Available online at www.sciencedirect.com
ScienceDirect
Virus Research 132 (2008) 140–144
Virus Research
www.elsevier.com/locate/virusres

Henipavirus susceptibility to environmental variables

Rhys Fogarty [a], Kim Halpin [b], Alex D. Hyatt [b], Peter Daszak [c], Bruce A. Mungall [b,*]

[a] *Department of Ophthalmology, Flinders Medical Centre, Adelaide, Australia*
[b] *Australian Animal Health Laboratory, CSIRO Livestock Industries, Private Bag 24, Geelong 3220, Australia*
[c] *Consortium for Conservation Medicine, 460 West 34th Street, New York, NY, USA*

Received 10 September 2007; received in revised form 15 November 2007; accepted 16 November 2007
Available online 31 December 2007

Abstract

The routes of henipavirus transmission between hosts are poorly understood. The purpose of this study was to measure the persistence of henipaviruses under various environmental conditions and thereby gain an insight into likely mechanisms of transmission. Henipaviruses survived for more than 4 days at 22 °C in pH-neutral fruit bat urine but were sensitive to higher temperatures and pH changes. On mango flesh, survival time varied depending on temperature and fruit pH, ranging from 2 h to more than 2 days. Desiccation of viruses substantially reduced survival time to less than 2 h. The sensitivity of henipaviruses to pH, temperature and desiccation indicates a need for close contact between hosts for transmission to occur, although under ideal conditions henipaviruses can persist for extended periods facilitating vehicle-borne transmission.

Keywords: Paramyxovirus; Hendra virus; Nipah virus; Henipavirus; Virology; Dessication; Urine; Fruit; Temperature; Transmission

1. Introduction

Hendra virus (HeV) and Nipah virus (NiV), currently the sole members of the genus *Henipavirus*, family *Paramyxoviridae*, are recently emerged zoonotic viruses causing encephalitic and respiratory illness in humans and livestock. Fruit bats of the genus *Pteropus* are the probable wildlife reservoir for both viruses. HeV is restricted to Australia and of the seven outbreaks to date all have involved infection of horses from unidentified sources, with subsequent direct transmission to humans on three occasions (Hanna et al., 2006; Hooper et al., 1996; Murray et al., 1995a). NiV outbreaks have occurred in Malaysia, Singapore, India and Bangladesh following various chains of transmission including intermediate host species (Chua et al., 2000), human-to-human transmission (ICDDRB, 2004b), possible bat-to-human transmission (Hsu et al., 2004) and vehicle-borne transmission (Luby et al., 2006).

In most cases, the route of transmission from bats to subsequent hosts has not been identified. The most likely route of HeV transmission to horses is through the ingestion of grass or partially eaten fruit contaminated with bat urine, saliva or other fluids. The coincidence of HeV outbreaks with birthing seasons of Australian fruit bat species (Field et al., 2001) and the isolation of HeV from the uterine fluid and aborted foetus of a wild grey-headed fruit bat (*P. poliocephalus* (Halpin et al., 2000)) indicate that this may be a significant route of HeV infection for horses. We have recently demonstrated vertical transmission of NiV in experimentally infected cats further supporting this route as potentially an important natural route of transmission (Mungall et al., 2007).

Two NiV outbreaks show a reasonably clear chain of transmission from bats to humans: Malaysia in 1998–99 and Tangail, Bangladesh in 2005. During the Malaysian outbreak, 265 people were infected, primarily through contact with infected pigs (Parashar et al., 2000). The pigs were probably infected by *P. vampyrus* bats that fed on fruit trees adjacent to the pig farms, either through direct exposure to urine or via saliva-contaminated partially eaten fruit dropped into the pigsties (Chua et al., 2002). In the Tangail outbreak, NiV transmission to humans apparently occurred via date palm sap (ICDDRB, 2005; Luby et al., 2006). The sap may have been contaminated

Abbreviations: NiV, Nipah virus; HeV, Hendra virus; EMEM, Eagles modified essential medium; TCID, tissue culture infectious dose; BSL4, Biosafety Level 4.

