Hendra and Nipah viruses: pathogenesis, animal models and recent breakthroughs in vaccination

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Abstract: Hendra and Nipah viruses are two highly pathogenic zoonotic members of the genus Henipavirus, family Paramyxoviridae, requiring work under biosafety level 4 conditions due to a lack of effective therapy and human vaccines. Several vaccine candidates were protective in animal models: recombinant vaccinia virus expressing Nipah virus (NiV) F and G proteins in hamsters against NiV; recombinant ALVAC–NiV F and G in swine against NiV; recombinant Hendra virus (HeV) soluble G protein (sG$_{HeV}$) against HeV and NiV in cats, ferrets, horses, and African green monkeys (AGM); recombinant vesicular stomatitis virus-based vectors expressing NiV F or G against NiV in hamsters and ferrets; measles virus-based NiV G vaccine candidate in hamsters and AGMs against NiV; and adenovirus-associated virus expressing NiG protein, which protected hamsters against NiV. The sG$_{HeV}$ was licensed for use in horses (Equivac HeV®) in 2012. It is the first vaccine candidate licensed against a biosafety level 4 agent. With the development of suitable animal models (ferret, hamster and, importantly, AGM), progress can be made toward development of a human vaccine.

Keywords: henipavirus, equine, swine, human infection, animal models, vaccine candidates

Introduction
Hendra and Nipah viruses: reservoir, transmission, epidemiology

Henipaviruses are considered to be bat viruses, found to be associated with bats from the families Pteropididae, Phystolomidae, and Mormoopidae. Pteropus bats serve as a reservoir for two zoonotic viruses: Hendra virus (HeV) and Nipah virus (NiV), classified into genus Henipavirus in the family Paramyxoviridae, together with the recently discovered nonpathogenic Cedar virus.

Bats infected with henipaviruses do not develop an apparent disease, as confirmed by experimental infections with HeV in pregnant and nonpregnant Pteropus poliocephalus and P. alecto,3–5 and NiV in P. poliocephalus.6 Experimentally or naturally infected bats, however, seroconvert and shed the virus in urine, saliva, and uterine fluid.4–10 There is a greater than 40% prevalence of antibodies to HeV in Australian pteropid bats11,12 with viral RNA and infectious virus detected in urine samples collected under the roosting flying foxes,10 in anteater colonies of P. alecto (fetus) and P. poliocephalus (fetus and uterine fluid).7 NiV antibodies were detected in Cynopterus brachyotis, Eonycteris spelaea, Scotophillus, P. vampyrus, P. hypomelanus, P. giganteus, and P. lylei; the reservoir status of the bats was confirmed by the detection of viral RNA or by virus isolation.8–13,15
The geographical locations of the up to date HeV and NiV outbreaks correspond to the geographical distribution of the natural reservoir species in Australia (Hendra) and Southeast Asia (Nipah), respectively (Figure 1).

HeV and NiV are able to successfully cross interspecies barriers and infect a wide range of host species; in this respect, they are unusual among paramyxoviruses. Spillover of HeV and NiV from their natural reservoir to humans, pigs, or horses with secondary transmission to humans is sporadic, and appears to be seasonal, possibly related to stress in bats due to their natural life cycle, such as mating and breeding, enhanced by environmental pressures due to a loss of habitat.

Henipaviruses were discovered in 1994 in Australia during an outbreak of severe respiratory disease in horses, with 14 animal deaths. During the outbreak, virus transmission also occurred to a horse trainer and a stable hand providing care for the sick horses. The etiological agent was initially named equine morbillivirus, and it was later renamed (and reclassified) after the geographical location of the first isolate to HeV. None of the subsequent sporadic outbreaks in horses was as extensive as the first recognized one. A total of 90 deaths in horses was recorded over the next 20 years (between 1994 and 2014), with most of the self-limiting outbreaks occurring between May/June and September/October. Horses are suspected to become infected by grazing on pastures contaminated by bat urine, food debris (masticated fruit), or birthing material. Horse-to-horse transmission occurs via respiratory secretions—either due to direct contact among horses, or as a fomite. The virus transmission is not considered very effective unless a superspreader is involved, as was the case in the initial outbreak, or in the 2008 outbreak at the veterinary clinic.

Similarly, transmission from horses to humans is not very efficient, since it requires a high degree of exposure to respiratory secretions (possible open abrasions or cuts on the skin, or direct inoculation of the mucosa—eg, in the eye). From the seven reported clinical cases of human HeV infections, four were fatal. All cases involved either veterinarians or people caring for very ill or moribund horses, or those performing necropsies of dead animals. However, the last two human cases were initially due to treating a horse that did not show clinical signs of Hendra disease at that time, but it was infectious to another horse prior to developing clinical signs. This corresponds with experimental findings of presymptomatic shedding in horses infected with HeV. Human-to-human transmission of HeV was not reported, and only one asymptomatic infection of a dog was reported in connection with one of the horse cases.

NiV was first isolated from the cerebrospinal fluid of a patient from the village of Nipah close to Ipoh in Malaysia during an outbreak of viral encephalitis in 1998/1999. The route of NiV transmission in this, and an associated outbreak in Singapore, was initially from bats to pigs via pig feed contaminated by bat urine or saliva. Transmission from pigs to humans, confirmed by sequence analysis of human and swine isolates, occurred by direct exposure to the contaminated body fluids or tissues of pigs, or it was airborne by large droplets, as supported by the detection of NiV in the upper and lower respiratory tract of the infected pigs. There were a total of 265 human cases with 105 deaths reported. The outbreak was controlled by culling in an excess of one million pigs, resulting in substantial economic loss.

