

Nipah Virus Survey of Flying Foxes in Malaysia

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Abstract

An outbreak of Nipah virus infection occurred in 1998 in Malaysia in which a total of more than 1 million pigs were culled and 109 people died from this disease. Samples were collected from frugivorous bats living in Malaysia that were considered to be a natural reservoir of Nipah virus. There were two kinds of fruit bats, the so-called flying fox and the small fruit bat. Samples were collected from small fruit bats and flying foxes caught by mist net traps. No antibody and no Nipah virus were detected from the samples of small fruit bat. However, an average 18% and 63% positive for antibody were detected from the samples of flying foxes living in the islands (Island flying fox) and peninsula of Malaysia (Malayan flying fox), respectively. Nipah virus was not isolated from either of the flying foxes. However, Nipah virus gene was detected from Malayan flying fox by real-time PCR. Reovirus like virus and other unknown viruses were isolated from Malayan flying foxes. From these results, flying fox was an important natural reservoir of Nipah virus and especially Malayan flying fox was considered to be more important as a natural reservoir.

Discipline: Animal health

Additional key words: natural reservoir

Introduction

Nipah virus infection was first recognized in Ipoh Malaysia at the end of 1996 as a respiratory disease of pigs⁷. Accompanied by the swine disease, human encephalitis occurred and 15 people died from this disease by October 1998 in Ipoh. At the time of the infection outbreak, this disease was considered to be Japanese encephalitis (JE) infection and JE vaccination for humans was applied in the affected area. Unfortunately, the disease could not be controlled and spread to areas near Kuala

Lumpur, accompanied by pig movement. The patients of this disease were limited to persons who had contact with affected pigs, and the age distribution of patients had a peak between 30–40 years old. From these facts, it was difficult to consider the disease to be JE. In May 1998, a new virus was isolated from the brain samples of patients who lived in the village of Nipah in Bukit Pelanduk by Dr. Chua^{2,15}. This virus was identified as a paramyxovirus in the same group as Hendra virus isolated in Australia¹⁴ by Center for Disease Control and Prevention (CDC) in Atlanta, USA. The antibodies of this virus were detected in the patients affected with encephalitis and the pigs

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affected with respiratory disease. The Malaysian government started a complete eradication program against this disease by culling all pigs suspected to have the disease. Finally, 283 people were infected and 109 had died by December 1999. Other than Malaysia, 11 people in Singapore who were attached to work in the slaughter of pigs imported from Malaysia were infected and one died⁶. The causal agent of this disease was named Nipah and classified in a new family named Henipavirus in *paramixoviridae*¹³. The epidemiological study of Nipah virus infection was performed and antibody detection in many kinds of wild animals and domestic animals was conducted in Malaysia. As a result, an antibody against Nipah virus was detected in 5 species of bats. However, the antibody against Nipah virus was found mainly in frugivorous bats belonging to *Megachiroptera* species⁹. Fruit tree orchards are planted in the surroundings of the pig farms and these orchards are used as feeding sites by the frugivorous bats. Therefore, we considered frugivorous bats to be the most suspected natural reservoir for Nipah virus and studied the antibody detection and virus isolation from them. This study describes a method for collecting samples from wild frugivorous bats and the results of antibody detection against Nipah virus, virus isolation and viral genome detection.

Materials and methods

1. Cells

RK-13 cells (derived from rabbit kidney cells) and Vero cells (derived from green monkey kidney cells) were used for the serum neutralization test and virus isolation. Both cells were maintained in MEM medium containing 5% fetal bovine serum and 0.0292% L-Glutamin adjusted

to pH 7.4 by 7% Na₂HCO₃. Cells cultured within 5 days were used for the serum neutralization test (SNT), and 1 day cultured cells were used for virus isolation.

2. Virus

Nipah virus, strain VRI-ASI, isolated from affected swine in 1999 in Malaysia was passaged in Vero cells and cloned by a plaque purification method 3 times⁸ prior to being used for SNT.

3. Serum neutralization test (SNT)

Serum samples were diluted 5 times by MEM medium and 25 µL of each serum sample was 2-steps diluted on 96 well plastic plates. Twenty-five µL of Nipah virus adjusted to the titer of around 200 TCID₅₀/0.1 mL was added into each of the diluted serum samples and incubated for 1 h at 37°C. After incubation, 50 µL of Vero cell suspension (around 5 × 10⁵/mL) was added into each of the serum and virus mixtures and incubated for 3 days at 37°C in a 5% CO₂ incubator, and then checked for any cytopathic effect (CPE). The titer of SNT was decided to be the maximum dilution of each sample that inhibited CPE. For SNT, the process after serum dilution was performed in a biosafety level-3 (BSL-3) facility using a safety cabinet that was completely isolated and equipped with rubber gloves.