* Corresponding author. Tel.: +61 3 52275431; fax: +61 3 52275555.
E-mail address: Bruce.Mungall@csiro.au (B.A. Mungall).

0168-1702/$ – see front matter.
doi:10.1016/j.virusres.2007.11.010

by urine, faeces or saliva from *P. giganteus* which are known to feed on the sap during harvesting.

Little is known about the transmission routes for the remaining NiV outbreaks, although preliminary analysis of the 2007 outbreaks (Kushtia, Bangladesh and Nadia, India) suggests likely human to human transmission (S. Luby, personal communication). The absence of identifiable intermediate hosts may be evidence of bat-to-human transmission. In at least three outbreaks human-to-human transmission is likely to have occurred (Chadha et al., 2006; Gurley et al., 2007; ICDDRB, 2003; ICDDRB, 2004a); however, the mode of transmission is uncertain.

A greater understanding of potential henipavirus modes of transmission may clarify the events that lead to viral spillover into new species and assist in controlling or averting outbreaks. Knowledge of virus survival in the environment is also critical in controlling human-to-human and nosocomial transmission.

The purpose of this study was to define the ability of henipaviruses to survive under a range of environmental conditions and thereby gain an understanding of the likely mechanisms of viral transmission between hosts. While we intended to mimic the natural conditions relevant to bat viral transmission as closely and accurately as possible, certain limitations on fruit selection due to seasonal availability and limitations inherent in the procurement of bat urine and saliva necessitated some generalizations.

2. Materials and methods

2.1. Viruses and titrations

HeV was isolated in Vero cells from the lung of a horse infected in the Brisbane outbreak in October 1994 (Murray et al., 1995b) and was passaged five times in Vero cells followed by triple plaque purification and a further five passages in Vero cells (Hyatt and Selleck, 1996). NiV was isolated in Vero cells from the brain of a human fatally infected in the 1998–99 Malaysian outbreak and was passaged three times in Vero cells then double plaque purified and passaged a further three times in Vero cells (Shiell et al., 2003).

For titrations, serial 10-fold dilutions of samples were made in EMEM and 25 μl transferred to five wells of a 96-well microtitre plate. Vero E6 cells in EMEM containing 10% fetal calf serum were added (2×10^4 cells/well). Plates were incubated at 37 °C for 5–7 days and wells displaying cytopathic effect were scored as infected. Virus titre was calculated using the Reed–Meunch method (Reed and Muench, 1938) and the limit of detection was 126 $TCID_{50}$/ml virus. All work with live virus was carried out under Biosafety Level 4 (BSL-4) conditions.

2.2. Effect of pH on virus survival

The pH of 0.01 M PBS was adjusted using NaOH or HCl to provide stock buffers in the range, pH 1–13. Viruses were diluted 1:100 in each pH buffer ($n = 3$) then incubated at 22 °C for 60 min and samples collected for titration in Vero cells.

2.3. Effect of desiccation on virus survival

Viruses were desiccated by spreading 10 μl onto a polystyrene dish and air drying at 22 °C for 15 min ($n = 3$). Dishes were stored at 22 or 37 °C and virus recovered at intervals by resuspension in 250 μl PBS followed by titration in Vero cells.

2.4. Virus survival in urine

HeV was diluted in a pooled urine sample from an Australian species (*P. alecto*) with a pH of 7. NiV was diluted in a pooled urine sample from a Malaysian species (*P. vampyrus*). The *P. vampyrus* urine was used either at its natural pH of 2 or following adjustment to pH 7 with NaOH to compensate for pH effects on virus survival. Viruses ($n = 3$) were incubated at 22 or 37 °C for up to 96 h and samples collected at intervals for titration in Vero cells. Both *P. alecto* and *P. vampyrus* had been extensively studied in recent years (Halpin and Middleton, unpublished data) enabling the procurement of sufficient quantities of urine for these experiments but unfortunately, urine from additional species was not available at the time of testing.

