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Review

# Recent progress in henipavirus research: Molecular biology, genetic diversity, animal models

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ABSTRACT

Nipah and Hendra virus are members of a newly identified genus of emerging paramyxoviruses, the henipaviruses. Both viruses have the ability to cause severe pulmonary infection and severe acute encephalitis. Following their discovery in the 1990s, outbreaks caused by these zoonotic paramyxoviruses have been associated with high public health and especially economic threat potential. Currently, only geographic groupings in Asia and Australia have been described for the henipaviruses. However, while few viral isolates are available and more detailed characterization is necessary, there has been recent evidence that divergent henipaviruses might be present on the African continent. This review endeavours to capture recent advances in the field of henipavirus research, with a focus on genome structure and replication mechanisms, reservoir hosts, genetic diversity, pathogenesis and animal models.



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## 1. Introduction

Nipah and Hendra virus (NiV and HeV) are members of a newly identified genus of emerging paramyxoviruses, the henipaviruses. Both agents are able to infect a range of wild and domestic animal species and cause severe disease in humans, with a high case fatality rate. NiV, but not HeV, has been observed to spread from person to person. Given the lack of effective therapeutics and vaccines, these viruses are considered as public health concerns and listed as category C priority pathogens for biodefense research by the National Institute of Allergy and Infectious Diseases. Infection with these viruses in domestic animals such as pigs and horses can also cause severe disease, resulting in significant economic loss. During the NiV outbreak in Malaysia, more than 1 million pigs were culled, resulting in economic losses totaling far more than their export value of US$100 million (Lam, 2003). NiV could therefore be a devastating agent of bioterroism if used against the swine industry (Lam, 2003). Henipaviruses are considered "overlap" select agents and regulated by both Centers for Disease Control and Prevention and by the Animal and Plant Health Inspection Service of the United States Department of Agriculture. In this review, we summarize recent advances in henipavirus research, focusing on genome structure and replication mechanisms, reservoir hosts, genetic diversity, pathogenesis and animal models.

## 2. The henipaviruses

### 2.1. Classification

The family *Paramyxoviridae* is classified into two subfamilies, the *Pneumovirinae* and the *Paramyxovirinae*. The pneumoviruses comprise two genera, *Pneumovirus* and *Metapneumovirus*, and includes important human and animal pathogens, such as bovine and human respiratory syncytial viruses. The second subfamily,

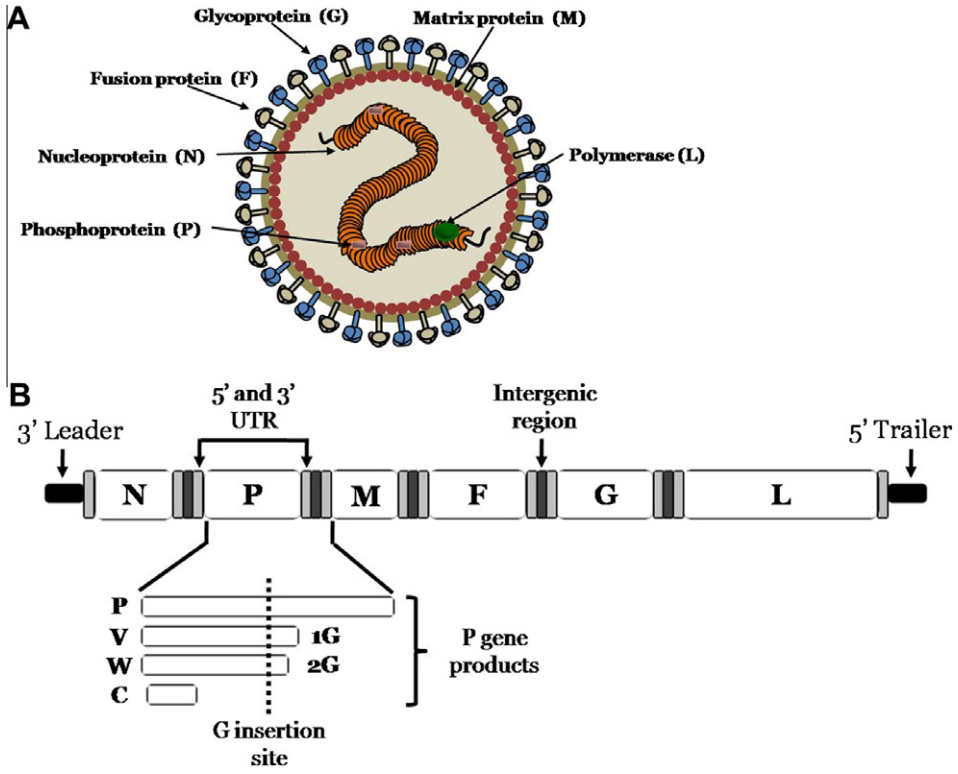


**Fig. 1.** Henipavirus virion structure and genome organization. (A) Schematic representation of a henipavirus virion, illustrating the M protein lining the inner surface of the lipid envelope, the viral glycoproteins decorating the virion surface, and the helical ribonucleoprotein (RNP) complex in association with the polymerase and phosphoprotein enclosed within. (B) Schematic representation of henipavirus genome organization. The polycistronic nature of the P gene is indicated.

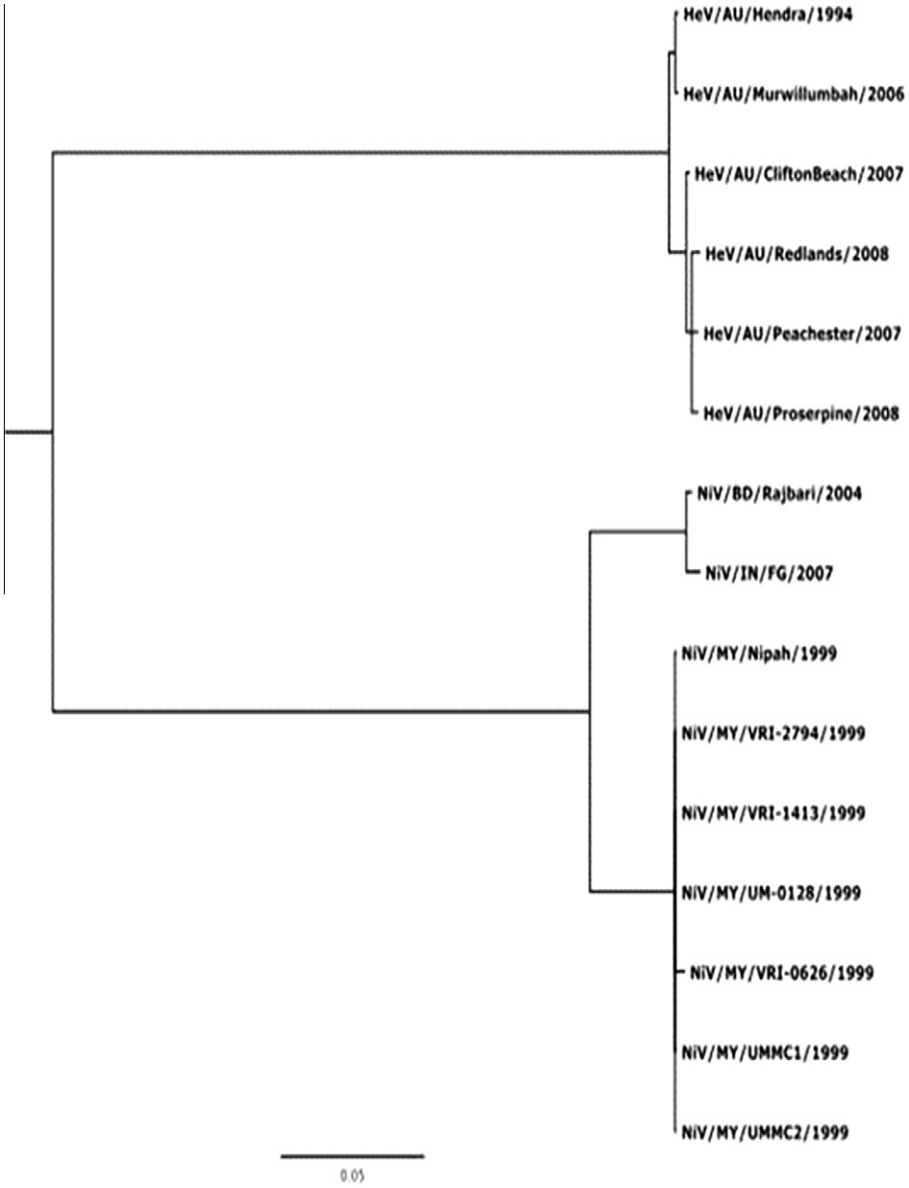


**Fig. 2.** Phylogenetic tree based on the Nipah and Hendra N open reading frames (ORF). Sequences were aligned using the ClustalW multiple sequence alignment function of MacVector® version 8.0 (Accelrys, Cary, NC) with default gap penalties. Phylogenetic analyses of the aligned genomic sequences were estimated with Bayesian analysis (MrBayes v3.1.0) where 4 MCMC tree searches of 40 million generations each were run simultaneously sampling 1 in 100 trees and computing a 50% majority-rule consensus tree out of the last 90,700 sampled trees, where the initial 10% of trees removed as burn in. Bootstrapping obtained by the Bayesian analysis was used to place confidence values on grouping within the consensus tree. Available GenBank accession numbers for HeV are: Hendra/1994 AF017149, Murwillumbah/2006 HM044318; Clifton Beach/2007 HM044321; Redlands/2008 HM044317; Peachester/2007 HM044319; Proserpine/2008 HM044320. Available GenBank accession numbers for NiV are: Rajbari/2004 AY988601; FG/2007 FJ513078; Nipah/1999 AF212302; VRI-2794/1999 AJ564621; VRI-1413/1999 AJ564622; UM-0128/1999 AJ564623; VRI-0626/1999 AJ627196; UMMC1/1999 AY029767; UMMC2/1999 AY029768.