4. Small fruit bats and flying foxes sample collection

Small fruit bats (*Cynopterus brachyotis* and *Eonycteris spelaea*), Island flying foxes (*Pteropus hypomelanus*) and Malayan flying foxes (*Pteropus vampyrus*) were mainly caught using mist net traps. For the capture of small fruit bats, the mist net was set surrounding a rambutan tree in the evening (around 4:00 pm to

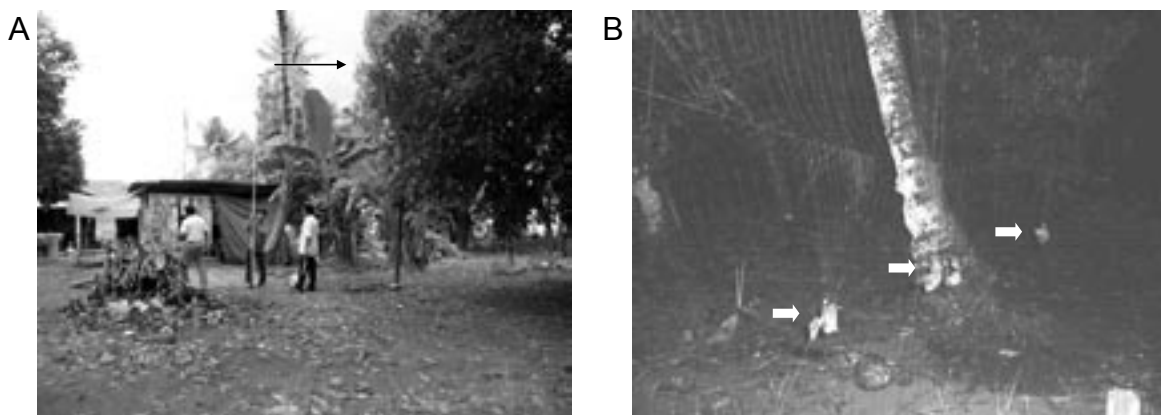


Fig. 1. Mist net trap used for the small fruit bats

The trap was set in the garden of a farmer's house in Ulu Piah, Ipoh. A: The mist net trap was set surrounding a rambutan tree (see arrow, right side of picture). B: The small fruit bats caught on the mist net trap were immediately removed and put into a cage. A total number of about 100 fruit bats could be caught in a period of approximately 4 h.

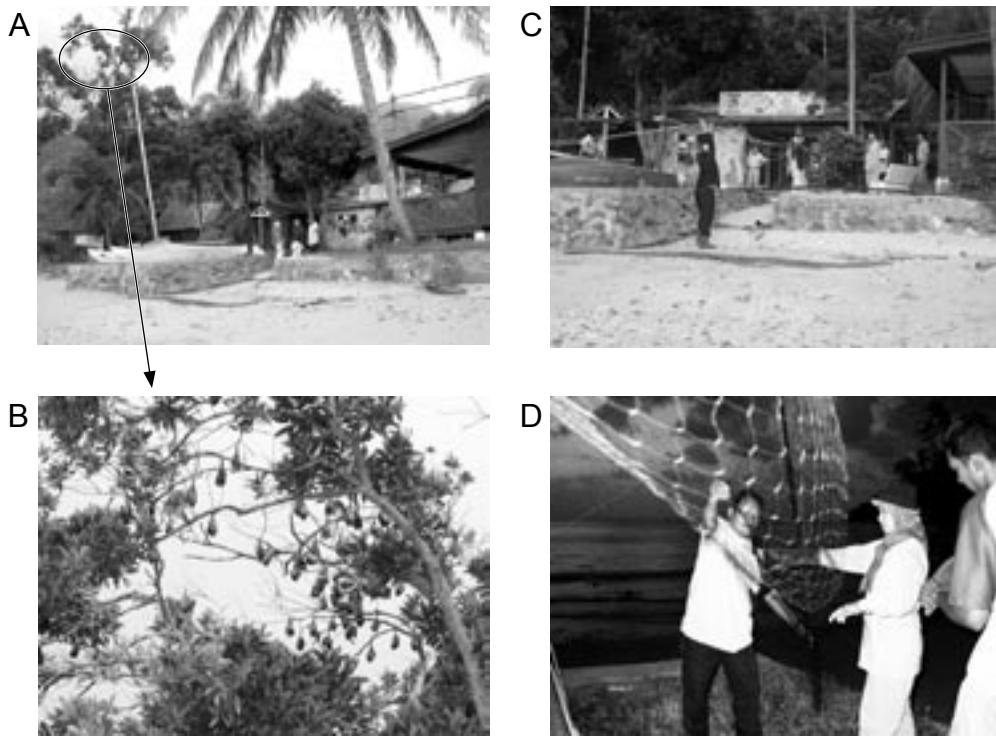


Fig. 2. Mist net trap used for the Island flying fox

A: Circle indicates the roosting site of Island flying foxes. B: Enlarged picture of Island flying foxes roosting site shown in the circle marked in A. C: Mist net trap prepared beside the roosting site. D: Flying foxes were caught by the mist net trap when they flew from their roosting site.

