การสำรวจภาวะการติดเชื้อไวรัส Nipah ของค้างคาวในประเทศไทย

นางสาว สุภาภรณ์ วัชรพฤษาดี

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SURVEY FOR NIPAH VIRUS INFECTION AMONG BATS IN THAILAND

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สุภาภรณ์ วัชรพฤษาดี : การสำรวจภาวะการติดเชื้อไวรัส Nipah ของค้างคาวในประเทศไทย (SURVEY FOR NIPAH VIRUS INFECTION AMONG BATS IN THAILAND) อ. ที่ปรึกษา : ศ.นพ. ธีระวัฒน์ เหมะจุฑา 126 หน้า. ISBN 974-53-2435-3.

การศึกษาภาวะการติดเชื้อไวรัส Nipah ของค้างคาวในประเทศไทย ระหว่าง เดือนมีนาคม พ.ศ. 2545 ถึง เดือนกุมภาพันธ์ พ.ศ. 2547 ได้ทำการสำรวจค้างคาว 12 ชนิด ใน 9 จังหวัดได้แก่ สิงห์บุรี อยุธยา กรุงเทพฯ ระยอง ชลบุรี ฉะเชิงเทรา ปราจีนบุรี ราชบุรี และสุราษฎร์ธานี เป็นจำนวน 1,304 ตัว โดยตรวจหาภูมิคุ้มกันแอนติบอดีย์ต่อเชื้อไวรัส Nipah โดยวิธี ELISA ในตัวอย่างเลือดค้างคาวจำนวน 1,054 ตัวอย่าง พบผลบวก 82 ตัวอย่าง คิดเป็นร้อยละ 7.8% จำแนกเป็นตัวอย่างจากค้างคาวแม่ไก่ภาคกลาง (*Pteropus lylei*) จำนวน 72 ตัวอย่าง ค้างคาวแม่ไก่เกาะ (*P.hypomelanus*) จำนวน 4 ตัวอย่าง และ จำนวนละ 1 ตัวอย่างจาก ค้างคาวแม่ไก่ป่าฝน (*P.vampyrus*) และ ค้างคาวหน้ายักษ์สามหลืบ (*Hipposideros larvatus*) การตรวจหาสารพันธุกรรมของเชื้อไวรัส จากตัวอย่างน้ำลายค้างคาวจำนวน 1,286 ตัวอย่าง และตัวอย่างเยี่ยวค้างคาวจำนวน 1,282 ตัวอย่าง โดยวิธีการรวมกลุ่มตัวอย่าง 10 ต่อ 1 จากตัวอย่างชนิดเดียวกัน ที่เก็บได้จากค้างคาวชนิดเดียวกัน ในเวลาและสถานที่เดียวกัน จำนวนชนิดตัวอย่างละ142 กลุ่มตัวอย่าง พบสารพันธุกรรมของเชื้อไวรัส Nipah (RNA)ใน 2 กลุ่มตัวอย่างน้ำลาย และ 6 กลุ่มตัวอย่างเยี่ยว การถอดรหัสพันธุกรรมความยาว 181 นิวคลีโอไทด์ จากกลุ่มตัวอย่างน้ำลายของค้างคาวหน้ายักษ์สามหลืบพบว่ามีความเหมือนกับสายพันธุ์ของไวรัสที่ทำให้เกิดโรคระบาดในประเทศมาเลเซีย รหัสพันธุกรรมจากกลุ่มตัวอย่างที่เหลือได้แก่ น้ำลายและเยี่ยวของค้างคาวแม่ไก่ภาคกลางมีความเหมือนกับสายพันธุ์ของไวรัสที่ทำให้เกิดโรคระบาดในประเทศบังคลาเทศ โดยที่มีเพียง 3 จังหวัด (สิงห์บุรี ราชบุรี และสุราษฎร์ธานี) ที่ไม่พบหลักฐานการติดเชื้อ จากข้อมูลเหล่านี้อาจกล่าวได้ว่าค้างคาวในประเทศไทยเป็นแหล่งรังโรคตามธรรมชาติของไวรัส Nipah และอาจมีความเสี่ยงต่อการแพร่ไปยังสัตว์และคนซึ่งต้องการการเฝ้าระวังอย่างเข้มงวด

สาขาวิชา..ชีวเวชศาสตร์(สหสาขาวิชา).....ลายมือชื่อนิสิต.....

ปีการศึกษา..2548.....ลายมือชื่ออาจารย์ที่ปรึกษา.....

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Surveillance for Nipah virus (NV) infection in bats was conducted among bat population in 9 provinces in Thailand. During March 2002 to Febuary 2004, 1,304 bats of 12 species were captured. Of 1,054 blood samples, 82 (7.8%) were positive for NV antibody by enzyme linked immunosorbent assay (ELISA). All but 6 (4 from *Pteropus hypomelanus*, and 1 each from *P.vampyrus* and *Hipposideros larvatus*) antibody positive samples were from *P.lylei*. Saliva (n = 1,286) and urine (n = 1,282) specimens were pooled on an average of 1:10, according to species and geographical locations of collection. Of 142 pools of each saliva and urine specimens, Nipah virus RNA could be demonstrated in 2 and 6 pools from saliva and urine specimens, respectively. The 181 nucleotide sequences of the nucleoprotein gene obtained from one saliva pool of *H.lavatus* was identical to those published sequences reported from Malaysia whereas those from one saliva and 6 urine pools from P.lylei shared 92% homology but identical to Bangladesh strain. These data suggested an existence of naturally infected Nipah virus infection in Thai bats.

Field of study Biomedical Sciences (Inter-Department) Student's signature Supaporn Wacharapluesadee

Academic year...2005.....Advisor's signature... Thiravat Hemachudha

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LIST OF ABBREVIATIONS

AY	Ayutthaya
BK	Bangkok
bp	base pair
CB	Chon Buri
CC	Cha Choeng Sao
Cs	*Cynopterus sphinx*
ELISA	Enzyme linked immunosorbent assay
Em	*Emballonura monticola*
Es	*Eonycterus spelaea*
Ha	*Hipposideros armiger*
Hl	*Hipposideros lavatus*
HV	Hendra virus
IC	Internal control
IgG	Immunoglobulin G
km	Kilometer
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimolar
Ms	*Megaduma spasma*
N	Nucleoprotein
nt	Nucleotide

NV	Nipah virus
OD	Optical density
PC	Pra Chin Buri
pg	Picogram
Ph	*Pteropus hypomelanus*
Pl	*Pteropus lylei*
Pv	*Pteropus vampyrus*
RB	Ratcha Buri
RNA	Ribonucleic acid
Rs	*Rousettus leschenault*
RT-PCR	Reverse transcription polymerase chain reaction
RY	Ra Yong
s	Second
SB	Sing Buri
Sh	*Scotophilus heathi*
SP	Saliva pool
SR	Surat Thani
Tp	*Tadarida plicata*
ug	Microgram
ul	Microliter
uM	Micromolar
UP	Urine pool
UV	Ultra violet