the paramyxoviruses, includes five genera, *Avulavirus*, *Morbillivirus*, *Respirovirus*, *Rubulavirus* and *Henipavirus*. This subfamily includes the very important Newcastle disease, measles, parainfluenza and mumps viruses, as well as Nipah and Hendra viruses. Other emerging members of the *Paramyxovirinae* (e.g. Tioman, Menangle or Beilong virus) do not cluster into these 5 genera. The classification is based on genome organization, morphologic criteria, the sequence relationship of the encoded proteins and their biological activities.

### 2.2. Virion structure

The NiV and HeV virions display morphological features typical for members of the subfamily *Paramyxovirinae*. The virus particles range in size from approximately 40–600 nm, and are pleomorphic in shape, varying from spherical to filamentous (Hyatt et al., 2001; Murray et al., 1995b). They possess a lipid envelope, which encloses a nucleocapsid. The surface of the virus envelope is covered with projections, representing the fusion and attachment proteins. In the case of NiV, a single layer of surface projections has been described, while for HeV the projections are distinctive, double-fringed spikes (Hyatt et al., 2001). The nucleocapsid core is composed of the genomic RNA, the nucleocapsid protein, phosphoprotein and polymerase. The matrix protein is located inside the envelope and stabilizes the virion structure (Fig. 1A).

#### 2.2.1. The nucleocapsid protein

The N protein (592 amino acids) is the most abundant viral protein in infected cells, encapsulates the viral RNA, and plays an important role during the transcription and replication of viral

mRNA and genomic RNA (Halpin et al., 2004). There is 92.1% sequence identity between the NiV and HeV N protein and the variable region is limited to 42 amino acids within the C-terminus (Harcourt et al., 2000; Wang et al., 2001; Yu et al., 1998) (Fig. 2). The N protein has strong immunogenic properties, which are utilized for the development of diagnostic assays (Eshaghi et al., 2005a,b; Juozapaitis et al., 2007; Tan et al., 2004; Yu et al., 2006).

NiV N has the ability to self-assemble into herringbone-like structures in the absence of viral RNA and other viral proteins when expressed in recombinant form in *Escherichia coli*, and morphologically resemble the structure of isolated RNPs from virus particles (Tan et al., 2004). Ong and colleagues have shown that deletion of the C-terminal 129 amino acids completely abolishes the formation of the herringbone-like particles and results in the formation of spherical aggregates (Ong et al., 2009). Further, they suggest that the first 29 amino acids from the N-terminus of the NiV N protein do not play an important role in the assembly of the capsid, but that amino acids at positions 30–32 are crucial for formation of the capsid. These findings have not been reported previously. Four highly conserved and hydrophobic regions in the middle of the primary structure are involved in N–N interactions and required for capsid assembly.

For the transcription and replication of paramyxovirus genomes, the polymerase complex (L protein associated with the P protein) needs to bind to the nucleocapsid. One of the binding sites of the P protein was mapped to a region (amino acids 468–496) within the C-terminal domain of the NiV N protein (Chan et al., 2004). Interestingly, NiV and HeV N and P are able to form heterologous complexes, indicating the close phylogenetic relationship between these 2 viruses. A recent study by Huang and colleagues reported that NiV N undergoes a rapid phosphorylation/dephosphorylation turnover at Ser451 and that this phosphorylation plays an important role in virus transcription and replication (Huang et al., 2011). Further, this phosphorylation is not important for the direct N–P interaction.

*2.2.2. The phosphoprotein*

The henipavirus P protein gene (NiV P: 709 amino acids, HeV P: 707 amino acids) is the least conserved gene within the henipavirus genome (67.6%) and is approximately 100–400 amino acids longer compared to other paramyxovirus Ps. The protein is phosphorylated at multiple sites and the interaction between P and N is essential for paramyxovirus genome replication and transcription. Since P also needs to interact with L to carry out RNA synthesis, it plays a central role during virus replication. Chan and colleagues have shown that there are at least 2 independent N-binding regions in NiV P, which are located at the N-terminus (amino acids 3–220) and the C-terminus (amino acids 636–709) (Chan et al., 2004).

The unedited henipavirus P gene transcript encodes the P protein. A separate transcript containing a single G nucleotide inserted at the editing site generates the V protein (HeV: 461 amino acids, NiV: 458 amino acids) (Fig. 1B). The W protein is generated, when two G nucleotides are inserted (453 amino acids). Both proteins share their amino termini with the P protein but each possesses a unique carboxyl-terminal domain. The P gene also contains an alternative second short overlapping open reading frame upstream of the editing site, which in the P, V, and W mRNAs encodes the C protein (166 amino acids). The C protein is unique and does not share any detectible sequence similarity with the P protein.

As for the other paramyxoviruses, the henipavirus C, V, and W proteins are known to play important roles in the viral life cycle, regulation of viral transcription and replication (Sleeman et al., 2008), and function as interferon antagonist (these functions are described in more detail later in this review). Lo et al. showed that C, V, and W can be detected in purified NiV virions (Lo et al., 2009). In NiV infected cells, the 4 proteins have different subcellular localizations; P is located throughout the cell and the plasma membrane and V and C are located in the cytoplasm, while W is the protein located either in the nucleus or in the cytoplasm, depending on the cell type (Lo et al., 2009, 2010).

*2.2.3. The matrix protein*

The henipavirus M protein (392 amino acids) has been shown to play an important role during the life cycle of the virus. It mediates virus assembly and budding process, and is one of the 4 major structural proteins found in the virus particle. If M is expressed alone in the cell, it results in the formation and release of virus-like particles (VLPs) in the absence of other viral proteins (Ciancanelli and Basler, 2006; Patch et al., 2007). Patch et al. have shown that the YPLGVG sequence located in the N-terminal region of NiV M is required for budding (Patch et al., 2008). Further, a potential late-domain YMYL motif has been identified, which abolished VLP release when mutated (Ciancanelli and Basler, 2006). Recently, Wang and colleagues reported that a nuclear-cytoplasmic trafficking of NiV M, associated with post-translational modification, plays an important role for the budding process (Wang et al., 2010). The nuclear-cytoplasmic trafficking is regulated by a putative bipartite nuclear localization signal (NLS) and a leucine-rich nuclear export signal. Ubiquitination of a conserved lysine located in the NLS was found to be crucial for both nuclear import and export, but also for the plasma membrane targeting of NiV M and its incorporation into the virus particle (Wang et al., 2010).

*2.2.4. The fusion protein*

The F protein (546 amino acids) represents one of the surface membrane glycoproteins and is required for the fusion of the viral envelope with the host cell membrane. The paramyxovirus F protein is an oligomeric type I membrane protein, classified as a class I fusion protein and synthesized in an inactive $F_0$ precursor state. Cleavage and activation of the henipavirus F protein is different from other paramyxoviruses. Most *Paramyxovirinae* are either activated by extracellular trypsin-like proteases that recognize a single basic residue at the cleavage site (e.g. Sendai virus F) or by furin-like proteases that are localized in the trans-Golgi network of many eukaryotic cells (recognizing a dibasic cleavage site as found in measles virus). The cleavage site in the henipavirus F protein contains an apparent monobasic residue, which is neither cleaved by trypsin (Moll et al., 2004) nor furin (Michalski et al., 2000). For HeV F the basic residue is lysine in the sequence VGDVKLAG, while in NiV F the lysine is replaced by arginine (Harcourt et al., 2000; Michalski et al., 2000).