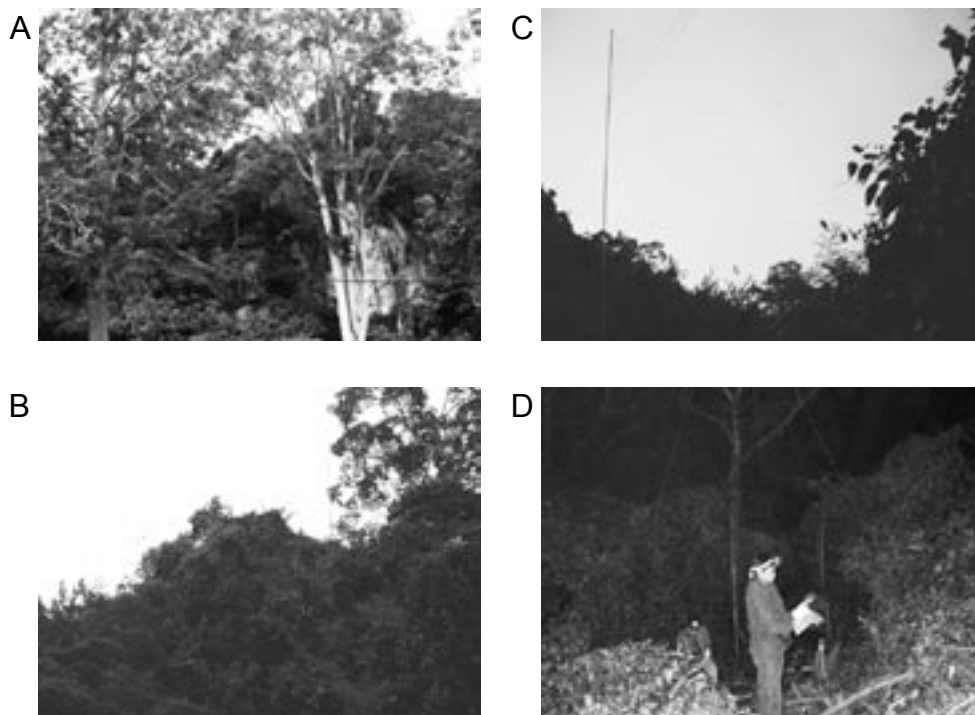


Fig. 3. Mist net trap used for the Malayan flying foxes

A: Feeding site of Malayan flying foxes. The tree in the right side of the picture bore fruit. B: The mist net trap was set beside the tree. C: Malayan flying foxes flew to feeding sites at around 7 pm. D: Flying foxes were caught by the mist net trap when they flew down to the feeding site.

4:30 pm), and bats were collected from 7:00 pm to 11:00 pm (Fig. 1). For the capture of Island flying fox, the mist net was set near a tree below which the flying foxes were roosting, and bats were collected from 7:00 pm to 8:00 pm when they flew out from their roosting site and from 6:00 am to 7:30 am when they came back to their roosting site (Fig. 2). For the capture of Malayan flying fox in Lenggong, Perak State the mist net was set near a tree that the flying foxes came to eat fruits from, and bats were collected from 7:00 pm to 6:00 am (Fig. 3). The hunters of the Malaysian Voluntary Home Guards shot the Malayan flying foxes in Kampong Gajyah, Wang Kelian and Kuala Berang and samples were collected from the bats. Small fruit bats were anesthetized by chloroform and were sacrificed by heart puncture. A mixture of ketamine and xylazine applied intravenously was used to anesthetize the flying foxes and they were bled via the vein under the wing. The liver, kidney and lung of the small fruit bats were collected and used for virus isolation. A tracheal swab (Tsw), urinal swab (Usw) and rectal swab (Rsw) were collected from all flying foxes caught in this study. The lung, liver, kidney, spleen, intestine, tonsil, and salivary gland were collected from Malayan flying foxes.

5. Virus isolation

The virus isolation samples collected from the small fruit bats and flying foxes were homogenized and centrifuged at 4°C, 3,300 × g for 15 min. The supernatants of the samples were inoculated into RK-13 cells cultured in 24 well plastic plates. Five days after inoculation, the supernatant of RK-13 cells were inoculated into both RK-13 and Vero cells. The passage through RK-13 and Vero cells was repeated 3 times. The virus isolation was done in a BSL-2 facility, but when CPE appeared in the inoculated cell culture, all manipulation was done in a BSL-3 facility after that.

