet. Fomites, such as bowls and toys, are a common way for the virus to spread in places where multiple dogs are housed. The virus can also be passed from an infected dog to a healthy dog on the clothing and shoes of staff members. If an infected dog coughs or sneezes, the virus can remain in the air for an extended time before settling on surfaces. The virus can remain infectious on surfaces for up to 48 hours and on clothing for up to 24 hours. Once a dog is infected with the virus, it takes approximately 2 to 4 days for the dog to begin exhibiting clinical signs. The optimal shedding time for the virus is often during the incubation period, before clinical signs develop. This means that a seemingly healthy dog can spread the disease to other dogs before the owner realizes that the dog is sick. Viral shedding drastically declines within the first 4 days that the patient exhibits clinical signs. However, viral shedding can continue for up to 10 days after clinical signs develop. In 20% to 25% of cases, dogs have not exhibited clinical signs while shedding the virus (102).

3.16 Clinical signs

The challenge with CIV is that, like many respiratory pathogens, the clinical signs are often very similar. In fact, in most cases, a clinician would be hard pressed to distinguish a CIV infection from those agents that cause “kennel cough”. Virtually all CIV cases in the canine pet population investigated are linked to shelters, boarding kennels, or “doggie” day care centers, a feature not different from kennel cough. Distinctive characteristics of CIV infections is the degree of morbidity within the facility. For kennel cough, a few dogs exhibit clinical signs, because prior exposure and vaccination reduce the attack rate. For CIV, virtually all dogs are susceptible, regardless of age, and attack rates of 60% to 80% are not unusual. The signs associated with most CIV infections are not pathognomonic (94). Dogs infected with CIV can have general respiratory clinical signs, including lethargy, fever, nasal discharge, and coughing. In mild cases of infection, clear nasal discharge quickly changes to mucopurulent discharge. A dry,

nonproductive cough or a soft, moist cough can also be seen. A low-grade fever (up to 40°C) may be seen in dogs with mild infection. Some dogs may develop a more severe form of influenza that results in pneumonia. Dogs at risk for severe CIV infection can be very young, very old, already immunocompromised by another disease, and/or malnourished (poor body condition). Up to 20% of infected dogs develop severe CIV infection, resulting in a fever > 40°C and an increased respiratory rate. Even young, healthy dogs can develop severe influenza, and patients that exhibit the clinical signs can die if they are not treated quickly (102).

3.17 Diagnosis

The diagnostic methods we have discussed for the EIV are also applicable for the CIV. A thorough patient history is important when dealing with a suspected case of influenza. Clinical signs, along with determining the patient's exposure to other dogs, especially group housing, can alert veterinary staff to the possibility of exposure to influenza infection. Being aware of recent influenza outbreaks in the area is important when pursuing a diagnosis for a patient with respiratory signs. A complete blood count, a chemistry panel, and thoracic radiography are beneficial diagnostic tools. In patients with viral infection, leukopenia can be seen on a complete blood count, or leukocytosis may develop with secondary pneumonia. Thoracic radiographs can show a broncho-interstitial pattern and/or consolidation of lung lobes. If a secondary bacterial infection is present, culture and sensitivity testing of various areas in the respiratory tract can help determine appropriate antibiotic therapy (102). Influenza can be diagnosed from a nasal swab of the patient. PCR is used as the initial screening test to look for H3N8 and H3N2. Virus isolation can also be attempted from the same nasal swab. Paired serum samples can also be collected, where an initial serum sample is compared to another serum sample, collected 2-3 weeks later, to look for antibodies in the serum against influenza (103).

3.18 Treatment

All the general considerations made for the EIV are applicable to CIV. Other than supportive care, there is no specific treatment for CI. Antiviral drugs are typically reserved for use in humans, while oseltamivir is not specifically recommended in companion species due to unknown effectiveness (103). Antibiotics can be used to prevent or treat secondary bacterial

infection. The antibiotic choice should be based on culture and sensitivity testing results. For patients that develop pneumonia, hospitalization is usually required along with appropriate isolation procedures. These patients often benefit from intravenous fluids, oxygen therapy, and nebulization. Caution should be used when administering antitussives. In patients with a productive cough, antitussives are contraindicated. Duration of treatment depends on the severity of the illness. A cough can persist for 10 to 21 days, even with medical treatment (102).

3.19 Prevention

3.19.1 Biosecurity

Minimizing exposure to dogs with unknown health statuses during outbreaks (especially in high density dog areas such as dog parks, shelters, kennels, etc.) is the most practical measure for prevention. After identifying an infection, the focus should be on preventing further spread of the virus. Quarantine of any suspect animals should begin as soon as clinical signs are observed and be continued for a minimum of 21 days if H3N2 is suspected due to prolonged shedding of that virus (a minimum of 7 days is recommended for H3N8 cases). Any surfaces, tools, toys, bedding, clothing, etc., that the affected animal came in to contact with should be cleaned and disinfected with a disinfectant designed to kill IAV. Normal laundering of bedding items in a washer and dryer is sufficient to kill the virus. Moving infected animals to a separate air space, preferably with independent ventilation, may also help to reduce spread within an affected facility due to direct or aerosol transmission. If physical separation in another room is not possible, consider leaving several empty cages between the healthy and sick animals, and try to house the sick animals down-wind from the healthy animals as this will help reduce exposure to aerosolized viral particles (103).

3.19.2 Vaccination

Nobivac Canine Flu H3N8 (Merck Animal Health) and Vanguard CIV (Pfizer Animal Health), which are killed virus vaccines that protect against the H3N8 virus, have been approved by the FDA for vaccination against CIV(102). Combined (bivalent) vaccines that contain both the H3N2 and H3N8 strains are also available. The most concerning virus is currently the H3N2 strain, because it is still circulating widely in the USA (96). For Nobivac Canine Flu H3N8, two

doses given 2 to 4 weeks apart have been shown to produce a fivefold increase in a dog's immunity to the virus. After the initial vaccination series, a booster is administered annually. Vanguard CIV requires two doses that are administered 3 weeks apart, and a booster is administered annually. Dogs vaccinated with Vanguard CIV that contract the virus have a shorter viral shedding period: an average of 0.4 days compared with an average of 5.2 days in unvaccinated dogs. Vaccinated dogs that become infected with CIV develop clinical signs that are less severe than those seen in unvaccinated dogs. Dogs that should be vaccinated against CIV include those that go to kennels, grooming or daycare facilities, or shows or those that are exposed to dogs that have recently been group housed (102).

3.20 Public health and concluding remarks

The CIV/human virus reassortant reported for the H3N2 CIV is something about we should be alert. Such a threat results from the extensive and sustained contacts between humans and their pet dogs, which would result in significant human exposure to CIV if the viruses were to spread widely among household dogs. Furthermore, it is possible that additional reassortments between canine and human viruses could occur in dogs or humans or possibly other hosts, potentially creating a new human IV for which there may be little or no natural immunity. It is clear that these risks may vary depending on the virus, with H3N2 CIV seemingly posing a greater risk that H3N8 CIV. In addition, to date there have been no documented transmissions of H3N8 EIV or CIV to humans despite the close interactions between them since the first appearance of EIV in the early 1960s, although serological positivity has been demonstrated, or between humans and dogs since 2000. However, direct experimental inoculation of human volunteers with EIV in the 1960s showed that infection did occur, but only at low levels. These results suggest that there are high intrinsic barriers to the establishment of H3N8 in humans, such that onward transmission in two mammalian hosts (horses and dogs) does not guarantee successful infection of another species (humans). In contrast, the observation of H3N2 CIV-human virus reassortants in dogs shows that coinfection of one of the hosts (likely dogs) is possible and hence a cause for concern (95). It seems to be that viruses of the H3 subtype have proven to be highly adaptable and are able to recruit avian, mammalian, as well as human hosts. While the canine H3N8 and H3N2 viruses are genetically and antigenically different from strains currently circulating in humans, potential transmission of these or similar viruses to the human population from infected

pet dogs cannot be excluded. A careful and intensified monitoring of canine and equine populations for IV infections is advisable, especially during outbreaks in other species; however, organizing such surveillance is more of a challenge than might be expected. Dogs in industrialized countries are mostly healthy, but suffer from a range of respiratory diseases that make it more difficult to recognize influenza. In contrast with food animals and horses, where public safety and international travel, respectively, have driven surveillance, there has been little inducement to develop surveillance of dogs for infectious diseases (92).

The emergence of CIV, like the emergence of any IV lineage in a novel mammalian host population, may in this way constitute a direct or indirect pandemic risk, but it is clear that adaptation to mammals should not be seen as a direct conduit to emergence in humans. In fact, there are important host barriers that have yet to be understood or breached. A better understanding of the ecological, evolutionary, and molecular mechanisms of influenza emergence is essential to accurately determine which viruses pose a risk to human health (95).

The spate of emerging zoonotic diseases in the 21st century, as exemplified by IA infections, has led to the emergence of the *One World, One Health* philosophy. Many different organizations around the world are now recognizing that surveillance should also include companion animals. This constitutes a welcome development. However, surveillance is ultimately only a tool for targeting prevention and control strategies (92).

4. The Hendra Virus (HeV) and its spillover from horses to humans

"The original emergence of HeV didn't seem very dire or newsworthy unless you happened to live in eastern Australia. It couldn't match an earthquake, a war, a schoolboy gun massacre, a tsunami. But it was peculiar. It was spooky. Slightly better known now, at least among disease scientists and Australians, and therefore slightly less spooky. Hendra virus still seems peculiar. It's a paradoxical thing: marginal, sporadic, but in some larger sense representative" (1).

Part 1: The complete spillover event of the HeV

4.1 History of a new emerging disease

In September 1994, a sudden outbreak of an acute respiratory syndrome in thoroughbred horses in a training complex in Hendra, Brisbane, in the state of Queensland, Australia, resulted in an immediate shutdown of the horse racing industry. The syndrome was characterized by severe respiratory signs and high mortality and seemed to be caused by an unknown virus. It caused the deaths of thirteen horses and a trainer at a training complex (104). The *index case* or *patient zero* was attributed to a mare, 'Drama Series', that was brought in from a paddock in Cannon Hill, was housed with 19 other horses after falling ill, and died two days later. Subsequently, all of the horses became ill, with 13 dying. The remaining six animals were subsequently euthanized as a way of preventing relapsing infection and possible further transmission (105). Meanwhile, Vic Rail, the horse's trainer, had taken sick and so had the stable hand. It seemed at first that they each had a touch of a bad flu. Rail went into the hospital, worsened there and, after a week of intensive care, his organ had failed and he died. Autopsy showed that his lungs were full of blood, other fluid and (upon examination by electron microscopy) some sort of virus. The stable hand, a man named Ray Unwin, survived, as well as Peter Reid, the veterinarian of the mare, although he had been working on the same suffering horses amid the same bloody froth. The authorities began wondering whether the cause could be an exotic virus, such as the one responsible for *African horse sickness* (AHS), a disease carried by biting midges in sub-Saharan Africa. AHS virus affects mules, donkeys, and zebras as well as horses, but it hadn't been reported in Australia and it isn't directly contagious from horse to horse. But for real, Queensland's pestiferous midges don't generally come biting in September, when the

weather is cool. Therefore, AHS did not appear to be a fit (1). Based on its characteristics, the virus was first described as “Equine morbillivirus”, but further analysis demonstrated that the virus was an undescribed member of the family *Paramyxoviridae*, named

Hendra virus, the place in Brisbane suburb where the outbreak occurred, but we’ll get back to that. There had been one previous HeV spillover events in Mackay, which was responsible for the death of two horses and a mild-influenza-like illness in one human the previous month (August 1994), but the cause of the infection was not recognized as a new strain at the time. We will better describe this case in its peculiarity later (104).