CHAPTER I

INTRODUCTION

1. Background and Rationale

The emergence of zoonotic viruses maintained by wildlife reservoirs has complicated the study of cross-species transmission. In this regard, bats have been associated with a number of newly recognized zoonotic agents, including lyssavirus, Hendra (HV) and Nipah (NV) viruses (1-3). Hendra and NV are members of the *Paramyxoviridae* family. Their biological properties and genomic organization are closely related and classified as a new genus, the *Henipavirus* (4). NV caused a major outbreak in swine and humans in Malaysia between September 1998 and April 1999. This resulted in 265 human cases with 106 deaths (5, 6). The outbreak was controlled only after the new NV etiology was realized. Pigs were identified as the source of the virus infecting humans. More than 1 million pigs were culled (5). The virus was named NV after Kampung Sungai Nipah (Nipah River village) where the first virus isolate had originated. The genesis of the outbreak was traced to bats. A seroepidemiological study in Malaysia implicated 4 fruit bat species, *Pteropus hypomelanus, P.vampyrus, Cynopterus brachyotis, Eonycteris spelaea* and an insectivorous bat, *Scotophilus kuhli* (7,8). NV was also identified and isolated from bat urine samples of *P. hypomelanus* (9). The presence of NV was demonstrated in Cambodia during 2002 and in Bangladesh and India in 2001 and 2003-2004 (10, 11). In Febuary 2004, the reemergence of NV killed at least 35 people in Bangladesh.

Unlike its first appearance in Malaysia, NV may result in person to person transmission, or the disease may result after direct contact with bat saliva from eating contaminated fruits (11, 12). Recently, antibodies to NV antigen were detected in two *P.giganteus* adult females from Bangladesh (13).

Bats are found in all parts of Thailand. Their habitats are close to humans for example, some of their roosts are located in temple or garden. Furthermore, these places are promoted to be tourist attraction sports or become places for collecting of bat feces to produce organic fertilizer. Such a close proximity to human posses risk by mean of either direct contact with or via consumption of contaminated fruits (with urine, saliva or feces). NV surveillance in pigs industries and back yard racing pigs is routinely performed annually by National Institute of Animal Health, Department of Livestock Development. No information whether Thai bats are infected with NV even though NV has been reported in Asia. Survey of NV infection among bats in Thailand is extremely important to obtain baseline data for surveillance and to estimate risk to humans and animals.

2. Objectives

1. To determine whether there is NV infection in Thai bats.

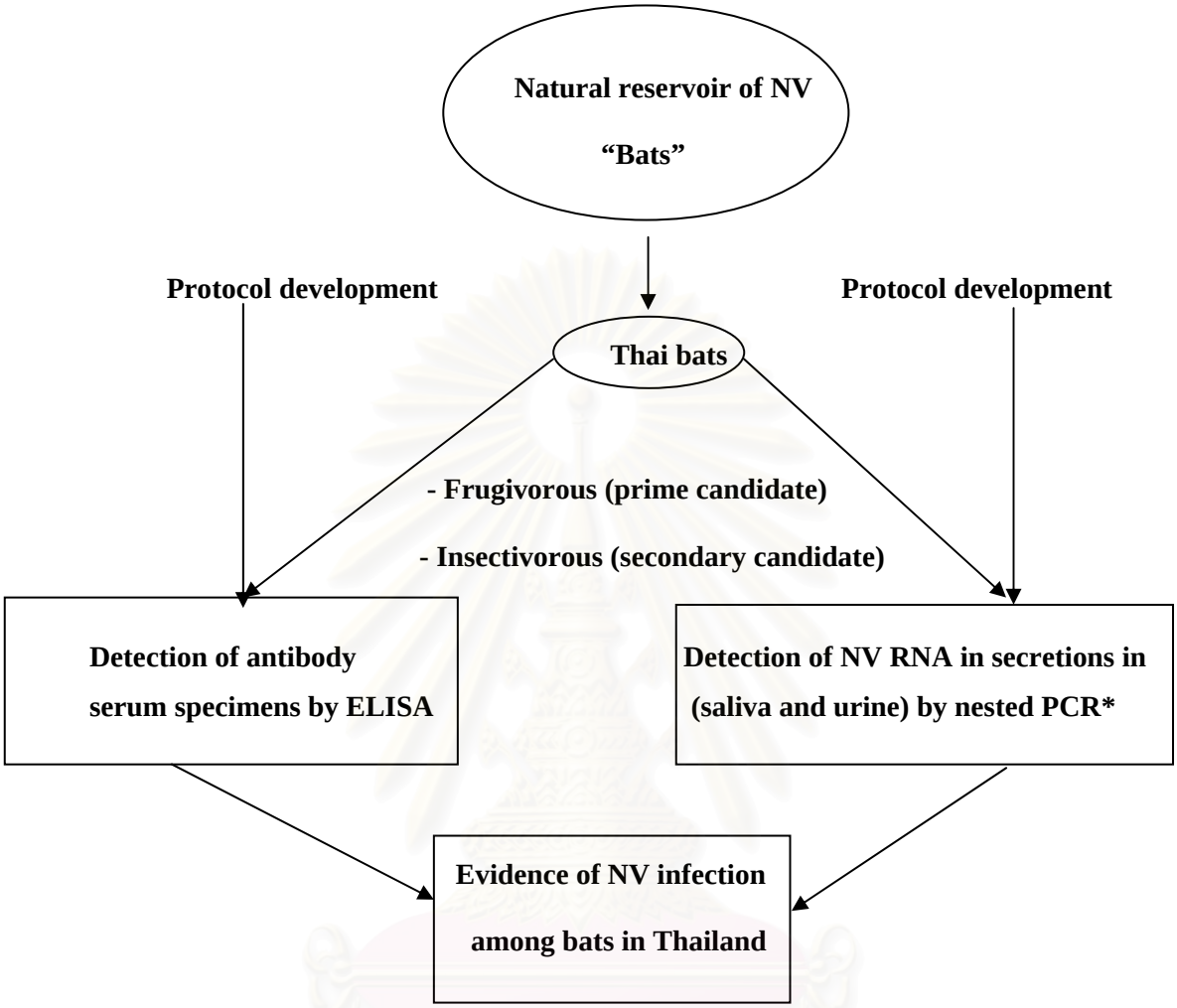
2. To determine prevalence of NV infection in bats according to geographical regions.