2.5. Virus survival in fruit juice and flesh

Cubes of mango flesh (1 cm^3) were placed in tubes, 10 μl of virus ($n = 3$) pipetted onto the surface and samples stored at 22 or 37 °C for up to 48 h. Virus was recovered by vortexing the sample in 0.5 ml PBS and the supernatant was titrated in Vero cells.

Virus survival was measured in juices from mango, lychee and pawpaw. As mentioned previously, the seasonal availability of fruits in Australia relevant to henipavirus infections prevented a wider range of fruits specific to the tropical locations of Nipah virus outbreaks from being tested in this study. Juices were extracted and passed through a 0.2 μm filter. Viruses were diluted in juice ($n = 3$) and incubated at 22 or 37 °C for up to 96 h. Samples were collected at intervals for titration in Vero cells.

2.6. Calculation of half-lives

Virus survival data was collated and half-life was calculated using non-linear regression analysis based a one phase exponential decay model using GraphPad Prism software (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Effect of pH on virus survival

Both HeV and NiV exhibit an extremely broad tolerance to extremes of pH with viable virus recovered after a 60 min incubation in solutions ranging from pH 3 to 11 for NiV and pH 4 to 11 for HeV (Fig. 1).

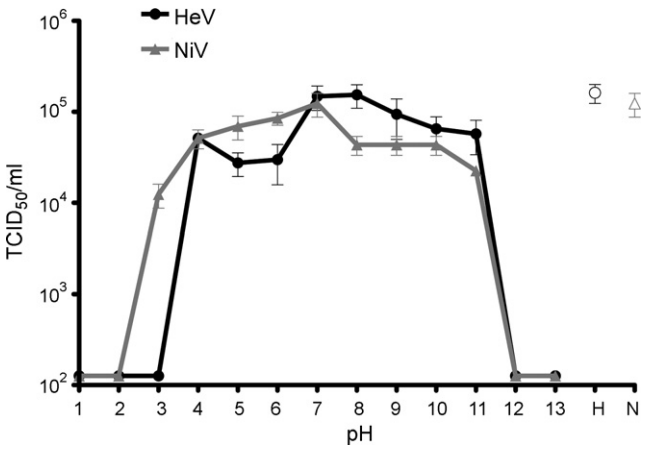

Fig. 1. Henipavirus survival for 60 min at 22 °C in PBS at various pH. H/N—open symbols represent control virus (Hendra and Nipah virus, respectively) samples kept in growth medium (EMEM) for an equivalent period. Data are presented as mean ± S.E.

3.2. Effect of desiccation on virus survival

Henipaviruses were rapidly inactivated by desiccation at both 22 and 37 °C. Both viruses survived for less than 15 min at 37 °C (Fig. 2) while HeV decreased by more than 3 logs within 30 min (half-life of 1.2 min) and NiV decreased by more than 2 logs within 60 min (half-life of 1.45 min) at 22 °C. Limitations inherent in virus recovery under BSL4 biocontainment provided logistical restrictions to a much broader evaluation of viral dessication on other surfaces. Non-porous polystyrene media was chosen to enable maximum virus recovery, and thus represents a "best case" scenario for recovery from viral dessication.

3.3. Virus survival in urine

Experimental infections have shown that urine is a significant route of henipavirus excretion from fruit bats (Middleton et al., 2007) (Halpin et al., unpublished). Therefore, the persistence of henipaviruses in fruit bat urine was examined. Recent experimental studies with *P. alecto* and *P. vampyrus* enabled sufficient urine to be collected for these studies; however, urine from additional species was unavailable at the time of testing. HeV incubated in *P. alecto* urine (pH ~ 7) survived for more than 4 days at 22 °C with a half-life of 19 h, but at 37 °C was virtually inactivated in less than 1 day with the half-life reduced to 3 h (Table 1, Fig. 3).

Table 1
Henipavirus survival in fruit bat urine

Virus	Fruit bat species	Urine pH	Half-life at 22 °C (h)[a]	Half-life at 37 °C (h)[a]
HeV	*P. alecto*	7	18.8	3
NiV	*P. vampyrus*	7	17.8	1.8
NiV	*P. vampyrus*	2	<0.5	<0.5

[a] Values are the mean of three separate experiments.