September 1994								
	7	9	13	14	15	16	17	19-26
Horses								
Cannon Hill (Paddock)	2 horses moved							
Hendra (Stables)		Mare died				2 horses moved		10 horses dead 1 horse recovered
Hendra (Neighbouring property)		1 horse moved						1 horse dead 1 horse recovered
Kenilworth (150 km distant)								1 horse dead 1 horse recovered
Samford (Paddock)								1 recovered
			New South Wales					
Humans								
Stable hand				Becomes ill				Slow recovery
Trainer					Becomes ill		Hospitalized	Died

Table 2: Chronology of equine and human cases of disease attributed to HeV infection
(Murray et al, 1995)

In October 1995 near Mackay in central Queensland, almost 1 000 km north of Brisbane, a second HeV outbreak in horses was retrospectively diagnosed after the HeV-attributed death of a thoroughbred stud owner who suffered a relapsing encephalitic disease. This second outbreak chronologically preceded the Brisbane outbreak by several weeks, and resulted in the death of two horses. The first horse, a 10-year-old heavily pregnant thoroughbred mare died on August 1, 1994 after exhibiting severe respiratory distress, ataxia, and marked swelling of the cheeks and supraorbital fossa over a 24h period. The owner assisted in necropsies of the horses and, within three weeks, was admitted to hospital suffering from meningitis. He recovered, but 14 months later developed neurological signs and died. For this reason, the outbreak was diagnosed retrospectively by the presence of HeV in the brain of the patient. The second horse, a 2-year-old colt in an adjoining paddock was reported to have had direct contact (through the fence) with the dead mare. The colt died 11 days later, again after a 24h clinical course, during which he exhibited aimless pacing, muscle trembling and hemorrhagic nasal discharge. Histopathological examinations performed at the time were inconclusive in both cases. In January 1999, four and a half years after the previous cases, a new fatal case of HeV infection was reported in a horse near Cairns, in north Queensland (104). In late 2004, in two separate incidents in north Queensland, a further two horses were fatally infected and a human case non-fatally infected. The diagnosis was confirmed in one horse and presumptive in the second, based on history, clinical signs, gross pathology, and on the detection of a rising antibody titre to HeV in the attending veterinarian (105). Between the years 1994-2010, HeV appeared infrequently in the horse population of Queensland and New South Wales, with an amount of fourteen events recorded, in which four additional humans became infected. The first of these was in the 2004, when a veterinarian who performed the necropsy on a Hendra-infected horse became infected. After an influenza-like illness, the veterinarian recovered with no relapse. The next human infections occurred in 2008, when a veterinarian and a veterinarian nurse who were involved in the necropsy and treatment of Hendra-infected horses respectively both became infected with HeV. The veterinarian died from the disease while the nurse recovered, albeit with lasting neurological deficits. Another veterinarian became infected in 2009 and died from the disease. Two individuals who were potentially exposed to the infection in 2010 were treated with the experimental human monoclonal antibodies (mAb) 102.4 (106). In the period between 20 June 2011 and 28 August 2011, a further seventeen events were identified, during which twenty-one

horses died. It is not clear why there was a sudden increase in the number of spillover events between June and August 2011. In fact, HeV outbreaks typically seem to have a *seasonal trend,* between May and October, sometimes called the "Hendra Season". One theory attributes the causes to the 'Cyclone Yasi' and the summer season flooding in Queensland over 2010/2011. All of these factors corroborated another theory for which links the emergence of the virus to fruit bats. During the 2011 HeV emergency, the presence of the virus in a dog was confirmed via an antibody test. The equine industry and Government authorities were on high alert (107). Another eight spillover events occurred in 2012, and seven more cases occurred in 2013. Despite the increase in the spillover events in horses during these years, there have been no further human infections (106). From 2013 until today, the HeV incidents in horses were: 4 in 2014, 3 in 2015,1 in 2016, 4 in 2017,1 in 2018, 1 in 2019, 1 in 2020 with extended geographic locations (between far north Queensland to north New South Wales, Australia) (108). The average response cost and the economic loss due to horse deaths as a result of HeV infection are estimated to be A$30,660 per horse (109).

4.2 The origin of HeV

Shortly after the discovery of HeV in 1994, the search for the natural reservoir began. There was no evidence of HeV infection was found in 168 individuals from more than 16 species of rodents, marsupials, birds, amphibians and insects tested. However, the small sample size for any particular species limited meaningful interpretation of the negative findings, and indicated the need for a more targeted approach to wildlife surveillance.

In a subsequent study for the prioritization of possible host species for surveillance, the following criteria were applied: the target species should be present in both outbreak locations; the species should be capable of moving between the two locations, or have overlapping, mixing populations spanning the two locations, and contact between the target species and horses should be plausible. Several species of nomadic birds and pteroid bats flying foxes met the criteria. A higher priority for further investigation were given to flying foxes based on the apparent mammalian predilection of the virus, and reports of Paramyxovirus infections in bats elsewhere. As a means of screening wild flying fox populations , opportunistic sampling of sick or injured wild flying foxes in temporary captivity was employed. While primarily a methodology of convenience, it was recognized that the potentially positive bias of the

opportunistic sample could maximize the likelihood of detecting evidence of infection (assuming infection with HeV predisposed flying foxes to becoming 'sick or injured'). In April 1996, anti-HeV antibodies were identified in a black flying fox in central Queensland, and within weeks, in grey-headed flying foxes (*Pteropus poliocephalus*), little red flying foxes (*Pteropus scapulatus*), and spectacled flying foxes (*Pteropus conspicillatus*) at several locations in Queensland. Two years after the first reported outbreak of HeV infection in horses in Brisbane, in September 1996 a Hendra-like virus was isolated from the reproductive tract of an apparently healthy, pregnant grey-headed flying fox euthanized after becoming entangled on a wire fence. Comparison of the bat isolate (tentatively called *bat paramyxovirus* at the time) with the isolate from horses showed it to be indistinguishable from HeV by a range of tests. A serologic survey of 1043 non-randomly sampled flying foxes of the above four mainland Australian species collected from multiple Queensland locations between 1996 and 1998 revealed a crude HeV seroprevalence of 47%. Similar frequencies were also identified in other samples taken at locations across the Australian mainland range of flying foxes during the same period. In addition, in a retrospective serological survey of Australian flying foxes, anti-HeV antibodies were identified in sera collected in 1982, the earliest sample tested. The described occurrence and frequency of anti-HeV antibodies in flying foxes is compatible with an endemic pattern of infection Australia-wide. This interpretation is supported by the absence of gross pathology or attributable illness in naturally infected or experimentally infected flying foxes, indicating that infection in flying foxes may be largely sub-clinical. These features identify flying foxes as the probable natural host of HeV (104).

Initial virological investigations in flying-foxes had limited success, but virus and/or viral RNA was detected in fetal tissues and fluids, prompting a hypothesis that infection and transmission was associated with the reproductive cycle in flying-foxes. More recent studies have established urine as the primary route of excretion in flying-foxes, with blood, feces, nasal discharge and saliva having a lower and decreasing viral load. These findings support a urine-oronasal mode of transmission between flying-foxes, facilitated by the frequent exposure to the urine of co-roosting individuals, and the recognized self-grooming behavior of flying-foxes.

4.3 Etiological agent

Taxonomy

Initially, AHS was suspected as the cause of the Hendra outbreak. The etiology was confirmed by the isolation of a virus from the tissues of naturally and experimentally infected horses, and by reproduction of the disease in experimentally infected horse by the intranasal or parenteral routes. An identical virus was isolated from human cases and a two-way cross-neutralization has been demonstrated between viruses and convalescent antisera obtained from horses and humans. The virus was firstly called "equine morbillivirus", but genetic analysis later showed that its most appropriate classification is as the prototype member of a new genus within the *Paramyxoviridae* family (110).

HeV, together with the NiV, another virus that emerged in Malaysia, jumping from fruit bats to pigs and then to humans, are the sole members of a new Genus, *Henipavirus*, in the Family *Paramyxoviridae,* Subfamily *Paramyxovirinae,* Order Mononegavirales (111).

The *Paramyxovirus* family comprises major human and animal pathogens such as the measles virus (MeV), mumps virus (MuV), the parainfluenzaviruses, Newcastle disease virus (NDV), and the highly pathogenic zoonotic HeV and NiV viruses. There is little serological cross-reaction between HeV and other known paramyxoviruses (112).

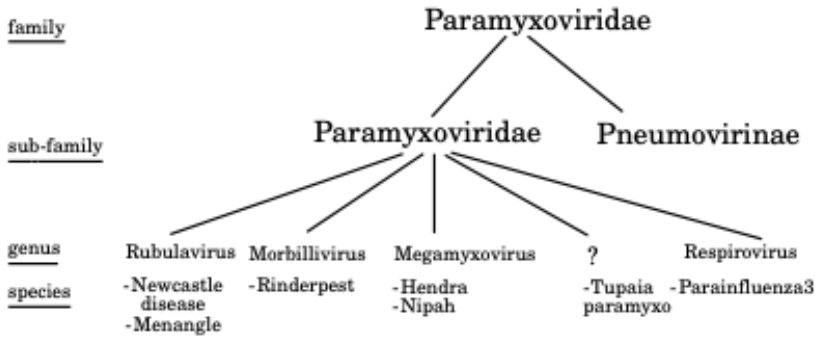

Figure 11: Hendra virus in relation to some other viruses of the family
Paramyxoviridae('Hendra (Equine Morbillivirus)', Barclay A., Paton D. (2000))

Virion morphology and characteristics

According to the description of the Paramyxovirus family made by Rima et al., virions are 150 nm or more (up to 500nm) in diameter, pleomorphic, but usually spherical in shape in vitreous ice. Virions consist of a lipid envelope surrounding a nucleocapsid. The envelope is derived directly from the host cell plasma membrane by budding and contains two transmembrane glycoproteins. These are present as homo-oligomers and form spike-like projections, 8–12 nm in length, spaced 7–10 nm apart (depending on virus genus affiliation). Also, depending on the genus, one or two additional transmembrane proteins may be present. One non-glycosylated membrane or matrix protein is associated with the inner face of the envelope. The virus nucleocapsid consists of negative-sense virus genome RNA and the nucleocapsid protein (N). The nucleocapsid has helical symmetry and is approximately 18 nm in diameter with a 7 nm pitch; its length can be up to 1000 nm in viruses of some genera. The ribonucleoprotein (RNP) complex in the virion consists of the nucleocapsid together with the polymerase-associated or phosphoprotein (P) and the L protein. Multiploid virions are also found, although the vast majority of virions contain a single functional genome (111).

The virus grows in a variety of cell types such as MDCK, BHK, RK13, LLC-MK2, and MRC5. It also grows in cells derived from birds, reptiles, amphibians, fish, and embryonated chicken eggs. The virus lacks neuraminidase activity and agglutination of erythrocytes (111).

Nucleic acids and genome organization

Virions contain a single molecule of linear, negative-sense, single stranded RNA that is not infectious alone but is infectious if the RNP complex is introduced into the cytoplasm (111).
The HNV genome is markedly longer than that of other members of the *Paramyxoviridae* (typically 15.1–15.9 kb). The genome of HeV is 18,234 nucleotides long and arranged as 39-N–P/V/C–M–F–G–L-59 (113) . This genome can encode 10–12 proteins. For viral RNA to start transcription and replication, it is necessary an integral ribonucleoprotein complex of RNA and associated proteins, which have a helical symmetry. Viral genes are mostly monocistronic, the derived mRNAs producing a single viral protein. The entire genome has recently been sequenced revealing that it is the largest of all known *Paramyxoviridae.* Genetic studies have demonstrate that HeV is either most similar to members of the *Morbillivirus* genus, or

intermediate between the *Respirovirus* and Morbillivirus genera. Amino-acid homology between HeV and morbilliviruses ranged from 17–21% (P protein) to 40–42% (M protein), compared to 6–10% and 8% respectively for other *Paramyxoviridae*. Studies of the G protein revealed low sequence homologies with other *Paramyxoviridae*, but found that the structure of the protein was most similar to the G protein of *Respiroviruses*. For these reasons it has been concluded that HeV represents the prototype of a unique genus (110).

Proteins

As said above, the genome is composed of six genes, of which the genes from 3' to 5', include the nucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F), attachment glycoprotein (G), and the large polymerase (L).

- Nucleocapsid protein (N): the N protein of HeV is responsible for encapsidating the viral genome. The N proteins exhibits a herringbone-like ultrastructure, visible under electron microscopy. It has two sites that interact with the P protein; one at the N-terminus and the other mapped to the last 29 C-terminal amino acids.
- Large polymerase protein (L): it is an RNA-dependent RNA polymerase which has many different enzymatic functions. The HeV L protein is like other paramyxovirus L proteins and is responsible for initiation, elongation, and termination of viral mRNA transcription and genome replication.
- Phosphoprotein (P): it is critical for genome replication as part of the RNA-dependent RNA polymerase complex while in association with the L protein. The P protein has two independent N protein interaction sites: one located at the N-terminus and other at the C-terminus within amino acids 636-709. Recent studies have indicated that the X domain of the HeV P is important for interacting with the intact nucleocapsids which is the first step in polymerase complex interaction. While the P gene encodes the full-length P protein for HeV, there are additional non-structural proteins encoded in the P gene: C, V, W and the small basic (SB) proteins.
- Matrix protein (M): provides structure to the virion while interacting with the cytoplasmic tail of the F protein, the ribonucleoprotein complex and the inner leaflet of

the viral membrane. The HNV M protein is also involved in the budding of virions from the host cell recruiting host factor that enhance virus release to the sites of virus assembly.

- Attachment glycoprotein (G):upon expression at the cell surface, the HeV G monomers assemble into tetramers and these function as the attachment protein on the surface of the virion by interacting with a receptor. Structurally, the N-terminus of G begins the cytoplasmatic tail of the protein, followed by the transmembrane region, a large globular head formed by the C-terminus. The receptor for HeV is ephrin-B2, which is not a strong receptor, although there is still some ability for the HeV G to bind. The HeV G-ephrinB2 interaction of the two causes conformational changes in the globular head domain, which trigger the activation of the F protein. This protein has a fundamental role as it has been used as the basis of vaccines against HeV infections and is currently the target of therapeutic mAb.
- Fusion protein (F):is responsible for mediating the fusion of the virus membrane and host cell membrane in order to release virus genome into host cells. This fusion activation for the HNV F proteins is dependent on specific sequences in the cytoplasmatic tail. The F proteins are synthesized as inactive precursor that are called F_0 and are generally cleaved by host cell protease to produce the disulfide-linked biologically active F_1 and F_2 subunits. These HeV F_0 are cleaved by cathepsin L in acidic endosomes after endocytosis from the cell surface before incorporation into virions (111,114).

Lipids

Lipids in the virus envelope are derived from host cell plasma membrane (111).

Carbohydrates

Virions are composed of approximately 6% carbohydrate by weight; composition is dependent on the host cell. Fusion and RBP proteins are glycosylated by *N*-linked carbohydrate side chains (111).

