

Brief communication (Original)

Genetic characterization of Nipah virus from Thai fruit bats (*Pteropus lylei*)

Supaporn Wacharapluesadee^a, Thongchai Ngamprasertwong^b, Thongchai Kaewpom^a, Peerapat Kattong^a, Apaporn Rodpan^a, Sawai Wanghongsa^c, Thiravat Hemachudha^a

^aWHO Collaborating Centre for Research and Training on Viral Zoonoses, Faculty of Medicine, Chulalongkorn University and King Chulalongkorn Memorial Hospital, Department of Medicine, Chulalongkorn University, Bangkok 10330, Thailand, ^bDepartment of Biology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand, ^cMinistry of Natural Resources and Environment, Bangkok 10400, Thailand

Background: Nipah virus (NiV) is an emerging zoonosis of paramyxovirus that causes febrile encephalitis with high death rates in humans. It has been successfully isolated and genetically characterized in three species of fruit bats including *Pteropus hypomelanus*, *P. vampyrus* in Malaysia, and *P. lylei* (Lyle's fruit bat) in Cambodia. Little is still known about NiV genetic information in Lyle's fruit bats in Thailand.

Objective: Partial NiV sequences obtained from 61 isolates from nine bat roosting sites, in Central Thailand during 2002–2008 were genetically characterized to determine the diversity of NiV found in Thai bats.

Methods: All samples were collected from Lyle's fruit bats, except for two from insectivorous bats. NiV RNA was extracted directly from the bat urine, saliva or serum specimens, and a fragment of the nucleoprotein (N) gene was amplified by hemi-nested RT-PCR and 357 base pairs were sequenced for phylogenetic analysis.

Results: The NiV isolates from bats found in Thailand form a monophyletic group with those from Malaysia, Bangladesh and Cambodia. It is divided into two closely related lineages: Clade A and B. Clade A represents the majority of those found in Thailand (52/61).

Conclusion: The genetic divergence of NiV isolates obtained from Lyle's fruit bats in a small geographic region might indicate that the ancestor of this group of virus has circulated in *Pteropus* fruit bats for a long time. More viral surveillance studies in *Pteropus* bat populations in this region might help to clarify the genetic distribution of bat-NiV in *Pteropus* and allow risk assessment in NiV outbreaks from fruit bats.

Keywords: Bats, Nipah virus, nucleoprotein gene, phylogenetic

Nipah virus (NiV) is an emerging (or long circulating, but only now recognized) paramyxovirus that causes human encephalitis. It had a reported fatality rate greater than 40% during the original outbreak in Malaysia and Singapore in 1998–1999 [1-4] and 75%–100% in a Bangladeshi outbreak during 2001–2012 [5, 6]. NiV belongs to a new genus, *Henipavirus*, of the Paramyxoviridae family. The natural reservoir for NiV is thought to be flying fox species, genus *Pteropus* [7]. NiV has been successfully isolated and the whole genome of

the virus was determined from 3 species of fruit bats, *P. hypomelanus* and *P. vampyrus* in Malaysia and *P. lylei* in Cambodia [7-9]. Although NiV has not caused any human outbreaks in Thailand, 3 species of *Pteropus* bats have been reported to be infected by NiV. They are *P. hypomelanus*, *P. vampyrus* and *P. lylei* [10]. Previously, partial NiV sequences obtained from *P. lylei* in Thailand [10-12] suggested that both Malaysian and Bangladesh like-NiV strains circulate in this bat species. Little is still known about NiV genetic information in bats. We report data from our ongoing surveillance of bats for NiV during 2002–2008 in Thailand, focusing on *P. lylei*.