NiV was first identified as an etiological agent of human encephalitis and severe pulmonary disease in the early outbreaks of 2001 in Bangladesh, and retrospectively also in India’s West Bengal. The virus causes seasonal, relatively small outbreaks with mortality rates as high as 75% compared to the 40% seen in the outbreak in Malaysia, with an
estimated 400 cases between 2001 and April 2013 in this area of the Indian subcontinent.\textsuperscript{36–40} Outbreaks in West Bengal and Bangladesh start in December and cease by April, overlapping with the palm sap harvest and the breeding season of \textit{Pteropus} spp. bats. Stress associated with breeding leads to increased shedding of NiV in bats, resulting in a high risk for spillover of the virus.\textsuperscript{14,21,41–43} Consumption of fruit or, importantly, of the date palm sap contaminated with bat saliva and/or urine is the primary risk factor for human NiV infection in Bangladesh and India.\textsuperscript{13,15,44,45} There is epidemiological evidence that in a few cases in Bangladesh, NiV may have been transmitted from infected domestic animals (cow, goat, pig) to humans.\textsuperscript{13,21} One important characteristic of the outbreaks in India and Bangladesh caused by the NiV-B lineage of the virus is person-to-person transmission.\textsuperscript{13,36,38,39} In cases where human-to-human transmission was documented, the clinical disease developed 6–11 days postexposure.\textsuperscript{46,47} The transmission requires very close contact with an ill or deceased person, usually by family members or attending medical staff, and larger clusters were found to be associated with a superspreader.\textsuperscript{39} NiV RNA was detected by reverse transcription polymerase chain reaction in the urine of several patients.\textsuperscript{36}

In 2014, an outbreak of a henipavirus (suspected to be NiV based on serology and partial genome sequence) occurred in the Philippines with horse-to-human and human-to-human transmission, resulting in 17 human cases with 82\% case-fatality, the death of ten horses, and possibly one dog and several cats, which were fed the horse meat. It is suspected that similarly to infections with HeV in Australia, the source of horse infections were bats from the family Pteropodidae roosting near one of the villages. The outbreak started by the sudden death of several horses due to a neurological disease, and the first human cases were infected during the slaughter and consumption of undercooked horse meat. Several human cases (primarily involving the medical staff), however, acquired the infection through human contact.\textsuperscript{48} There is field evidence that horses, cats, and dogs were infected with NiV also during the 1998/1999 outbreak in Malaysia.\textsuperscript{26,49}

**HeV and NiV: classification, virus proteins, and virus–host interaction**

HeV and NiV virus belong to the genus \textit{Henipavirus}, subfamily Paramyxovirinae, family Paramyxoviridae (enveloped, nonsegmented, negative-strand RNA viruses). Their genome organization resembles viruses found in the \textit{Respirovirus} and \textit{Morbillivirus} genera.\textsuperscript{50–52} A prominent molecular feature of henipaviruses is their large genome when compared to other paramyxoviruses: 18,234 nucleotides for HeV and 18,246 nucleotides for NiV.\textsuperscript{53} Nucleotide variation and the associated amino acid variation range detected between different NiV isolates, along with some differences in clinical factors, led to the classification of NiV into two genotypes: Malaysia (NiV-M) and Bangladesh (NiV-B).\textsuperscript{54–58}

The genomic RNA of henipaviruses is encapsidated by the nucleoprotein (N), with the phosphoprotein (P), and RNA-dependent RNA polymerase (large protein; L)
being minority proteins of the nucleocapsid as well.\textsuperscript{59} Figure 2 illustrates the structural arrangement of the NiV nucleocapsid in the virion. Infected hosts generally produce good non-neutralizing antibody titers against the N protein, making detection of anti-N antibodies suitable for enzyme-linked immunosorbent assay or Luminex assay, and of interest for the differentiating infected from vaccinated animals (DIVA) strategy in veterinary vaccine development.\textsuperscript{60,61} Location of the N gene at the very 3′ end of the genome makes it a sensitive target for RNA detection in infected cells due to the abundance of the transcripts from it.\textsuperscript{62}

The nucleocapsid interacts with matrix (M) protein, located at the inner surface of the virion’s lipid envelope. The M protein, which drives the virus budding, also interacts with the cytoplasmic tale of the fusion (F) protein, thus stabilizing the virion.\textsuperscript{51,63,64} The F protein (type I membrane protein) and the attachment G glycoprotein (type II membrane glycoprotein) are embedded in the lipid bilayer of the envelope of the form projections on the virion surface and elicits the development of neutralizing antibodies\textsuperscript{65,66} in all infected hosts, including bats. That makes the F and G proteins primary candidates as immunizing antigens in vaccine development.\textsuperscript{67}

Henipavirus fusion is a pH-independent process, but it requires proteolytic cleavage of the F protein (F\textsubscript{0}) into two subunits (F1 and F2). The F\textsubscript{0} precursor expressed on the cell surface is endocytosed in clathrin-coated vesicles, cleaved by endosomal proteases known as cathepsins, and the functionally mature F protein is transported back to the plasma membrane,\textsuperscript{68} where it forms trimers.\textsuperscript{69} Direct plasma membrane fusion and macropinocytosis were identified as the henipavirus mode of cell entry,\textsuperscript{59,70,71} and this may be cell type specific. Plasma membrane fusion of infected cells with uninfected ones further facilitates virus spread throughout the host, and contributes to the pathology of henipavirus disease. Epithelial and endothelial syncytia, and multinucleated cells (mostly macrophages and dendritic cells), were observed in the tissues of humans and other host species.\textsuperscript{6,49,72–75}