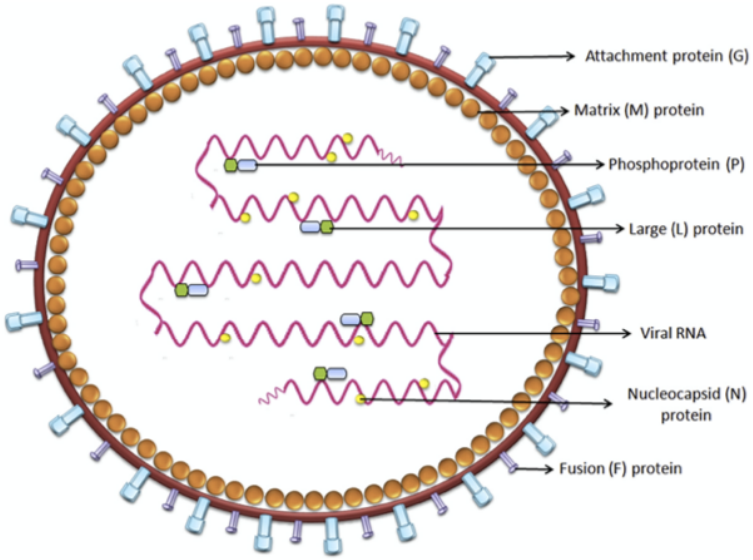

Figure 12: Structure of HeV
('Hendra Virus Infection in Horses: A Review on Emerging Mystery Paramyxovirus', Khusro A. et al. (2020))

Since HeV is an RNA-virus, it is capable of mutation. As a result, since its first appearance, it has changed many times. Recently (April 2021), Australian scientists have identified a new strain of the deadly HeV that was the cause of a previously unexplained horse death in September 2015.

The newly recognized variant, identified by the Australian veterinarian-led research project, 'Horses as Sentinels', had not been detected previously by routine biosecurity testing in horses. The new strain shares ~99% sequence identity with the 2015 horse case strain, and has been detected in grey-headed flying fox samples from Adelaide, South Australia, in 2013. Partial sequences of the variant have also been detected in flying foxes in other states (115).

4.4 Transmission of HeV

It is not an highly contagious disease, but has very high mortality (horses 75%) (109).

In Horses

HeV can be transmitted from flying fox to horse or horse to horse. As already said, flying foxes are the reservoir host of the virus. As fruit bats are attracted by fruiting trees, they will be more likely present in places where they can find their food source. If infected bats feed on fruits, there is high possibility of contamination. The contaminated fruits are dropped from the trees

during consumption, therefore making it likely that healthy horses ingest them or inhale the contaminants while grazing and get infected with HeV. The exact mode of transmission is unknown yet, but the most common mode of transmission of HeV is horses' contact with urine, saliva, fluids, or any other excretions of infected flying foxes. Although most of the spillover events of HeV in horses have involved only a single animal, in each of the two largest equine outbreaks there is evidence of horse-to-horse transmission (109). The horse-to-horse transmission in the 1994 Hendra, Queensland outbreak was probably due to direct contact with an infected horse. In the 2008 Redlands, Queensland outbreak, direct contact is also most likely, although there is a possibility of fomite transmission (106). Aerosol transmission apparently is not a major mode of spread. In the original outbreak, a 5-km (3-mile) radius around the infected stables encompassed many other training stables, and the area was close to two major Thoroughbred racing and training tracks (64). Experimental evidence indicates that HeV, under some conditions, can remain on environmental surface for several days. It has also been demonstrated that HeV RNA can be detected in the nasal secretions of experimentally infected horses up to 2 days before the presentation of clinical signs. It is interesting to note that in each of the two outbreaks where horse-to-horse transmission occurred, horse-to-human transmission also occurred (106) .

In Humans

Transmission of HeV to humans has occurred infrequently. All humans who were infected have been in close proximity to extremely sick or dead horses which were infected with HeV (106). In this case, since the reservoir host is represented by flying foxes, horses play the role of "amplifying host". An amplifying host is an organism in which an infectious agent (such as a virus or bacterium) that is pathogenic for some other species is able to replicate rapidly and to high concentrations. The amplifying host tends to increase the spread of infectious pathogens and may be intermediate to or also serve as the pathogen reservoir (116).

It is likely that transmission of the virus to humans occurred through exposing the mucous membranes or non-intact skin to either respiratory or nasal secretions, or to blood or urine from the infected horses. Before the the discovery of HeV, there was no known zoonotic virus that could infect both horses and humans. Therefore, veterinarians and horse owners typically did not wear much personal protective equipment. Since HeV killed three veterinarians in 2004,

2008 and 2009, the Australian veterinarian community has become more informed and concerned about the risk that HeV presents. Hendra infection now being considered a work-related illness. As a consequence, more personal protective equipment is being used when working with horses that might have HeV. This may also explain why no human cases of HeV have been seen since 2009, when the number of spillover events has increased dramatically at the same time.

There is no evidence of human-to-human transmission or human-to-horse or direct bat-to-human transmission, and there is also no evidence of human-to-horse transmission. Extensive surveillance of humans who were close to infected horses, as well as bat caretakers who handle Australian flying foxes, have shown no seroconversion. Thus, it is likely that there have been no human sub-clinical cases (106).

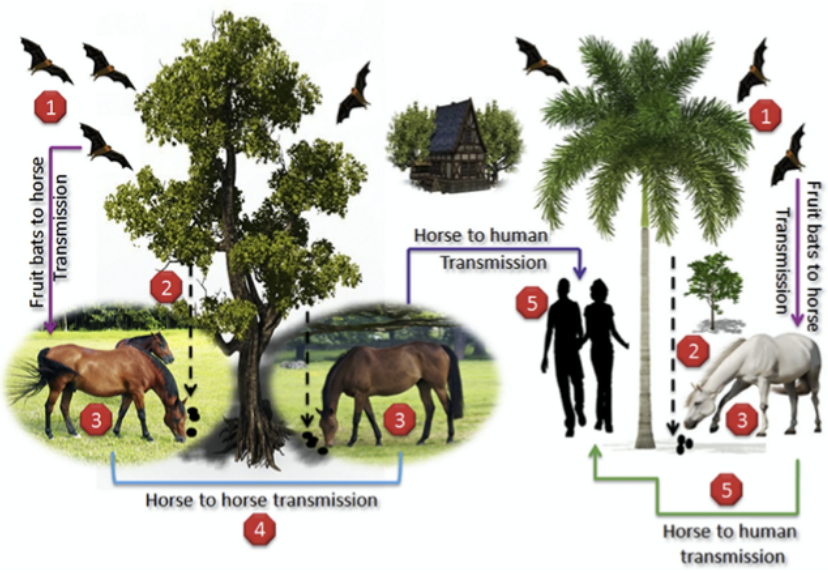

Figure 13: Transmission of HeV
('Hendra virus infection in Horses: a review on emerging mystery paramyxovirus', Khusro A. et al. (2020))

Other species

As the reservoir host, in fruit bat the infection is subclinical. At the moment, the only species besides horses, humans, and bats to show evidence of infection in nature are dogs. In June 2011 in Boonah, Queensland, a dog was found to have antibodies against the HeV. No viral RNA was detected in the dog and it had not shown any clinical signs of disease. There was also no evidence that the dog can transmitt the virus to horses. In order to ensure that the dog was not a

carrier of the virus, the animal was euthanized, as is the practice for all horses found to be positive for HeV. Similarly, a second dog showed serological evidence of the virus in the July 2013 spillover event in Macksville, Queensland (106). It has been shown experimentally that cats suspended in cages above feed troughs used by infected horses did not become infected. However, infected cats kept in similar cages transmitted infection to one of three contact horses. Guinea pigs are also susceptible and develop a fatal illness starting from seven days after inoculation with HeV (113).

4.4.1 Characteristics and risk factor of the HeV cross-species transmission

The factors that favor spillover have been extensively outlined in chapter 1. In the specific case of HeV, *P. alecto* and *Pteroid conspicillatus* appear to represent the primary source of spillover infection to horses, and horses within their geographic range are putatively at a greater risk of exposure than horses that are outside their range (106).

Bats forage for food over large areas, although the reason the urbanization of coastal Australia has led to the formation of fairly stable flying fox colonies around large urban and suburban areas in eastern Australia where food sources are relatively consistent throughout the year (106).

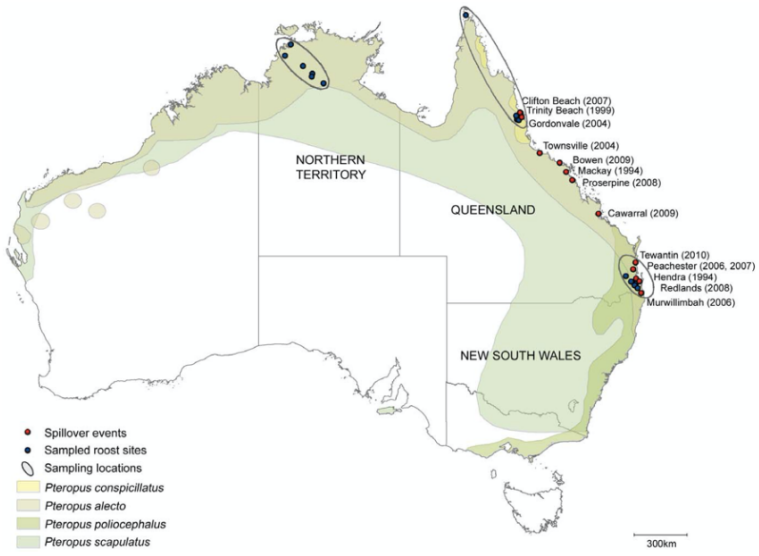

Figure 14: Map of Australia with flying fox distribution, Hendra virus spillover locations and flying fox sampling locations
('Hendra Virus Infection Dynamics in Australian Fruit Bats', Field H. et al.(2020))

Further, HeV infection and excretion varies over space and time (117). Field et al. reported variability in detection associated with roost location and year (118) and more recently reported the same variability, but additionally showed that the within-year pattern of infection and excretion varied with location, with the winter peak in virus detection evident in some regional locations while largely absent in others. This winter peak of excretion in flying foxes parallels a winter peak of equine cases, and this suggest that infection prevalence in flying foxes is the fundamental driver for infection risk in horses. However, HeV infection in horses is quite rare, suggesting infrequent exposure and/ or a complex causal web. Thus, while the total reported equine cases underline the low likelihood of infection, the consequence of infection (for the case horse, and for horses and humans in effective contact) is high. A limited number of researches have sought to identify spatial and temporal risk factors associated with spillover of HeV from flying-foxes to horses (117).

Among them, one study found a positive spatial association with postcodes containing flying fox roosts, and a temporal association with seasonal low rainfall. Another study found out a similar association with proximity to flying fox roosts, with a statistically significant increased risk of equine cases within 7 km of a known roost. The HeV has a sensitivity to pH, temperature and desiccation, supporting the contention that climatic variables can contribute to infection risk for horses. Studies on the effect of temperature on virus survival in the environment concluded that temperature was not a primary driver for equine infection, but along with other environmental factors that influenced HeV survival in the environment, it was likely a component of the causal web (117).

As seen in Chapter 1, Plworight et al. proposed physiological stress as a risk factor for HeV infection in flying foxes. This could explain also the amount of average of cases in 2011 due to 'Cyclone Yasi': the hurricane may have destroyed food sources and increased the stress level of fruit bats that may have shed more virus (22). Among other stress factors it is interesting to note that the period of pregnancy for flying foxes in eastern Australia is typically between April and September with birth usually occurring in September or October. Of these, the majority of the cases occurred in the months between May and October, corresponding with the time when female flying foxes are pregnant and giving birth. It is therefore possible that pregnancy represents another stress factor that induces more viral shedding. Five of the spillover events, however, occurred outside of this timeframe suggesting that the pregnancy in flying foxes is a

factor in HeV transmission, but not strictly necessary (106). In fact, more recent analysis suggest that immunologically naïve sub adult *P. alecto* could play an important role in maintaining HeV infection at a population level. Contrary to previous studies, we found no association between HeV infection and pregnancy or lactation, and therefore no support for reproductive stress as a driver for infection or recrudescing infection associated with pregnancy. The study also identified an association between the BCS and infection that plausibly could support a role for immune system competence in HeV infection in flying-foxes (119).

Risk factors associated with the development of HeV infections depend on (14) (120) :

Viral load	**Horse exposure**	**Horse susceptibility**
Local bat density Abundance of bat food in trees Rate and duration of bat feeding visits Infection prevalence or shedding rates Viral load excreted	Husbandry Pasture equality Green forage under trees Nutritional status Horse behaviour	Innate immunity Acquired immunity Route of exposure Gender: female Breed: Thoroughbred Age: < 8 years Late pregnancy

Table 3: Risk factor in Hendra Virus infection

Part 2: The HeV infection in horses

We have already discussed about the etiological agent, the epidemiology and the mode of transmission of the virus. We will now elaborate on others aspects of the infection that specifically concern horses.

4.5 Clinical Signs

Approximately 75-80% of horses infected with HeV die.

In horses, but also in humans, HeV manifests itself in a respiratory or a neurologic form.

The incubation period in natural horse cases is usually between 8 and 11 days with a maximum of 16 days. The clinical course of the disease is very acute, and the time between the onset of signs until death being usually between 1 and 3 days (110).

In the acute onset of the disease, the horses show fever (up to 41°C), depression, inappetence, tachycardia, tachypnea, dyspnea, facial edema, aimless pacing, muscle fasciculation, and ataxia. Death follows within 48 to 72 hours in approximately 75% of cases. In animals which are terminally ill, a copious frothy nasal discharge may also be seen as a reflection of severe pulmonary edema. Some affected horses may be found dead. In horses that survive the acute infection, clinical recovery may appear to be complete but, as the Australian national policy requires the euthanasia of convalescent horses, no long-term follow-up has been carried out on such cases, especially with respect to the potential for recrudescence of virus replication in the central nervous system (121).