6. Electron microscope observation

The samples that showed CPE were cultured in 250 mL plastic flasks and the supernatant fluid (25 mL) was collected and centrifuged at 4°C, 1,450 × g for 10 min. Formalin was added into the fluids at a final concentration of 0.1%, and the infected fluids were kept at 4°C for 3 days in a BSL-3 facility. After disinfection of the surface of the sample containers, the samples were brought out from the BSL-3 facility. The inactivated infected fluids were centrifuged at 4°C, 70,409 × g for 90 min. The pellets were suspend with 100 µL of distilled water, and one drop of each sample was mounted on a 400-mesh carbon-coated grid, and negatively stained with 2% phosphotungstic acid, pH 7.4 for 1 min. The samples were examined in a JEM-1200 EX electron microscope (JEOL

Ltd. Tokyo, Japan).

7. RT-PCR and real-time PCR

The specimens for RT-PCR and real-time RT-PCR were collected from 28 Malayan flying foxes caught in Lenggong, Perak State. The Tsw, Usw and Rsw of these 28 flying foxes, a total of 84 samples, were examined by RT-PCR and real-time RT-PCR. The RNA was extracted from the specimens (swab samples and organs) by using TRIZOL LS reagent (Invitrogen) and then used for RT-PCR and real-time RT-PCR. Positive controls of Nipah virus were prepared using supernatant and infected Vero cells. The negative control was prepared using uninfected Vero cells. The RT-PCR was carried out following the literature¹⁰. The real-time RT-PCR was carried out as follows by using the MiniOption Two-Color Real-Time PCR Detection System (BIO-RAD Laboratories). Two point five µL of the RNA templates of 84 samples was mixed with 1.0 µL of the reverse Nipah virus primer (5' CTG CTGCAGTTCAGGAAACATCA3') and heated at 95°C for 5 min and then immediately put on ice and kept at 4°C for 5 min. Twenty five µL of real-time PCR reagents (iScript One-Step Reaction Mix: SYBR GRN-BIORAD, BIO-RAD Laboratories), 1.0 µL of the forward Nipah virus primer (5' AGGTCATTTTGAGCAGGTTT 3'), 0.5 µL of reverse transcriptase, and 20µL of DNase and RNase free water were mixed and set at thermal profile as follows. First heating was 50°C for 20 min, then 95°C for 5 min and 50°C for 30 sec were repeated for 40 times, after that for melting curve analysis, 95°C for 1 min and 55°C for 1 min and 80 cycles of 0.5°C at an increment of 10 sec each.

Results

1. Small fruit bats and Flying foxes

Body length of the small fruit bat was up to 10 cm (from head to tail). The small fruit bats are distributed everywhere in Malaysia. A total of 112 fruit bats were caught and the samples were collected from 98 survivors. The Island flying fox (*Pteropus hypomelanus*) and the Malayan flying fox (*Pteropus vampyrus*) were caught and studied (Fig. 4). Body length of the Island flying fox averaged 17 cm (from head to tail), and the body weight averaged 500 g. The hair color of the Island flying fox was dark brown. Body length of the Malayan flying fox averaged 25 cm (from head to tail), and body weight averaged 900 g. The hair color of the Malayan flying fox was almost black but the back was partially dark brown. The roosting sites of Island flying foxes are distributed on the seashore of islands surrounding peninsular Malaysia (Fig. 5). The roosting sites of Malayan flying foxes are distrib-

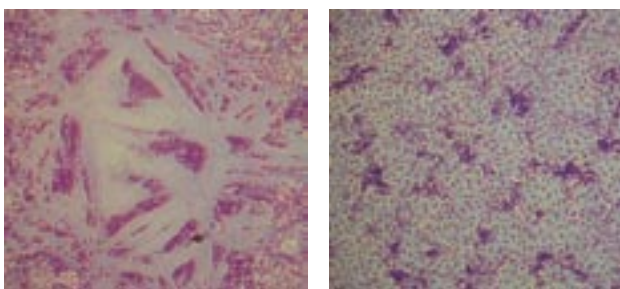


A: Island flying fox

B: Malayan flying fox

Fig. 4. Flying foxes in Malaysia

- A: Body length of the Island flying fox (*Pteropus hypomelanus*) is approximately 17 cm (from head to tail) and body weight is approximately 500 g. The hair color of the Island flying fox is dark brown.
- B: Body length of the Malayan flying fox (*Pteropus vampyrus*) is approximately 25 cm (from head to tail) and body weight is approximately 900 g. The hair color of the Malayan flying fox is almost black with a partially brown back.



A

B

Fig. 7. CPE of the isolates

The CPE of isolates identified by microscope observation are shown.

- A: The isolate from the rectal swab sample of No. 3 flying fox captured in Wang Kelian, Perlis State passed through Vero cells. Syncytial type CPE with multinuclear giant cells was observed. May-Grünwald Giesma stained ($\times 200$).
- B: The isolate from the spleen of No. 5 flying fox captured in Wang Kelian passed through RK-13 cells. Aggregation type CPE was observed, and aggregate cells were observed everywhere. May-Grünwald Giesma stained ($\times 200$).