The inactive precursor $F_0$ protein traffics to the cell surface, subsequently is internalized back in the cell through endocytosis (Diederich et al., 2005; Meulendyke et al., 2005), where it is cleaved by cathepsin L in acidic endosomes (Pager et al., 2006; Whitman et al., 2009). The activated and disulphide-linked heterodimers ($F_1 + F_2$ subunits) are then recycled back to the cell surface. Endocytosis of $F_0$ is mediated by the presence of YXXF motifs in the cytoplasmic tail of F. During this process the fusion peptide will be positioned at the newly formed N-terminus of $F_1$. Fusion and henipavirus entry result from the interaction of the attachment glycoprotein (G) with F, triggering this F protein cascade as reviewed in (Lee and Ataman, 2011). Between NiV and HeV the sequence identity of the F gene is 88.1%.

*2.2.5. The glycoprotein*

The G protein represents the second surface membrane glycoprotein, is required for cell attachment, and a type II membrane protein. It consists of an N-terminal cytoplasmic tail domain, a transmembrane domain, a stalk region and a globular head domain. In contrast to other *Paramyxovirinae* attachment proteins,

the henipavirus G protein neither displays hemagglutinin nor neuraminidase activity. The NiV and HeV G protein globular head forms a six-bladed β-propeller structure, similar to other *Paramyxovirinae* attachment proteins, which is involved in receptor binding (Bowden et al., 2008a,b, 2010; Xu et al., 2008). The stalk region is most likely involved and responsible for the oligomerization of G's on the virion surface.

The henipavirus G protein uses the protein receptors ephrin-B2 and ephrin-B3 (Bishop et al., 2007; Bonaparte et al., 2005; Negrete et al., 2005, 2006). Ephrin-B2 is expressed on microvascular cells and neurons, while ephrin-B3 is not expressed in the endothelium but is found in the brain and brain stem (reviewed in: (Melani and Weinstein, 2010)) (Attwood et al., 2012; Benson et al., 2005; Gale et al., 2001; Lein et al., 2007; Liebl et al., 2003; Shin et al., 2001; Singh et al., 2012). This expression pattern correlates with the cellular tropism of henipaviruses and contributes to the pathophysiology (Hooper et al., 2001; Wong et al., 2002). The binding sites for ephrin-B2 and ephrin-B3 are overlapping on NiV G and have recently been localized at the top of the globular head domain (Bowden et al., 2008a). This receptor binding position is similar to the sialic acid binding sites on the hemagglutinin-neuraminidase attachment proteins of other paramyxoviruses. The X-ray crystallographic structure of the complex with ephrin-B3 reveals an extensive area of protein–protein interaction and the insertion of a portion of the receptor into the central cavity of NiV G. Further, it has been reported that NiV has a 30-fold higher affinity for ephrin-B3 than HeV (Bossart et al., 2008). The severe brain stem neuronal dysfunction seen in fatal NiV encephalitis could be explained by the fact that ephrin-B3 but not ephrin-B2 is expressed in the brain stem (Negrete et al., 2007). Henipaviruses can use ephrin-B2 and ephrin-B3 from a variety of species, which most likely accounts for their unusually wide host range (Bossart et al., 2008).

#### 2.2.6. The polymerase

The henipavirus L protein represents the largest of the viral proteins, predicted to consist of 2244 amino acids, and contains the 6 domains found within the polymerases of all non-segmented negative-sense RNA viruses (Harcourt et al., 2001). However, in contrast to other paramyxoviruses, the predicted catalytic site, located in motif C of domain III, has the sequence GDNE and not the typical GDNQ sequence (Harcourt et al., 2001; Poch et al., 1990). Mutating the glutamic acid to glutamine in NiV L does not affect replication of a NiV minigenome (Magoffin et al., 2007). Further, mutational analysis revealed that the amino acid at the E position most likely has no importance for the catalytic activity of the henipavirus polymerase (Magoffin et al., 2007).

### 2.3. Genome organization

Like all paramyxoviruses, NiV and HeV are single-stranded, negative-sense RNA viruses that replicate entirely in the cytoplasm (Fig. 1B). Henipaviruses are closely related to the respiro- and morbilliviruses, coding for six genes and nine viral proteins, but they differ from the respiroviruses in that they lack a neuraminidase function. However, several genetic features distinguish henipaviruses from other paramyxoviruses. Their genome length of about 18 kb (18,246 nt for NiV, and 18,234 nt for HeV) is approximately 2700 nt longer than others in the family. The genomes of NiV isolates from the Bangladesh strain (NiV-B) have a length of 18,252 nt, 6 nt longer than the originally sequenced NiV Malaysian strain (NiV-M) (Harcourt et al., 2005). The additional 6 nt are located in the 5′ untranslated region of the F gene. The NiV and HeV genomes are multiples of six, confirming the "Rule-of-Six" (Halpin et al., 2004). However, henipaviruses are unique in having extra genomic nucleotides (in the form of long untranslated regions), mostly located at the 3′ end in 5 out of the 6 transcription units, except for the viral polymerase gene (Halpin et al., 2004).

The 3′ leader sequence is 55 nt in length and identical to other members of the *Paramyxoviridae*. The 5′ trailer sequence is 33 nt in length and tends to be more variable compared to other paramyxoviruses. The leader and trailer function as promoters for the positive and negative strands, respectively. The 12 terminal 3′ and 5′ terminal nucleotides are highly conserved and complementary to each other. The organization of the henipavirus genes is conserved and each gene is separated by three nucleotides (intergenomic GAA). The open reading frame encodes for the nucleoprotein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), glycoprotein or attachment protein (G), and RNA polymerase (L). All henipavirus genes are monocystronic, except for the P gene. Through RNA editing, the P gene also encodes for the nonstructural V, C, and W proteins (Fig. 1B).

### 2.4. Cell entry and replication

Entry of paramyxoviruses into target cells occurs via fusion at the plasma membrane, endocytosis (for RSV and NDV) or even macropinocytosis (for NiV) (Diederich et al., 2008; Pernet et al., 2009). Entry is mediated by the glycoprotein and fusion protein (Tamin et al., 2002). The henipavirus attachment protein, which lacks both the hemagglutinin and neuraminidase activity found in many paramyxovirus attachment proteins, interacts with the cellular receptor to trigger the F protein-promoted fusion. Two cellular receptors for NiV and HeV are ephrin-B2 and ephrin-B3 (Bishop et al., 2007; Bonaparte et al., 2005; Negrete et al., 2005, 2006). Receptor binding results in a conformational change of the closely associated G and F proteins (Aguilar et al., 2009).

The extracellular domain of the $F_1$ subunit comprises two α-helical heptad repeat regions (HR1 and HR2), which fold into a 6-stranded helical bundle and bring the viral and host cell membranes into a fusion state. Upon receptor binding, F fusion activity is promoted by the glycoprotein by a yet not defined mechanism, resulting in a conformational change, which allows the hydrophobic fusion peptide of $F_1$ to penetrate the membrane of the host cell. These steps also take place during the formation of syncytia in infected cells. A detailed description of the complex henipavirus fusion process has recently been reviewed (Lee and Ataman, 2011).

After the viral membrane has fused with the cellular plasma membrane, the genome is released into the cytoplasm. The contacts between the M protein shell and the N proteins needs to be disrupted prior release of the helical nucleocapsids into the cytoplasm, but the driving for this uncoating step is not known yet. Transcription and viral RNA replication in henipavirus infection are believed to follow the same strategy as in other paramyxoviruses. The genomic negative (−) sense RNA (vRNA) is tightly associated with the N proteins and the RNA polymerase complex and serves as the functional template for both transcription and replication. In the first phase of the infection cycle, before viral protein synthesis will be initiated, the RNA polymerase complex, which was packaged in the virion, copies the vRNA template and generates short uncapped leader RNAs and capped and polyadenylated mRNAs, which encode each of the viral proteins (called primary transcription).

There is only a single promoter at the 3′ end of the genome for the polymerase and transcription of the viral mRNAs follows the "stop-start" model first described for vesicular stomatitis virus (Schnell et al., 1996). The viral mRNAs are not produced in equimolar amounts and a transcription gradient from the N to the L gene exists (3′-N-P-M-F-G-L-5′) (Wright et al., 2005). After these mRNAs are translated into the viral proteins, viral genome replication

begins. The vRNA is now used as a template to produce positive (+) sense antigenomes (cRNA), which are then used as templates to produce new vRNAs. These genomes can be used as templates for mRNA synthesis, for replication, or can be assembled into virions. The translated viral proteins are then performing their specific roles in regulating viral genome replication, virion assembly and budding and inhibition of the host immune response.