3. To determine whether there is a preponderance of bat species in terms of susceptibility to NV infection.

4. To determine whether NV RNA can be demonstrated in urine and saliva of infected bats and whether this shedding can be constantly or episodically observed.

3. Conceptual Framework

It is believed that certain species of fruit bats are the natural reservoir of NV. These bats live in subtropical and tropical areas of Africa, India, Southeast Asia, Australia, and all but the easternmost islands of Pacific. Survey of NV from Thai bats will be performed in both frugivorous and insectivorous bats. Blood will be collected for antibody assay and saliva and urine will be collected for PCR assay of virus RNA. A new method for detection of NV RNA with internal control will be developed. NV epidemiology in Thai bats will be analyzed based on data obtained from serological data and viral RNA study. The diagram of conceptual framework is shown in figure 1.



* Verification of PCR results and analyzing of virus characteristics by genome sequencing.

Figure1. Conceptual framework

4. Assumption

1. Bats being caught by using mist net (fruit bats) or other means (insectivorous bats) will be assumed to be healthy.

2. Blood volume obtained should be variable depending on size of bat.

3. Urine and saliva volume obtained from swabbing technique may vary depending on bat condition. In similar, total volume after pooling of urine or saliva specimens may also be variable.

5. Limitation

1. This is a cross-sectional descriptive study designed to determine the prevalence of NV infection in surveyed areas at targeted time point which may not cover the entire seasons of the year. Therefore, this may not reflect the true incidence nor the seasonal preference.

2. The number of captured bats at each site may be unpredictable and variable due to many factors, such as roost habitats, nature of bats (whether they are strict wildlife or live closely to human perimeter), species and availability of resources (e.g. manpower) during each survey.

3. The amount of blood, saliva and urine from each bat species may be variable depending on size of bat; fruit bats are usually bigger and easier to collect samples from them as compared to the insectivorous bats.

6. Operational Definitions

1. **Enzyme-Linked Immunosorbent Assay** (ELISA or enzyme-immunoassay-EIA) is a method to detect the presence of a certain substance in a sample. It utilizes antibodies specific to the substance (antigen); these antibodies are linked to an enzyme which causes a chromogenic or fluorogenic substrate to produce a signal.

2. **RT-PCR** is an abbreviation for Reverse Transcription-Polymerase Chain Reaction. RT-PCR is a technique in which an RNA strand is "reversely" transcribed into its DNA complement, followed by amplification of the resulting DNA using a polymerase chain reaction (PCR).

3. Nested PCR is a double amplification technique using two pairs of PCR primers for a single locus. The first pair amplified the locus as seen in any PCR experiment. The second pair of primers (**nested primers**) binds within the first PCR product and produces a second PCR product that will be shorter than the first one. The logic behind this strategy is that if the wrong locus is mistakenly amplified, the probability for a second amplification should be very low.

4. **Duplex PCR** is the method to amplify and differentiate two target genes in single PCR reaction by using two primer pairs of specific genes.

5. **Prevalence** is the measure of a condition in a population at a given point in time.

6. Incidence is the number of new occurrences of a condition (or disease) in a population over a period of time.

7. Expected Benefit and Application

This study is for the first time that evidence of NV infection in bat species in Thailand will be identified. These data are necessary for surveillance management of both human and animal health. It should be useful not only to prevent the spread of the disease, but also to protect swine industries as occurred in Malaysia. The method for NV RNA detection will also be developed based on multiplex PCR with internal control. It will become an essential tool for NV RNA detection during the NV outbreak due to its simplicity and its applicability to variety of specimens.

8. Obstacles and Strategies to solve the problems

Sample size based on calculation from previous figures as reported in neighboring country is 3,500. However, it is expected that such sample size may not be achieved. This may be compromised by various factors, for example, roost distribution, amount of bats in each colony, time and budget. In order to obtain a meaningful data while not being jeopardized by the smaller sample size, analysis will be done in parallel to the ongoing activities of field survey. Adaptation or modification of the survey method, selection of sites and species and sample size to be collected will be adjusted accordingly.

9. Ethical Considerations

Bat capture and the whole process of bat survey in this study have been approved by the National Park, Wildlife and Plant Conservation Department, Ministry of Natural Resources and Environment.

10. Research Methodology

1. Locations to be surveyed: Bat roosts in Thailand.

2. Population: Fruit- and insect eating bats of various species from locations known as tourist sites for viewing or as places for collection of bat feces to produce organic fertilizers (data obtained from National Park, Wildlife and Plant Conservation Department, Ministry of Natural Resources and Environment).

3. Sample size: According to the previous reports from neighboring countries, the prevalence of NV infection by ELISA method was 11.5%, 10.5%, and 8.9% in Cambodia, Bangladesh and Malaysia, respectively. The mean of prevalence was 10.3%. Calculating from this prevalence number with a 10% acceptable error, the approximate sample size would be 3,500.

4. Sampling method: By accidental sampling method, larger fruit bats will be mist-netted near sunset when leaving their roosts for night feeding or before dawn upon return. Insectivorous bats in caves will be captured during the day using fine-mesh, long-handled butterfly nets.

5. Sample collection: Captured bats will be anesthetized and blood, saliva and urine samples will be collected as previously described (1). Bats will be released after recovery from sedation. The amounts of blood will vary from 0.2 ml to 1.0 ml, according to the size of the animals. Blood specimens will be centrifuged on site for 20 min at 5,000 rpm. Serum samples will be transported at -20 ^{o}C and stored at -70 ^{o}C. Saliva and urine will be obtained by swabbing. These samples will be stored in tubes containing 1.0 ml of NucliSens lysis buffer containing guanidine

thiocyanate (Biomerieux, Boxtel, The Netherlands) for transport and storage. Specimens will be kept at 2-8 ^{o}C in the field. Upon arrival at the laboratory, swabs will be thoroughly mixed by vortex and applied tightly to the tube walls; the liquid from approximately 10 individual samples from the same species, colony and the time of capture will be saved into the same pool. The pooled specimens will be frozen to -70^{o}C until analysis.