Henipavirus fusion by the F protein also requires, in addition to cleavage of the F protein, activation of this protein by the second viral glycoprotein, the G protein.\textsuperscript{70} Henipavirus G glycoproteins form covalently linked dimers noncovalently associated into tetramers.\textsuperscript{77–79} In contrast to attachment glycoproteins of other paramyxoviruses, henipavirus G proteins lack both hemagglutinin and neuraminidase activities.\textsuperscript{66,80} HeV and NiV glycoprotein G is the virus attachment protein recognizing ephrin B2 and ephrin B3 as cellular receptors,\textsuperscript{81,82} and it may also bind with lower affinity to a C-type lectin on endothelial cells in the lymph nodes and liver.\textsuperscript{78} Ephrin B2 is important in mammalian host development and it is a highly conserved protein across mammalian species, expressed on lymphocytes, neurons, smooth muscle cells, and endothelial cells surrounding small arteries, corresponding with virus distribution in an infected host as determined by immunohistochemistry.\textsuperscript{30,56,73,83–85} The cellular function of ephrin B2 is to regulate processes such as neurogenesis, angiogenesis, proliferation, and remodeling, as well as immune activation and bone formation.\textsuperscript{86–88} HeV G protein has a somewhat lower affinity for ephrin B3 compared to the NiV G protein.\textsuperscript{91} This may play a role in the somewhat different pathology in human infections, where NiV with its higher affinity for this receptor than HeV, causes fatal neurological disease with severe brain stem dysfunction,\textsuperscript{34,73} although the last human fatal case of HeV had extensive brain involvement.\textsuperscript{25} Interestingly, in experimentally infected swine, HeV invasion of the central nervous system (CNS) was limited in the early stages to the olfactory bulb alone,\textsuperscript{75} and with a lower inoculation dose, HeV did not reach the CNS (Pickering B, Weingartl HM, unpublished data, 2015). Using ubiquitous and conserved proteins as receptors, henipaviruses not only have a wide host range, but they also infect a wide range of tissues and organs within each individual host species.

The P gene of henipaviruses encodes three nonstructural proteins (C, V, and W) in addition to the phosphoprotein P. The P protein is critical for virus replication,\textsuperscript{89,90} and through its interaction with cellular proteins, it also modulates cell signaling. Some of the functions encoded in the N terminus of the protein are shared with the V and W proteins due to an identical N-terminal portion of the three proteins. The V protein is produced by the cotranscriptional addition of one nontemplated Gs at the editing site, and the W protein by insertion of two nontemplated Gs.\textsuperscript{91–94} The proteins are located in the cytoplasm, except for the W protein being detected also in the nucleus in some cell types. The C protein is encoded by a separate internal open reading frame within the P gene,\textsuperscript{91,92,95} and it localizes predominantly into the perinuclear region.\textsuperscript{94,96} The P, W, and V proteins hinder the interferon JAK–STAT signaling pathway by binding to STAT1 and preventing its translocation into the nucleus.\textsuperscript{96–98} Nuclear localization of NiV W impairs the TLR3/TRIF pathway by blocking TRIF-mediated activation of interferon regulatory factor (IRF)-3 responsive promoter, ultimately interfering with the induction of interferon (IFN)-β and other molecules controlled by this pathway.\textsuperscript{99} NiV V proteins bind to the MDA5 helicase along with LGP2 to suppress RIG-I-like (RLR) signaling, thereby inhibiting the downstream signaling events also leading to IFN-β synthesis.\textsuperscript{100,101} NiV C exhibits inhibitory activity
against TRL7/9-dependent IFN-α induction by binding to IKKs and inhibiting phosphorylation of IRF-7, as well as influencing IFN-β and antiviral gene expression. A study by Mathieu et al suggested that the C protein can regulate cytokine balance in transfected cells. The nonstructural proteins are involved in the NiV life cycle by regulating replication and evading the innate immune response, as confirmed by in vivo reverse genetics studies in animal models.

The evasion of the IFN system appears to be cell specific, since NiV will induce IFN type I and other innate cytokines in endothelial cells, an important in vivo target for henipaviruses, and there is the possibility that cells may employ alternative pathways to establish an antiviral state. The molecular aspects of henipavirus replication and its interaction with the host cell are – with some differences between HeV and NiV, and between individual nonreservoir host species – reflected in the clinical disease, pathogenesis, pathology, and immune response.

**Infections in natural, nonreservoir hosts with henipaviruses**

**Human henipavirus infections**

With only few human HeV disease cases, the clinical symptoms and pathology of henipavirus infections in humans are summarized mainly based on clinical infections with NiV, since there appear to be common general characteristics and similarities between Hendra and Nipah infections, as well as between NiV infections with the Malaysia and Bangladesh genotype. Symptomatic infections present mainly as severe acute encephalitis, with severe pulmonary involvement often being reported from Bangladesh. Infections with a virus causing the more severe respiratory form may be potentially associated with increased shedding and the observed human-to-human transmission with the NiV-B genotype. Disease onset is characterized by fever and headache, followed by varying degrees of altered consciousness in the majority of cases. Some patients suffer from nausea, vomiting, muscle pain, and involuntary muscle movements. Fatal cases, death likely due to severe brain stem involvement generally occurred within 1 week or 2 weeks after the onset of symptoms. The neurological signs are supported by pathological findings: brain damage due to the infection of small blood vessels accompanied by vasculitis with thrombosis; hemorrhage and frequent adjacent necrosis; as well as direct infection of specific groups of neurons. Henipaviruses can persist in the CNS of some of the infected individuals causing neurological relapse. In late-onset encephalitis, the target cells are neuronal only, with no vascular involvement.

Although there is only a low level of viremia detected in humans, viremic spread of NiV appears to be the main route of CNS invasion in humans, and the virus can be isolated from the cerebrospinal fluid. NiV replicates to a low level in human dendritic cells, and it can spread mechanically through attachment to monocytes and lymphocytes and putatively via cell-free viremia. Infection of the endothelial cells of the small blood vessels is a hallmark of henipavirus infections, and it contributes to virus spread into the parenchyma of several organs – importantly, also through the blood–brain barrier and the blood–air barrier contributing to virus spread and pathology in the brain and lungs. Epithelial cells can also be infected with henipavirus, interestingly either from the basal membrane or by fusion with the neighboring epithelial cells, supporting a hypothesis for viremic spread to the lungs besides via the airway route.