The newly synthesized N proteins associate with the nascent vRNA and form the nucleocapsid structure, followed by the association of the RNA polymerase complex. The two viral surface proteins are synthesized in the endoplasmic reticulum and undergo conformational maturation steps. To produce infectious henipavirus particles, the non-fusogenic precursor form of the fusion protein ($F_0$) must undergo a proteolytic conformational maturation process, which results in the fusiogenically active and disulfide-linked heterodimer $F_1 + F_2$. In this activated state, the fusion peptide is positioned at the newly formed N-terminus of $F_1$ (reviewed in: (Dutch, 2010) and (Smith et al., 2009)). The series of conformational changes from the metastable, pre-fusion conformation to the stable, post-fusion state, releases energy, which is utilized to drive the energetically demanding process of membrane fusion between virus and host cell membrane.

Virion assembly occurs at the plasma membrane of the infected cell. The nucleocapsid, M, G and $F_1 + F_2$ proteins are transported to the host cell plasma membrane, where the budding process will be initiated. The M protein plays a major and organizing role during this process. While lining up at the inner surface of the plasma membrane, it is believed to concentrate the RNPs, as well as the surface glycoproteins through interaction with their cytoplasmic tail domains, at the site of assembly. This has been shown by the ability to generate VLPs containing the M, F and G proteins (Ciancanelli and Basler, 2006; Patch et al., 2007; Walpita et al., 2011; Wolf et al., 2009). However, the viral glycoproteins do not enhance the efficiency of M-driven VLP release from transfected cells (Ciancanelli and Basler, 2006; Patch et al., 2007). Further, it has been shown that the NiV F and G proteins have intrinsic exocytosis activities, resulting in the formation of VLPs (Ciancanelli and Basler, 2006; Patch et al., 2007). The N, P, C, M, F, G and L proteins are incorporated in the released virions (Lo et al., 2009; Wang et al., 2001).

### 2.5. Virulence factors

As with other members of the family *Paramyxoviridae*, the henipaviruses have developed molecular mechanisms to antagonize host cell interferon (IFN) induction and signaling pathways. The P gene encodes proteins with anti-IFN activities, and is unusually long in the henipaviruses (reviewed in: (Fontana et al., 2008)). As mentioned earlier, the P, C, V and W proteins can be translated from the P gene; they have all been shown to exhibit anti-IFN activities through inhibition of at least the Janus kinases/signal transducers and activators of transcription (JAK/STATs) [V and W proteins] and Toll-like receptor (TLR)-3 pathways [W protein] (Ciancanelli et al., 2009; Fontana et al., 2008; Park et al., 2003; Rodriguez and Horvath, 2004; Rodriguez et al., 2002; Shaw et al., 2005, 2004). Surprisingly, the W protein shows a very strong IFN-inhibitory activity, attributed to its atypical nuclear localization (Shaw et al., 2005, 2004).

Despite their common STAT1-binding domain, the NiV V and P proteins act by retaining STAT1 in the cytoplasm, while the W protein sequesters STAT1 in the nucleus, creating both a cytoplasmic and a nuclear block for STAT1. Currently, the mechanism by which the C protein blocks IFN activities remains unknown (Shaw et al., 2004). Seto et al. showed that human monocyte-derived dendritic cells infected with recombinant Newcastle disease virus expressing NiV P, W, and V lack an early antiviral cytokine response and that this almost complete suppression is due to NiV W, followed by V and then P (Seto et al., 2010). Interestingly, production of some inflammatory cytokines occurs at early time points, which might contribute to increased vascular permeability and virus dissemination.

Lo and colleagues investigated the antiviral and inflammatory response of human neurons and human primary endothelial cells to infection with NiV (Lo et al., 2010). While no antiviral response could be detected in neurons, a robust IFN-β response was detected in endothelial cells, which correlated with the cellular localization of the W protein. In endothelial cells, NiV W was primarily found in the cytoplasm, while it localized to the nucleus of neurons. This indicates that the IFN response in endothelial cells is not inhibited, due to the reduced amount of W in the nucleus. Further, they could show that infection of endothelial cells resulted in a release of pro-inflammatory chemokines (such as MCP-1, IP-10, IL-8, and IL-6), which were able to induce T-lymphocyte and monocyte chemotaxis. These findings are consistent with the vasculitis observed in natural infections.

Similar to the findings by Lo et al. that NiV-infected endothelial cells can generate a functional IFN-β response, are results described by Virtue et al. (2011). The authors demonstrated that infections with henipaviruses are only resulting in a partial block of the IFN signaling pathways, which is in contrast to the antagonism of the innate immune response observed in transfected cells, where a complete block of the IFN response has been described (Ciancanelli et al., 2009; Lo and Rota, 2008; Shaw et al., 2005). It was shown that transfections with individual henipavirus genes (encoding the V, W, or C proteins), results in a greater amount of expressed P protein products and consequently complete block in the IFN signaling, than compared to naturally infected cells. It was suggested that unbound STAT1 proteins are still present in infected cells, which could likely be activated, and subsequently result in an initiation of the IFN response. Indeed, treatment of HeV-infected HEp-2 cells with IFN-β resulted in a reduction of infected cells by approximately 85%, indicating the potential use of IFN as a postexposure therapeutic for henipavirus infections (Virtue et al., 2011).

A study by Yoneda and colleagues using recombinant NiV lacking either the C, V or W protein showed that the IFN response in infected cells is suppressed to a similar level as observed with wild-type NiV, indicating that the individual accessory proteins do not have any significant effect on IFN suppression (Yoneda et al., 2010). NiV lacking the V or C protein does not cause any clinical signs of disease in the Syrian golden hamster model and 100% of challenged animals survive. However, animals infected with NiV lacking the W protein develop a fatal outcome of disease, indicating that the V and C proteins have a key role during the life cycle of NiV (Yoneda et al., 2010). The authors also suggested that the attenuated outcome of infection in hamsters with recombinant NiV lacking the V and C protein could be due to the reduced replication ability of the viruses *in vivo* which appeared to be below the level needed to induce clinical disease.

### 2.6. Antigenic variation

The HeV and NiV attachment glycoprotein G and fusion protein F are essential for virus binding and entry and as such are the primary targets for protective antibody responses. The 83% and 88% amino acid identity between HeV and NiV G and F, respectively, results in cross-reactivity between these viruses but not with other members of the family *Paramyxoviridae*. While cross-reactivity is observed in sera from HeV- and NiV-infected cases, their distinct geographic distributions assure high specificity for serological diagnostics. This cross-reactivity has proved beneficial in the development of therapeutics and vaccines. Several potential therapies targeting the henipavirus G and F proteins have been shown to

completely protect animals from lethal disease, including recombinant NiV or HeV soluble G-based subunit vaccines, vaccinia and canarypox virus-based vectors carrying the G or F proteins of NiV, and neutralizing antibodies (Bossart et al., 2009; Guillaume et al., 2004a,b, 2009; Mungall et al., 2006; Weingartl et al., 2006).

Several neutralizing epitopes have been identified on the HeV and NiV G. Based on monoclonal antibody studies, 4 sites appear to contribute to a major discontinuous epitope on the base of the globular head (aa 183–185, 417, 447 and 570), similar to the location of immunodominant epitopes found in other paramyxoviruses (White et al., 2005). Three additional epitopes identified include residues 289/324, 191–195 and 385–386 (White et al., 2005). Monoclonal antibodies have also been developed against NiV G and were used to identify important neutralizing epitopes. Seven residues W504, E505, N557, Q530, T531, A532, and E533 form a contiguous site on the top surface between the central shallow depression and the rim of the globular head (Guillaume et al., 2006). These epitopes overlap the receptor binding domain and therefore the primary mechanism of neutralization appears to be direct blocking of the virus–receptor interaction.

Epitope mapping for monoclonal antibodies directed against the fusion glycoprotein has not been reported.

The high mutation rate of RNA viruses is a major problem in developing therapeutic MAbs, especially against viruses that emerge from animal reservoirs. Neutralization escape mutants have been generated in the laboratory in an effort to map epitopes of neutralizing monoclonal antibodies, emphasizing this concern during natural infection (Guillaume et al., 2006) (Rockx; unpublished data).

## 3. Epidemiology and clinical illness

### 3.1. Geographic range

To date, outbreaks of NiV have been recognized in peninsular Malaysia, Singapore, Bangladesh and India, while outbreaks of HeV have not been reported outside of Australia. The range of *Pteropus* bats covers the geographic locations of all known outbreaks (Luby et al., 2009a).