6. Detection of Nipah virus antibodies: IgG antibodies to NV will be assayed by indirect ELISA at Chulalongkorn University Hospital, using a protocol developed by the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia. NV antigen will be extracted from infected Vero E6 cells with detergent, and inactivated by gamma irradiation ($5x10^{6}$ rad). Sera will be tested against both NV and a negative control antigen extracted from uninfected E6 cells given the same irradiation dosage. Antigen will be used at a dilution of 1:2,000 as determined by checkerboard titration against known positive samples which has already been determined by CDC, USA. Bat sera will be diluted with serum diluent containing 5% skim milk (Difco, Becton Dickinson, MD, USA) by 4-fold dilution from 1:100 to 1:6400. IgG in bats will be detected by using protein A/G conjugated with horseradish peroxidase (HRPO) (Pierce Biotechnology, Inc. IL, USA) at a dilution of 1:1,500. ABTS [2, 2'-azino-bis-(3-benzthiazoline-6-sulfonic acid)] (KPL, Inc. MD, USA) will be used as a substrate in the detection step. The resultant color, in the presence of HRPO, is intense blue-green, and the optical density (OD) will be read at a wavelength of 410 nm. The OD values of the mock antigen will be subtracted from those of positive antigen to give an adjusted OD value.

7. Detection of Nipah virus RNA: Duplex - Nested RT-PCR (duplex nRT-PCR): Total RNA will be extracted from saliva and urine swabs by using the silica-guanidine thiocyanate protocol, NucliSense Isolation Reagent (Biomerieux, Boxtel, The Netherlands). After being washed with 70% ethanol, followed by acetone, the silica particles will be dried at 56 ^{o}C, the RNA will be eluted in 50 ul of elution buffer at the same temperature and stored at -70 ^{o}C. A non infectious NV cell lysate will be used as control and RNA will be extracted as described above. The RNA plasmid, 1.2 kb Kanamycin (Promega,Madison, Wis) will be introduced as internal control (IC) RNA in the duplex RT-PCR mixture containing both NV and IC primers. This will be performed by adding 2000 molecules of RNA plasmid prior to single step RT-PCR reaction.

NV nucleoprotein (N)-specific primers for reverse transcription and PCR are modified from previous data (3). The external primers for first round PCR are as follows: NP1F, 5' CTT GAG CCT ATG TAT TTC AGA C 3'; NP1R, 5' GCT TTT GCA GCC AGT CTT G 3'. The NV internal primers for nested PCR are NP2F 5' CTG CTG CAG TTC AGG AAA CAT CAG 3' and NP2R, 5'ACC GGA TGT GCT CAC AGA ACT G 3'. Preparation of external and nested IC primers follows previously described protocol (14), CONINT1F (CTG GCC TGT TGA ACA AGT CT) and CONINT1R (GAT CTG ATC CTT CAA CTC AGC) are used as external and 1UPS (GCC ATT CTC ACC GGA TTC AGT CGT C) and 1DS (AGC CGC CGT CCC GTC AAG TCA G) as nested primers. To allow the amplification of the IC-RNA to proceed in the same tube without inhibiting NV amplification, varieties of ratio between IC-RNA and IC primer will be assessed. This will allow an IC to be

visualized as the upper and NV products as lower bands (of 323 and 227 bp respectively).

Single-step RT-PCR will be performed using the One Step RT-PCR kit (Qiagen Inc., CA, USA). Five ul of extracted sample and 2 ul of IC RNA (total of 2,000 molecules) will be added to a final volume of 50 ul. The 1xRT-PCR buffer contained 2.5 mM $MgCl_2$, 400 uM each dATP, dCTP, dGTP and dTTP, 0.6 uM of NP1F and NP1R primers and 0.2 uM of CONINT1F and CONINT1R primers, 10U of RNase inhibitor and 2 ul of One Step RT-PCR enzyme mix. The amplification will be performed on a MJ Research PTC100 thermal cycler (GMI, Inc. Ramsey, Minnesota, USA). The thermocycler will be programmed for both reverse transcription and PCR as follows: 30 min at 50^{o}C for reverse transcription followed by 15 min at 95^{o}C for HotStarTaq DNA polymerase activation and reverse transcriptase inhibition, and 30 repetitive cycles of 1 min of denaturation at 94^{o}C, 1 min of annealing at 55 ^{o}C and 1 min of elongation at 72^{o}C. Elongation will be extended for 10 additional min in the last cycle.

For nested PCR, 1ul of the primary amplification products will be added to a new PCR mixture to a final volume of 50 ul of 1X magnesium-free PCR buffer, 2 mM $MgCl_2$, 0.2 mM each dATP, dCTP, dGTP and dTTP, 0.6 mM of NP2F and NP2R primers, 0.2 mM 1UPS and 1DS primers, 2.5 U of Taq DNA polymerase (Promega). The thermal profile will be performed by 5 min denaturation at 94 ^{o}C and 35 repetitive cycles of 30 s of denaturation at 94^{o}C, 30 s of annealing at 55 ^{o}C and 1 min of elongation at 72^{o}C. Elongation will be extended for 10 additional min in the last cycle. The 15ul of PCR product will be sized by gel electrophoresis in 2 %

agarose containing 0.5 ug/ml of ethidium bromide in TBE (Tris-borate-EDTA) buffer and observed under UV light. Standard precautions will be taken to avoid carryover contamination. Pipetting will be performed with aerosol-resistant tips, and different biosafety cabinets will be used for master mix preparation, sample and extract handling, and nested reaction. Product detection will be undertaken in a different laboratory room.

All samples with positive results will be tested again without IC-RNA and primers. A single band of PCR products will be gel purified and extracted with the QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA, USA). Sequencing of amplified products will be performed using primers NP2F and NP2R by Applied Biosystems 310 DNA sequencer (Applied Biosystems, Foster City, CA, USA), according to the manufacturer protocol. Multiple sequence alignments were generated with Clustal X 1.81 program.

CHAPTER II

REVIEW OF RELATED LITERATURES

1. Theory and Concept

Several novel viruses associated with bats of the genus *Ptropus* (sub-order *Magachiroptra*) in Australia and Southeast Asia cause encephalitis as well as respiratory tract infection of varying degree in animals and humans (Appendix A, B). These viruses include Hendra and Nipah viruses (genus *Henipavirus*, family *Paramyxoviridae*) and Australian bat lyssavirus (genus *Lyssavirus*, family *Rhabdoviridae*). Generally, strategies for disease prevention and control in the spillover host are directed at minimizing direct or indirect contact with the natural host, improving farm-gate and on-farm biosecurity, and better disease recognition and diagnosis. Effective management strategies in natural host are predicated on an understanding of the ecology of the disease in the natural host, and the identification and avoidance of factors putatively associated with emergence, such as habitat loss, land use change and demographic shifts.

Nipah virus was first identified in 1999 as the primary etiologic agent in a major outbreak of encephalitis and pneumonia in pigs and humans in Malaysia (15, 16). Respiratory and neurological syndromes were seen in pigs (17). The predominant clinical syndrome in human was encephalitis, with clinical signs of fever, headache, myalgia, drowsiness and disorientation. Coma ensued in many cases within 48 hours after brain symptoms developed (5, 18). Approximately 1.1 million pigs were culled to contain the outbreak. Of 265 reported human cases, 106 were

fatal. Direct contact with infected pigs was identified as the predominant mode in causing human infection (3, 18).