People infected with henipaviruses develop antibodies against the virus that may last for years. Based on serological evidence, subclinical infections were reported in Malaysia and Singapore.

**Henipaviruses in horses**

NiV is suspected to infect horses in the 2014 outbreak in the Philippines; however, the etiological agent was identified retroactively through human samples, and thus no pathological examination or sample collection took place. Only partial sampling (brain, spinal cord) was conducted for one horse in the Malaysian outbreak with immunohistological evidence of virus infection.

HeV-infected horses develop severe, acute, febrile respiratory disease sometimes accompanied by facial swelling, ataxia and, terminally, copious frothy nasal discharge, increased rectal temperature (41°C), and profuse sweating, which can resemble African horse sickness, for example. Neurological signs with clinical features such as hypersensitivity, ataxia, disorientation, head pressing, and stranguria predominated in more recent outbreaks. Shedding of the virus in oral secretions in HeV-infected horses prior to the onset of clinical signs began to be suspected during the 2008 outbreak, and it was confirmed experimentally starting 48 hours postinfection. After the onset of a clinical disease, the virus can also be detected in urine and feces.

Similar to human cases, predominant lesions in the HeV infection of horses include vasculitis with syncytial cells in the vascular endothelium, fibrinoid degeneration, and necrosis, most markedly in the lymphatic vessels and small
blood vessels in major organs such as the lungs, brain (including meninges), spleen, lymph nodes, and kidney, but which are also detectable in the nasal mucosa, liver, heart, stomach, intestine, uterus, and ovaries. Pulmonary lesions in horses are particularly extensive, with severe necrotizing alveolitis with marked fibrinous alveolar exudates, corresponding with severe respiratory clinical signs in infected animals. Syncytial cells were observed in addition to endothelial cells in respiratory epithelial and lymphoid cells as well. Viral antigen was detected in the affected organs and tissues, including the brain and choroid plexus. 

While some HeV-infected animals remain asymptomatic, horses that have survived an acute HeV infection and that have seroconverted may have moderate focal nonsuppurative meningoencephalitis, with the HeV antigen and viral RNA still detectable several weeks after the resolution of clinical signs. Persistent infection of horses has not been identified. 

### Henipaviruses in pigs

HeV can, under experimental conditions, infect swine by the oronasal route, causing a disease similar to NiV, although with limited invasion of the CNS, however, natural infections of pigs with HeV have not been reported. 

In contrast, NiV-infected pigs were the source of human infections in the first recognized outbreak in Malaysia in 1998/1999. The virus appears to be highly contagious in pigs with an infection rate close to 100% in affected farms, but with low mortality (1%–5%). Direct, and possibly also airborne, exposure to secretions from infected animals was one of the presumed modes of transmission of NiV among pigs, supported by the detection of NiV in the epithelium of the upper and lower respiratory tract, as well as in the lumen of the airways. Another important route of infection is oral by ingestion of contaminated material (especially from bat to pig), likely resulting in less severe clinical signs observed in the field. Generally, the disease was mild with nonspecific clinical signs which, in addition, varied depending on the age of the pig. However, if pigs developed severe disease, neurological and/or respiratory signs were the most frequently observed. In the experimentally infected animals, severe clinical signs were observed in subcutaneously, nasally, or oronasally inoculated pigs compared to orally inoculated ones. About 20%–40% of piglets developed CNS signs requiring euthanasia, either due to viral encephalitis or to secondary bacterial meningitis. Pathological observations, especially microscopical, correspond with human or horse NiV infections. 

Besides the ability of the swine host to mount an effective protective immune response in the majority of animals in a natural setting, there are two other characteristics that are different from human or horse infections with NiV: direct invasion of the brain via the cranial nerves, and transient immunosuppression early postinfection, linked to the ability of NiV to infect a range of porcine immune cells, such as dendritic cells, monocytes, macrophages, natural killer cells, and CD8+ T-cells. This, together with the ability of henipaviruses to evade the IFN system, may lead to delayed or modified immune cell signaling, in turn leading to a less efficient innate immune response and less efficient transition to the adaptive immune response. Nevertheless, pigs develop protective neutralizing antibodies against Nipah only with a short delay. A protective immune response in pigs requires both humoral and immune cell memory (Pickering B, Weingartl HM, unpublished data, 2015), in agreement with the pilot data from a previous vaccine efficacy study. Serum antibodies against NiV appear to be long lasting, as suggested by the postoutbreak investigation in Malaysia.

The current understanding of routes of infection and virus spread throughout the host infected with henipaviruses is briefly summarized in Figure 3. Despite some differences in the pathogenesis and pathology between species, there are common characteristics of henipavirus infections, such as the development of viremia; the involvement of the respiratory, central nervous, and immune systems; vasculitis; the formation of synctia in the endothelium; the infection of dendritic cells, etc. Considering the routes of infection, mucosal immunity may be important early postinfection; however, this has not been widely explored, even in terms of vaccine design.

### Animal models of henipavirus infection employed in vaccine development and efficacy studies

Approval of human vaccines requires that the vaccine candidate is found to be efficacious in at least two animal models representing a human disease, preferably including a nonhuman primate model, while for veterinary vaccines, the requirement is efficacy in the target species. However, vaccine efficacy testing for large animals under biosafety level 4 conditions is extremely difficult and constrained. Consequently, the two animal models rule was considered when licensing the HeV vaccine for horses (Equivac HeV®) in Australia, based on the outcomes of protection studies in ferrets against...
Animal models, vaccine candidates, and henipaviruses

HeV, and cats, ferrets, and nonhuman primates against NiV. In addition to horses, guinea pigs and ferrets were used in parallel to complement the efficacy testing.