In contrast, NiV incubated in *P. vampyrus* urine (pH 2) was inactivated in less than 30 min at both 22 and 37 °C (Table 1). However, in *P. vampyrus* urine with the pH adjusted to 7, the half-life of NiV was 18 h at 22 °C and 2 h at 37 °C.

3.4. Virus survival in fruit juice and flesh

Viral transmission from bats to intermediate hosts via partially eaten fruit has been posited for both NiV (Chua et al., 2002) and HeV (Field et al., 2001) and NiV has been isolated from a swab of partially eaten fruit in the wild (Chua et al., 2002). Therefore, the survival of henipaviruses on fruit flesh and in fruit juice was examined. Survival time on fruit pulp and in fruit juices varied depending on the type and pH of the fruit. As shown in Table 2, virus survival on mango flesh decreased with increasing acidity. At pH 5, half-lives for HeV and NiV were 22 and 30 h, respectively, compared to 0.3 and 1.4 h at pH 3.

Viruses incubated in juice from lychees showed greater persistence than with either pawpaw or mango juice with two- to three-fold longer half-lives and survival for more than 3 days (Fig. 4). While these results do provide a general indication of

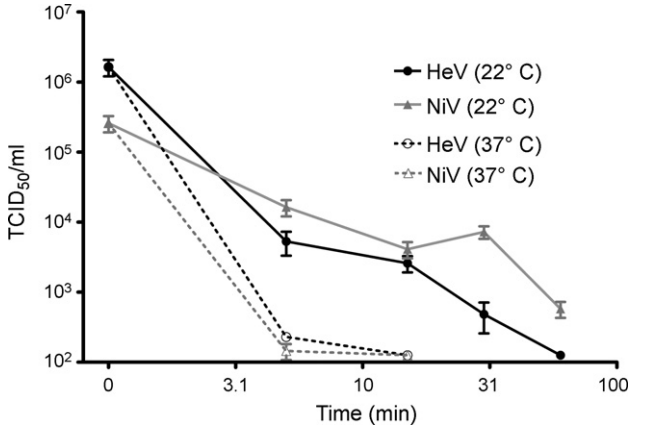

Fig. 2. Henipavirus survival after desiccation. Viruses were air dried for 15 min at 22 °C (time 0) then resuspended in growth medium and titrated at 5, 15, 30 and 60 min. Data are combined from three separate experiments and are presented as mean ± S.E.

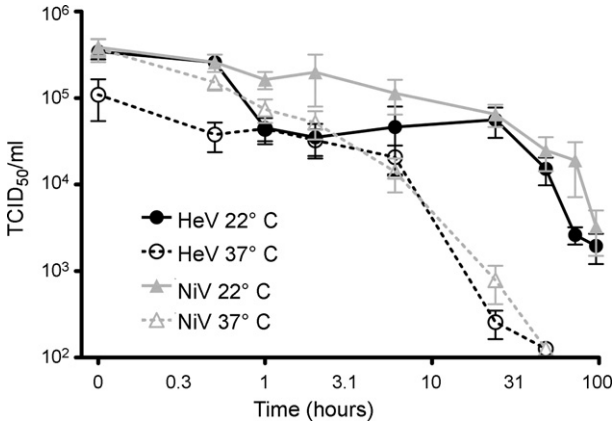

Fig. 3. Henipavirus survival in fruit bat urine. Viruses were incubated in *P. alecto* urine (pH ~ 7) at 22 or 37 °C. Data are combined from three separate experiments and are presented as mean ± S.E.

Table 2
Henipavirus survival on mango flesh

Mango pH[a]	HeV half-life (h)		NiV half-life (h)	
	22 °C	37 °C	22 °C	37 °C
3.5	0.3	0.4	1.4	0.2
4.5	3.5	0.5	5.5	0.5
5	22.4	5.9	30.3	2.2

[a] pH of mango flesh was determined at the time of virus inoculation only.