Recently, NV has been established as the cause of fatal febrile encephalitis in human patients in Bangladesh during the winter of 2001, 2003, and 2004 (13, 19-21). An NV- like virus was identified as the cause of the outbreaks in 2001 and 2003 on the basis of serologic testing (13). Two outbreaks consisting of 48 cases of NV were detected in 2004 in 2 adjacent districts (30 km apart) of central Bangladesh (Rajbari and Faridpur) with a case fatality rate of nearly 75% (22). Although antibodies to NV were detected in fruit bats from the affected areas in 2004, an intermediate animal host was not identified, suggesting that the virus was transmitted from bats to humans. Human-to human transmission of NV was also documented during the Faridpur outbreak (20,21). Genetic characterization of new strain of NV isolated during the outbreak in Bangladesh in 2004 has been studied, and confirmed that NV was the etiologic agent responsible for these outbreaks (22).

Effective disease management requires an understanding in the epidemiology of the disease (knowledge of its cause, mechanisms in maintenance and transmission, host range of the etiologic agent, and the nature of host-pathogen relationship), an ability to detect disease (surveillance and diagnostic capabilities) and political/public/industry support. Broadly, current strategies for the management of the recently emerged bat-associated agents are directed at minimizing direct or indirect contact with the natural host, monitoring intermediate hosts, and improving biosecurity on farms and better disease recognition and diagnosis (23).

Preliminary wildlife surveillance revealed serological evidence of infection in four species of flying foxes, *Pteropus vampyrus, P.hypomelanus*

P.giganteus and *P.lylei* (20). The recent isolation of virus from *P.hypomelanus* and *P.lylei* strengthens the contention that flying foxes are a natural of host of NV (9, 25). Thailand is bordered by Malaysia in the south and Cambodia in the southeast. To date, there has been no NV infections in humans reported in Thailand. Surveillance in swine by enzyme linked immunoadsorbent assay (ELISA) showed negative results (26). Estimates suggest approximately 112 bat species in Thailand; 18 are fruit bats and 94 are insectivorous bats (27, Appendix C, D). Given the fact that NV has caused several outbreaks in the region, it is extremely important to obtain baseline data for surveillance, and to plan for future public health assessment of its impact.

2. Review of Related Literatures

2.1 Overview

NV is a newly recognized zoonotic virus “discovered” in 1999. It has caused diseases in humans and animals, through contact with infected animals. NV is closely related to another recently recognized zoonotic virus, Hendra virus (HV), which caused a first outbreak in Australia, in 1994 (28). Both viruses have been associated with the disease in humans and animals. Although only a few focal outbreaks were reported, the ability of these viruses to infect a wide range of hosts and to produce such an exceptional high fatalities in humans has made them a major public concern.

2.2 Virology

Result of genomic analysis of NV led to categorizing NV as virus in the family *Paramyxoviridae*, subfamily *Paramyxovirinae*. This has also been supported by other characteristics, such as their syncytial-formation, cytopathic effect in cell cultures, and their ultrastructural morphology (5). Its biological properties and genomic organization classify NV and the closely related Hendra virus, into a new genus of Henipavirus (Appendix E). NV contains a single-stranded (-) RNA of 18,246 nucleotides associated with the viral proteins of the replicative complex; the nucleoprotein (N), the phosphoprotein (P), and the polymerase (L) enclosed by a lipid bilayered envelope containing the attachment protein (G) and the fusion protein (F).

2.3 Natural host

Surveillance for the reservoir hosts involving domestic animals (eg. dog, cat, goat, chicken and fish) and wildlife (wild boar, rodent, bird and bat) was undertaken with special emphasis on fruit bats, given the fact that *Pteropid* bats were prime reservoir of HV (19,30) as well as their close phylogenetic relationship between NV and HV (3, 31). Sero-surveillance confirmed that domestic animals, notably dogs, cats, goats and horses were infected with the virus. Investigation showed that pigs were primarily infected with NV and then subsequently spread the virus to other species. However, all were “dead-end” host (32). No neutralizing antibody to NV was found in wildlife except for bats. These included *Pteropus hypomelanus, P.vampyrus, Cynopterus brachyotis, Eonycteris spelaea* and *Scotophilus kuhli* (24). NV was also successfully isolated from urine samples collected from island fruit bats (*P.hypomelanu*) and from partially eaten fruits (9). Sero-epidemiological survey of NV infection in bats was also done in other Asian countries; *P.lylei* and *P.giganteus* from Cambodia and Bangladesh respectively were species found infected with NV (10, 13), whereas no evidence of NV infection was found in fruit bats from Indonesia (33).

2.4 Outbreaks of NV

NV first came to light as the causative agent of a major disease outbreak in pigs and humans in Peninsula Malaysia between September 1998 and April 1999. This resulted in 265 human cases with 106 fatalities, and the eventual culling of more than one million of pigs (3, 5). Almost all patients were in close contact with pigs, especially those associated with pig farming or involved in the

transport or slaughter of pigs. The outbreak later spread to involve abattoir workers in Singapore, where pigs from Negri Sembilan (outbreak area in Malaysia) had been sent for slaughter (34, 35). A virus was first isolated from brain tissue of a fatal human case and found to be a novel agent closely related to HV (3, 5). The virus was called Nipah after Kampong Sungai Nipah (Nipah River village), the origin of the first outbreak (3, 36). Two encephalitis outbreaks, caused by Nipah/Hendra-like viruses, occurred in separate areas in western Bangladesh in 2001 and 2003. In contrast to previous experience in Australia, Malaysia and Singapore, in which no human-to-human transmission had occurred, outbreak in Bangladesh suggested the possibility of such transmission. Exposure to animals may have contributed to disease transmission to humans, nevertheless, the ultimate source of origin remained to be zoonotic reservoir in bats. During February 2004, WHO reported another outbreak of NV encephalitis which spread across 6 districts of Bangladesh and claimed 17 deaths of 23 human cases - a mortality rate of 74%. A second outbreak in the Faridpur district in Bangladesh killed 18 of a total of 30 cases - a 60% mortality rate (as of April 19th). Common signs of infection include flu-like symptoms (fever, headache, vomiting), seizures, loss of consciousness, and coma.