### 3.2. Genetic variation

As RNA viruses, HeV and NiV likely produce quasispecies during replication; however no data are available on mutation rates or hotspots and no genetic shift or drift has been reported. Sequence comparisons of viruses isolated from CSF and throat swabs from the same patient revealed differences at 4 nucleotide positions, 2381 (T to C) at the 3′ non-coding region of the P/V/C gene, 11315(G to A) at the 3′ non-coding region of the L gene, 16160 (C–T) at the coding region of the L gene and 16345 (T–C) at the coding region of the L gene (Chan et al., 2001). Predicted amino acid identities between NiV-M and HeV range from 92% to 67%, whereas amino acid identities between NiV-M and NiV-B range from 100% to 92%.

Partial genome sequencing from outbreak isolates has show that HeV isolates remain genetically identical over time (Halpin et al., 2000). Despite the overall genome showing genetic stability, diversity of HeV is observed primarily in hypervariable regions between strains circulating within the flying fox populations at one time in multiple locations (Smith et al., 2011). One such region is the carboxyl terminal of the N protein gene. The presence of multiple variants circulating at one time in multiple locations suggests that spill-over events do not occur due to specific isolates but rather due to other factors that could include environmental stressors, pregnancy and urbanization (Smith et al., 2011).

For NiV, the ranges of nucleotide and amino acid variation of sequences within the Malaysia genotype (NiV-M) were 0.19–2.21% and 0.18–3.67%, respectively, and within the Bangladesh genotype (NiV-B) were 0.28–1.06% and 0.28–0.56%, respectively (Lo et al., 2012). In Malaysia, very limited variation was observed between human NiV isolates and isolates obtained from bats years later (AbuBakar et al., 2004; Chan et al., 2001; Chua et al., 2002; Harcourt et al., 2000). Interestingly, molecular evidence suggests that at least two major strains of NiV in pigs were circulating during the 1998 NiV outbreak in Malaysia: one strain from the initial outbreak in the north and the other strain from the subsequent outbreak approximately 4 months later in the south (AbuBakar et al., 2004). No data are available to indicate whether these 2 strains represent 2 independent introductions of NiV into the pig population, or if the latter strain evolved from the initial NiV strain. In Bangladesh, genetic heterogeneity in 4 human isolates suggests multiple introductions of NiV in the human population from bats (Luby et al., 2009b). Recently, a 729 nucleotide region of the N protein gene of NiV has been proposed that can be used for genotyping (Lo et al., 2012).

### 3.3. Epidemiology and clinical manifestations

#### 3.3.1. Hendra virus

HeV was the first member of the genus to be identified as the causative agent of an acute respiratory disease in horses in 1994

**Table 1**
Chronology of Hendra virus outbreaks. *Source*: World Health Organization[a]; ProMED-mail archive number: [b]20090910.3189; [c]20100520.1673; [d]20110818.2512, 20111013.3061; [e]20120106.1001359.

| Year | Australian state | Equine cases | Human cases | | |
|---|---|---|---|---|---|
| | | | Cases | Deaths | Case fatality (%) |
| 1994[a] | Queensland | 2 | 1 | 1 | 100 |
| 1994[a] | Queensland | 21 | 2 | 1 | 50 |
| 1999[a] | Queensland | 1 | 0 | 0 | N/A |
| 2004[a] | Queensland | 1 | 1 | 0 | 0 |
| 2004[a] | Queensland | 1 | 0 | 0 | N/A |
| 2006[a] | Queensland | 1 | 0 | 0 | N/A |
| 2006[a] | New South Wales | 1 | 0 | 0 | N/A |
| 2007[a] | Queensland | 1 | 0 | 0 | N/A |
| 2007[a] | Queensland | 1 | 0 | 0 | N/A |
| 2008[a] | Queensland | 5 | 2 | 1 | 50 |
| 2008[a] | Queensland | 3 | 0 | 0 | N/A |
| 2009[b] | Queensland | 2 | 1 | 1 | 100 |
| 2010[c] | Queensland | 1 | 0 | 0 | N/A |
| 2011[d] | Queensland (9 properties) | 12 | 0 | 0 | N/A |
| 2011[d] | New South Wales (8 properties) | 10 | 0 | 0 | N/A |
| 2012[e] | Queensland | 1 | 0 | 0 | N/A |
| Total | | 63 | 7 | 4 | 57 |

**Table 2**
Chronology of Nipah virus outbreaks. *Source*: World Health Organization[a]; ProMED-mail archive number: [b]{Lo et al., 2012}; [c]20110308.0756.

| Year | Country | Human cases | | |
|---|---|---|---|---|
| | | Cases | Deaths | Case fatality (%) |
| 1998–1999[a] | Malaysia | 265 | 105 | 40 |
| 1999[a] | Singapore | 11 | 1 | 9 |
| 2001[a] | India | 66 | 49 | 74 |
| 2001[a] | Bangladesh | 13 | 9 | 69 |
| 2003[a] | Bangladesh | 12 | 8 | 67 |
| 2004[a] | Bangladesh | 29 | 22 | 76 |
| 2004[a] | Bangladesh | 36 | 27 | 75 |
| 2005[a] | Bangladesh | 12 | 11 | 92 |
| 2007[a] | Bangladesh | 7 | 3 | 43 |
| 2007[a] | Bangladesh | 8 | 5 | 63 |
| 2007[a] | India | 5 | 5 | 100 |
| 2008[a] | Bangladesh | 3 | 3 | 100 |
| 2008[a] | Bangladesh | 8 | 3 | 38 |
| 2010[b] | Bangladesh | 17 | 15 | 88 |
| 2011[c] | Bangladesh | unknown | 35 | unknown |
| Total | | $\geqslant$506 | 290 | $\leqslant$57 |

and to date there have been 17 outbreaks in Australia, with at least one occurrence per year since 2006, most recently in January 2011 (Table 1). Following an incubation period of 4–16 days, horses typically develop an acute febrile illness with rapid, progressive respiratory system compromise and high fatality rates (Field et al., 2000; O'Sullivan et al., 1997; Selvey et al., 1995; Westbury, 2000). More recently, outbreaks have occurred with clinical features reflecting primarily central nervous system involvement (Field et al., 2010). Acute respiratory syndrome is followed by death in 1–3 days.

Despite the frequent outbreaks of HeV infection in horses, only 7 human cases have been identified, of which 4 were fatal (O'Sullivan et al., 1997; Playford et al., 2010; ProMED-mail, 2009; Selvey et al., 1995; Wong et al., 2009). Human infections have been reported only for people who have close contact with infected horses. Strict quarantine guidelines require veterinarians to wear gloves, facial masks, and full body clothing when handling sick horses. All patients initially presented with influenza-like illnesses (ILI) after an incubation period of 7–16 days. Lethal cases either developed pneumonitis and died from multiorgan failure or developed encephalitic manifestations (mild confusion, ataxia) and seizures.

### 3.3.2. Nipah virus

The first human cases of NiV infection were identified during an outbreak of severe febrile encephalitis in Malaysia and Singapore in 1988/1999 (Chua et al., 1999). A total of 276 patients with encephalitis were reported with 106 fatalities (38%) (Chua et al., 2000a). Since this outbreak, recurrent outbreaks of NiV in Bangladesh and India have involved more than 120 people (2004; Chadha et al., 2006) (Table 2). These outbreaks were associated with significantly higher case fatality rates of 67–92% (Luby et al., 2006). The increased case fatality rate in Bangladesh may be due to inherent strain-specific differences between Malaysian and Bangladesh strains of NiV, or a lower level of supportive medical care available compared to Malaysia and Singapore.

In the majority of cases, the incubation period has been reported to be 4 days to 2 weeks; however, a delay of 2 months between exposure and the onset of illness has been reported {Goh et al. 2000}. Following the onset of clinical signs of disease, NiV infection can cause rapid, acute encephalitis with a high mortality (reviewed in: (Lo and Rota, 2008)). Interestingly, the incubation period during the Bangladesh outbreaks has been markedly shorter, with clinical signs and symptoms starting between 6 and 11 days (Hossain et al., 2008; Lo and Rota, 2008). For all outbreaks, the majority of patients initially develop influenza-like signs and symptoms, including fever, headache, myalgia and vomiting (reviewed in: (Lo and Rota, 2008)). This can be followed by dizziness, drowsiness, reduced levels of consciousness, and neurological signs that indicate acute encephalitis. In severe cases, encephalitis and seizures can occur, which can progress to coma within 24–48 h. Interestingly, a higher prevalence of respiratory disease was observed during the Bangladesh outbreaks (reviewed in: (Lo and Rota, 2008)) with cases experiencing atypical pneumonia and developing an acute respiratory distress syndrome (ARDS) (Hossain et al., 2008). Consistent with ARDS, chest radiographs revealed diffuse bilateral opacities covering the majority of the lung fields (Hossain et al., 2008).