Equine and swine target species models

The two large animal models used in vaccine efficacy testing are both based on studies looking into the susceptibility, pathogenesis, and transmission described in the previous section. Oronasal inoculations with 2×10^6 median tissue culture infective dose (TCID\textsubscript{50})/animal of HeV in a horse model are fatal,\textsuperscript{3,16,17,24,119,120,126} in contrast to NiV infection in a pig model, where only 20%-40% of animals require euthanasia due to CNS signs. This putative fatality rate is already much higher than the one observed during the 1998/1999 outbreak in Malaysia, and a lethal model in this species may not be possible. However, all animals in the existing model are infected and have a high virus load in their tissues and oronasal secretions.\textsuperscript{30,31,119,124} NiV vaccine efficacy trials in young pigs (4 weeks of age at immunization) were conducted employing this model using intranasal (IN) inoculation with
2.5 × 10^5 plaque forming units (PFU) per piglet at the age of 10 weeks.124

**Naturally infected species models**

Cats can be naturally infected with NiV,48,49 which makes them an a priori suitable candidate as a model for vaccine efficacy studies. Cats have been shown to be highly susceptible to HeV or NiV infection under experimental conditions with clinical and pathological features similar to human disease: HeV-infected cats exhibit fever and changes in behavior, accompanied with respiratory disease often leading to death. All routes of inoculation – oral, nasal, and subcutaneous – lead to acute clinical disease and death using doses of 5 × 10^3 or 5 × 10^4 TCID_{50} per animal. In-contact animals were also infected and featured similar outcomes as the inoculated ones. HeV produced significant respiratory signs, including dyspnea with increased temperature, typically dropping 24 hours before death. Severe interstitial pneumonia, vasculitis (lung, gastrointestinal, and lymphoid systems), and syncytia in the endothelium were detected postmortem.5,127,128 Cats infected with NiV (5 × 10^2–5 × 10^4 TCID_{50} per animal) oronasally or subcutaneously had, on postmortem examination and in addition to lesions observed with HeV, necrosis of the lymphatic tissues, degeneration of neurons, and moderate meningitis.123,129 Experimental infection of a pregnant cat (unintentional) provided further evidence that the virus can be transmitted to the fetus.130

Since dogs can be naturally infected with henipaviruses, and although they are not considered a secondary reservoir,131 there is a possibility that ferrets (another canine species) may be a suitable model for henipavirus, which was eventually considered.132,133 Several protection or vaccine efficacy studies employing different vaccine platforms demonstrated that ferrets are a reliable challenge model for henipaviruses.126,134–136 It appears that a minimal NiV infectious dose to infect all inoculated ferrets oronasally is 5 × 10^3 TCID_{50} with highest virus load in the lungs and spleen, detected in all animals infected with this and one log_{10} higher dose. All four animals in this study by Bossart et al.132 also had the virus in the olfactory section of the brain. NiV was detected mainly in the neurons, and detection of the virus antigen in ependymal epithelium and the arachnoid membrane of the meninges would indicate viremic spread of the virus into the CNS. The HeV model (oronasal inoculation with 5 × 10^3 TCID_{50}) was described by Pallister and colleagues.133,134 Recently, a recombinant HeV-expressing GFP has been reported to facilitate further pathogenesis studies.137

Both models represent human henipavirus histopathology well, with typical multisystemic vasculitis including the lung, spleen, and, to a lesser extent, the brain. The lung involvement also warranted the use of the HeV/ferret model for the equine vaccine efficacy trials as a control.126,134

**Small animal models**

Following the initial outbreak of HeV in Australia, an early experiment focusing on species susceptibility to HeV tested many animal models including mice, guinea pigs, rats, chickens, rabbits, cats, and dogs, with only guinea pigs and cats developing the disease,128 although it has to be noted that the dogs were vaccinated against canine distemper, a virus from the closely related genus Morbillivirus. Further studies, unfortunately, determined that the outcome of guinea pig infections with henipaviruses is not consistent, and that the guinea pig model is not reliable for vaccine efficacy studies of HeV vaccine candidates, and is even less reliable for efficacy studies of NiV vaccine candidates.4,6,74,138 Interestingly, work by Williamson et al.4 showed that HeV was transmissible to the guinea pig fetus during gestation.

Mice, although originally found to be refractory to both HeV and NiV,4,74,128 are being recently developed as disease models, employing animals with an impaired immune response. For example, IFN-α receptor knockout, or aged C57Bl/6 and BALB/c mice were reported to be susceptible to henipavirus infections.139,140 An intriguing mouse model to study human lung tissue pathogenesis in NiV infections has been developed using human lung xenograft in immunodeficient NSG mice.141 These models would not be considered suitable for vaccine efficacy studies due to impairments in their immune response. However, genetically defined mice are an excellent tool to elucidate specific aspects of henipavirus pathogenesis.140,142 Mice with functional immune responses were used in a number of proof of principle immunization studies for different vaccine candidate platforms.143–145

Golden (Syrian) hamster is the first and currently the only small animal model used for vaccine efficacy testing.146 The original model was developed to mimic human NiV encephalitis73 and, more recently, it was modified to simulate the respiratory involvement during henipavirus infections for both NiV and HeV147 and to study transmission.148–150 The first pathogenesis study in hamsters with HeV indicated that the animals are highly susceptible to the virus: a dose of 10^2 PFU per animal was 100% fatal when inoculated intraperitoneally (IP). Inoculation with 100 PFU indicated that there may be age differences in susceptibility, with survivors in the older
animals group.\textsuperscript{151} IN and IP inoculations of hamsters with higher doses both result in clinical signs and death, although delayed course of the disease relative to the IP inoculation was observed with the same dose of virus administered IN.\textsuperscript{74} IN inoculation with a high dose of NiV, and especially HeV, resulted in severe acute respiratory distress in the infected hamsters.\textsuperscript{147} High IP NiV dose or IN dose (range of $10^7$–$10^8$ PFU/TCID\textsubscript{50}) resulted in acute encephalitis. In the high-dose IN inoculation, the virus accessed the brain via cranial nerves from the nasal cavity early postinoculation,\textsuperscript{152} similar to the neuroinvasion of NiV in pigs.\textsuperscript{30} The high-dose inoculated animals developed tremor and limb paralysis 24 hours before death, with virus detected in the urine, brain, spinal cord, lungs, kidney, spleen, liver, and heart. Low-dose (10 PFU and 10\textsuperscript{2} PFU) IN inoculation was not uniformly fatal, and survivors developed high levels of neutralizing antibodies by 29 days postinoculation (dpi).\textsuperscript{74} The IN inoculation with a low dose of HeV or NiV resulted in systemic spread and invasion of the brain by the virus crossing the blood–brain barrier.\textsuperscript{147} There may be some differences in the pathogenesis between the Malaysian and Bangladesh genotypes of NiV, as different lesion severities were observed in IP-inoculated animals between the two, but not in the IN-infected animals.\textsuperscript{57,58}