Correspondence to: Supaporn Wacharapluesadee, WHO Collaborating Centre for Research and Training on Viral Zoonoses, Faculty of Medicine, Chulalongkorn University and King Chulalongkorn Memorial Hospital, Bangkok 10330, Thailand. E-mail: spwa@hotmail.com

Materials and methods

Our teams of faculty members and graduate students from Chulalongkorn University, Queen

Savabha Memorial Institute, and Department of National Parks, Wildlife and Plant Conservation have been surveying Thai bats for the presence of NiV since 2002. The principal site has been Wat Luang in the province of Chonburi consisting of a traditional village, a Buddhist monastery and a large area of rainforest trees, including padauk and tamarind trees with an estimated 135 roosting trees and 12,000 *P. lylei* (Lyle's fruit bats). Villagers and monks have been living in close proximity and no outbreaks of neurological disease, likely to have represented NiV infection, has been reported by the inhabitants or the local health center. The research team has developed close relationships with villagers and monks, which includes provision of some basic health services and collection of data about the bats. Active ongoing work includes collecting blood and urine samples from bats and village residents. Blood samples were collected from bats captured by netting and venipunctures from wing veins with great care and without harming the

animals, which are protected by law. Pooled urine samples were collected using the plastic sheet method under bat's roost sites as shown on **Figure 1C** [13]. We also collected samples from different bat species and locations as shown on **Table 1**. Nine bat roosting sites were included in this study, in the provinces of Chachoengsao, Chonburi, Prachinburi, Saraburi, Singburi, Angthong, Ayuttaya, Srakaew, and Bangkok, of Central Thailand during 2002–2008 (**Figure 2**).

NiV RNA was extracted directly from bat urine, saliva or serum specimens by Boom's technique using a commercial available extraction kit (bioMérieux, France). Fragments of nucleoprotein (N) gene were amplified by hemi-nested RT-PCR and sequenced as previously described [11]. The partial sequences of N gene containing 357 base pairs (bp) at position 1197–1553 (GenBank accession no. NC_002728) were analyzed by a neighbor-joining distance method and bootstrap values were determined using 1000 replicates.

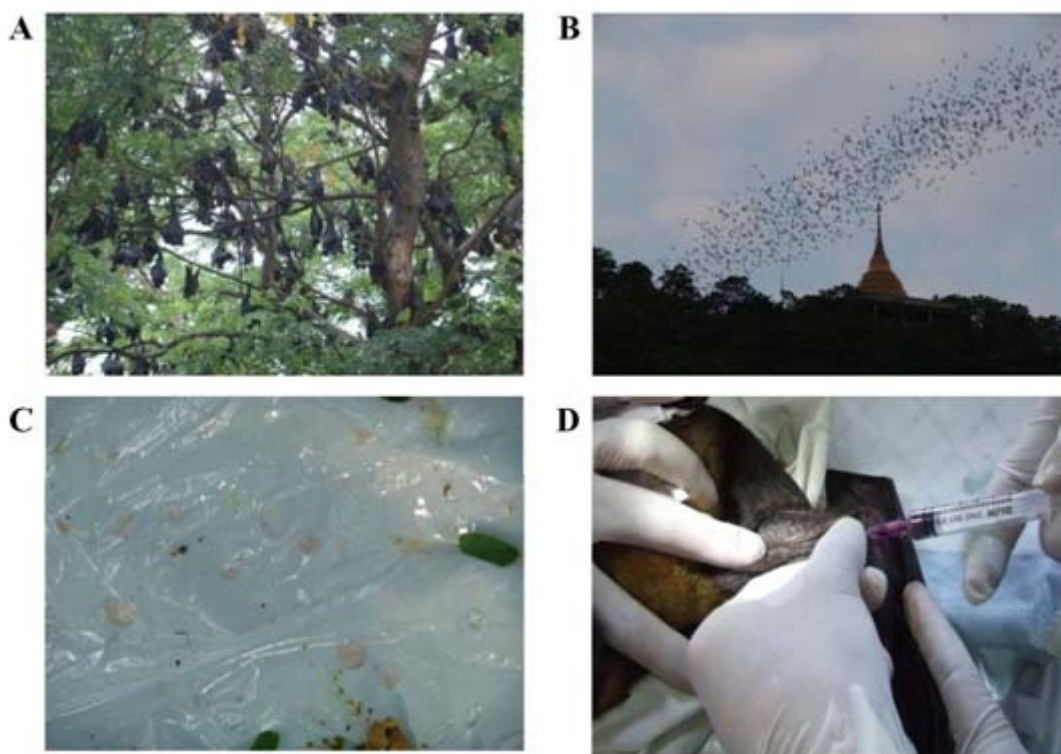


Figure 1. **A:** Fruit bats (*Pteropus lylei*) on roosted tree at Wat Luang, Chonburi province, **B:** Insectivorous bats flying out of cave at Khao Chong Phran, Ratchaburi province, **C:** Bat urine drops on plastic sheet, **D:** Drawing blood from bat's wing vein