The majority of patients who survive acute NiV encephalitis make a full recovery. However, about 22% are left with residual neurological sequelae such as persistent convulsions and personality changes. Approximately 8% of patients who recover subsequently develop relapse encephalitis, whereas 3% initially with asymptomatic or non-encephalitic infection develop delayed onset encephalitis (Tan et al., 2002). In the long term, persistent neurological dysfunctions are observed in more than 15% of people.

### 3.4. Reservoir hosts

HeV was first identified as the causative agent of an outbreak of acute respiratory disease in horses in 1994 (Selvey et al., 1995). To date there have been 33 outbreaks in Australia, with at least one occurrence per year since 2006, most recently at least 18 outbreaks in 2011, involving 21 horses and 1 outbreak in 2012 (http://www.promedmail.org archive 20110830.2666 and 20120106.1001359) (Broder, 2012; Hess et al., 2011; Marsh et al., 2010; Playford et al., 2010). Every outbreak has involved horses as the initially infected host, and there have been a total of 7 human cases due to exposure to infected horses.

NiV emerged as the cause of porcine respiratory and encephalitis syndrome in pigs in Malaysia in 1998 (1999). Pigs primarily exhibited respiratory disease with only a few animals showing neurological signs; a large percentage of asymptomatic infections was observed (Weingartl et al., 2005). Up to 95% of sick animals recovered. Unlike the limited host range and interspecies transmission observed for most paramyxoviruses, henipaviruses display a wide host range, with NiV naturally infecting pigs, humans, horses, dogs and cats, and HeV naturally infecting horses, dogs and humans (http://www.promedmail.org archive 20110802.2324) (Chua, 2003; Chua et al., 2000a; Mills et al., 2009; O'Sullivan et al., 1997; Parashar et al., 2000).

The natural reservoirs for NiV and HeV are fruit bats from the genus *Pteropus* (Halpin et al., 2011, 2000; Reynes et al., 2005; Williamson et al., 1998; Yob et al., 2001). Serological evidence of NiV infection was found in 5 of 14 bat species in Malaysia. The highest seroprevalence was observed in *Pteropus hypomelanus* (31%) and *Pteropus vampyrus* (17%) (Yob et al., 2001). Infectious NiV was isolated from urine or saliva of *P. hypomelanus* and *Pteropus lylei* in South and South East Asia (Reynes et al., 2005; Wacharapluesadee and Hemachudha, 2007; Wacharapluesadee et al., 2005). In Australia, all 4 local *Pteropus* species showed serologic evidence of HeV infection (Young et al., 1996). Experimental inoculation of *Pteropus* bats with either NiV or HeV results in infection in the absence of clinical disease, supporting their roles as reservoirs (Middleton et al., 2007; Williamson et al., 1998). *Pteropus* bats are not found in continental Africa, but henipavirus-specific antibodies and viral RNA have been detected in *Eidolon helvum* in Ghana; infectious virus has not been isolated from these animals (Drexler et al., 2009; Hayman et al., 2008).

While the dynamics of NiV and HeV in their reservoir hosts are poorly understood, some studies show that pregnant and lactating

females have significantly higher risk of infection, which may explain the temporal associations between NiV and HeV outbreaks and flying fox birthing periods (Plowright et al., 2008). Both NiV and HeV are transmitted horizontally via feces, urine or saliva (Sohayati et al., 2011) and the highest risk of infection is associated with animals that show evidence of nutritional stress, suggesting that environmental processes that alter food sources, such as habitat loss and climate change, may increase NiV and HeV infection and transmission (Plowright et al., 2008). In addition, some data suggests that bats are persistently infected, shedding virus intermittently which will allow viral circulation in a population, leading to a steady rate of infection in susceptible individuals in bat colonies (Sohayati et al., 2011).

### 3.5. Transmission cycles

The only known route of HeV transmission to humans has been direct contact with contaminated body fluids from infected horses. No evidence for direct bat-to-human, or human-to-human transmission has been found. During the initial NiV outbreaks in Malaysia and Singapore, the primary mode of transmission to humans was believed to be pig-to-human as the initial outbreak involved occupational exposure to pigs (Chew et al., 2000; Parashar et al., 2000; Premalatha et al., 2000; Sahani et al., 2001). Transmission was thought to have occurred via respiratory droplets or secretions from infected pigs or their contaminated tissues.

During the Bangladesh outbreaks, epidemiological studies have identified several routes of NiV direct transmission from bat reservoirs to human. A frequently implicated route was the consumption of fresh date palm sap (Luby et al., 2006). Date palm sap is believed to be contaminated by *Pteropus* bats that visit the date palm trees and lick the sap, or by contamination of the date palm sap by feces. A second route of transmission was believed to be direct contact with NiV-infected bat secretions (Montgomery et al., 2008). In addition, several NiV outbreaks in Bangladesh have resulted from human-to-human transmission (Blum et al., 2009; Gurley et al., 2007a; Homaira et al., 2010). Nosocomial transmission was suspected in up to 75% of cases and supported by the detection of NiV RNA on hospital surfaces (Gurley et al., 2007b).

One explanation for the differences in clinical presentation observed between the NiV outbreaks in Malaysia and Bangladesh may be the differences in routes of transmission and different infectious doses associated with these routes. In addition, the limited numbers of human cases of HeV infection suggest that the transmission efficiency may differ between NiV and HeV.

### 3.6. Pathogenesis in humans

Infections caused by henipaviruses are characterized by their ability to infect multiple organ systems and by a remarkable range of species that are susceptible to infection. Due to the higher incidence of NiV infections in humans, the pathology has been described in more detail for NiV than HeV, although they have very similar features (Hooper et al., 2001; Tan and Chua, 2008; Wong et al., 2009). The clinical hallmarks of both NiV and HeV infections are a predominantly neurological or respiratory tropism, depending on the host. Magnetic resonance imaging scans of human patients display widespread focal lesions, mainly localized in the white matter, and abnormal electroencephalograms (Goh et al., 2000; Lee et al., 1999; Lim et al., 1999). Further, most patients also suffer segmental myoclonus, which together with the multifocal lesions, appears to be unique to NiV infections (Goh et al., 2000). Anti-NiV antibodies can be detected in the CSF of 31% of the patients and in the serum of 76%, but no correlation with the outcome of the disease can be made (Chua et al., 2000b). Interestingly, henipavirus infections can also result in a chronic course of disease after an initial non-encephalitic or asymptomatic infection, causing relapsing or late-onset encephalitis up to 4 years after infection (Tan and Chua, 2008; Tan et al., 2002; Wong et al., 2009).

Histopathologic changes in fatal human HeV and NiV infections include systemic vasculitis and parenchymal necrosis in the central nervous system, while in the lungs, pathologic findings mainly include vasculitis, fibrinoid necrosis, alveolar hemorrhage, pulmonary edema and aspiration pneumonia. Other organs affected include the heart, kidney and spleen, which show generally mild or focal inflammation. The development of syncytial multinucleated microvascular endothelial cells is characteristic for both HeV and NiV infections. The endotheliotropism at the end stage of disease suggests that the virus can spread to other organs by the hematogenous route. A recent study by Mathieu and colleagues suggested the ability of NiV to hijack leukocytes as a vehicle to spread throughout the host and to transinfect host cells (Mathieu et al., 2011). They showed that NiV is not able to infect but efficiently binds to human lymphocytes, and that co-culturing with permissive endothelial cells results in virus amplification and infection of these target cells. Further, mononuclear leukocytes isolated from infected hamsters can transfer lethal infection to naïve animals.

At present, the details of the pathogenesis and histopathological changes mediated by HeV or NiV infection in humans is derived from only the terminal phase of the disease course and therefore a relevant animal model is needed that mimics the disease progression seen in humans.

### 3.7. Diagnostic tests

The laboratory diagnosis of HeV and NiV infection makes use of serologic tests, quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), virus isolation and electron microscopy (Daniels et al., 2001). Serologic tests include the detection of HeV- and NiV-specific antibodies in serum or cerebral spinal fluid (CSF) using an enzyme-linked immunosorbent assay (ELISA) or an immunofluorescence assay (IFA) with recombinant glycoproteins or inactivated virus particles as antigens. Based on a panel of sera from human cases involved in an outbreak of NiV, the relative sensitivity and specificity of a recombinant NiV N protein ELISA were 99% and 98%, respectively (Yu et al., 2006). Serum neutralization tests are accepted as reference serological tests but typically require BSL-4 containment. Several surrogates for neutralizing titers in samples have been developed. A neutralization assay was developed using the Bio-Plex protein array system where the interaction of ephrin-B2 and a soluble G of HeV or NiV, each coupled to a different microsphere, could be blocked by neutralizing antibodies in a dose-dependent fashion (Bossart et al., 2007b). The benefit of this method is the lack of infectious agents needed. Alternatively, recombinant vesicular stomatitis viruses pseudotyped with the surface glycoproteins F and G of HeV and NiV have been used in neutralization assays (Kaku et al., 2009; Tamin et al., 2009).