Nonhuman primate models

Squirrel monkeys were found to be only partially susceptible to NiV, with most challenged animals not developing clinical signs – even with a high challenge dose – and viral RNA was detectable in only some organs of some animals.\textsuperscript{153} The African green monkey (AGM) model well resembles disease and pathology in humans, and the animals are susceptible to both HeV (dose in a range of $10^3$ PFU per animal) and NiV (dose range of $10^4$ PFU) infection via the intratracheal route.\textsuperscript{85,154–156} NiV infection in this model was well analyzed, indicating pulmonary and CNS involvement, with microscopic brain lesions and antigen distribution in correlation with findings in humans. The model does not appear to be 100\% lethal, even with the IP dose of $10^3$ TCID\textsubscript{50} but all inoculated animals are infected with detectable virus in swabs, urine, and multiple organs, and they develop viremia.\textsuperscript{154,156} The oronasal route of inoculation with $10^4$ or $10^5$ TCID\textsubscript{50} was also not uniformly lethal.\textsuperscript{157} The amount of viral RNA detected in peripheral blood mononuclear cells (PBMCs) is rather significant ($10^4$–$10^7$ copy numbers/mL),\textsuperscript{156} and it may indicate viral replication in some subpopulations of AGM leukocytes. Lymphocyte depletion in the spleen, lymph nodes, and tonsils was observed in IP-inoculated AGMs.\textsuperscript{157} Interestingly Geisbert et al\textsuperscript{154} found more virus associated with the red blood cell fraction than in PBMCs and granulocytes. A somewhat higher dose of HeV per animal ($4\times10^3$ TCID\textsubscript{50}) resulted in uniform lethality in the study involving 12 AGMs. The animals developed disease and pathology consistent with human disease.\textsuperscript{85} However, vaccine efficacy studies indicated that the HeV-infected AGM model is similar to NiV, not a lethal challenge model.\textsuperscript{155} Nevertheless, the availability of the AGM model is crucial for the development of human vaccines, and additional correlates of infection can be developed for protection studies – eg, a lack of azotemia.\textsuperscript{156}

Vaccine candidates

Reports on vaccine candidates tested in animals for their efficacy against NiV or HeV challenge are summarized in Table 1. As many of them can be considered for both human and veterinary vaccines, there is no distinction made in Table 1 based on their intended use.