Table 1. GenBank accession number and information of collected samples during 2002–2008

No.	Collection date	Location	Specimen type	Sampling method	GenBank accession no.	Group	Species	Reference
1184	Feb 18–20, 2004	Wat Luang, Chonburi	serum		FJ648075	B1	<i>P. lylei</i>	<i>This study</i>
1248	Feb 18–20, 2004	Wat Luang, Chonburi	serum		FJ648076	B1	<i>P. lylei</i>	<i>This study</i>
20	Mar 12–16, 2002	Kram Island, Chonburi	saliva	pool	DQ061851	M6	<i>H. larvatus</i>	<i>This study</i>
126	Feb 18–20, 2004	Wat Luang, Chonburi	saliva	pool	DQ061852	B1	<i>P. lylei</i>	<i>This study</i>
61	Jan 7–9, 2003	Wat Pho, Chachoengsoa	urine	pool	DQ061853	B7	<i>P. lylei</i>	<i>This study</i>
101	Sep 3–5, 2003	Nongjok, Bangkok	urine	pool	DQ061854	B1	<i>P. lylei</i>	<i>This study</i>
126	Feb 18–20, 2004	Wat Luang, Chonburi	urine	pool	DQ061855	B1	<i>P. lylei</i>	<i>This study</i>
132	Feb 18–20, 2004	Wat Luang, Chonburi	urine	pool	DQ061856	B1	<i>P. lylei</i>	<i>This study</i>
133	Feb 18–20, 2004	Wat Luang, Chonburi	urine	pool	DQ061857	B1	<i>P. lylei</i>	<i>This study</i>
134	Feb 18–20, 2004	Wat Luang, Chonburi	urine	pool	DQ061858	B1	<i>P. lylei</i>	<i>This study</i>
3	Apr 9, 2005	Wat Luang, Chonburi	urine	plastic	EF070182	M	<i>P. lylei</i>	11
8	May 10, 2005	Wat Luang, Chonburi	urine	plastic	EF070183	B1	<i>P. lylei</i>	11
14	May 10, 2005	Wat Luang, Chonburi	urine	plastic	EF070184	B1	<i>P. lylei</i>	11
24	May 11, 2005	Wat Luang, Chonburi	urine	plastic	EF070185	B1	<i>P. lylei</i>	11
27	May 11, 2005	Wat Luang, Chonburi	urine	plastic	EF070186	B1	<i>P. lylei</i>	11
35	May 11, 2005	Wat Luang, Chonburi	urine	plastic	EF070187	B1	<i>P. lylei</i>	11
47	May 11, 2005	Wat Luang, Chonburi	urine	plastic	EF070188	B1	<i>P. lylei</i>	11
48	May 11, 2005	Wat Luang, Chonburi	urine	plastic	EF070189	B1	<i>P. lylei</i>	11
53	May 11, 2005	Wat Luang, Chonburi	urine	plastic	EF070190	B1	<i>P. lylei</i>	11
72	Jun 12, 2005	Wat Luang, Chonburi	urine	plastic	EU603724	B1	<i>P. lylei</i>	12
414	Dec 14, 2005	Wat Pho, Chachoengsoa	urine	plastic	EU603725	M	<i>P. lylei</i>	12
561	Jan 13, 2006	Wat Luang, Chonburi	urine	plastic	EU603726	M1	<i>P. lylei</i>	12
823	Mar 10, 2006	Wat Pho, Chachoengsoa	urine	plastic	EU603728	M5	<i>P. lylei</i>	12
840	Apr 6, 2006	Wat Luang, Chonburi	urine	plastic	EU603729	B2	<i>P. lylei</i>	12
841	Apr 6, 2006	Wat Luang, Chonburi	urine	plastic	EU603730	B2	<i>P. lylei</i>	12
842	Apr 6, 2006	Wat Luang, Chonburi	urine	plastic	EU603731	B2	<i>P. lylei</i>	12
986	May 5, 2006	Ayuttaya	urine	plastic	EU603732	B1	<i>P. lylei</i>	12
1027	May 11, 2006	Wat Luang, Chonburi	urine	plastic	EU603727	B2	<i>P. lylei</i>	12