Various qRT-PCR assays have been developed for the detection of viral RNA in serum, CSF or throat swabs, targeting the phosphoprotein, matrix protein, or nucleoprotein (Chang et al., 2006; Guillaume et al., 2004a; Smith et al., 2001; Wacharapluesadee and Hemachudha, 2007; Wright et al., 2005). Virus isolation by cell culture has historically been the gold standard for diagnosis, and requires work to be carried out under BSL-4 conditions, severely limiting its practicality. Both HeV and NiV grow efficiently in a wide range of cell lines including Vero cells with development of characteristic syncytia (Aljofan et al., 2009). Cytopathic effect are usually observed within 3 days, but 2 passages are recommended before discontinuing attempts at virus isolation (Daniels et al., 2001).

During the initial NiV outbreak in Malaysia, the diagnosis was confirmed by transmission electron microscopy of negatively stained preparations of CSF (Chow et al., 2000). Once NiV or HeV has been isolated in cell culture, the viruses can be visualized by negative-contrast electron microscopy, and immunoelectron microscopy provides rapid information on the virus morphology and antigenic reactivity (Daniels et al., 2001; Hyatt et al., 2001).

## 4. Experimental infection of natural hosts and laboratory animals

The natural amplifying hosts of NiV and HeV are bats, horses and pigs. However, both viruses grow in a wide range of cell culture systems of mammalian, reptile, avian, amphibian and even fish origin (Halpin et al., 2007). Henipaviruses are also able to amplify in various animal species, and show a broad tissue tropism. This paragraph summarizes existing animal models developed to study the pathogenesis caused by henipaviruses and to evaluate therapeutics, antivirals and vaccines. More detailed descriptions of individual animal models are reviewed in Weingartl et al. (2009) and Williamson and Torres-Velez (2010).

### 4.1. Bats

Grey-headed fruit bats (*Pteropus poliocephalus*) infected with HeV and NiV do not develop any clinical disease but became seropositive (Middleton et al., 2007; Williamson et al., 1998). No HeV can be isolated from challenged bats (Williamson et al., 1998) but can be detected in infected fruit bats in late gestation in the kidneys, heart, spleen, and fetus (Williamson et al., 2000). In NiV-infected bats, virus can be detected in urine, while neutralizing antibodies are present in the serum (Middleton et al., 2007).

### 4.2. Pigs

Pigs infected with NiV or HeV, developed mainly respiratory syndromes, but rare and mild neurological syndromes also occur (Berhane et al., 2008; Li et al., 2010; Middleton et al., 2002). Oral infection of pigs with NiV generally does not result in development of severe clinical signs, while pigs inoculated via the subcutaneous route develop neurological and respiratory signs of disease (Middleton et al., 2002). However, excreted NiV can be detected from the oropharynx as early as 4 days post infection (Middleton et al., 2002). It has also been shown that NiV can be spread from pig-to-pig by nasal secretions, indicating that field transmission of NiV between pigs most likely occurs via respiratory and oropharyngeal secretions (Berhane et al., 2008; Li et al., 2010; Middleton et al., 2002; Weingartl et al., 2005). Weingartl and colleagues could show that NiV is detectable in nasal swabs at 2 days post-infection and in the pharyngeal swabs one day later (Weingartl et al., 2005). Their data also confirmed that the nasal cavity likely functions as a site of NiV entry and replication, however, this route of initiation of infection might be specific to pigs, due to their food sniffing and rooting behavior.

Pigs infected with HeV via the oronasal or intranasal route develop respiratory illness and signs of mild neurologic disease; virus is detected in nasal, oral, and ocular swabs (Li et al., 2010). In contrast to NiV-infected pigs, HeV-infected pigs also appear to shed virus via the rectal route. Vasculitis, with bronchiolar and endothelial syncytial cells is present (Li et al., 2010; Middleton et al., 2002). Viral antigen is present in endothelial cells, smooth muscle cells, airway epithelium, cranial nerves and renal glomeruli. Approximately 20% of pigs infected with NiV via the intranasal, oral and ocular routes develop neurological signs of disease but virus is present in the nervous system of both sick and healthy animals (Weingartl et al., 2005). In these animals, viral antigen is detected in brain, cerebrospinal fluid, cranial nerves, and trigeminal ganglion, indicating that NiV could invade the porcine central nervous system via the cranial nerves (mainly via the olfactory nerve) and by crossing the blood–brain barrier. While HeV currently seems to demonstrate an increased virulence for the respiratory tract, NiV appears to have to stronger ability to invade the CNS of pigs (Li et al., 2010).

It was recently shown that peripheral blood mononuclear cells (PBMC) play a role in the viremic spread of the virus. It was suggested that infection of T cells carrying CD6 marker, a strong ligand for the activated leukocyte cell adhesion molecule ALCAM (CD166) highly expressed on the microvascular endothelial cells of the blood–brain barrier could explain the preferential tropism of NiV for small blood vessels of the CNS (Stachowiak and Weingartl, 2012).

### 4.3. Horses

Horses experimentally infected with HeV developed severe respiratory signs of disease (increased respiratory and heart rate, labored breathing), high fever and weakness (Murray et al., 1995a,b). Depending on the challenge dose, animals die within 4 days of infection (Murray et al., 1995a). Histologically, interstitial pneumonia with alveolar edema associated with hemorrhage, alveolar thrombosis and necrosis, and necrosis of the walls of small blood vessels is observed. Syncytia in endothelial cells of blood vessel walls and bronchial epithelium are positive for viral antigen. Virus can be detected in the lung, spleen, kidney, brain, lymph nodes and blood (Williamson et al., 1998). HeV can also be isolated from saliva and urine (Williamson et al., 1998). A recent study by Marsh and colleagues, reported that horses exposed to HeV via the oronasal route resulted in systemic disease (Marsh et al., 2011). HeV RNA was detected in nasal swabs as early as 2 days post challenge, indicating that systemic spread of the virus may be preceded by local viral replication in the nasal cavity or nasopharynx. A disease transmission study using cats, horses and fruit bats suggested that HeV was not highly contagious (Williamson et al., 1998). The authors observed limited transmission of HeV among horses; while the virus could be transmitted through contact from infected cats to horses, no transmission could be detected from bats to horses, from horses to horses or from horses to cats.

### 4.4. Hamsters

The Syrian golden hamster is highly susceptible to henipaviruses and represents their only reliable small rodent animal model (Guillaume et al., 2009; Rockx et al., 2011; Wong et al., 2003). The development of clinical signs mimics the ones observed in humans, such as acute encephalitis and acute respiratory distress, and animals normally succumb to infection 4–17 days post infection. However, disease progression is dependent on challenge dose and route of inoculation (Rockx et al., 2011; Wong et al., 2003). Development of respiratory disease followed by radiographic imaging shows that moderate interstitial infiltrates and consolidation are evident at early stages of disease, progressing to pulmonary infiltrates throughout the lung and formation of air bronchograms (Rockx et al., 2011). In the final stages of infection, animals present imbalance, limb paralysis, lethargy, muscle twitching, and breathing difficulties. Vasculitis and thrombosis are prominent in multiple organs including brain, lung, liver, kidney and heart, while endothelial syncytial cells can only rarely be detected. Infectious virus is detected in lung, brain, spleen, kidney, heart, spinal cords and urine but not in serum (Wong et al., 2003). Viral RNA and antigen have been detected in the brain, lung, liver, kidney, spleen and heart. In terms of vascular and parenchymal le-

sions, the brain is the most severely affected organ. Neurons found in the vicinity of vasculitis show eosinophilic inclusion bodies in the cytoplasm, which are often positive for viral antigen and RNA. Other parenchymal changes include ischemia and edema. In the lung, areas of parenchymal inflammation, often associated with vasculitis, can be observed. Fibrinoid necrosis of lung parenchyma is rare and focal. Inflammatory cells detected in the affected lung tissues consist of macrophages, lymphocytes and neutrophils. In the kidneys, viral antigen was detected in the occasional glomerulus, tubule and covering epithelium of the renal papilla. Interestingly, similar to infection in pigs, NiV can bind to PBMC without infecting them and transfer of these cells to naïve animals will result in fatal infection. This suggests that NiV can also use hamster PBMC to spread throughout the host (Mathieu et al., 2011).