Both neurologic and respiratory signs have been a feature of HeV infection in horses since the original outbreak in 1994 where, although the dominant clinical presentation was respiratory disease, two convalescent seropositive horses exhibited myoclonic twitches. However, in later years, the appearance of field cases of acute HeV infection were even more strongly associated with signs that localized in the respiratory system. If we consider the non-specific nature of many other clinical signs, differential diagnosis, especially from more common disorders such as pneumonia, pleuropneumonia, and colic, was and remains challenging and complex. In contrast to a respiratory syndrome presentation, a multi-horse outbreak developed in an equine referral practice in 2008, where the predominant clinical signs were attributable to the

involvement of the central nervous system. These included ataxia, disorientation, hypersensitivity, head tilt, facial nerve paralysis, strangury, head pressing, and circling. This event highlighted need to also consider HeV in the differential diagnosis of neurological disease in horses. It also seems that horses presenting neurologic manifestations of acute HeV infection had pre-existing lesions of the head (corneal lesion, nasal granuloma and mandibular fracture). Therefore, there was potential for HeV exposure in a manner that may have bypassed mucosal protective mechanisms, thereby influencing the course of infection (121).

4.6 Pathogenesis

In horses, HeV is predominantly pneumotropic but may also be neurotropic (64).
According to the description of Khusro et al., initially, HeV enters a host's cell by binding with ephrin-B2 (a receptor present on neurons, smooth muscle, and endothelial cells surrounding small arteries). In fact, the virus attachment protein (G) binds with the receptor ephrin-B2. After the attachment, the fusion protein (F) of virus is cleaved into two linked polypeptides (F1 and F2) and then initiates endocytosis by fusing with the host cell membrane. The viral ribonucleocapsid is then released into the cytoplasm after the fusion between the viral envelope and the host cell membrane. The transcription of viral mRNAs is initiated by the polymerase complex, which is mainly composed of polymerase (L) and phosphoprotein (P). As translation of viral mRNA occurs, viral proteins accumulate in the cell, and the polymerase switches from transcription to genome replication. Nucleoprotein (N) encapsidates the newly made genomes and polymerase complexes become associated with packaged nucleocapsids. The glycoproteins are synthesized in the endoplasmic reticulum, mature in the Golgi network and then transported to the cell membrane. The fusion (F) glycoprotein processing occurs in the endosome. The interaction with the matrix (M) protein is carried out by cytoplasmic tails of the F and G glycoproteins, which initiates virus maturation and budding, thereby causing infections (109).

During early infection of HeV in horses, the vascular lesions cause edema, hemorrhage of vessel walls, fibrinoid degeneration with pyknotic nuclei in endothelial and tunica media cells, and numerous giant cells (syncytia) in the endothelium. In the later stages of infection, the virus reaches various tissues like the vascular endothelium of subarachnoid and cerebral vessels, renal glomerulus and pelvis, lamina propria of the stomach, spleen, various lymph nodes, and

myocardium. The virus causes progressive destruction of alveolar walls with the appearance of alveolar and intravascular macrophages. In addition, the virus also causes neuronal necrosis and focal gliosis (109).

4.7 Pathological lesions

In the preclinical stage of infection, viral genetic material can be recovered on nasal swabs from experimental horses after as little as two days post-exposure to HeV by oral and nasal routes. Gene copy numbers in nasal secretions steadily increase through the incubation period and into the clinical phase of infection, consistent with local replication in the upper respiratory tract or nasopharynx. Then viremia sets, followed rapidly by the onset of fever, and soon afterwards viral genome can also be recovered from oral secretions and urine. Signs of systemic illness develop shortly after that as HeV replication becomes more widely established in tissues and organs.

Unfortunately, there is comparatively little pathologic data available from field post-mortems and most information has been recorded from experimental studies. In peracute cases, there may be few gross abnormalities at post-mortem examination. Where described, post-mortem lesions in acutely affected animals have included pulmonary edema, congestion and consolidation, with blood-tinged foam in the airway, dilation of subpleural lymphatic, subpleural hemorrhage, congestion of intra-abdominal lymph nodes, and enlarged, edematous submandibular, sternal, and bronchial lymph nodes (121). The pericardial sac may contain up to 100 ml of serous fluid. In some horses with HeV infection, extensive subcutaneous hemorrhages were observed, but these may have been agonal (64).

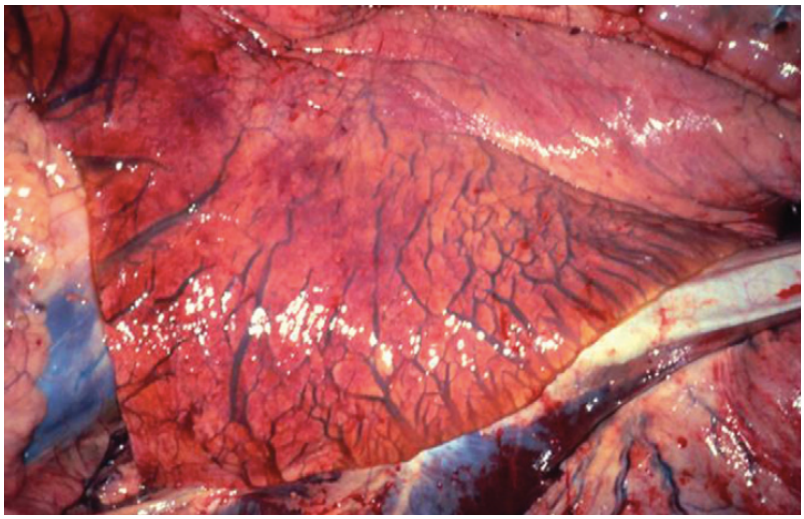

Figure 15: Gross lesions in lung of horse experimentally infected with Hendra virus, showing pneumonia and dilation of lymphatics
('Equine infectious disease', Sellon& Long (2007))

The dominant microscopic lesion in both natural and experimental infected horses is vasculitis that affects predominantly smaller blood vessels in a wide range of tissues including lung, brain (and meninges), lymphoid tissues, kidney (glomeruli), and female reproductive tract but also nasal mucosa, adrenal gland, liver, heart and gastrointestinal tract. Necrotizing lymphadenitis is common, as is extensive lung involvement including widespread necrotizing alveolitis with marked fibrinous alveolar exudates. Syncytial cells are regularly identified within renal glomeruli, lymphoid tissues, vascular endothelium, lymphatic endothelium, and in alveolar walls. Virus may also be recovered from the fresh carcass, especially from lung, kidney and lymphoid tissues but also brain and spinal cord, cerebrospinal fluid, meninges, upper respiratory tract, heart and adrenal gland (121).

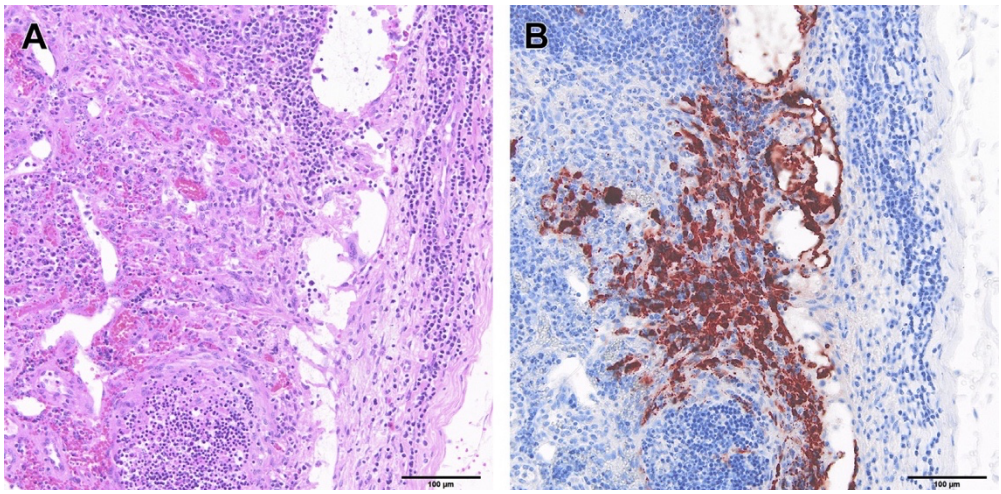

Figure 16: Microscopic lesions caused by HeV

(A) Histologic section of lymph node from horse with acute HeV infection showing lymphadenitis with syncytial cell formation (hematoxylin and eosin, original magnification x 20). (B) HeV antigen in section adjacent to ('Hendra Virus' D. Middleton, (2014))

A few cases of HeV that have clinically recovered from acute disease (associated with the development of virus neutralizing antibodies) have been then euthanized in line with Australian national policy. Each had shown neurological signs during the clinical phase of infection and, at the time of post-mortem examination, had mild to moderately severe, focal, nonsuppurative meningoencephalitis with gliosis and perivascular cuffing; low levels of HeV genome were also recovered from the brain. The significance of these findings with respect to the possibility of virus persistence in the equine brain is not yet understood (121).

4.8 Diagnosis

The probability of detecting high cases of HeV-positive horses depends on sampling and testing a maximum number of sick horses. The virus can only be isolated in limited laboratories because HeV is a biosafety level 4 (BSL4) pathogen (109).

- Direct diagnosis – antigen detection:
    - Clinical Laboratory: Initially, the clinical signs and postmortem findings of Hendra infection in horses were suggestive of acute AHS or EIV. Inoculation of HeV experimentally into horses reproduced the syndrome, and specific antibody was detected in all recovered horses and two infected humans during the first outbreak (64).

- Post-mortem examination: Post-mortem examination of a recently dead horse is a particularly hazardous activity as the HeV viral load is highest at this time, but it may be appropriate when atypical disease is observed or when confirmation of the diagnosis is essential, for example after a human exposure. Necropsy can be safely conducted by experienced and suitably equipped operators with a predetermined plan and appropriate infrastructure for preventing exposure of personnel, for carcass removal, and for environmental decontamination. However, if those criteria cannot be met then suitable risk reduction measures might include limiting specimen collection to tissues such as the superficial mandibular lymph nodes and a jugular vein blood sample, as well as swabs of nasal, oral and rectal orifices (121).
- Virus isolation: HeV may be isolated in monolayer cell cultures of Vero cells inoculated with filtered lung homogenates, although the virus can be isolated from other tissues and in cells such as primary equine fetal kidney cultured cells. Following inoculation of monolayer cell cultures, a cytopathic effect (CPE) was detected 3 days after inoculation. The CPE consisted of focal syncytium formation, which subsequently spread throughout the monolayer. Virus from a human case was isolated in LLC-MK2 and MRC5 cells, in which the CPE characterized by syncytium (multinucleate cell mass) was detected 12 days after inoculation (64).
- PCR: isolation of the virus followed by a PCR test can be a preliminary step. Blood in ethylenediaminetetraacetic acid, as well as nasal, oral, and rectal swabs are collected for PCR testing. Samples such as urine, conjunctival swabs, and swabs of other orifices (vaginal and urethral) can also be collected. Similar swab samples along with blood collected from the jugular vein and superficial submandibular lymph node are used for dead horses (109).
- Real-time PCR: For this test, RNA is extracted, either using a commercial, viral-RNA extraction kit or manually. Briefly, complementary DNA (cDNA) is generated from the viral RNA template before the cDNA is amplified using primers and probes targeting the M gene of HeV. The sensitivity of this assay

has been described as 1000 times better than that of conventional PCR and can be improved by the use of additional primers targeting different regions of the HeV genome (such as the P gene), to the point where direct culture becomes unnecessary. Several other, modified PCR-based assays, including some based on TaqMan or SYBR Green, are in use at diagnostic facilities in Australia (113).

- IHC: One of the most successful and basic tests for the detection of HeV post-mortem involves the use of a Hendra-specific monoclonal or polyclonal antiserum on formalin-embedded tissue, to detect HeV-specific antigen. Many different tissue samples can be used for such IHC, including spleen, lung, brain, mediastinal lymph nodes, kidney, uterus, placenta, and fetal matter. Even primary infection occurs in the vascular endothelium, HeV antigens may be cleared from the lung relatively soon after infection and tissue sampling should therefore not be confined to the lung. Following the post-mortem isolation of virus, electron microscopy can be used to demonstrate HeV in viral cultures (113).

- Indirect diagnosis – antibody detection:
  - Serum neutralization test: The serum neutralization test is the 'gold standard' reference test for HeV. The neutralization test works on the principle that the usual CPE of the virus, on a cell monolayer, will be blocked by the HeV-specific antibodies present in serum from an animal that, or patient who, is seropositive for HeV. In the standard assay, a test serum, which may be diluted 1:2 or even 1:5 if the serum sample is small, is incubated with HeV before the virus is added to a Vero-cell monolayer in culture. The cultures are checked for a CPE 3 after some days. In the last decade, the standard serum neutralization test has been modified to produce a rapid, immune plaque assay that takes advantage of the development of syncytia in HeV-infected Vero-cell cultures within 24 h of culture inoculation and allows for viral load to be quantified. This plaque assay can be used to test sera after any viruses present have been inactivated with methanol and gives a result after just 1 day of incubation.
  - ELISA: In situations requiring urgent, rapid detection, such as an outbreak, ELISA, however, is more commonly used. Although an indirect ELISA for the

detection of anti-HeV antibodies is the test that is most routinely employed, antigen-capture ELISA is rapidly gaining credence and Luminex-based tests are also being used (113).

Differential diagnosis

The differential diagnosis of the disease includes circulatory catastrophes, shipping or transit fever, poisonings, acute bacterial infections and intoxications such as anthracosis, pasteurellosis, legionellosis or botulism. Other viral diseases to be considered include equine pestis, AHS, Hantaan virus, EHV or highly EIV. Although gross pathological findings are not absolutely distinctive, a histopathological finding of vasculitis with the characteristic syncytia would be highly suggestive (110).