1045	May 11, 2006	Wat Luang, Chonburi	urine	plastic	EU603733	M2	<i>P. lylei</i>	12
1056	May 11, 2006	Wat Luang, Chonburi	urine	plastic	EU603734	B2	<i>P. lylei</i>	12
1061	May 11, 2006	Wat Luang, Chonburi	urine	plastic	EU620498	M3	<i>P. lylei</i>	12
1149	May 12, 2006	Angthong	urine	plastic	EU603735	B3	<i>P. lylei</i>	12
1190	May 12, 2006	Singburi	urine	plastic	EU603736	B1	<i>P. lylei</i>	12
1191	May 12, 2006	Singburi	urine	plastic	EU603737	B1	<i>P. lylei</i>	12
1192	May 12, 2006	Singburi	urine	plastic	EU603738	B1	<i>P. lylei</i>	12
1235	May 13, 2006	Saraburi	urine	plastic	EU603739	B1	<i>P. lylei</i>	12
1242	May 13, 2006	Saraburi	urine	plastic	EU603740	B4	<i>P. lylei</i>	12
1243	May 13, 2006	Saraburi	urine	plastic	EU603741	B4	<i>P. lylei</i>	12
1244	May 13, 2006	Saraburi	urine	plastic	EU603742	B4	<i>P. lylei</i>	12
1245	May 13, 2006	Saraburi	urine	plastic	EU603743	B1	<i>P. lylei</i>	12
1246	May 13, 2006	Saraburi	urine	plastic	EU603744	B1	<i>P. lylei</i>	12
1247	May 13, 2006	Saraburi	urine	plastic	EU603745	B1	<i>P. lylei</i>	12
1285	May 14, 2006	Prachinburi	urine	plastic	EU603746	B1	<i>P. lylei</i>	12
1286	May 14, 2006	Prachinburi	urine	plastic	EU603747	B1	<i>P. lylei</i>	12
1287	May 14, 2006	Prachinburi	urine	plastic	EU603748	B1	<i>P. lylei</i>	12
1354	Jun 9, 2006	Wat Luang, Chonburi	urine	plastic	EU603749	M4	<i>P. lylei</i>	12
1431	Jun 9, 2006	Wat Pho, Chachoengsoa	urine	plastic	EU603750	B5	<i>P. lylei</i>	12
1543	Jul 8, 2006	Chakan, Sakaew	urine	plastic	EU603752	M2	<i>Taphozous</i>	<i>This study</i>
1907	Oct 14, 2006	Wat Pho, Chachoengsoa	urine	plastic	EU603753	B1	<i>P. lylei</i>	12
2597	May 6, 2007	Wat Luang, Chonburi	urine	plastic	EU603754	B1	<i>P. lylei</i>	12
2611	May 6, 2007	Wat Luang, Chonburi	urine	plastic	EU603755	B4	<i>P. lylei</i>	12
2647	May 6, 2007	Wat Pho, Chachoengsoa	urine	plastic	EU603756	B4	<i>P. lylei</i>	12
2648	May 6, 2007	Wat Pho, Chachoengsoa	urine	plastic	EU603757	B4	<i>P. lylei</i>	12
2705	May 25, 2007	Wat Luang, Chonburi	urine	plastic	EU603751	B4	<i>P. lylei</i>	12
2707	May 25, 2007	Wat Luang, Chonburi	urine	plastic	EU603758	B4	<i>P. lylei</i>	12
2876	Apr 3, 2008	Wat Luang, Chonburi	urine	plastic	FJ648077	B1	<i>P. lylei</i>	<i>This study</i>
2878	Apr 3, 2008	Wat Luang, Chonburi	urine	plastic	FJ648078	B6	<i>P. lylei</i>	<i>This study</i>
2903	Apr 3, 2008	Wat Luang, Chonburi	urine	plastic	FJ648079	B4	<i>P. lylei</i>	<i>This study</i>
2905	Apr 3, 2008	Wat Luang, Chonburi	urine	plastic	FJ648080	B4	<i>P. lylei</i>	<i>This study</i>
2909	May 1, 2008	Wat Luang, Chonburi	urine	plastic	FJ648081	B6	<i>P. lylei</i>	<i>This study</i>
2918	May 1, 2008	Wat Luang, Chonburi	urine	plastic	FJ648082	B6	<i>P. lylei</i>	<i>This study</i>