### 4.5. Guinea pigs

The guinea pig has been shown to be a reliable model for experimental NiV and HeV infection; however, it is only of limited use due to the absence of respiratory disease (Hooper et al., 1997; Middleton et al., 2007; Torres-Velez et al., 2008; Williamson et al., 2000, 2001). HeV administered via the subcutaneous route induces a generalized vascular disease without pulmonary edema (Hooper et al., 1997). Inoculation of a higher dose more closely recapitulates human disease, even including encephalitis lesions (Williamson et al., 2001). Intradermal and intranasal injection does not result in development of disease but results in seroconversion. Intraperitoneal infection with NiV causes a clinical profile with similarities to human disease, but with reduced pulmonary signs (Torres-Velez et al., 2008). Histologically, a systemic vasculitis is observed, most severely affecting the brain, spleen, lymph nodes, ovary, uterus and urinary bladder. Vascular lesions are characterized by large numbers of macrophages and perivascular lymphocytes, as well as vascular fibrinoid necrosis. Syncytia formation is prominent in epithelial and endothelial cells, specifically of the kidney, lung and bladder (Hooper et al., 1997). Guinea pigs develop severe meningoencephalitis, with intracytoplasmic and intranuclear inclusions in neurons and microglia, and viral antigen can be detected in neurons, microglia, meninges, ependymal cells and blood vessels. Shedding of virus in urine has been described. Virus is also able to cross the placenta, associated with severe placentitis, and can be isolated from fetuses (Williamson et al., 2000).

### 4.6. Ferrets

Ferrets represent a model for henipavirus pathology that is similar to the hamster model, and reflects very closely the clinical signs of disease observed in humans (Bossart et al., 2007a, 2009; Pallister et al., 2009, 2011). Oronasally-infected ferrets develop both severe respiratory and neurological disease within 6–9 days post challenge, including pneumonia, hind limb paralysis, tremor, and depression, resembling the disease in humans (Bossart et al., 2009; Pallister et al., 2009). Ferrets develop fever, followed by depression, nasal discharge, coughing, dyspnea, and generalized tremors. Some ferrets have signs of nonsuppurative meningitis and virus can be isolated from their brains; however, fewer lesions are observed than in human brains. Viral antigen can be detected in small blood vessels and syncytial cells in tissues of multiple organs. Histologically, systemic vasculitis and hemorrhage due to endothelial infection and focal necrosis, pulmonary alveolitis, cystis, and thyroiditis are observed. Viral RNA can be detected in all tissues examined, such as kidney, lung, spleen, brain, liver, bladder, adrenal glands and bronchial lymph nodes. Lungs and lymphoid tissues appear to be the sites of extensive virus replication. Viral shedding is suggested by the presence of virus in pharyngeal and rectal swabs.

### 4.7. Cats

Cats are susceptible to NiV and HeV and develop a pulmonary syndrome, showing similar illness to that observed in humans and horses (Hooper et al., 1997; Middleton et al., 2002; Westbury et al., 1996). Despite the fact that virus can be detected in the brain, no neurological signs develop. After an incubation time of 4–8 days, infected animals develop severe pulmonary lesions with pulmonary edema, necrosis of alveolar walls, and vascular lesions. Viral antigen can be detected in many small blood vessels in the lung, kidney, spleen, gastrointestinal tract, and lymph nodes. Subcutaneously-, orally-, or intranasally-administered HeV results in the development of severe dyspnea and open-mouth breathing, and infectious virus can be isolated from the lung, brain, spleen, kidneys and urine (Westbury et al., 1995; Williamson et al., 1998). HeV can be transmitted between cats housed in the same cage and their urine can infect horses (Westbury et al., 1996; Williamson et al., 1998). Cats infected with NiV develop a stronger involvement of the upper and lower respiratory tract and virus can be isolated from the placenta, fetal tissues, and uterine fluids from an infected pregnant cat (Mungall et al., 2007).

### 4.8. Nonhuman primates

Two non-human primate (NHP) models have been developed and described for the henipaviruses. Squirrel monkeys (*Saimiri sciureus*) infected with NiV via the intranasal or intravenous route with doses as high as $10^7$ pfu develop clinical signs of disease similar to those described in humans (Marianneau et al., 2010). However, only 50% of the challenged animals develop clinical illness and most recover over the course of a study. Monkeys challenged intranasally show milder signs of disease compared to animals infected via the intravenous route. Initial clinical signs of disease include anorexia, depression and weight loss, which progress to an acute respiratory syndrome with dyspnea and hyperventilation. Involvement of the neurological system is indicated by uncoordinated motor movement, loss of consciousness and coma. Virus-specific RNA can be detected in lung, brain, liver, kidney, spleen and lymph nodes but is limited to animals challenged intravenously. Vasculitis and brain abnormalities are less evident compared to those described for humans.

African green monkeys (AGM, *Chlorocebus aethiops*) have recently been described as a NHP model for NiV and HeV (Geisbert et al., 2010; Rockx et al., 2010). In contrast to the squirrel monkey, AGMs represent a consistent and highly pathogenic model to study the pathogenesis of henipaviruses. Intratracheal challenge of animals with up to $1.3 \times 10^6$ pfu of NiV or $4 \times 10^5$ TCID50 of HeV results in a severe systemic infection with nearly 100% mortality. AGMs challenged with NiV succumb to disease within 9–12 days post infection, while the average time to death in HeV-infected animals is 8.5 days. Clinical signs include decreased activity and depression, and rapidly progress to severe respiratory and neurological disease, and generalized vasculitis, reflecting very accurately the clinical signs of human illness. The developed severe acute respiratory distress syndrome was associated with copious amounts of sanguinous fluid and froth and viral RNA could be detected in nasal and throat swabs. X-ray autoradiographs show developing pneumonia or congestion by day 7 post infection. At later time points, development into diffuse interstitial infiltrates and pericardial effusion are evident. Severe lesions in the lungs (covering up to 90% of the tissue in HeV-infected animals) are observed and show signs of pulmonary edema. Animals showing neurologic disease had severe congestion with evidence of meningeal hemorrhage and edema. Particularly the brainstem and neurons are infected. Prominent development of endothelial syncytia and vasculitis is observed and viral antigen is present in endothelial

and smooth muscle cells. The lung appears to the most severely affected organ, but antigen can be detected in every organ system tested, such as the kidneys, heart, brain, gall bladder, stomach, sex organs and skeletal muscles. Virus can also be isolated from plasma and whole blood samples, suggesting that the virus can spread via a hematogenous route. Overall, the observed clinical signs observed in AGMs resemble those observed in human cases.

### 4.9. Other animals

The susceptibility of other animals, such as mice, rats, rabbits, chickens, and dogs, to experimental infection with HeV has been evaluated as well but these species do not develop any clinical signs of disease (Westbury et al., 1995; Wong et al., 2003). Infection of Swiss mice with NiV by the IN or IP route does not result in apparent disease; however susceptibility has never been confirmed (Wong et al., 2003). Interestingly, HeV and NiV can be lethal if administered intracranially into suckling Balb/c mice (Mungall et al., 2006). The time to death can be decreased by passage of suckling mouse brains (Crameri et al., 2002). Embryonated chicken eggs have been tested as a model for the study of vascular and neuronal tropism of NiV (Tanimura et al., 2006). Injections into the yolk sac of 6 day old embryos results in the development of severe pathology within the CNS and uniform mortality. Injections into the allantoic sac of embryos give varying results with a lower mortality rate. While there are no reports on experimental inoculation of dogs with NiV, there are reports indicating that dogs can suffer from severe disease and fatal disease (Field et al., 2001; Mills et al., 2009). However, there is evidence that NiV is not spreading by dog-to-dog transmission and that dogs are not acting as an amplifying reservoir for NiV in the absence of infected pigs (Mills et al., 2009).

## 5. Goals for future research

Considerable progress has been made in elucidating the molecular mechanisms by which henipaviruses enter host cells and the major structural transitions of the henipavirus F protein during the fusion cascade. However, only limited information is available on how receptor binding affects the interaction between the F and G proteins, resulting in membrane fusion. More structural studies on the henipavirus F and G complex in conjunction with the receptors ephrin-B2 or-B3 are crucial.

Despite the availability of hamster, ferret and African green monkey models that closely mimic the disease progression seen in NiV and HeV infection of humans, detailed studies of the pathogenesis of henipavirus infection have been hampered by a lack of reagents and other limitations of these models. The production of reagents specific for hamsters and ferrets as well as the development of a lethal mouse model would greatly enhance research on pathogenesis, vaccines and therapies. In terms of clinical studies, the molecular determinants of henipavirus virulence in humans are still unknown, and the effect of viral genetic variation in the clinical outcome of disease has not been studied in detail. Full genome sequences are only available for a small number of isolates, and analyses of genome stability, mutation rates and hotspots have therefore been limited. The availability of a reverse genetics system for NiV and HeV will be crucial for identification of molecular determinants of virulence. Finally, the role of viral genetic variation in the efficacy of vaccines and antiviral therapeutics will be an important question for the future design of medical countermeasures.

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