2.5 Clinical features

The symptoms of the illness may range from non-existent, to mild or moderate (such as, influenza like symptoms with high fever and muscle pain) and severe as in the case of encephalitis. NV causes severe and rapidly progressive encephalitis which carried a high mortality rate; among these patients, some also had significant pulmonary symptoms (18, 36-37). The incubation period ranged from 4

days to 2 months. The main presenting features were fever, headache, myalgia, dizziness, drowsiness and vomiting (5, 18, Appendix F). There was a significant association between the presence of virus in cerebrospinal fluid and mortality (3). Distinctive clinical signs include segmental myoclonus, areflexia and hypotonia, hypertension, and tachycardia. Initial cerebrospinal fluid examination was abnormal in 75% of patients. Serology was helpful in confirming the diagnosis. Magnetic resonance imaging (MRI) showed distinctive changes of multiple, discrete, and small high signal lesions, best seen with fluid-attenuated inversion recovery (FLAIR) sequences (38). The key pathological findings included systemic endothelial infection and cell damage accompanied by vasculitis with syncytia in affected vessels (39). Recent report showed that NV could also cause an unusual form of encephalitis which occurred as late as 24 months after an initial exposure or the initial manifestation (of either encephalitis or meningitis) which had already subsided. Although the mortality of such late onset or relapse form was much lower, its sequele was reported to be significant. MRI showed multifocal or scattered abnormalities confined to gray matter of cerebral cortices.

2.6 Treatment

No drug therapies have yet been proven to be effective in treating NV infection in humans. Treatment usually relies on providing intensive supportive care. Treatment with an antiviral agent, Ribavirin, was attempted in a group of these acute encephalitic patients in an open-label trial (40). The results suggested that the drug might reduce the mortality with no serious side effects.

2.7 Bats as reservoir of emerging infectious diseases

Bats are unique in terms of their response to viral infections; they may remain asymptomatic and can sustain viral infection in the absence of overt disease (28). Evidence of a wide range of viral infections, including many arboviruses, rhabdoviruses, arenaviruses, reoviruses, and paramyxoviruses has been identified in bat species (41). The emergence of HV and NV represents a quantum leap, thus, reflecting the significance of bat-related viruses to human and animal health. Bats are found throughout the world in tropical and temperate habitats. The approximately 925 species of living bats make up around 20% of all known living mammal species (42). In Thailand, 112 species of bats have been found, including 18 of *Pteropid* or fruit bats whereas the rest are insectivorous bats. It has been estimated that Thai bats comprise of 10 % of bat population all over the world (27). Despite alarming reports of NV or HV outbreaks in animals and humans in neighboring countries, there has not been yet a single case report of transmission from bat to animal or human in Thailand (Thai MOPH).

Survey of NV infection among Thai bats is essential in order to estimate risk of transmission to other species and potential risk of causing outbreaks. If there appear NV infection in Thai bats, it is also important to know its prevalence according to geographical regions. Survey will be conducted among several frugivorous (in particular, flying foxes) and insectivorous bat species in many parts of the country including those tourist spots. Data acquired should be essential resources in the preparation of practice guidelines for public and health professionals.

CHAPTER III

MATERIALS AND METHODS

1. Collection of Specimens

From March 2002 through February 2004, seventeen trips to 15 sites in 9 provinces throughout central, eastern and southern Thailand were made (Figure 2). Sites were chosen on the basis of local reports of known bat colonies and after investigation by National Park, Wildlife and Plant Conservation Department, Ministry of Natural Resources and Environment. These locations were known as tourist sites for viewing bats or as places for collection of bat feces to produce organic fertilizers. Larger fruit bats were mist-netted near sunset when leaving their roosts for night feeding or before dawn upon return. Insectivorous bats in caves were captured during the day using fine-mesh, long-handled butterfly nets. Thick leather gloves were worn when bats were handled and transferred into individual cotton pouches for transportation and processing.

Of 1304 bats collected, 12 different frugivorous or insectivorous species were identified (Figure 2). All bats appeared healthy. At least 112 bat species (>20 million) are believed to be present in Thailand, according to estimates from a National Park, Wildlife and Plant Conservation Department in 2003. Eighty five percent are insectivorous and the rest are frugivorous.

Captured bats were anesthetized by administering a 0.2- 0.5 mg intramuscular injection of ketamine hydrochloride. Animals were identified to sex and by species, based on gross morphology (43). Animals were marked by hair or claw

clipping. Blood, obtained by direct cardiac puncture (Figure 3), was transferred from the collecting syringe into 1.5 mL microtubes and stored in an icebox until centrifugation. Serum was frozen at -20°C during transportation and stored in a freezer at -70°C. Saliva and urine were obtained by swabbing with sterile cotton swabs (Figure 3-4). These samples were stored in tubes containing 1.0 ml of NucliSens lysis buffer containing guanidine thiocyanate (Biomerieux, Boxtel, The Netherlands) for transport and storage. Specimens were kept at 2-8 ^{o}C in the field. Upon arrival at the laboratory, swabs were thoroughly mixed by vortex and applied tightly to the tube walls; the liquid from approximately 10 individual samples from the same species, colony and the time of capture was saved into the same pool. The pooled specimens were frozen to -70^{o}C until analysis. After recovery from sedation, bats were allowed to fly to their roosts.