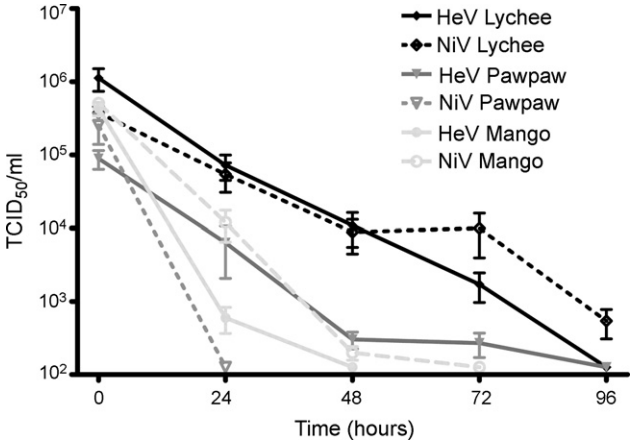

Fig. 4. Henipavirus survival in fruit juice at 22 °C. The pH of lychee juice was 4, pawpaw juice pH 6 and mango juice pH 5. Data are combined from three separate experiments and are presented as mean ± S.E.

virus survival in these naturally occurring fruits and juices, the lack of available bat saliva for experimental evaluations ensures some degree of artificiality with respect to interpretation of results.

4. Discussion

This study demonstrates that survival of henipaviruses in the environment is highly sensitive to temperature and desiccation. Under most conditions survival time was brief, with half-lives limited to a few hours, indicating that transmission to a new host requires close contact with an infected animal or exposure to contaminated material shortly after excretion. However, under optimal conditions henipaviruses can persist for a number of days and under these circumstances, vehicle-borne transmission may be possible.

This extended survival time may have contributed to the NiV outbreak in Tangail, Bangladesh in 2005 where there was a strong epidemiological link between human NiV cases and drinking raw date palm sap (Luby et al., 2006). Fruit bats are known to drink from the pots in which the sap is collected and fruit bat excrement in or on the pots is sometimes observed. The conditions under which date palm sap is collected and consumed provide an opportunity for NiV transmission. The sap is collected at night during the coldest months of the year when the average minimum temperature is 13–15 °C. The pH of palm sap is near neutral (Aidoo et al., 2006) and it is consumed within a few hours after sunrise before it ferments. While date palm sap was not available for testing here, under these conditions, the current study indicates that there is likely to be only minimal loss of viral titre before consumption.

The sensitivity of henipaviruses to environmental conditions may contribute to their observed low transmissibility under most circumstances. Attempts to recreate transmission events in a laboratory setting have been largely unsuccessful. A study by Westbury et al. (1996) found HeV transmission to one of two cats in contact with HeV infected cats and none of two cats housed contiguously with infected cats indicating low transmissibility. Similarly, Williamson et al. (1998) demonstrated one incidence of cat-to-horse transmission but were unable to demonstrate transmission from bats to bats (using *P. poliocephalus*), bats to horses, horses to horses or horses to cats. They also noted an absence of live virus in urine collected from the floor of horse stalls despite high titres in bladder urine from the horses, indicating viral inactivation shortly after excretion.

The sensitivity of henipaviruses to the pH of fruit bat urine and fruit flesh may be relevant to the timing of henipavirus outbreaks. Changes in the diet of fruit bat populations due to season or bat migration may influence urine pH and therefore henipavirus persistence after excretion through this route. The marked difference in urine pH between *P. alecto* and *P. vampyrus* despite being fed similar diets may indicate that certain species of fruit bat are more suited to henipavirus dispersal. Similarly, variations in fruit pH may reflect differences in their suitability for transmitting virus. We were unable to evaluate the effect of bat saliva on fruit pH and subsequent virus survival in this study such that any conclusions regarding oral transmission of virus by bats still remain to be tested scientifically.