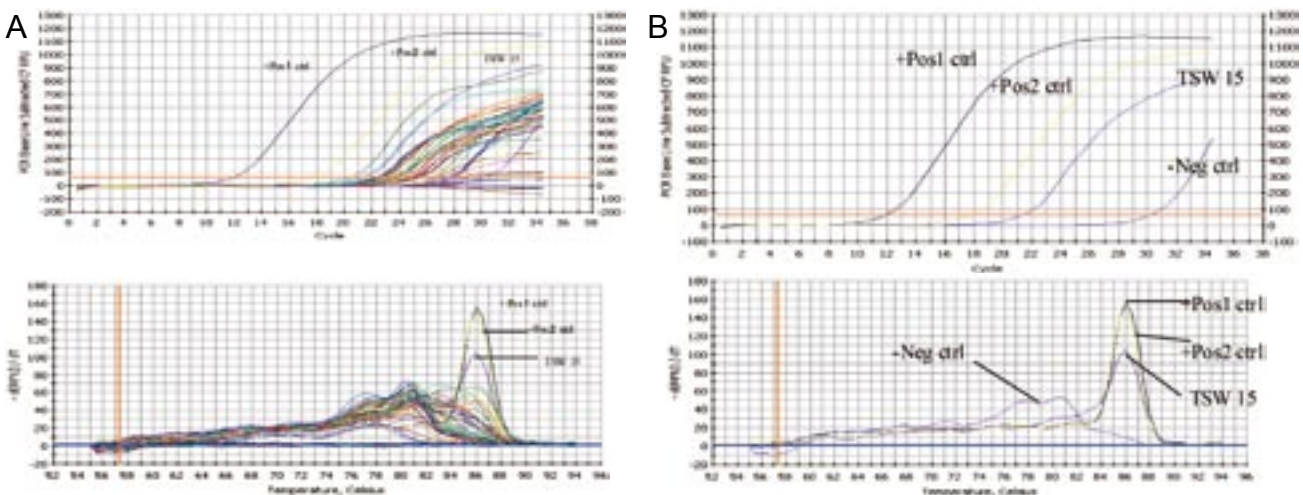


Fig. 8. Results of real-time RT-PCR

- A: Total results of real-time RT-PCR. Pos1 ctrl: Positive control of Nipah virus sample prepared from infected Vero cells. Pos2 ctrl: Positive control of Nipah virus sample prepared from supernatant of infected Vero cells. TSW 15: The tracheal swab sample of No. 15 flying fox caught in Lenggong.
- B: Selected results of real-time RT-PCR. Neg ctrl: Negative control prepared from uninfected Vero cells. Threshold cycle (Ct) values of Pos1 ctrl and Pos2 ctrl were 11.7 and 17.6 respectively and that of TSW 15 was 20. The peak of the specific RT-PCR product was observed at 86°C melting temperature in Pos1 ctrl, Pos2 ctrl and TSW 15.