An important strategy, when considering the protection of human population against zoonotic viruses, is interruption of the transmission cycle at the level of the intermediate host, or natural reservoir, if practical. Abolition or a very significant reduction of shedding following the challenge of vaccinated animals is considered a critical requirement for the efficacy of veterinary vaccines. The advantage of the vaccination of livestock or pets is that the vaccination also protects them. The second advantage of this approach is that the licensing of veterinary vaccines can generally take less time than the licensing of human vaccines. Licensing of the Hendra vaccine for horses (Equivac HeV\textsuperscript{85}©, Zoetis, Inc., Florham Park, NJ, USA) in Australia in November of 2012 is the best example. Following the fatal human case of HeV infection, Australian health authorities decided in 2009 to pursue rapid licensing of an equine vaccine for HeV. It took a total of 2 years from the decision to licensing. The successful candidate, soluble HeV G protein (sG\text sub{HeV}), which was first tested in cats in 2006,\textsuperscript{129} was shown to be protective against HeV in ferrets\textsuperscript{134} prior to testing in horses.\textsuperscript{126} Following the successful protection experiments in AGMs against both HeV and NiV,\textsuperscript{151,158} sG\text sub{HeV} may be potentially considered as a vaccine candidate for humans in the future. Even though the AGM model is not 100\% lethal, additional correlates of protection and infection can be employed to assess protection,\textsuperscript{154–156,158} as is currently being considered for swine NiV efficacy tests, where a lack of viral RNA presence in tissues – and, importantly, a lack of shedding postchallenge – as well as cellular and humoral immune response are considered (Pickering B, Weingartl HM, unpublished data, 2015).\textsuperscript{124} Very interestingly, vaccination with sG\text sub{HeV}
<table>
<thead>
<tr>
<th>Vaccine candidate</th>
<th>Immunization dose, route, schedule</th>
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<th>Model</th>
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<tr>
<td>VV NiG</td>
<td>10^9 PRU, SC, boost at 1 month</td>
<td>NIV</td>
<td>Hamster</td>
<td>Full 7/7 or 8/8 based on survival, no clinical disease, at 30 dpc</td>
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<td>VV NiF</td>
<td>10^9 PRU, SC, boost at 1 month</td>
<td>IP; 10^4 PFU</td>
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<tr>
<td>VV NiF + G</td>
<td>5×10^4 + 5×10^4; PFU, SC, boost at 1 month</td>
<td>4 months</td>
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<tr>
<td>ALVAC Ni F</td>
<td>10^9 PRU, IM, boost at 14 dpv</td>
<td>NIV</td>
<td>Pig, 9–10 weeks old</td>
<td>Full 4/4 and 4/4; residual RNA in nasal/throat swabs (NiF), OLF, TGG, TRA at 6–7 dpc</td>
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<td>ALVAC Ni G</td>
<td>10^9 PRU, IM, boost at 14 dpv</td>
<td>IN, 2.5×10^5 PFU; 28 days</td>
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<tr>
<td>ALVAC Ni F + G</td>
<td>10^9 + 10^8 PFU, IM, boost at 14 dpv</td>
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<tr>
<td>Recombinant He G: sG_{HeV}</td>
<td>100 μg sG/Quil A/DEAE–dextran/Montanide; SC, boost at 13 d and 28 d</td>
<td>NIV</td>
<td>Cat</td>
<td>Full 4/4, no viral RNA at 24 dpc</td>
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<tr>
<td>Recombinant Ni sG</td>
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<td>SC, 5×10^6 TCID_{50}; 104 days</td>
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<td>Recombinant He G: sG_{HeV}</td>
<td>5 μg, 25 μg, or 50 μg sG_{HeV} + CpG–ODN 2007 + alhydrogel; SC; boost at 21 d</td>
<td>NIV</td>
<td>Cat</td>
<td>Full 6/6 at 21 dpc; no antigen, but residual RNA in brain; RNA in oral swabs + urine</td>
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<td>Recombinant HeG: sG_{HeV}–CpG</td>
<td>4 μg, 20 μg, or 100 μg sG_{HeV} + ODN 2007 + alhydrogel; SC; boost at 20 d</td>
<td>HeV</td>
<td>Ferret</td>
<td>Full 6/6; no virus (RNA) in tissues at 21 dpc; residual RNA in nasal wash for 4 μg dose</td>
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<td>Recombinant He G: sGHeV–alum–CpG</td>
<td>10 μg, 50 μg, or 100 μg sG_{HeV} + ODN 2006 + alhydrogel; IM; boost at 21 d</td>
<td>NIV</td>
<td>AGM (4–6 kg)</td>
<td>Full 9/9 survival, no clinical signs, no gross pathology or virus (RNA) at 28 dpc</td>
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<tr>
<td>Recombinant He G: sG_{HeV}</td>
<td>4 μg, 20 μg, or 100 μg sG_{HeV} + CpG–ODN 2007 + alhydrogel; SC; boost at 20 d</td>
<td>NIV</td>
<td>Ferret</td>
<td>Full 5/5; no clinical signs, sterile immunization at 20 dpc</td>
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<td>Recombinant He G: sG_{HeV}</td>
<td>50 μg or 100 μg sG_{HeV} + adjuvant (Zoetis); IM</td>
<td>HeV</td>
<td>Horse</td>
<td>Full 7/7, no clinical signs, no virus (or RNA, Ag) in tissues, no shedding at 7–9 dpc; serum VNT prechallenge: 256–4096</td>
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<td>Recombinant He G: sG_{HeV}–alum</td>
<td>100 μg sG_{HeV} + alhydrogel/animal; IM; boost at 21 d</td>
<td>HeV</td>
<td>Horse</td>
<td>Full 3/3, no clinical signs, no infectious virus shedding (one horse low transient RNA copy numbers) Serum VNT prechallenge: 16–32</td>
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<tr>
<td>Recombinant He G: sG_{HeV}–alum–CpG</td>
<td>100 μg sG_{HeV} + ODN 2006 + alhydrogel; IM; boost at 21 d</td>
<td>NIV</td>
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<td>Recombinant He G: sG_{HeV}–alum</td>
<td>5×10^4 TCID_{50}; 28 days</td>
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<tr>
<td>Recombinant He G: sG_{HeV}–alum–CpG</td>
<td>194 d</td>
<td>HeV</td>
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<tr>
<td>Vaccine</td>
<td>Dose</td>
<td>Route</td>
<td>Challenge</td>
<td>Animal</td>
<td>Protection</td>
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<tr>
<td>rVSV–ZEOV GP–Ni F</td>
<td>$10^3$ PFU/animal</td>
<td>IP, single dose</td>
<td>NiV</td>
<td>Hamster</td>
<td>Full 6/6 (reduction in viral load in tissues)</td>
</tr>
<tr>
<td>rVSV–ZEOV GP–Ni G</td>
<td>$10^5$ PFU/animal</td>
<td>IP, single dose</td>
<td>NiV</td>
<td>Hamster</td>
<td>Partial protection: 2/6 survival</td>
</tr>
<tr>
<td>rVSV–ZEOV GP–Ni N</td>
<td>$10^7$ PFU, IM, single dose</td>
<td>NiV</td>
<td>Ferret</td>
<td>Full 15/15, viral RNA in blood at 6 dpc; one rVSV–ΔG–NiV/F–GFP RNA in spleen at 21 dpc</td>
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<tr>
<td>rVSV–ΔG–NiV/G–GFP</td>
<td>$10^7$ PFU, IM, single dose</td>
<td>NiV</td>
<td>Hamster</td>
<td>Full 20/20, no clinical signs, no virus (RNA) in tissues at 32 dpc</td>
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<td>rVSV–ΔG–GFP NiV/F + NiV/G</td>
<td>$10^7$ PFU, IM, single dose</td>
<td>NiV</td>
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<td>rVSV–ΔG–NiVF</td>
<td>$10^7$ PFU, IM, single dose</td>
<td>NiV</td>
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<td>Full 10/10</td>
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<tr>
<td>rVSV–ΔG–NiVG</td>
<td>$10^7$ PFU, IM, single dose</td>
<td>NiV</td>
<td>Ferret</td>
<td>Full 10/10</td>
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<tr>
<td>rMV Ed strain pMV–Ed–NiG</td>
<td>$2\times10^7$ TCID_{50}, IP, boost at 21 days</td>
<td>NiV</td>
<td>Hamster</td>
<td>Full 10/10</td>
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<tr>
<td>rMV HL strain pMV–HL–NiG</td>
<td>$2\times10^7$ TCID_{50}, IP, boost at 21 days</td>
<td>NiV</td>
<td>Hamster</td>
<td>Full 10/10</td>
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<tr>
<td>rMV Ed strain pMV–Ed–Ni G</td>
<td>$10^7$ TCID_{50}, SC, boost at 28 days</td>
<td>NiV</td>
<td>AGM (4–5 kg)</td>
<td>Full 2/2 with no fever and no pathology detected at 20/23 dpc; shedding not followed</td>
<td>160</td>
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<tr>
<td>Adenoassociated virus Ni G:</td>
<td>$6\times10^{11}$ genome particles; IM</td>
<td>NiV</td>
<td>Hamster</td>
<td>Full 6/6, no detectable RNA at 29 dpc</td>
<td>160</td>
</tr>
<tr>
<td>AAV8–NiV.G</td>
<td>$6\times10^{11}$ genome particles; IM</td>
<td>NiV</td>
<td>Hamster</td>
<td>Partial 3/6 survival (50%); no RNA in survivors at 29 dpc</td>
<td>160</td>
</tr>
</tbody>
</table>