Human-to-human NiV transmission has been observed or suspected in six outbreaks (2, 6, 12, 13, 14, S. Luby, personal communication). It is usually associated with close contact with a NiV patient who has respiratory symptoms. Except for the outbreak in Siliguri, India in 2001 (Chadha et al., 2006) nosocomial infections have not occurred despite an absence of barrier nursing methods and exposure to body fluids of NiV patients (Mounts et al., 2001). However, the outbreak in Siliguri demonstrates that nosocomial transmission may be a major source of infection and as demonstrated in the current study, the potential for vehicle-borne transmission exists. Therefore barrier nursing practices should be employed when dealing with HeV and NiV patients.

Acknowledgements

Financial support: RF, KH, ADH and PD are supported in part by a National Institutes of Health/National Science Foundation "Ecology of Infectious Diseases" (R01-TW05869) award from the John E. Fogarty International Center and by core funding to the Consortium for Conservation Medicine from the V. Kann Rasmussen Foundation.

References

Aidoo, K.E., Nout, M.J., Sarkar, P.K., 2006. Occurrence and function of yeasts in Asian indigenous fermented foods. FEMS Yeast Res. 6, 30–39.

Chadha, M.S., Comer, J.A., Lowe, L., Rota, P.A., Rollin, P.E., Bellini, W.J., Ksiazek, T.G., Mishra, A., 2006. Nipah virus-associated encephalitis outbreak, Siliguri, India. Emerg. Infect. Dis. 12, 235–240.

Chua, K.B., Bellini, W.J., Rota, P.A., Harcourt, B.H., Tamin, A., Lam Sai Kit, K., Ksiazek, T.G., Rollin, P.E., Zaki, S.R., Shieh, W.J., Goldsmith, C.S., Gubler, D.J., Roehrig, J.T., Eaton, B.T., Gould, A.R., Olson, J., Field, H.E., Daniels, P.W., Ling, A.E., Peters, C.J., Anderson, L.J., Mahy, B.W.J., 2000. Nipah virus: a recently emergent deadly paramyxovirus. Science 288, 1432–1435.

Chua, K.B., Lek, K.C., Hooi, P.S., Wee, K.F., Khong, J.H., Chua, B.H., Chan, Y.P., Lim, M.E., Lam Sai Kit, K., 2002. Isolation of Nipah virus from Malaysian Island flying-foxes. Microbes Infect. 4, 145–151.

Field, H.E., Young, P.L., Yob, J.M., Mills, J.N., Hall, L.S., MacKenzie, J.S., 2001. The natural history of Hendra and Nipah viruses. Microbes Infect. 3, 307–314.

Gurley, E., Montgomery, J.M., Hossain, M.J., Bell, M., Azad, A.K., Islam, M.R., Molla, M.A.R., Carroll, D.S., Ksiazek, T., Rota, P.A., Lowe, L., Comer, J.A., Rollin, P., Czub, M., Grolla, A., Feldmann, H., Luby, S.P., Woodward, J.L., Breiman, R. F., 2007. Person-to-person transmission of Nipah virus in a Bangladeshi community. Emerg. Infect. Dis. 13, 1031–1037.

Halpin, K., Young, P.L., Field, H.E., Mackenzie, J.S., 2000. Isolation of Hendra virus from pteropid bats: a natural reservoir of Hendra virus. J. Gen. Virol. 81, 1927–1932.

Hanna, J.N., McBride, W.J., Brookes, D.L., Shield, J., Taylor, C.T., Smith, I.L., Craig, S.B., Smith, G.A., 2006. Hendra virus infection in a veterinarian. Med. J. Aust. 185, 562–564.

Hooper, P.T., Gould, A.R., Russell, G.M., Kattenbelt, J.A., Mitchell, G., 1996. The retrospective diagnosis of a second outbreak of equine morbillivirus infection. Aust. Vet. J. 74, 244–245.

Hsu, V.P., Hossain, M.J., Parashar, U.D., Ali, M.M., Ksiazek, T.G., Kuzmin, I., Niezgoda, M., Rupprecht, C., Bresee, J., Breiman, R.F., 2004. Nipah virus encephalitis reemergence, Bangladesh. Emerg. Infect. Dis. 10, 2082–2087.

Hyatt, A.D., Selleck, P.W., 1996. Ultrastructure of Equine Morbillivirus. Virus Res. 43, 1–15.