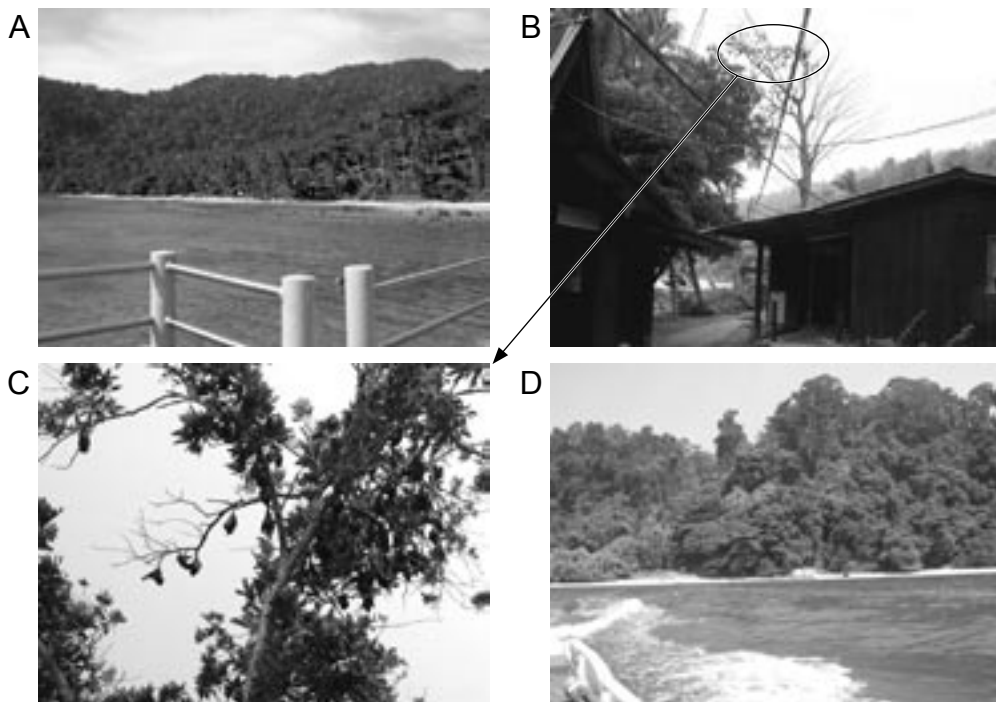


Fig. 5. Roosting site of the Island flying fox

Island flying foxes are distributed in islands surrounding peninsular Malaysia. A: Seashore of Air Batang, Tioman Island Johor State where the roosting sites of Island flying foxes are distributed. B: A roosting site of the Island flying foxes located very close to the seashore and village. C: Enlarged picture of the roosting site indicated by the circle in B. D: Seashore of Rumbia Island, Sembiran Islands Perak State. The roosting sites of the Island flying foxes are distributed in tree growing areas on the seashore of this small island.

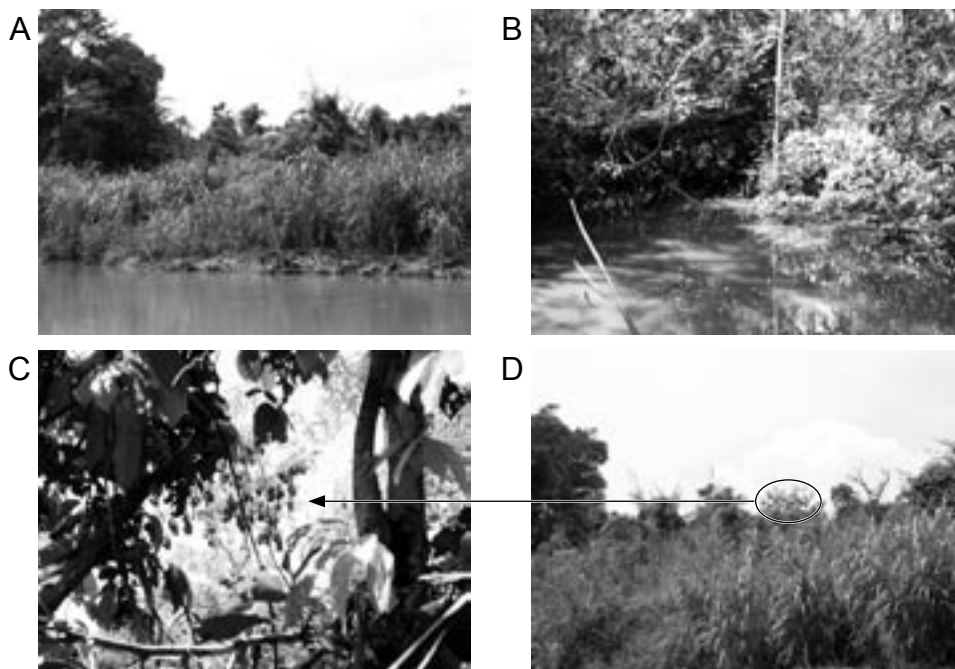


Fig. 6. Roosting site of Malayan flying fox

Malayan flying foxes are distributed not only in peninsular Malaysia but in Indonesia and Thailand as well. A: The roosting sites of Malayan flying foxes are located near the mouth of rivers. B: Trees that are Malayan flying foxes roosting sites grow in mangrove jungles where deep water covers the ground. C: A picture of Malayan flying foxes roosting site taken from a distance away from the bats because they are very sensitive to humans and fly away immediately even during their period of sleep. D: The roosting tree of the flying foxes was very far away from the riverbank. The circle indicates the roosting site of flying foxes shown in C.

uted in mangrove jungles located at the mouth of rivers (Fig. 6). A total of 102 Island flying foxes were caught by mist net and samples collected for SNT against Nipah virus and virus isolation. A total of 56 Malayan flying foxes were caught or shot down and samples collected for SNT and virus isolation.

2. SNT against Nipah virus

The results of SNT against Nipah virus in 98 sera from the small fruit bats were all negative for the virus (data not shown). The results of SNT against Nipah virus in the sera from the Island flying foxes are shown in Table 1. The sera collected from the Island flying foxes in Tioman Island in January 2004 showed a higher positive rate than those of the sample collected in April 2004. The high positive group showed clearly a high titer from 40 to 320 against Nipah virus, but the low positive group showed the minimum positive titer ($\times 5$). Two samples collected from the Island flying foxes caught in the Sembiran Islands showed negative. The average positive rate of SNT against Nipah virus of the sera from the Island flying foxes was 18%. The results of SNT against Nipah

virus in the sera from the Malayan flying foxes are shown in Table 2. The sera collected from the Malayan flying foxes in Lenggong and Kuala Berang showed a higher positive rate than the others. The sera collected from the Malayan flying foxes in Wang Kelian showed the lowest positive rate. The high positive group showed a titer from 20 to 320 against Nipah virus and the low positive group showed a titer from 5 to 10. The average positive rate of SNT against Nipah virus in the sera from the Malayan flying foxes was 63%.