4.9 Therapy

At the moment, there are no specific antiviral drugs available for the treatment of HeV infection in horses. However, over the past few years, several antiviral agents such as known antiviral drugs, small molecules, and peptides as well as recombinant monoclonal antibodies for passive immunotherapy have been tested which showed minimal or no positive effect. Studies showed a lack of effect of ribavirin on hamsters, revealing uncertainty on the use this drug toward the treatment of HeV infection. Chloroquine (an antimalaria drug), either alone or in combination with “ribavirin” was tested but showed no therapeutic impact on hamsters challenged with HeV (109).

4.10 Prevention

4.10.1 Vaccination

Because there is no effective antiviral drug on the market, vaccination of horses is considered as the sole emphatic way to prevent HeV infection. Vaccination helps reduces the possible risk of HeV transmission. The vaccine available for preventing HeV infection in horses consists of a synthetically produced glycoprotein (non-infectious protein component; G protein) which stimulates the horse’s immune system to produce antibodies against the virus. Because the vaccine does not contain live HeV, the possibility for the horse to get infected due to the

exposure through the vaccine is nil. At present, the only registered vaccine (*Equivac HeV*) is available to help prevent HeV disease in horses.

In May 2011, the Commonwealth Scientific and Industrial Research Organization (CSIRO) announced the development of a prototype vaccine for horses and launched the *Equivac HeV* vaccine in November 2012. *Equivac HeV* vaccine was released for administration by veterinarians late in 2012. By March 2013, scientists confirmed that horses were immune to lethal exposure of the HeV six months after vaccination.

The quantity of the vaccine is 1 ml and is injected IM in horses. It prevents the infection from four months of age. *Equivac HeV* vaccine is a "subunit" vaccine (containing only a small portion of the protein from the virus surface, not the live virus).

The vaccine triggers the antibodies production. If the horse is exposed to the virus, the antibodies will bind with the viral particles, thereby preventing them from active infection of HeV. The viral particles bound to the antibody are then eliminated by the immune system of the horses. Veterinarians inject two 1 ml doses into the side of the neck at an interval of three weeks, and antibodies are generated within three weeks of the second vaccine dose being administrated

Equivac HeV is a world-first commercial vaccine for a BSL4 disease agent. The vaccine is cost-effective and minimizes the chances of the HeV mutation and rapid transmission between horses. The Australian Veterinary Association currently recommends that all horses in Australia be vaccinated against HeV.

The vaccine also has short-term, mild side effects, including swelling and soreness at the site of vaccination, elevation of body temperature, lethargy, loss of appetite, muscle stiffness and swelling in the joints, skin rashes, and colic.

Recently, Tan et al. determined the antibody responses in 61 horses after administrating *Equivac HeV*. Horses were given a primary vaccination course comprising two doses administered 3-6 weeks apart and a third dose given 6 months after the second. This was followed by a booster vaccinations at 12 months intervals. A virus-neutralization test was adopted to assess antibody titers. Findings on some resarch revealed that the administration of *Equivac HeV*, using a primary vaccination course followed by annual booster vaccinations, showed an effective secondary immune response and acquired antibody responses that were consistent with protective immunity against HeV in the form of virus-neutralizing antibodies. No adverse events were observed after vaccine administration (122).

In another study, Schemann et al. evaluated the effect of *Equivac HeV* vaccine on Thoroughbred racing performance. The study concluded no effect of *Equivac HeV* vaccination on racing performance in Australian Thoroughbreds (123).

4.10.2 Biosecurity

Apart from vaccinating the horses, there are a plethora of other preventive approaches which can help not only to avoid the infection of HeV in horses but also confine the transmission of virus from infected horses to the healthy horses.

- Horses' feed and drinking water containers should not be kept under trees attracting flying foxes, but rather shifted under shelter.
- Horses should not be kept near paddocks where flowering/ fruiting trees may attract flying foxes. In case owners are unable to remove horses from the paddock, they should consider fencing for restricting access to flowering/fruiting trees.
- Items such as halters, lead ropes, and twitches coming into contact with the horses should be disinfected before using it for another horse.
- Wearing gloves, covering any cuts or grazes, and washing hands while cleaning contaminated equipment should be encouraged.
- The sick horse should be isolated from healthy horses and other animals until further action.
- Healthy horses should always be handled before handling sick horses with appropriate precautions.
- Traveling with, working on, and taking sick horses to other properties should be banned.
- Close contact with the horses under investigation, and other horses that have been in contact with it, until HeV has been ruled out should be avoided. If it is essential to contact with horses under investigation, ensure to take appropriate precautions.
- People with low immunity, and children should not be allowed to get contact with horses under investigation for HeV.
- Horses that are under examination for HeV should be moved away from the public areas.
- Horses under observation should be isolated from other healthy animals.

- Feed and water to any horses under HeV investigation should be provided from proper distance.
- Horses under HeV examination should be monitored from a distance and inform a veterinarian immediately if any health- related issue is notified.
- If a horse under HeV observation dies, avoid contact with the carcass and bury with proper precautions.
- If the horse is diagnosed as HeV negative, monitor the horses for few more days in isolation and observe for any kind of changes in the health status.
- If the horse is diagnosed as HeV positive, do not allow anyone to enter or leave the property without permission from the respective authority. Neighboring properties with horses should also be monitored for the risk of exposure to HeV. After the confirmation that animals are no longer infected with HeV, the quarantine on the property should be lifted.
- Personal protective equipment (PPE) should be worn by veterinarians if the horse is suspected for HeV infection. Veterinarians should wash their hands thoroughly and sanitize after removing the PPE. During any kind of physical support and help, the assistants should also wear PPE and sanitize hands thoroughly to avoid transmission of virus (124).

Community impact

The Hendra outbreak also represents an interesting scenario to evaluate the role of animal owners in biosecurity. Some studies aimed to identify factors influencing the uptake of safety practices, and it turns out that the best biosecurity measures were adapted by female owners who were also involved in either mainly competitive/equestrian sports (37%) or recreational horse activities (35%). 75% of owners indicated that they follow at least one-third of the recommended practices regularly when handling their horses, resulting in medium to high levels of biosecurity. Main factors associated with a higher level of biosecurity were high self-rated standards of biosecurity, access to personal protective equipment, absence of flying foxes in the local area, a good sense of control over HeV risk, the likelihood of discussing a sick horse with a veterinarian and likelihood of suspecting HeV in a sick horse.

The events also highlight the fundamental role of veterinarians in managing emerging disease, both with the vaccination practice, and making the owners understand the importance of biosecurity. The veterinarian's communication skills are fundamental in this situations (125) .

Biosecurity also depends on the control of wild flying foxes as we have already outlined. Flying foxes population play a critical role in the Australian environment through the pollination of native trees and the spreading of seeds. Without flying foxes, eucalypt forests and rainforests would cease to exist. Interaction between domestic animals and flying foxes can be exacerbated by pressure on flying fox populations, such as through loss of natural forest habitat, forcing flying foxes to seek urban and peri-urban food sources. The downside of managing flying fox populations has some impact on the country's agriculture (125).

4.10.3 Outbreak management as provided for by the Australian national public health guidelines

Australian national guidelines in preventing the spread of infection of HeV include:

- Respond to a confirmed equine case, or where heightened suspicion of infection in a horse exists as advised by the relevant animal health agency, immediately on notification:
    - ensure appropriate infection control measures are in place;
    - establish an incident management team to manage the public health response to any confirmed human or equine case;
    - close liaison with authorities is necessary – joint meetings should be held with the government to ensure a coordinated response. An initial meeting should be held within 24 hours of notification
- A confirmed case requires laboratory definitive evidence or laboratory suggestive evidence and epidemiological evidence and clinical evidence
- After confirmation:
    - Commence investigation immediately on notification of a confirmed equine case, or where notified by an animal health agency of heightened suspicion of infection in a horse on clinical and epidemiological grounds.
    - Liaise with the national government to:

- ensure appropriate infection control measures are in place for all confirmed or suspected equine cases. Biosecurity officers will determine if property quarantine, livestock quarantine (including the use of PPE) and/or travel restrictions for close contact horses is required. The decision will take into consideration if the horses have received an appropriate course of HeV vaccine;
- clarify timeline for results of laboratory testing when not already confirmed;
- establish whether/what communication has occurred with potential human contacts.

- Identify and manage human contacts.
- Isolation and restriction quarantining the property of the outbreak.
- Euthanizing the infected horse or horses: all the past confirmed equine cases have been euthanized in accordance with nationally-agreed policy, to prevent further risk of transmission, provided it is done humanely and the carcass is safely disposed of.
- Tracing of animals that have recently moved from the property, isolating and testing other animals that may have been exposed, and vaccinating other horses on the property (126).

Part 3: The HeV infection in human

HeV is one of the deadliest viruses to humans currently known in the world. As we have illustrated before, the first reported human cases of HeV date back to the first outbreak of the virus, when Vic Rail and his stable foreman, Ray Unwin, were involved in nursing the index case, and both fell ill with an influenza-like illness within one week of the first horse's death. The stable hand recovered but Rail died. Between then and 2013, there have been 7 identified humans cases, of which 4 died from the infection. We have also analyzed the fact that veterinarians are the most susceptible category to the virus.

Case report: the Mackay outbreak

In referring to the historical framework of the virus, it is interesting to analyze more in-depth the Mackay case. The second human death resulting from HeV infection occurred in Mackay, 1000 km (600 miles) north of Brisbane. In August 1994, a 36-year-old sugarcane farmer assisted his wife, a veterinarian, in performing a postmortem examination on two horses that died 10 days apart on their property. Retrospective diagnosis of HeV as the cause of death of the horses was performed on paraffin-embedded, formalin-fixed tissue blocks. Ten days after the second horse died, the farmer was admitted to the hospital with meningitis, from which he apparently recovered. Fourteen months later he was again hospitalized, and he died 25 days later after the development of seizures and paralysis of increasing severity. Postmortem examination revealed meningoencephalitis with areas of necrosis throughout the cortex. Vascular thrombosis and occasional multinucleate giant cells were present in the brain. The brain tissue was positive for HeV, even though the virus was not isolated. Both the cerebrospinal fluid and brain tissue were positive for HeV on PCR, and sequencing of the PCR products showed the sequence to be identical to the HeV sequence of the first isolate. During the hospitalization period, there was a rise in HeV neutralizing antibody from 1:16 at admission to 1:5792 terminally. The veterinarian who assisted with the postmortem examination did not develop clinical disease and was antibody negative. This case shows us that the clinical course and pathology of this second fatal human case were very different from the first human case, in whom the predominant signs were respiratory, and death occurred within 2 weeks after infection (64).

As in the case of horses, the etiological agent, the epidemiology and the mode of transmission of the virus have been already discussed.

4.11 Clinical Signs

The disease has a rough fatality rate of about 57%.

After the identification of the first four human cases, the incubation period was initially estimated at 6-8 days, however, the later cases presented with longer incubation period of up to 21 days.

HeV infection in humans causes two distinct, but not mutually exclusive, syndromes: acute respiratory syndrome (ARDS) and meningoencephalitis. The symptoms may include fever, headache, dry cough, sore throat, breathing difficulties, dizziness, unusual sleepiness, and confusion. Fatal complications also included septic pneumonia.

- Case 1: August 1994, 35 years old male farmer. He was exposed by close contact with respiratory secretions and blood of infected horses by assisting in the necropsy of an infected horse. It is reported that he had non-intact skin on arms and hands. He developed an influenza-like illness (pharyngitis, headache, drowsiness, vomiting) and aseptic meningitis (neck stiffness) in August 1994, with initial recovery followed by fatal relapse and death of encephalitis on the 25th day of hospitalization. In this case, the incubation period was of 6-7 days.
- Case 2: September 1994, 40 years old male stable hand. He had close contact with respiratory secretions of an infected horse. He developed an influenza-like illness (fever, pharyngitis, myalgia, headaches, lethargy, and vertigo). The incubation period was of 8 days. He fully recovered, with no relapse.
- Case 3: September 1994, 49 years old male horse trainer. He had close contact with respiratory secretions of an infected horse by non-intact skin. He developed an influenza-like illness and dyspnea with subsequent multiorgan failure (respiratory and renal failure), arterial thrombosis in right leg and fatal cardiac arrythmia. The incubation period was of 7 days and he died after 13 days of illness.

- Case 4: October 2004, 25 years old female veterinarian. She was infected by performing a necropsy of an infected horse. She developed an influenza-like illness (fever, dry cough, pharyngitis, myalgia, lethargy and cervical lymphadenopathy). The incubation period was of 7 days. She recovered.
- Case 5: July 2008, 33-year-old male veterinarian. He had close contact with respiratory secretions of an infected horse while he was performing a necropsy. He developed an influenza-like illness, thrombocytopenia, subsequent encephalitis and eventual unconsciousness. The incubation period was of 9-16 days. He died on the 45th of illness.
- Case 6: July 2008, 21-year-old female veterinarian nurse. She also had close contact with respiratory secretions of infected horses. She developed an influenza-like illness with subsequent encephalitis on day the 12th of illness. The incubation period was of 9-16 days. She recovered but she had persistent neurological deficits.
- Case 7: August 2009, 51 years old male veterinarian. He got infected by close contact with respiratory secretions of infected horses. He developed encephalitis following 5 days of post-exposure prophylaxis with intravenous ribavirin and hydroxychloroquine. In this case, the incubation period was of 11-12 days. He died on the 19th day of illness.