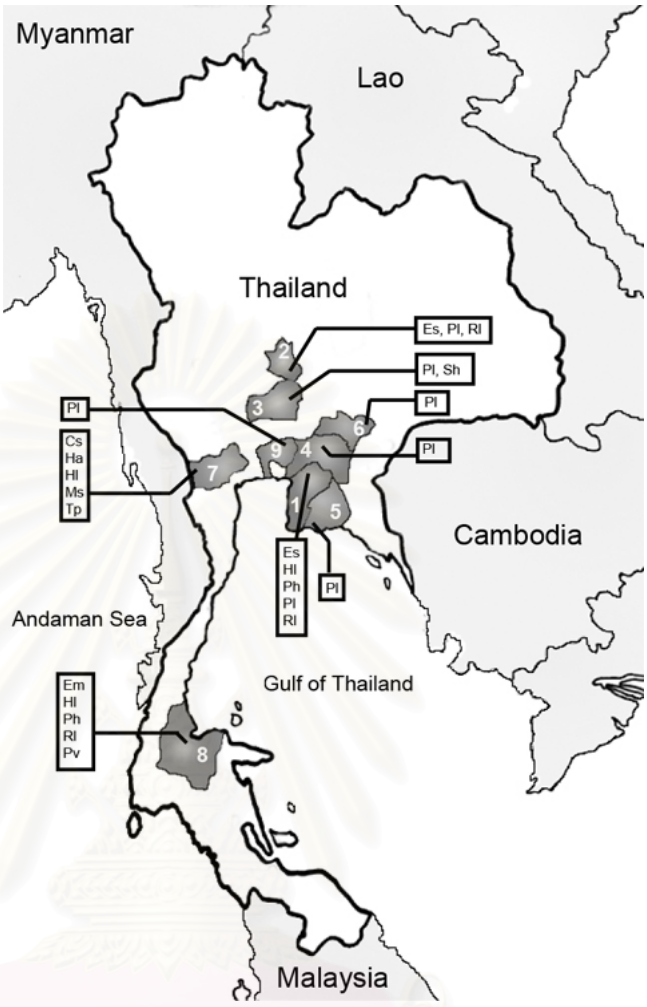


Figure 2. Map showing the localities in Thailand where bats have been captured. 1.Chon Buri (CB); 2 Sing Buri (SB); 3. Ayutthaya (AY); 4. Cha Choeng Sao (CC); 5. Ra Yong (RY); 6. Pra Chin Buri (PC); 7. Ratcha Buri (RB); 8. Surat Thani (SR); 9. Bangkok (BK). Species analyzed: Cs = *Cynopterus sphinx*, Em = *Emballonura monticola*, Es = *Eonycterus spelaea*, Ha = *Hipposideros armiger*, Hl = *Hipposideros lavatus*, Ms = *Megaduma spasma*, Ph = *Pteropus hypomelanus*, Pl = *Pteropus lylei*, Pv = *Pteropus vampyrus*, Rs = *Rousettus leschenault*, Sh = *Scotophilus heathi*, Tp = *Tadarida plicata*

Figure 3. Bats were capture by mist-net. The 12-15 meter high of mist-net were set near bat roost in the evening. Bats were mist-netted near sunset when leaving their roosts for night feeding or before dawn upon return.

Figure 4. Three types of specimens were collected from each bat. (A) Blood was collected by cardiac puncture and saliva was collected by swabbing from its mouth. (B) Bat urine was collected by swabbing on urethral opening; urination was usually evident following a 5-10 s irritation at such area.

2. Detection of Nipah virus antibodies

IgG antibodies to NV were assayed by indirect ELISA at Chulalongkorn University Hospital, using a protocol developed by the Center for Disease Control and Prevention (CDC), Atlanta, Georgia (Appendix G). An IgG ELISA was performed using a Nipah antigen extracted from infected Vero E6 cells with detergent, and inactivated by gamma irradiation ($5x10^6$ rad). Sera were tested against both Nipah antigen and a control antigen extracted from uninfected E6 cells given the same irradiation dose. Antigen was used at a dilution of 1:2,000 as determined by checkerboard titration against known positive samples. IgG in bat was detected using a recombinant chimeric protein antigen (Protein A/G) conjugated with horseradish peroxidase (HRPO), which detected only IgG subclasses of antibody. All conjugates were used at dilutions determined by checkerboard titration with known positive sera for each species.

2.1 **Protocol for NV IgG ELISA assay**

Apply Antigen

1. Add 100 ul antigen diluted in coating solution (PBS, PH 7.4) to a-d rows and control antigen in e-h rows.
2. Incubate overnight at 4 ^{o}C.
3. Wash 3 times with wash buffer (PBS with Tween -20 (0.1%), pH 7.4)
4. Empty plate and tap out residual liquid.

Test sera (with positive and negative controls)

1. Dilute primary serum to 1:25 with master plate diluent (5% skim milk in PBS, pH 7.4). Heat inactivate at 56 ^{o}C for 30 min.
2. Add 300 ul blocking solution serum diluent (wash buffer with 5% skim Milk, pH7.4) to each well.
3. Add 33 ul diluted primary serum antibody to first row (a-row for NV antigen and e-row for control antigen). Prepare a 1:100 dilution.
4. Prepare 4 fold dilution from 1:100 to 1:6,400 (from a- to d- rows or e- to h- rows).

Reaction with Primary Antibody

1. Incubate 1 hour at 37 ^{o}C.
2. Empty plate, tap out residual liquid.

Wash Procedure

1. Fill each well with wash buffer.
2. Aspirate plate to empty.
3. Repeat 3 times. Tap out residual liquid

Secondary Antibody Solution

1. Add 100 ul diluted Protein A/G HRPO conjugated to each well.

2. Incubate 1 hour at 37 ^{o}C.

3. Empty plate and wash 3 times.

4. Tap residual liquid from plate.

Reaction with Substrate

1. Dispense 100 ul ABTS / H_2O_2substrate into each well.

2. Incubate at 37 ^{o}C for 30 min.

3. Read plate with plate reader at 410 nm.

2.2 Interpretation of IgG assay

The OD values of the mock antigen were subtracted from those of positive antigen to give a “net” positive or adjusted OD value. Cut-off OD for each adjusted OD is 0.2. Titer was assigned to the serum based on the last dilution which was counted as positive. Sera with titer less than 1:400 were considered as “not positive”. Sum of ODs through the dilution series was also used as a measure of reactivity. In IgG assay, an adjusted OD sum of 0.95 was used as the cutoff for consideration of positivity.

3. Detection of NV RNA

3.1 Duplex nested reverse transcription (duplex nRT-PCR) technique for detection of NV RNA

An internal control system was included in RT-PCR method to avoid false-negative results due to handling errors or the presence of enzyme inhibitors. The RNA plasmid was introduced as internal control (IC) in the duplex RT-PCR mixture containing both nipah primers and IC primers.

3.1.1 Nipah virus and internal control (IC) RNA

Non infectious nipah virus cell lysate was kindly provided by Dr. Pierre E Rollin from Center for Disease Control and Prevention (Atlanta, Ga) and RNA was extracted using NucliSens extraction kit (BioMerieux). 1.2kb Kanamycin positive control RNA (Promega, Madison, Wis) was used to check for the presence of PCR inhibitor. This was performed by adding 2,000 molecules directly to single step RT-PCR reaction.

3.1.2 Primer design

Nipah virus N-specific primers as described by Dr. Chua et al (2000) were used as nested primers, NP2F position 1290-1313 and NP2R position 1495-1516. External primers were designed from GenBank according to Nipah virus complete genome accession number NC_002728 (NP1F position 1175-1196 and NP1R position 1554-1572). IC external and nested primers were prepared as previously described by Echevarria et al (2001). Briefly, CONINT1F and CONINT1R were used as external and 1UPS and 1DS as nested primers.