3. Virus isolation and real-time RT-PCR

No virus was isolated from the samples of the small fruit bats and the Island flying foxes. Viral agents were isolated from the samples of the Malayan flying foxes caught in Wang Kelian, Perlis State. The properties of the isolates from the samples of the Malayan flying foxes caught in Wang Kelian are shown in Table 3. Viral agents were isolated from the rectal swab of No. 3 flying fox (3 Rsw), and from the spleen (5 SP), the urinal swab (5 Usw), the rectal swab (5 Rsw), and the intestine of No. 5 flying fox (5 Int). The isolates from 3 Rsw, 5 Usw, 5 Rsw,

Table 1. Serum neutralization test of Island flying foxes

Location of capture (Month of capture)	Total no. of animals	No. of positives	No. of high positives (40–320)*	No. of low positives (5)*	Positive rate (%)
Tioman Island, Johor (January 2004)	50	11	8	3	22
Tioman Island, Johor (April 2004)	50	7	6	1	14
Sembiran Islands, Perak (May 2004)	2	0	0	0	0
Total	102	18	14	4	18

*: The range of the titer of the serum neutralization test.

Table 2. Serum neutralization test of Malayan flying foxes

Location of capture (Month of capture)	Total no. of animals	No. of positives	No. of high positives (20–320)*	No. of low positives (5–10)*	Positive rate (%)
Lenggong, Perak (June, 2004)	28	19	13	6	68
Kampong Gajyah, Perak (February, 2004)	7	4	2	2	57
Wang Kelian, Perlis (March, 2004)	6	2	1	1	33
Kuala Berang, Kuala Terengganu	15	10	7	3	67
Total	56	35	23	12	63

*: The range of the titer of the serum neutralization test.

Table 3. Summary of isolates from Malayan flying foxes captured in Wang Kelian, Perlis

Isolate	Growth (CPE type)		Plaque formation		Electron Microscope observation
	Vero ^{a)}	RK-13 ^{a)}	Vero	RK-13	
3 Rsw	+ (syncytial)	+ (syncytial)	+	+	— ^{b)}
5 SP	—	+ (aggregation)	—	—	— ^{b)}
5 Usw	+ (syncytial)	+ (aggregation)	+	+	Reo like ^{c)}
5 Rsw	+ (syncytial)	+ (aggregation)	+	+	Reo like
5 Int	+ (syncytial)	+ (aggregation)	+	+	Reo like

3 Rsw: Rectal swab sample of No. 3 flying fox. 5 SP: Spleen of No. 5 flying fox. 5 Usw: Urinal swab sample of No. 5 flying fox.

5 Rsw: Rectal swab sample of No. 5 flying fox. 5 Int: Intestine of No. 5 flying fox.

a): Type of cells used for virus isolation. b): No obvious viral particle was observed. c): Reovirus like particles were observed.

and 5 Int showed syncytial type CPE with multinuclear giant cell formation in Vero cells. However, the isolate from 5 SP did not grow in Vero cells and showed aggregation type CPE in RK-13 cells (Fig. 7 B). The isolate from 3 Rsw showed syncytial type CPE in RK-13 cells and Vero cells but it showed differences (Fig. 7 A) to those of the isolates from 5 Usw, U Rsw and 5 Int. All isolates were confirmed not to be Nipah virus by RT-PCR. The isolates from 5 Usw, 5 Rsw and 5 Int showed reovirus particles by electron microscope observation. The isolate from 5 SP made no plaque on RK-13 and Vero cells. The tracheal swab sample of No. 15 (TSW 15) Malayan flying fox caught in Lenggong, Perak State showed positive results in real-time RT-PCR (Fig. 8). The peak of the RT-PCR product of TSW 15 was observed at 86°C melting temperature which was the same position as that of the positive controls. The threshold cycle (Ct) values of TSW 15 and the positive controls were 20.7, 17.6 and 11.7, respectively.

Discussion

The outbreak of Nipah virus infection posed a big threat to people not only in Malaysia, but also in the world. This is because the encephalitis disease killed more than 100 people and mortality of infected patients was more than 40% in Malaysia¹³. The origin of this fatal disease was obscure at the time of the outbreak, and the epidemiological study for detecting the antibody against Nipah virus has been done in 237 bats, 18 wild boars, 16 hunting dogs used to hunt wild boar, and 25 rodents. As a result of this research, 4 species of frugivorous bats had the antibody against Nipah virus⁹. Therefore, we studied the antibody against Nipah virus in these 4 species of frugivorous bats, and tried to clarify the origin of the disease.