Six of the patients initially exhibited respiratory symptoms and influenza-like illness. The seventh patient was suspected to have been exposed to HeV and was therefore given a post-exposure prophylaxis regimen consisting of a 5-day course of IV ribavirin and oral hydroxychloroquine. This patient did not develop any influenza-like illness or respiratory symptoms, but developed encephalitis shortly after the 5-day regimen ended, and possibly died. This treatment likely prevented the respiratory illness, but did not prevent the virus from spreading systemically, infecting the nervous system, and causing death. Of the three survivors, two completely recovered from the influenza-like illness, without having any neurological symptoms and without any relapse. The third survivor developed encephalitis after the initial influenza-like symptoms and still suffers from some neurological deficits as a result of the illness. The other four patients all died following the infections. One of these recovered from the initial illness (influenza-like symptoms and aseptic meningitis), but 13 months later developed encephalitis, which was eventually fatal. This patient had no second exposure to HeV. This suggests that HeV can remain dormant in a human for an extended period of time

followed by later reactivation. The third case developed severe respiratory symptoms and multiorgan failure (renal and respiratory failure) but developed no neurological symptoms before dying of a fatal cardiac arrhythmia. The last patient developed acute encephalitis within days of presenting influenza-like symptoms. The encephalitis was eventually fatal.

From the cases observed to date, HeV infection in humans seems to always lead to initial influenza-like illness, following which a patient may recover with or without encephalitic relapse, develop acute encephalitis, which may or not prove fatal, or die from multiorgan failure as the disease goes systemic (106).

4.12 Pathogenesis

As mentioned previously while discussing the HeV horse infection, the receptor for the HeV G protein is ephrin-B2. Ephrin-B2 is a ligand and functions as a receptor tyrosine kinase which binds to the receptors EphB4 and EphB2 causing bidirectional signaling cascades in the cells respectively. This signaling though ephrin-B2 results in cell adhesion and repulsion which plays a role in chemotaxis and cell migration. The cellular receptor for HeV entry, ephrin-B2, is mainly expressed in neuronal and endothelial cells *in vivo*. With the distribution of receptor expression affecting the tropism of the virus, it is no surprise what are the cells that show the most pathology during HeV infection (106).

The respiratory epithelium is an important first line of defense and actively involved in inflammation and host defense against infectious diseases. The first fatal human case of HeV infection resulted in severe respiratory disease in which the lungs had gross lesions of congestion hemorrhage and edema associated with histological chronic alveolitis. Overall, histopathological changes of the tracheal/bronchial epithelium were uncommon. In experimental animal models, viral antigen is firstly detectable in the bronchi and alveoli, primarily targeting the bronchial epithelium and type II pneumocytes. It has recently been demonstrated that HNV can efficiently infect epithelial cells from the lower human respiratory tract and replicate to high titer. HNV infection of the respiratory epithelium results in the induction of inflammatory cytokines which then results in the recruitment of immune cells and can progress to an ARDS-like disease. Infection of lower respiratory tract epithelium results in differential inflammatory response depending on the sites of infection. HNV infection of the

small airway epithelium results in the induction of key inflammatory mediators such as IL-6, 8, IL-1α, MCP-1, G-CSF, GM-CSF and CXCL10. Interestingly, inflammatory cytokine expression was significantly lower in tracheal/bronchial epithelium in identified cases. Many of these cytokines in HNV infection play a role in ARDS and are also highly expressed during infection with other virulent respiratory viruses, such as H5N1 and SARS-CoV.

During the late stages of disease, virus replication spreads from the respiratory epithelium to the endothelium in the lungs. The infection can sometimes trigger a prominent vasculitis in small vessels and capillaries as characterized by endothelial syncytium and mural necrosis. Large vessels are usually not affected. HNV can then enter the bloodstream and disseminate throughout the host in either free form or by binding to host leukocytes. In addition to the lungs, other important target organs are the brain, spleen and kidneys, and viremia following respiratory infection can lead to multi-organ failure. Interestingly, HNV has been shown to bind to CD3+ leukocytes without entry or replication of the virus. It is currently unknown whether binding of HNV or infection of human leukocytes will affect the phenotype of the cells, such as increased CD6 expression, thereby preferentially homing to the CNS (127).

Entry into the CNS is thought to occur through two distinct pathways: anterogradely via the olfactory nerve and/or via the hematogenous route through the choroid plexus and cerebral blood vessels. Infection of the CNS in humans is characterized by vasculitis, thrombosis, parenchymal necrosis, and presence of viral inclusion bodies. Plaques with necrosis are found in both the gray and white matter and vasculitis, thrombosis, and parenchymal edema and inflammation are found in the vicinity of these plaques. Inflammatory cells found in the CNS primarily consist of neutrophils, macrophages, lymphocytes, and reactive microglia. HNV antigen can typically be detected in neurons and neuronal processes and endothelial cells. Occasionally, viral antigen is also detected in ependymal cells and rare glial cells in the white matter (127).

HNV infection of the CNS and the development of neurological signs are linked with the disruption of the blood-brain barrier (BBB) and expression of TNF-α and IL-1β. These pro-inflammatory cytokines have been shown to play a role in increasing the permeability of the blood-brain barrier, as well as the induction of neuronal injury and death. While the source of TNF-α and IL-1β expression in the brain is currently unknown, they can be released by microglia, which are also infected by HNV. However, whether disruption of the BBB is a direct

cytopathic effect of virus replication in the microvasculature or an indirect effect through expression of TNF-α and IL-1β by bystander cells such as neurons and microglia remains unclear (127).

4.13 Pathology

Autopsies were performed only on three of the four fatal cases of human HeV infection. The findings of these exams showed the general hallmarks of human HeV infection as disseminated vasculitis with extensive endothelial cell involvement which led to thrombosis, ischemia and necrosis. Parenchymal cell infection, particularly the brain, lung, kidney, and heart was also noted. Although there were many similarities, the exact pathology varied between the three cases.

Because the first patient died from relapsed encephalitis without respiratory or systemic disease, pathological changes occurred only in the CNS, particularly in the cerebral cortex, although a few small lesions occurred in the pons, cerebellum and spinal cord. There were numerous inflammatory lesions in the cerebral cortex, with massive infiltration of macrophages, lymphocytes and few plasma cells. Prominent perivascular cuffing was observed. These lesions showed several neuronal losses, with the proliferation of glial cells and reactive blood vessels. Viral antigen was observed in surviving neurons. Was also observed severe inflammation of the meninges.

The third case, who died from lung and kidney failure with cardiac arrhythmias and arterial thrombosis, exhibited pathological changes and arterial thrombosis, as well as pathological changes throughout many organ systems, with widespread vasculitis. In this patient, there was severe inflammation and necrosis in the lungs with extensive macrophage and inflammatory cell infiltrates in the intra-alveolar spaces. Viral antigens were detected in the alveolar type II pneumocytes and intra-alveolar macrophages. Inflammation and necrosis were also seen in the glomeruli and around the tubules of the kidneys. Even though this patient showed no clinical signs of encephalitis, necrotic plaques were found throughout the brain. Although the lesions were much smaller and less numerous that those seen in the first case, the same type of neuronal loss was evident and viral antigen could be detected in the surviving neurons.

The seventh case, despite of dying from acute encephalitis without prior respiratory symptoms, did show systemic vasculitis with inflammation of the endothelium in the heart, kidneys,

pituitary gland, mesenteric arteries, coronary arteries, brain and meninges. Multifocal panencephalitis with cortical, cerebellar and white matter involvement was observed. Viral antigen was present in neurons and glial cells. Multiple vasculitis-induced infarcts were observed in the brain (106).

4.14Diagnosis

HeV infection can be suspected in any case of fever, which had recent close exposure to an ill or dead horse, in an endemic region, which died from an infection with signs of respiratory or neurological involvement. This especially when the horse is confirmed to have been infected with HeV. As we discussed previously, the initial symptoms and clinical features of human HeV infection are non-specific. Due to this, even if HeV infection is suspected, a diagnosis of HeV infection can only be made by laboratory diagnostic methods of BSL-4.

- Laboratory diagnostic: the methods we have presented in the analysis of diagnostic options for horses are valid also for humans.
- Medical imaging:
    - Chest x-rays: the only patient present with ARDS was the third case. A chest x-ray taken shows diffuse alveolar shadowing with bilateral alveolar and interstitial infiltration. In the fifth and seventh case, the chest x-rays initially showed no anomalies, but also showed bilateral alveolar infiltration towards the end of the disease. In the second and sixth patient, x-rays were normal during the course of their illness, but each presented only minor or no respiratory symptoms. For the other cases, chest x-rays weren't performed.
    - Magnetic resonance imaging (MRI): MRIs were performed on all four patients present with encephalitis. A limited MRI study was performed on the first case. Although he initially presented aseptic meningitis, CT scan showed no abnormalities. When this patient relapsed with encephalitis, an MRI finding on day 6 of hospitalization showed widespread multifocal involvement of the neocortex with sparing of the subcortical white mater. This pattern was not seen in other types of encephalitis. More detailed MRI studies were conducted on the fifth and seventh cases. The lesions were confined to the basal ganglia and

cerebral cortex, with white matter and the cerebellum being spared. Lesions occurred in the precuneus in all three cases. After developing these initial lesions, the only survivor, who had fever and smaller lesions than the other cases, showed gradual resolution of the cortical lesion, while cases five and seven showed rapid progression of the cortical lesions, with lesion eventually appearing in the white matter as well. This progression was correlated with a deterioration in each patient's clinical condition. A perfusion study was performed in case seven, which showed reduced blood flow and blood volume in the areas of the lesion consistent with vasculitis-induced acute ischemia, which might explain the lesions.

- Electroencephalography (EEG): in the fifth case, which turned out to be fatal, EEG initially showed bilateral, high voltage, slow waves with epileptiform activity. As the disease progressed, this deteriorated to an absence of stable background rhythm with slow wave activity and periodic sharp discharges, with the development of an epileptogenic focus. In the survived patient with encephalitis, EEG showed severe, diffuse, encephalopathy with high-amplitude slow waves, which improved concurrently with the patient's clinical symptoms.
- Experimental diagnostics: includes pseudotype viruses, liquid protein array, microsphere suspension array (106).

4.15 Therapy

There are no approved or licensed therapeutics for treating HNV infection or disease in people, and antiviral approaches against the HNV that have been tested in animal cases are few, among which the ribavirin. In fact this drug is a well-known first line treatment strategy for suspected viral infections of unknown etiology. It exhibits antiviral activity against a wide variety of both RNA and some DNA viruses. *In vitro* studies have shown that ribavirin is effective against both Hendra and Nipah replication. The anti-malarial drug chloroquine was also shown earlier to block the critical proteolytic processing needed for the maturation and function of the HeV F glycoprotein. Not surprisingly, chloroquine was later shown to inhibit Nipah and Hendra infection in cell culture. In the examples of the recorded human HeV cases, three individuals

were treated with ribavirin and, of these, two succumbed to the disease and one survived. Chloroquine was administered along with ribavirin to one HeV-infected individual in 2009 with no apparent clinical benefit. Three additional patients received ribavirin treatment in combination with chloroquine after suspected exposure to HeV contaminated secretions from infected horses. While all three individuals survived, infection was not confirmed and therefore it remains unknown whether the treatment had any effect. In the absence of other therapies, ribavirin may be an option for the treatment of HNV infections. However, more recent studies have revealed no therapeutic benefit of either drug.

In contrast, passive immunotherapy with polyclonal or mAb specific for the viral envelope glycoproteins has proved successful. Currently, the only reported and effective post-exposure therapy against HeV or NiV infection and one that has the opportunity to be approved in the near future for use in people has been a human mAb known as m102.4 which was isolated from a recombinant naïve human phage-displayed Fab library. The m102.4 mAb has exceptionally potent neutralizing activity against both NiV and HeV and its epitope maps to the ephrin receptor binding site (128). When administered post-exposure to laboratory animals, m102.4 has been shown to prevent acute Nipah and Hendra-associated morbidity and mortality in ferrets and in African Green monkeys, although infection is not prevented. In addition, amelioration of disease signs in both species is optimal when m102.4 is administered within 24 hours of exposure to virus, prior to the detection of Hendra viral RNA in the blood or its recovery from oropharyngeal secretions, and before the onset of fever or other clinical signs (121).

4.16Prevention and control

4.16.1 Vaccination

As it is difficult to determine which vaccine platform would be best for the human population against deadly pathogens such as HeV, it is important to develop and test a number of platforms to combat an outbreak. One such new platforms is based on the recombinant adeno-associated vaccine vector expressing the closely related NiV G protein. This vaccine was shown to protect hamsters 100% from lethal NiV challenge, though the efficacy was only 50% for HeV. While a result of 50% protection against HeV challenge is encouraging, there is currently only one vaccine, which protects multiple animals against the infection. The HeV sG subunit vaccine is

an engineered, secreted version of the full G protein in which the transmembrane and cytoplasmic tail domains have been deleted from the N-terminus. When this protein is expressed in mammalian cells, it is released from cells, and the sG has been shown to retain the natural characteristics of the membrane bound version with the ability to oligomerize into dimers and tetramers, which retain the ability to bind to ephrin-B2. This type of vaccine has been successfully used in horses since 2012 (106).

At present time, however, there is no tested vaccination for human use. Currently (2021), based on the same immunogen, the HeV attachment glycoprotein ectodomain, a subunit vaccine formulation for use in people is now in a Phase I clinical trial. The study reports that a single dose vaccination regimen of this human vaccine formulation protects against otherwise lethal challenges of either HeV or NiV viruses in a nonhuman primate model. The data suggests that this human vaccine could be utilized as efficient emergency vaccine to disrupt potential spreading of Nipah disease in an outbreak setting and hopefully also for HeV (129).

4.16.2 Public Health measures

Considering the high rate of mortality and the potential risk to public health, HeV is a real concern for the Australian government, but also for all the other health authorities around the world. As mentioned above, the HeV represents a significant example of a virus with a high epidemic risk and its propagation in other continents could have disastrous effects. For this reason, it is fundamental for Australia to put in practice effective contagion control measures.