ICDDRB, 2003. Outbreaks of encephalitis due to Nipah/Hendra-like viruses, Western Bangladesh. Health Sci. Bull. 1, 1–6.

ICDDRB, 2004a. Person-to-person transmission of Nipah virus during outbreak in Faridpur District. Health Sci. Bull. 2, 5–9.

ICDDRB, 2004b. Nipah encephalitis outbreak over wide area of Western Bangladesh. Health Sci. Bull. 2, 7–11.

ICDDRB, 2005. Nipah virus outbreak from date palm juice. Health Sci. Bull. 3, 1–5.

Luby, S.P., Rahman, M., Hossain, M.J., Blum, L.S., Husain, M.M., Gurley, E., Khan, R., Ahmed, B.-N., Rahman, S., Nahar, N., Kenah, E., Comer, J.A., Ksiazek, T.G., 2006. Foodborne transmission of Nipah virus, Bangladesh. Emerg. Infect. Dis. 12, 1888–1894.

Middleton, D.J., Morrissy, C.J., van der Heide, B.M., Russell, G.M., Braun, M.A., Westbury, H.A., Halpin, K., Daniels, P.W., 2007. Experimental Nipah Virus infection in Pteropid Bats (Pteropus poliocephalus). J. Comp. Pathol. 136, 266–272.

Mounts, A.W., Kaur, H., Parashar, U.D., Ksiazek, T.G., Cannon, D., Arokiasamy, J.T., Anderson, L.J., Lye, M.S., 2001. A cohort study of health care workers to assess nosocomial transmissibility of Nipah virus, Malaysia, 1999. J. Infect. Dis. 183, 810–813.

Mungall, B.A., Middleton, D.J., Crameri, G.S., Halpin, K., Bingham, J., Eaton, B.T., Broder, C.C., 2007. Vertical transmission and fetal replication of Nipah virus in an experimentally infected cat. J. Infect. Dis. 196 (6), 812–816.

Murray, K., Rogers, R., Selvey, L., Selleck, P., Hyatt, A., Gould, A., Gleeson, L., Hooper, P., Westbury, H., 1995a. A novel morbillivirus pneumonia of horses and its transmission to humans. Emerg. Infect. Dis. 1, 31–33.

Murray, K., Selleck, P., Hooper, P., Hyatt, A., Gould, A., Gleeson, L., Westbury, H., Hiley, L., Selvey, L., Rodwell, B., et al., 1995b. A morbillivirus that caused fatal disease in horses and humans. Science 268, 94–97.

Parashar, U.D., Sunn, L.M., Ong, F., Mounts, A.W., Arif, M.T., Ksiazek, T.G., Kamaluddin, M.A., Mustafa, A.N., Kaur, H., Ding, L.M., Othman, G., Radzi, H.M., Kitsutani, P.T., Stockton, P.C., Arokiasamy, J.T., Gary Jr., H.E., Anderson, L.J., 2000. Case-control study of risk factors for human infection with a new zoonotic paramyxovirus, Nipah virus, during a 1998–1999 outbreak of severe encephalitis in Malaysia. J. Infect. Dis. 181, 1755–1759.

Reed, L.J., Muench, H., 1938. Simple method for estimating fifty percent end points. Am. J. Hyg. 27, 493–497.

Shiell, B.J., Gardner, D.R., Crameri, G.S., Eaton, B.T., Michalski, W.P., 2003. Sites of phosphorylation of P and V proteins from Hendra and Nipah viruses: newly emerged members of Paramyxoviridae. Virus Res. 92, 55–65.

Westbury, H.A., Hooper, P.T., Brouwer, S.L., Selleck, P.W., 1996. Susceptibility of cats to equine morbillivirus. Aust. Vet. J. 74, 132–134.

Williamson, M.M., Hooper, P.T., Selleck, P.W., Gleeson, L.J., Daniels, P.W., Westbury, H.A., Murray, P.K., 1998. Transmission studies of Hendra virus (equine morbillivirus) in fruit bats, horses and cats. Aust. Vet. J. 76, 813–818.