Plastic = samples were collected on plastic sheets, pool= samples were pooled (10) before testing



Figure 2. Location in Thailand where bat specimens were collected in this study CC = Chachoengsao, CB = Chonburi, PC = Prachinburi, SR = Saraburi, SB = Singburi, AT = Angthong, AY = Ayuttaya, SK = Srakaew, BK = Bangkok

Results

Phylogenetic analysis was conducted from 61 NiV isolates. **Table 1** shows data of all isolates including bat's location, species and source of NiV RNA. All samples except for two were collected from fruit bats, *P. lylei*. One was collected from pooled saliva of insectivorous bats, *Hiposiderous larvatus* from Kram island, Chonburi province (GenBank accession no. DQ061851), and the other from the pooled urine of *Taphazus* insectivorous bats from one cave located in Srakaew province (GenBank

accession no. EU603752). Two of the 59 Lyle fruit bat samples were isolated from serum (GenBank accession nos. FJ648075 - FJ648076), one was from the pooled saliva from ten bats (GenBank accession no. DQ061852), six from pooled bat's urine (GenBank accession nos. DQ061853-8) and 50 from the pooled urine samples which were collected by the plastic sheet method (GenBank accession nos. EF070182-070190, EU603724-603751, EU603753-603758 and FJ648077 - FJ648082).

Phylogenetic analysis was based on the 357 bp partial sequences deduced from the current ($n = 20$) and all previously published NiV isolates available in the GenBank database. They totaled 61 from Thailand and seven from other Asian countries and led to the construction of a phylogenetic tree (**Figure 3**). The sequences from 61 NiV isolates were grouped into group B1 to B7 and M1 to M7 and all sequences within one group were identical (**Table 1**). The NiV isolates from bats found in Thailand form a monophyletic group with those from Malaysia, Bangladesh and Cambodia. From the topology of the phylogenetic tree, NiV is divided into two closely related lineages, clade A and B. Both the topology of the phylogenetic tree and the bootstrap values indicate the genetic difference between NiV clade A and B. Both NiV clades were persistent in Lyle's fruit bat populations of Thailand throughout the whole study period, although clade A represents the majority of those found in Thailand (52/61).

Discussion

Bats have been reported to be the primary reservoirs of NiV [14]. **Figure 3** shows the phylogenetic analysis of 357 bp of N gene of NiV found in Thai bats. Viruses of clade A were all detected exclusively in *P. lylei* fruit bats, while those of clade B were detected from at least three different species of fruit bats, *P. lylei*, *P. hypomelanus*, *P. vampyrus* and two insectivorous bats including *H. larvatus* and *Taphozous* genus (**Figure 3**). The sequence of Thai NiV isolates in clade A share 98%–99% nucleotide sequence homology to the NiV isolate from Bangladesh patient (GenBank accession no. AY988601), whilst those in clade B share 99%–100% nucleotide homology to the NiV isolate from *P. hypomelanus* in Malaysia (GenBank accession no. AF376747). The nucleotide sequence identity between clade A and B was slightly less than that within clade A or B. For example, NiV group B2 (EU603727) versus group M3 (EU620498) shared 93% identity;