Considering the poor outcomes of humans infected with HeV, and because there are no approved therapeutics to treat HeV infection, it is extremely important to prevent humans from being infected by the virus. Due to the large population size, extensive geographical range, and the nomadic nature of the Australian flying foxes, it is impractical to attempt control interventions, such as vaccination or culling, in the flying foxes population. There is no evidence of direct bat-to-human transmission, with the second measure being the prevention of horse-to-human transmission. Preventing bat-to-horse transmission is important, because if no horses are infected with HeV, it becomes highly unlikely that any humans are infected. In this regard, we have already discussed the ways in which we can prevent bat-to-horse transmission. Even if all horses in the endemic area are vaccinated for HeV, there remains the possibility that outbreaks will continue to occur in the equine population with possible spillover into humans. In order to

prevent further horse-to-human transmission, public awareness of HeV must be increased, especially among veterinarians and horse owners. In fact, as we have already highlighted, although the number of spillover events in horses has been very high since 2011, no humans contracted the virus since then. This is likely due to the considerable efforts made to educate veterinarians about recognizing the signs of HeV infection and to consider it in the differential diagnosis of sick horses, as well as the more frequent use of appropriate protective PEE when working with sick horses.

The control measures delineated in the Australian National guideline for public health for horses are similar for humans. These measures includes:

- Identification of contacts.
- Contact definition: People who have had close contact with a symptomatic confirmed human case or person where heightened suspicion of infection exists on clinical and epidemiological grounds as determined by the relevant public health unit (including household or household-like contacts, sexual partners, and anyone with direct or indirect exposure of skin or mucous membranes to body fluids).
- While there is no evidence of human-to-human transmission, standard, contact and droplet precautions should be in place for all visitors and health-care workers caring for symptomatic persons suspected or known to be infected with HeV. Additional precautions may be ordered at the discretion of the treating infectious diseases physician.
- Airborne precautions should be implemented during any aerosol-generating procedures.
- Confirmed cases should avoid close contact with animals during acute illness.
- Confirmed cases should never subsequently donate blood or any other tissue, even if they recover. Local public health units should liaise with the Australian Red Cross Blood Service to record HeV infection status for cases.
- For all human contacts of confirmed equine cases:
    - assess exposure and current health status;

- if exposure is classified as high or as having classification uncertainty (medium or high) using the exposure assessment form, liaise with an infectious diseases physician as soon as practicable for consideration of post-exposure prophylaxis;
- if assessed as medium exposure, discuss as soon as possible with other appropriately-experienced public health practitioners and infectious diseases physician/s to reach consensus on exposure assessment;
- refer any symptomatic people to appropriate care;
- provide information about the Hendra virus;
- counsel about risk;
- provide advice about testing recommendations;
- provide advice about self-monitoring of the contact's health and advise the person to seek early medical advice if they develop fever or respiratory or neurological symptoms within three weeks of exposure, phoning ahead of the visit so that appropriate infection control measures can be put in place (124).

Conclusion

Cross-species transmissions are an increasingly common event capable of causing health crises all over the world. Also, horses should be considered in their spillover potential, giving cause for concern to public health authorities. The pandemic potential of diseases that come from horses may not be as obvious as for other species: in develop countries, the horse is now almost exclusively recognized for its sporting abilities and, as such, is considered like other companion animals. For these reasons, it necessitates its own particular focus. Today we hardly find intensive horse farming conditions, where numerous animals coexist in the same environment, and in which ideal circumstances are created for a pathogen to jump into another specie and trigger a global emergency. Despite this consideration, no less attention should be paid to its potential risk. In fact, precisely because the horse is considered as a pet animal, this somehow increases the possibility of contact between horses and humans, but also between horses and the other companion animals. This creates a different but possible situation for a virus to emerge. In addition, we must also consider the occupational risk for equine veterinarians.

The spillover of EIV to dogs, and of the HeV from horse to human further underlines the need of including the horse and all the related activities in the global surveillance and prevention program for EID. Moreover, it is imperative to opt for a 'One Health' approach in the surveillance of novel infectious diseases. This is because animal and human health should be considered as two sides of the same coin, and as such, indispensable to each other. In developing this vision, it is also essential to recognize the role of veterinarians, who act as a bridge between the animal and the human world, not only in the realm of public health but also in informing and instructing the owners on these risks.

Bibliography

1. David Quammen. Spillover: Animal Infections and The Next Human Pandemic. 527th ed. Vol. Adelphi Edizioni. 2014.
2. Childs JE, Richt JA, Mackenzie · J S. Introduction: Conceptualizing and Partitioning the Emergence Process of Zoonotic Viruses from Wildlife to Humans.
3. Merriam-Webster: Definition of the term "zoonosis." https://www.merriam-webster.com/dictionary/zoonosis.
4. Woolhouse MEJ, Haydon DT, Antia R. Emerging pathogens: The epidemiology and evolution of species jumps. Vol. 20, Trends in Ecology and Evolution. Elsevier Ltd; 2005. p. 238–44.
5. Wang LF, Anderson DE. Viruses in bats and potential spillover to animals and humans. Vol. 34, Current Opinion in Virology. Elsevier B.V.; 2019. p. 79–89.
6. Lloyd-Smith JO, George D, Pepin KM, Pitzer VE, Pulliam JRC, Dobson AP, et al. Epidemic dynamics at the human-animal interface. Vol. 326, Science. 2009. p. 1362–7.
7. lo Iacono G, Cunningham AA, Fichet-Calvet E, Garry RF, Grant DS, Leach M, et al. A Unified Framework for the Infection Dynamics of Zoonotic Spillover and Spread. PLoS Neglected Tropical Diseases. 2016 Sep 2;10(9).
8. Robin A Weiss & Anthony J McMicheal. Social and enviromental risk factors in the emergence of incetious disease. Nature. 2004;10:570–5.
9. Wasik BR, de Wit E, Munster V, Lloyd-Smith JO, Martinez-Sobrido L, Parrish CR. Onward transmission of viruses: How do viruses emerge to cause epidemics after spillover? Vol. 374, Philosophical Transactions of the Royal Society B: Biological Sciences. Royal Society Publishing; 2019.
10. Romero-Tejeda A, Capua I. Virus-specific factors associated with zoonotic and pandemic potential. Vol. 7, Influenza and other Respiratory Viruses. 2013. p. 4–14.
11. Liu P, Jiang JZ, Wan XF, Hua Y, Li L, Zhou J, et al. Are pangolins the intermediate host of the 2019 novel coronavirus (SARS-CoV-2)? Vol. 16, PLoS Pathogens. Public Library of Science; 2020.

12. World Health Organization. https://www.who.int.
13. Raina K.Plowright CRPHMPJHAIKALG and JOL-S. Pathways to zoonitc spillover. Nature. 2017;15:502–8.
14. Plowright RK, Eby P, Hudson PJ, Smith IL, Westcott D, Bryden WL, et al. Ecological dynamics of emerging bat virus spillover. Vol. 282, Proceedings of the Royal Society B: Biological Sciences. Royal Society of London; 2014.
15. Haydon DT, Cleaveland S, Taylor LH, Laurenson MK. Identifying Reservoirs of Infection: A Conceptual and Practical Challenge. Vol. 8. 2002.
16. Johnson CK, Hitchens PL, Pandit PS, Rushmore J, Evans TS, Young CCW, et al. Global shifts in mammalian population trends reveal key predictors of virus spillover risk. Proceedings of the Royal Society B: Biological Sciences. 2020 Apr 8;287(1924).
17. Subudhi S, Rapin N, Misra V. Immune system modulation and viral persistence in bats: Understanding viral spillover. Vol. 11, Viruses. MDPI AG; 2019.
18. de La Cruz-Rivera PC, Kanchwala M, Liang H, Kumar A, Wang L-F, Xing C, et al. The IFN Response in Bats Displays Distinctive IFN-Stimulated Gene Expression Kinetics with Atypical RNASEL Induction. The Journal of Immunology. 2018 Jan 1;200(1):209–17.
19. Plowright RK, Peel AJ, Streicker DG, Gilbert AT, McCallum H, Wood J, et al. Transmission or Within-Host Dynamics Driving Pulses of Zoonotic Viruses in Reservoir–Host Populations. Vol. 10, PLoS Neglected Tropical Diseases. Public Library of Science; 2016.
20. Gerow CM, Rapin N, Voordouw MJ, Elliot M, Misra V, Subudhi S. Arousal from hibernation and reactivation of Eptesicus fuscus gammaherpesvirus (EfHV) in big brown bats. Transboundary and Emerging Diseases. 2019 Mar 1;66(2):1054–62.
21. Davy CM, Donaldson ME, Subudhi S, Rapin N, Warnecke L, Turner JM, et al. White-nose syndrome is associated with increased replication of a naturally persisting coronaviruses in bats. Scientific Reports. 2018 Dec 1;8(1).
22. Letko M, Seifert SN, Olival KJ, Plowright RK, Munster VJ. Bat-borne virus diversity, spillover and emergence. Vol. 18, Nature Reviews Microbiology. Nature Research; 2020. p. 461–71.

23. Parrish CR, Holmes EC, Morens DM, Park E-C, Burke DS, Calisher CH, et al. Cross-Species Virus Transmission and the Emergence of New Epidemic Diseases. Microbiology and Molecular Biology Reviews. 2008 Sep;72(3):457–70.
24. Kuchipudi S v, Nelli RK, Gontu A, Satyakumar R, Surendran Nair M, Subbiah M. Sialic Acid Receptors: The Key to Solving the Enigma of Zoonotic Virus Spillover. Viruses. 2021 Feb 8;13(2):262.
25. Borremans B, Faust C, Manlove KR, Sokolow SH, Lloyd-Smith JO. Cross-species pathogen spillover across ecosystem boundaries: Mechanisms and theory. Vol. 374, Philosophical Transactions of the Royal Society B: Biological Sciences. Royal Society Publishing; 2019.
26. Plowright RK, Reaser JK, Locke H, Woodley SJ, Patz JA, Becker DJ, et al. Land use-induced spillover: a call to action to safeguard environmental, animal, and human health. The Lancet Planetary Health [Internet]. 2021 Mar; Available from: https://linkinghub.elsevier.com/retrieve/pii/S2542519621000310
27. Wolfe ND, Dunavan CP, Diamond J. Origins of major human infectious diseases. Vol. 447, Nature. Nature Publishing Group; 2007. p. 279–83.
28. Domingo E, Holland JJ. RNA VIRUS MUTATIONS AND FITNESS FOR SURVIVAL [Internet]. 1997. Available from: www.annualreviews.org
29. Jones KE, Patel NG, Levy MA, Storeygard A, Balk D, Gittleman JL, et al. Global trends in emerging infectious diseases. Nature. 2008 Feb 21;451(7181):990–3.
30. Delamater PL, Street EJ, Leslie TF, Yang YT, Jacobsen KH. Complexity of the basic reproduction number (R0). Emerging Infectious Diseases. 2019 Jan 1;25(1):1–4.
31. Holmes EC, Rambaut A, Andersen KG. Pandemics: Spend on surveillance, not prediction comment. Vol. 558, Nature. Nature Publishing Group; 2018. p. 180–2.
32. http://www.globalviromeproject.org/our-approach. Global Virome Project.
33. Naji RK, Hussien RM. The Dynamics of Epidemic Model with Two Types of Infectious Diseases and Vertical Transmission. Journal of Applied Mathematics. 2016;2016.
34. Introduction to Epidemic Modelling. Available from: http://www.stat.columbia.edu/~regina/research/notes123.pdf

35. Hethcote HW. Three Basic Epidemiological Models.
36. Ball F. Deterministic and Stochastic Epidemics with Several Kinds of Susceptibles [Internet]. Vol. 17. 1985. Available from: https://www.jstor.org/stable/1427049?seq=1&cid=pdf-
37. Chowell G, Sattenspiel L, Bansal S, Viboud C. Mathematical models to characterize early epidemic growth: A review. Vol. 18, Physics of Life Reviews. Elsevier B.V.; 2016. p. 66–97.
38. Ellwanger JH, Kaminski V de L, Chies JAB. Emerging infectious disease prevention: Where should we invest our resources and efforts? Vol. 12, Journal of Infection and Public Health. Elsevier Ltd; 2019. p. 313–6.
39. Hinma AR. Global progress in infectious disease control. Vol. 16, Vaccine. 1998.
40. The COVID-19 pandemic: A time for veterinary leadership in one health [Internet]. Available from: www.avma.
41. Eloit M. Strengthening veterinary services through the oie pvs pathway Executive Summary The Case for Investment in Veterinary Services.
42. OIE - World Organisation for Animal Health. https://www.oie.int.
43. A Tripartite Guide to Addressing Zoonotic Diseases in Countries Taking a Multisectoral, One Health Approach.
44. Old List A OIE. https://www.oie.int/en/animal-health-in-the-world/the-world-animal-health-information-system/old-classification-of-diseases-notifiable-to-the-oie-list-a/.
45. Marabelli R, Ferri G, Bellini & S. Management of animal health emergencies: general principles and legal and international obligations. Vol. 18, Rev. sci. tech. Off. int. Epiz. 1999.
46. Regolamento di polizia veterinaria DPR n. 320 8 febbraio 1954.
47. A consistent set of good practices for preparing, delivering, and learning from animal health and welfare and veterinary public health simulation exercises for Veterinary Services guidelines for simulation exercises guidelines for simulation exercises.
48. World Health Organization. Managing epidemics : key facts about major deadly diseases.