**Notes:** Administration/inoculation route: IT, SC, IP, IN, and ON. *Not given. Challenge day is always in relation to the first vaccination. End, provided time of tissue collection. Protection given as “Full 6/6” indicates six animals out of six survived.

**Abbreviations:** VV, vaccinia virus; PFU, plaque forming unit; SC, subcutaneous; NiV, Nipah virus; IP, intraperitoneal; dpc, days postchallenge; IM, intramuscular; dpv, days postvaccination; IN, intranasal; NiF, NiV F protein; NiG, NiV G protein; OLF, olfactory bulb; TGG, trigeminal ganglion; TRA, trachea; DEAE, diethylaminoethyl; TCID_{50}, median tissue culture infective dose; ODN, oligodeoxynucleotide; AGM, African green monkey; HeV, Hendra virus; IT, intratracheal; ON, oronasal; VNT, virus neutralization titer; LD_{50}, median lethal dose.
protein was found to be protective against NiV in number of species, but adenoassociated virus vector expressing the NiV G protein yielded only partial protection against HeV challenge, although it was fully protective against the NiV challenge. An adjuvant suitable for the target species has to be part of the vaccine formulation for the sG_{HeV}-based vaccine candidates to offer full protection: shedding of viral RNA was detected in cats, but not in other tested species vaccinated with sG_{HeV} and challenged with NiV.

Several recombinant virus vaccine candidates were also developed, some of them with no or limited replication in the target host, such as ALVAC, adenoassociated virus, or VSV-delta G requiring G_{Ind} for replication. The very first vaccine candidate tested was live recombinant vaccinia virus expressing NiV F or G protein. Besides demonstrating full protection, this study also indicated that neutralizing antibodies are protective in hamsters after passive transfer.

The canarypox-based ALVAC candidate was fully protective with sterile immunity in the swine as a target species when both F and G antigen-expressing ALVAC preparations were used for immunization, as no viral RNA was detected in swabs or tissues. Vaccination employed a primary and boost immunization regimen for vaccinia, as well as for the canarypox-based vaccine candidate. The next set of recombinant virus candidates based on VSV attempted to eliminate the need for boost, and when coding for the NiV F or G protein, those candidates were protective against NiV challenge, importantly also in AGMs during a pilot vaccine efficacy study. Immunization with rVSV protein expressing NiV N protein generating only non-neutralizing antibodies was partially protective in the hamster model, perhaps suggesting some role for non-neutralizing antibodies as well. More likely though, this indicated that the cellular immune response plays a role in protection. One of the recent vaccine candidates based on the measles virus platform is protective both in hamsters and in AGMs, and it is likely intended for human use.

Conclusion
Vaccine efficacy studies clearly indicate that the prevention of henipavirus disease by vaccination would be the best measure, considering that although numerous antivirals have been tested up to date in vitro, the in vivo efficacy was not very encouraging. Ribavirin and chloroquine were tested experimentally in animals, and they were also used for the clinical emergency treatment of humans; however, there was no conclusive benefit. The ability of henipaviruses to inhibit IFN response at the level of induction and signaling may, to a certain extent, explain the lack of effectiveness of ribavirin treatment.

Neutralizing antibodies have long been recognized as protective, and they are considered for postexposure treatment. Administration of the monoclonal antibody m102.4, which targets an epitope in the G protein receptor binding site and is thus effective against both HeV and NiV, provided full protection. Timing of the administration and the amount of antibody required for full protection, unfortunately, limit the use of this approach.

There are essentially no studies focusing on adaptive immune responses against henipaviruses. Our limited understanding comes from vaccination/immunization studies and they have focused on correlates of protection. For quite some time, the protection offered by vaccination was considered to be solely due to the presence of high levels of neutralizing antibodies prior to the challenge. Indications that adaptive cellular response may be important came from a study employing live recombinant vaccine ALVAC in swine. Vaccinated pigs had good IFN-γ response in vaccinated PBMCs, indicative of immune cell memory. Later, a study in hamsters using immunization with the rVSV-NiN protein showed partial protection despite a lack of neutralizing antibodies, again supporting the role of the cellular immune response in protection. In addition, protection against HeV was demonstrated in horses with low neutralizing antibody titers of only 16/32. Overall, it appears that depending on the host species, elicitation of both humoral and cell immunity memory may be required for vaccine efficacy.

Vaccination is, without a doubt, the best option for the protection of humans and animals against henipaviruses. With the Equivac HeV® vaccine licensed for use in horses in Australia in 2012, HeV became the first biosafety level 4 agent against which a vaccine was made available.

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