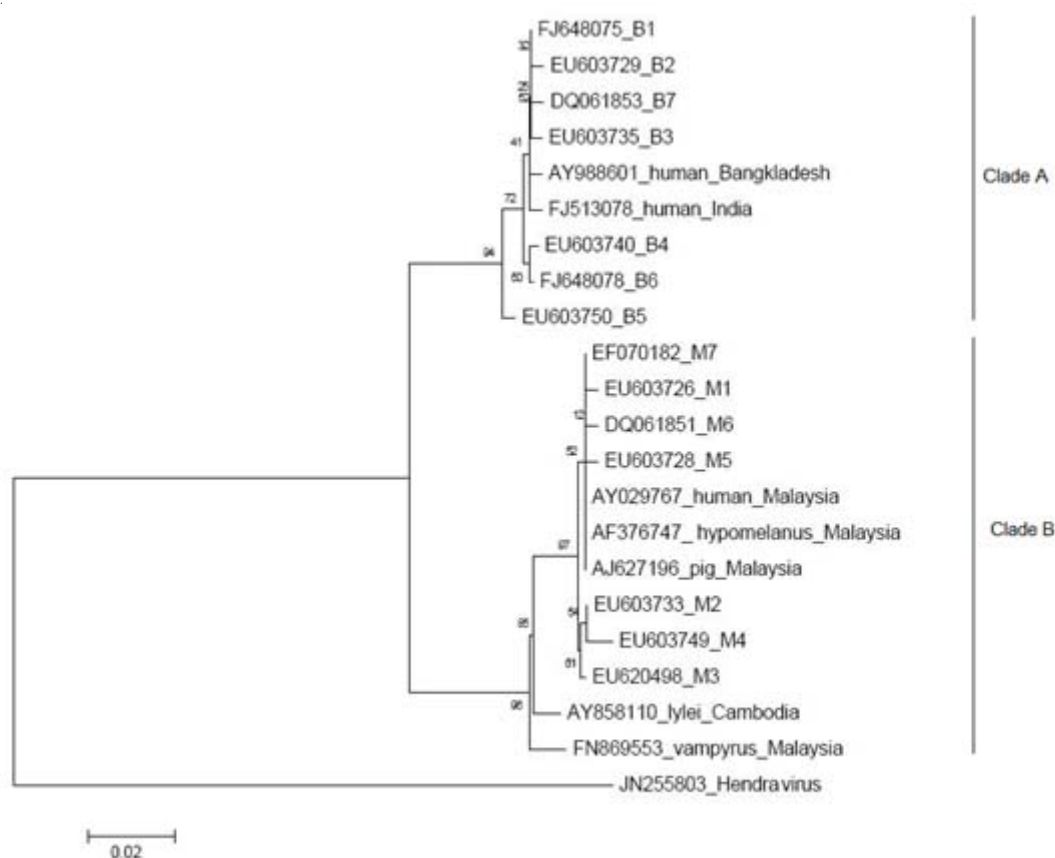


Figure 3. Phylogenetic tree illustrating the relationships of Nipah virus from Thai bats to all known Nipah viruses isolated from *Pteropus* genus (FN869553 from *P. vampyrus* in Malaysia, AY858110 from *P. lylei* in Cambodia, and AF376747 from *P. hypomelanus* in Malaysia (patients (AY988601 from Bangladesh, FJ513078 from India, and AY029767 from Malaysia), and pig (AJ627196). The phylogenetic tree was constructed using the neighbor-joining distance method and bootstrap values were determined using 1000 replicates. Hendra virus (GenBank accession no. AF017149.2) was used as the outgroup. NiV sequences Thailand were grouped by their identity into groups B1-B7 and M1-M6, details are shown in **Table 1**.

both of them were isolated from pool urine of *P. lylei* fruit bat at the same colony. The genetic heterogeneity between viruses detected from the same location on a single sampling occasion, e.g. EU60327, EU603733-4 and EU620498, suggested that there were multiple genetic variants co-circulating within bats at a single location.

No unusual mortality or illness was detected in bats during the surveillance. The seasonal prevalence previously found during the reproductive period [12] may suggest that this virus establishes persistent infections in these bats and is recrudescence during weak immune responses due to reproductive stress.

The genetic divergence of NiV isolates obtained from Lyle's fruit bats in a small geographic region might indicate that the ancestor of this group of viruses has circulated in *Pteropus* fruit bats for a long time, a period long enough for the precursors to spin out several genetically distinct viruses. Interestingly, the NiV genetic sequences from Lyle's fruit bats in this study were distinct from NiV isolated from Lyle's fruit bat in Cambodia [15]. This might be another evolutionary lineage of NiV. The range of *P. lylei* comprises Cambodia, Thailand and Vietnam, but there has been no disease outbreaks reported in these countries even though NiV has been found in Lyle's fruit bats, which are hunted for food in Cambodia and live in close proximity to humans [8]. Lyle's fruit bats are one of the major reservoirs of NiV clades A and B and are the major population of flying foxes in Thailand. More viral surveillance studies in Lyle's fruit bat populations in this region might help to clarify the genetic distribution of bat-NiV in Lyle's fruit bat and allow better risk assessment for NiV outbreaks.