49. Improving pandemic preparedness and management Research and Innovation Independent Expert Report. Available from: http://europa.eu
50. Lönker NS, Fechner K, Wahed AA el. Horses as a crucial part of one health. Veterinary Sciences. 2020 Mar 1;7(1).
51. Bender JB, Tsukayama DT. Horses and the risk of zoonotic infections. In: Veterinary Clinics of North America - Equine Practice. 2004. p. 643–53.
52. Kumar B, Manuja A, Gulati B, Virmani N, Tripathi BN. Zoonotic Viral Diseases of Equines and Their Impact on Human and Animal Health. The Open Virology Journal. 2018 Sep 14;12(1):80–98.
53. Angenvoort J, Brault AC, Bowen RA, Groschup MH. West Nile viral infection of equids. Veterinary Microbiology. 2013 Nov 29;167(1–2):168–80.
54. Epicentro; ISS. Febbre West Nile https://www.epicentro.iss.it/westnile/documentazione-italia.
55. CESME IGCT. Newsletter CESME: Autumn-summer period 2020. https://www.epicentro.iss.it/westnile/bollettino.
56. Field HE. Hendra virus ecology and transmission. Vol. 16, Current Opinion in Virology. Elsevier B.V.; 2016. p. 120–5.
57. Zacks MA, Paessler S. Encephalitic alphaviruses. Vol. 140, Veterinary Microbiology. 2010. p. 281–6.
58. Morens DM, Taubenberger JK. Historical thoughts on influenza viral ecosystems, or behold a pale horse, dead dogs, failing fowl, and sick swine. Influenza and other Respiratory Viruses. 2010 Nov;4(6):327–37.
59. Landolt GA. Equine influenza virus. Vol. 30, Veterinary Clinics of North America - Equine Practice. W.B. Saunders; 2014. p. 507–22.
60. Singh RK, Dhama K, Karthik K, Khandia R, Munjal A, Khurana SK, et al. A comprehensive review on equine influenza virus: Etiology, epidemiology, pathobiology, advances in developing diagnostics, vaccines, and control strategies. Vol. 9, Frontiers in Microbiology. Frontiers Media S.A.; 2018.
61. van Maanen C, Cullinane A. Equine influenza virus infections: An update. Veterinary Quarterly. 2002 Jan 1;24(2):79–94.

62. International Commitee on Taxonomy of Viruses (ICTV) - Taxonomy of Orthomyxoviridae Family. https://talk.ictvonline.org/taxonomy/p/taxonomy-history?taxnode_id=201903953.
63. Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. Evolution and ecology of influenza A viruses. Vol. 56, Microbiological Reviews. 1992. p. 152–79.
64. Sellon DC, Long MT. Equine infectious diseases. Saunders/Elsevier; 2007. 653.
65. J. H. Gilliespie JFT ediz. italiana a cura di FS. Malattie infettive degli animali domestici di Hagan e Bruner.
66. International Commitee on Taxonomy of Viruses (ICTV) - ICTV 9th Report (2011). https://talk.ictvonline.org/ictv-reports/ictv_9th_report/negative-sense-rna-viruses-2011/w/negrna_viruses/209/orthomyxoviridae.
67. Potter CW. A history of influenza.
68. 1968 flu pandemic -- Britannica Online Encyclopedia.
69. Rozo M, Gronvall GK. The reemergent 1977 H1N1 strain and the gain-of-function debate. mBio. 2015 Aug 11;6(4).
70. CDC: Center for Disease Control and Prevention. 2009 H1N1 Pandemic. https://www.cdc.gov/flu/pandemic-resources/2009-h1n1-pandemic.html.
71. CDC: Center for Disease COntrol and Prevention. Information on Swine Influenza. https://www.cdc.gov/flu/swineflu/index.htm.
72. Alexander DJ, Brown IH. History of highly pathogenic avian influenza. Vol. 28, Rev. sci. tech. Off. int. Epiz. 2009.
73. Cullinane A, Newton JR. Equine influenza-A global perspective. Vol. 167, Veterinary Microbiology. 2013. p. 205–14.
74. Mucha V, Hollý J, Varečková E, Kostolanský F. Avian influenza A virus adaptation to the equine host and identification of host-specific markers. Acta Virologica. 2018;62(3):266–76.
75. Virus de la grippe équine: épidémiologie, diagnostic et vaccination. F Laabassi, B Mamache; Revue Med Vet. 2014;165:31–43.
76. Myers C, Wilson WD. Equine Influenza Virus. Clinical Techniques in Equine Practice. 2006 Sep;5(3):187–96.

77. OIE Equine influenza. https://www.oie.int/en/disease/equine-influenza-2/.
78. Rush BR. Equine Influenza Etiology and Epidemiology [Internet]. 2020. Available from: https://www.merckvetmanual.com/respiratory-system/respiratory-diseases-of-horses/equine-influenza#
79. Equine Influenza : An Overview [Internet]. Available from: www.veterinaryworld.org
80. Yang H, Xiao Y, Meng F, Sun F, Chen M, Cheng Z, et al. Emergence of H3N8 equine influenza virus in donkeys in China in 2017. Veterinary Microbiology. 2018 Feb 1;214:1–6.
81. Pavulraj S, Bera BC, Joshi A, Anand T, Virmani M, Vaid RK, et al. Pathology of equine influenza virus (H3N8) in murine model. PLoS ONE. 2015 Nov 1;10(11).
82. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals [Internet]. 2009. Available from: http://oie.int/our-scientific-
83. Rash A. Diagnosis of equine influenza. Veterinary Record. 2017 Jul 29;181(5):113–4.
84. Arthur RJ, Suann CJ. Biosecurity and vaccination strategies to minimise the effect of an equine influenza outbreak on racing and breeding. Australian Veterinary Journal. 2011 Jul;89(SUPPL.1):109–13.
85. Paillot R. A systematic review of recent advances in equine influenza vaccination. Vol. 2, Vaccines. MDPI AG; 2014. p. 797–831.
86. OIE Terrestrial Animal Code. Equine influenza (infection with equine influenza virus).
87. CVMP. ProteqFlu-Te; INN: vaccine against equine influenza and tetanus in horses [Internet]. Available from: www.ema.europa.eu
88. Equine Influenza Vaccination AAEP. https://aaep.org/guidelines/vaccination-guidelines/risk-based-vaccination-guidelines/equine-influenza.
89. FEI: table of contents foreword 5 FEI code of conduct for the welfare of the horse.
90. FISE: regolamento veterinario, Edizione in vigore dal 24 Febbraio 2021. 2021.
91. Xie T, Anderson BD, Daramragchaa U, Chuluunbaatar M, Gray GC. A review of evidence that equine influenza viruses are zoonotic. Vol. 5, Pathogens. MDPI AG; 2016.

92. E. Paul J. Gibbs and Tara C. Anderson. Equine and canine influenza: a review of current events.
93. Crawford PC, Dubovi EJ, Castleman WL, Stephenson I, Gibbs EPJ, Chen L, et al. Epidemiology: Transmission of equine influenza virus to dogs. Science. 2005 Oct 21;310(5747):482–5.
94. Dubovi EJ, Njaa BL. Canine Influenza. Vol. 38, Veterinary Clinics of North America - Small Animal Practice. 2008. p. 827–35.
95. Parrish CR, Murcia PR, Holmes EC. Influenza Virus Reservoirs and Intermediate Hosts: Dogs, Horses, and New Possibilities for Influenza Virus Exposure of Humans. Journal of Virology. 2015 Mar 15;89(6):2990–4.
96. Parrish CR, Voorhees IEH. H3N8 and H3N2 Canine Influenza Viruses: Understanding These New Viruses in Dogs. Vol. 49, Veterinary Clinics of North America - Small Animal Practice. W.B. Saunders; 2019. p. 643–9.
97. Daly JM, Blunden AS, Macrae S, Miller J, Bowman SJ, Kolodziejek J, et al. Transmission of Equine Influenza Virus to English Foxhounds [Internet]. Available from: www.cdc.gov/eid
98. Wen F, Blackmon S, Olivier AK, Li L, Guan M, Sun H, et al. Mutation W222L at the Receptor Binding Site of Hemagglutinin Could Facilitate Viral Adaption from Equine Influenza A(H3N8) Virus to Dogs. Journal of Virology. 2018 Sep 15;92(18).
99. Yamanaka T, Nemoto M, Tsujimura K, Kondo T, Matsumura T. Interspecies transmission of equine influenza virus (H3N8) to dogs by close contact with experimentally infected horses. Veterinary Microbiology. 2009 Nov 18;139(3–4):351–5.
100. Yamanaka T, Nemoto M, Bannai H, Tsujimura K, Kondo T, Matsumura T, et al. No evidence of horizontal infection in horses kept in close contact with dogs experimentally infected with canine influenza A virus (H3N8) [Internet]. 2012. Available from: http://www.actavetscand.com/content/54/1/25
101. Jarod Hanson. What you need to know about dog flu [Internet]. Available from: https://www.cdc.

102. Smith AL. Canine Influenza: What You Should Know for Educating Clients [Internet]. 2012. Available from: http://www.avma.org/
103. Jarod Hanson. What you to know about dog flu [Internet]. Available from: https://www.cdc.
104. Field H, Young P, Yob JM, Mills J, Hall L, Mackenzie J. The natural history of Hendra and Nipah viruses. 2001.
105. Hendra virus: the initial research (Queensland Primary Industries and Fisheries).
106. Chad E. Mire BAS and TWG. Pathogenesis of Hendra Virus in Humans. Human Emerging and Re-emerging Infections: Viral and Parasitic infections. 2016;1.
107. du Preez R. The science and mystery of hendra virus [Internet]. Available from: https://www.abc.net.au/news/2011-09-12/hendra-virus-explained/2881140?nw=0
108. Summary of Hendra virus incidents in horses. 1995.
109. Khusro A, Aarti C, Pliego AB, Cipriano-Salazar M. Hendra Virus Infection in Horses: A Review on Emerging Mystery Paramyxovirus. Vol. 91, Journal of Equine Veterinary Science. W.B. Saunders; 2020.
110. Barclay AJ, Paton DJ. Hendra (Equine Morbillivirus). Vol. 160, Veterinary Journal. Bailliere Tindall Ltd; 2000. p. 169–76.
111. Rima B, Balkema-Buschmann A, Dundon WG, Duprex P, Easton A, Fouchier R, et al. Paramyxoviridae Family - ICVT. Vol. 100, Journal of General Virology. 2021.
112. Cox RM, Plemper RK. Structure and organization of paramyxovirus particles. Vol. 24, Current Opinion in Virology. Elsevier B.V.; 2017. p. 105–14.
113. Tulsiani SM, Graham GC, Moore PR, Jansen CC, van den Hurk AF, Moore FAJ, et al. Emerging tropical diseases in Australia. Part 5. Hendra virus. Vol. 105, Annals of Tropical Medicine and Parasitology. 2011. p. 1–11.
114. Barclay AJ, Paton DJ. Hendra (Equine Morbillivirus). Vol. 160, Veterinary Journal. Bailliere Tindall Ltd; 2000. p. 169–76.
115. New strain of horse-killing Hendra virus identified - Horsetalk.co.nz.
116. Merriam Webster. Definition of amplifying host. https://www.merriam-webster.com/dictionary/amplifying%20host.

117. Field HE. Hendra virus ecology and transmission. Vol. 16, Current Opinion in Virology. Elsevier B.V.; 2016. p. 120–5.
118. Field H, de Jong C, Melville D, Smith C, Smith I, Broos A, et al. Hendra virus infection dynamics in Australian fruit bats. PLoS ONE. 2011 Dec 9;6(12).
119. Edson D, Peel AJ, Huth L, Mayer DG, Vidgen ME, McMichael L, et al. Time of year, age class and body condition predict Hendra virus infection in Australian black flying foxes (Pteropus alecto). Epidemiology and Infection. 2019;147.
120. Marco Martini. Lyssavirus Virus emergenti.
121. Middleton D. Hendra virus. Vol. 30, Veterinary Clinics of North America - Equine Practice. W.B. Saunders; 2014. p. 579–89.
122. Tan RHH, Hodge A, Klein R, Edwards N, Huang JA, Middleton D, et al. Virus-neutralising antibody responses in horses following vaccination with Equivac® HeV: a field study. Australian Veterinary Journal. 2018 May 1;96(5):161–6.
123. Schemann K, Annand EJ, Reid PA, Lenz MF, Thomson PC, Dhand NK. Investigation of the effect of Equivac® HeV Hendra virus vaccination on Thoroughbred racing performance. Australian Veterinary Journal. 2018 Apr 1;96(4):132–41.
124. National Australian Public health Guidelines for Hendra Virus [Internet]. 2018. Available from: http://www.environment.nsw.gov.au/animals/flyin
125. Wiethoelter AK, Schembri N, Dhand NK, Sawford K, Taylor MR, Moloney B, et al. Australian horse owners and their biosecurity practices in the context of Hendra virus. Preventive Veterinary Medicine. 2017 Dec 1;148:28–36.
126. New South Wales HeV Control Guideline for Public Health Units Revision History, 2010. Available from: http://www.outbreak.gov.au/pages/hendra-virus.aspx
127. Escaffre O, Borisevich V, Rockx B. Pathogenesis of Hendra and Nipah virus infection in humans. Vol. 7, Journal of Infection in Developing Countries. 2013. p. 308–11.
128. Broder CC, Xu K, Nikolov DB, Zhu Z, Dimitrov DS, Middleton D, et al. A treatment for and vaccine against the deadly Hendra and Nipah viruses. Vol. 100, Antiviral Research. 2013. p. 8–13.

129. Geisbert TW, Bobb K, Borisevich V, Geisbert JB, Agans KN, Cross RW, et al. A single dose investigational subunit vaccine for human use against Nipah virus and Hendra virus. npj Vaccines. 2021 Dec 1;6(1).