From our results and previously investigated results (unpublished data) we showed that the sera from a total of more than 500 small fruit bats had no antibody against

Nipah virus and no Nipah virus was isolated from the small fruit bats (data not shown). This result differed from a previous study⁹. The reason for this difference is considered to be a non-specific reaction or detection of an antibody of a virus group similar to Nipah virus. This is because the positive reactions were observed in only one or two samples of small fruit bats, and the titer of the positive samples were found in the lowest dilution ($\times 2$ – $\times 5$). These positive reactions usually disappeared in repeat tests. The sera of the flying foxes showed a high positive rate of antibody against Nipah virus. The positive rate of the antibody against Nipah virus in the sera from the Malayan flying foxes (average 63%) was higher than that of the Island flying foxes (average 18%). Furthermore, the results of the real-time RT-PCR showed a positive in a sample from the Malayan flying foxes captured in Lenggong, Perak State. A clear positive result was observed in only one sample of 84 samples, but considerable positive results were observed in a few samples. Ct of the TSW 15 sample had a value 0.565–0.85 smaller than the positive control, but it showed a similarity to the Nipah virus gene product. In addition, the PCR product of TSW 15 was found in the same melting temperature (86°C) as the Nipah virus control. From these results, Malayan flying fox was strongly considered to be the origin of the disease. For further proof of the real natural reservoir of Nipah virus, it is necessary to isolate the Nipah virus from the flying foxes directly, but Nipah virus isolation has failed. The reason for the failure to isolate the virus was considered to be the periodical effect of sampling and the number of samples. A previous study of Nipah virus isolation from Island flying foxes indicated a periodical effect of sampling⁴. A periodical effect of the sampling was also observed in this study as shown in the results of the antibody positive rate in the Island flying foxes in Tioman Island. The positive rates for the antibody in the samples of flying foxes captured in January (22%) and April (14%) were different. Viruses were only isolated

from samples of the Malayan flying foxes captured in Wang Kelian, Perlis State in March. Three isolates were considered to be reovirus and possibly related to the Pulau virus that was isolated from Island flying foxes recently¹¹. Two isolates could not be identified to a specific virus by electron microscope observation. All five isolates were not Nipah virus. However, it is necessary to identify the isolates from Malayan flying foxes, to reveal the etiological role of flying foxes for other kinds of disease outbreak. The total numbers of flying foxes examined in a previous study of Nipah virus isolation from Island flying foxes and a recent study of Nipah virus isolation from Lyle's flying foxes (*Pteropus lylei*) were 588 and 769, respectively, which were larger than the number in our study (158)^{4,12}. More sampling was required to successfully isolate the Nipah virus from Malayan flying fox, but the sampling from this flying fox was difficult. The previous method for the virus isolation from Island flying foxes⁵ could not be applied for the Malayan flying foxes. The roosting sites of the Malayan flying foxes were located in mangrove jungles as shown in Fig. 6, therefore it was impossible to collect the dropped urine sample. We applied the capture or shooting method for sampling from the Malayan flying foxes. The capture of this flying fox was difficult to compare to the Island flying fox. The capture method of the Malayan flying fox as shown in Fig. 3 indicated the difficulty of deciding the period for virus isolation. The time of capture of the Malayan flying fox is controlled by the fruit season of their feeding site. However, a positive result of real-time RT-PCR was obtained in a sample of the Malayan flying fox captured in Lenggong, Perak State (same region of the first outbreak of Nipah virus infection in Malaysia). From these results, we strongly suggested the Malayan flying fox was an origin of the Nipah virus infection outbreak in Malaysia, even though we had no success in Nipah virus isolation from them.

Our results, strongly suggested the origin of the disease came from the Malayan flying fox, but there remains a question as to why the outbreak occurred in the period of 1996–1999. The reason for this is considered to be the pig production in Malaysia became active from 1990, because Singapore had closed down its pig farms entirely in 1990. Therefore, the pig industry in Malaysia has grown to be a big supplier of live pigs to Singapore, and the number of pig farms in Malaysia increased from this year. Ipoh, where the first outbreak of Nipah virus infection occurred, was previously famous for tin mining, and as a result sandy soils interspersed with large pools of water exist there. Such land is not suitable for large-scale plant agriculture, but it is particularly suitable for pig farming, and there has been a significant increase in capacity by the existing and new producers in Perak

State. Pig farms in Malaysia should have a buffer zone to avoid the odor and noise of the pigs. Most of the owners of pig farms in Ipoh planted fruit trees in this buffer zone¹. The jungle, which is the feeding site for flying foxes, has been reduced for pulp wood, pig farming and industrial plantations, and recently a severe El Niño southern oscillation (ENSO) provoked an abnormal weather pattern which reduced the production of fruit in the jungles of Malaysia³. Therefore, flying foxes came down to feeding sites close to the pig farms and Nipah virus spread from them to the pigs in the farms. Nipah virus is a fatal virus for humans, and the natural reservoir of this virus is considered to be a dangerous animal for humans. However, we consider that the flying fox is one of the victims of this disaster in Malaysia. The flying foxes have lived quietly in the jungles of Southeast Asia for so long a period without posing a threat to humans, but were forced to move to feeding sites close to the villages of humans due to recent changes in their environmental condition.

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