Acknowledgements

This research was sponsored by the Thailand Research Fund (DBG5180026, RDG5420089), the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission (HR1160A), the Thai Red Cross Society, Thailand and by the US Naval Health Research Center BAA-10-93 under Cooperative Agreement Number W911NF-11-2-0041. The views and conclusions contained in this document are those of the authors. The authors have no conflict of interest to report.

References

1. Chua KB, Bellini WJ, Rota PA, Harcourt BH, Tamin A,

Lam SK, et al. Nipah virus: a recently emergent deadly paramyxovirus. *Science*. 2000; 288:1432-5.

2. Chua KB. Nipah virus outbreak in Malaysia. *J Clin Virol*. 2003; 26:265-75.
3. Parashar UD, Sunn LM, Ong F, Mounts AW, Arif MT, Ksiazek TG, et al. 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*. 2000; 181:1755-9.
4. Paton NI, Leo YS, Zaki SR, Auchus AP, Lee KE, Ling AE, et al. Outbreak of Nipah-virus infection among abattoir workers in Singapore. *Lancet*. 1999; 354: 1253-6.
5. Luby SP, Hossain MJ, Gurley ES, Ahmed BN, Banu S, Khan SU, et al. Recurrent zoonotic transmission of Nipah virus into humans, Bangladesh, 2001–2007. *Emerg Infect Dis*. 2009; 15:1229-35.
6. Lo MK, Lowe L, Hummel KB, Sazzad HM, Gurley ES, Hossain MJ, et al. Characterization of Nipah virus from outbreaks in Bangladesh, 2008–2010. *Emerg Infect Dis*. 2012; 18:248-55.
7. Chua KB, Koh CL, Hooi PS, Wee KF, Khong JH, Chua BH, et al. Isolation of Nipah virus from Malaysian island flying-foxes. *Microbes Infect*. 2002; 4:145-51.
8. Reynes JM, Counor D, Ong S, Faure C, Seng V, Molia S, et al. Nipah virus in Lyle's flying foxes, Cambodia. *Emerg Infect Dis*. 2005; 11:1042-7.
9. Sohayati AR, Hassan L, Sharifah SH, Lazarus K, [Zaini CM](#), [Epstein JH](#), et al. [Evidence for Nipah virus recrudescence and serological patterns of captive *Pteropus vampyrus*](#). *Epidemiol Infect*. 2011; 139: 1570-9.
10. Wacharapluesadee S, Lumlertdacha B, Boongird K, Wanghongsa S, Chanhom L, Rollin P, et al. Bat Nipah virus, Thailand. *Emerg Infect Dis*. 2005; 11: 1949-51.
11. Wacharapluesadee S, Hemachudha T. [Duplex nested RT-PCR for detection of Nipah virus RNA from urine specimens of bats](#). *J Virol Methods*. 2007; 141:97-101.
12. Wacharapluesadee S, Boongird K, Wanghongsa S, Ratanasetyuth N, Supavonwong P, Saengsen D, et al. A longitudinal study of the prevalence of Nipah virus in *Pteropus lylei* bats in Thailand: evidence for seasonal preference in disease transmission. *Vector Borne Zoonotic Dis*. 2010; 10:183-90.
13. Chua KB. A novel approach for collecting samples from fruit bats for isolation of infectious agents. *Microbes Infect*. 2003; 5:487-90.

14. Yob JM, Field H, Rashdi AM, Morrissy C, van der Heide B, Rota P, et al. Nipah virus infection in bats (order Chiroptera) in peninsular Malaysia. *Emerg Infect Dis.* 2001; 7:439-41.
15. Harcourt BH, Lowe L, Tamin A, Liu X, Bankamp B, Bowden N, et al. Genetic characterization of Nipah virus, Bangladesh, 2004. *Emerg Infect Dis.* 2005; 11: 1594-7.