Primer name	5----->3
NP1F	CTT GAG CCT ATG TAT TTC AGA C
NP1R	GCT TTT GCA GCC AGT CTT G
NP2F	CTG CTG CAG TTC AGG AAA CAT CAG
NP2R	ACC GGA TGT GCT CAC AGA ACT G
CONINT1F	CTG GCC TGT TGA ACA AGT CT
CONINT1R	GAT CTG ATC CTT CAA CTC AGC
1UPS	GCC ATT CTC ACC GGA TTC AGT CGT C
1DS	AGC CGC CGT CCC GTC AAG TCA G

3.1.3 Duplex nRT-PCR and detection

Single-step RT-PCR was performed using the One Step RT-PCR kit (Qiagen Inc., Valencia, CA, USA). Five ul of extracted samples and 2 ul of IC RNA (total of 2,000 molecules) were added to a final volume of 50 ul of 1xRT-PCR buffer containing 2.5 mM $mgCl_2$, 400 uM each dATP, dCTP, dGTP and dTTP, 0.6 uM of NP1F and NP1R primers and 0.2 uM of CONINT1F and CONINT1R primers, 10U of RNase inhibitor and 2 ul of One Step RT-PCR enzyme mix. The amplification was performed on PTC100 (MJ Research, Watertown, Mass.) thermal cycler, programmed for both reverse transcription and PCR as follows: 30 min at 50^{o}C for reverse transcription followed by 15 min at 95^{o}C for HotStarTaq DNA polymerase activation and reverse transcriptase inhibition, and 30 repetitive cycles of 1 min of denaturation at 94^{o}C, 1 min of annealing at 55 ^{o}C and 1 min of elongation at 72 ^{o}C. Elongation was extended for 10 additional min in the last cycle.

For nested PCR, 1ul of the primary amplification products were added to a new PCR mixture to a final volume of 50 ul of 1X magnesium-free PCR buffer, 2 mM $MgCl_2$, 0.2 mM each dATP, dCTP, dGTP and dTTP, 0.6 mM of NP2F and NP2R primers, 0.2 mM 1UPS and 1DS primers, 2.5 U of Taq DNA polymerase (Promega, Madison, Wis). The thermal profile was as following orders: 5 min denaturation at 94 ^{o}C and 35 repetitive cycles of 30 s of denaturation at 94^{o}C, 30 s of annealing at 55 ^{o}C and 1 min of elongation at 72^{o}. Elongation was extended for 10 additional min in the last cycle.

The 15ul of PCR product was sized by gel electrophoresis in 2 % agarose containing 0.5 ug/ml of ethidium bromide in TBE (Tris-borate-EDTA) buffer and seen under UV light.

3.1.4 Interpretation

Samples showing both the 323-bp IC band and 227-bp Nipah virus specific band were considered as positive; those showing only IC band was judged negative; those showing no band were tested again and considered to contain enzyme inhibitors if no band was shown on repetition.

3.1.5 Duplex reaction optimization

To allow the amplification of the IC RNA to proceed in the same tube without inhibiting Nipah virus amplification a variety of IC RNA amount and ratios of IC and Nipah primers were assessed. Optimal condition was obtained at 2,000 molecules of IC with a ratio 1:3 of IC and Nipah primer ratio. These allowed the lower, Nipah-specific, band of 227 bp to be visualized and the upper, IC-specific, band of 323 bp was always detectable.

3.1.6 Sensitivity of the duplex and uncoupled nRT-PCR

Nipah RNA was serially 10-fold diluted of and RT-PCR was performed. For the Nipah/IC duplex reaction, 2 ul of 1,000 molecules IC RNA was added to 5 ul of diluted Nipah RNA as above, whereas 2 ul of DNase, RNase free water was added instead of IC RNA in uncoupled reaction. Duplex and uncoupled RT- and nested PCR were carried out as described in section 3.1.3, with an exception that only Nipah primers were present at a concentration of 1.0 uM in both RT-PCR and nested PCR steps.

3.1.7 Limit of detection of duplex nRT-PCR

Three dilutions of NV RNA from extracted cell lysate (3.7, 0.37, 0.037 pg/ul total RNA), were amplified by duplex nRT-PCR. The limit of detection of this technique was determined by visualized band intensity of PCR products under UV light.

3.1.8 Specificity of duplex nRT-PCR

The specificity of duplex nRT-PCR was tested with clinical samples from encephalitis patients collected at Molecular Biology Laboratory for Neurological Diseases, Chulalongkorn University Hospital. These samples had been tested and confirmed to contain enterovirus, Epstein-Barr virus (EBV), cytomegalovirus (CMV), varicella-zoster virus (VZV) and herpes simplex virus (HSV).

3.2 RNA Extraction

Total RNA was extracted from saliva and urine swabs by using the silica-guanidine thiocyanate protocol, NucliSense Isolation Reagent (Biomerieux, Boxtel, The Netherlands). Contents of 10 tubes containing 1 ml saliva or urine swab in lysis buffer were pooled. 50 ul of silica suspension was added to target RNA lysis buffer tube and incubated at room temperature for 10 min. Lysis buffer tubes were centrifuged at 2,000 G for 2 min and the supernatant was removed. After being washed 2 times with wash buffer and 2 times with 70% ethanol, followed by 1 time with acetone, the silica particles were dried at 56 ^{o}C. The nucleic acid was eluted in 50 ul of elution buffer at the same temperature and stored at -70 ^{o}C until analysis.

3.3 NV RNA detection from bat specimens by duplex nRT-PCR

Extracted nucleic acid from animal specimens was assayed for NV RNA by duplex nRT-PCR method as described above. Standard precautions were taken to avoid carryover contamination. Pipetting was performed with aerosol-resistant tips, and different biosafety cabinets were used for master mix preparation, sample and extract handling, and nested reaction. Product detection was undertaken in a different laboratory room. Samples showing both the 323-bp IC and 227-bp NV specific bands were considered positive; those showing only the IC band were considered negative; those showing no band were tested again and judged to contain enzyme inhibitors, if no band was shown on repetition. These invalid RNA specimens were further diluted to 1:5 and re-amplified. If the IC band was visualized, it confirms that these specimens contain inhibitors. The purpose of having this internal control

system was to assure the quality of the test. All samples with positive results were tested again without IC-RNA and primers for sequencing analysis.

3.4 Sequencing of positive PCR products

NV amplified product of 181- bp bands were sequenced for NV identification. A single band of PCR product was gel purified and extracted with the QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA, USA). Gel slices were dissolved in a solubilization buffer containing a pH indicator, and the mixture was applied to the MinElute spin column. Nucleic acids were adsorbed to the silica-gel membrane in the high-salt conditions provided by the buffer. Impurities were washed away and pure DNA was eluted with a small volume (10-20 ul) of low-salt buffer. Sequencing of amplified product was performed with the ABI PRISM big dye sequencing kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer indications. Both forward and reverse strings were sequenced using NP2F and NP2R as sequencing primers, respectively. Sequencing reactions were performed in a PTC100 (MJ Research, Watertown, Mass.) thermal cycler; program consisted of a first-denatueation cycle of 3 min followed by 25 cycles of 10 s of denaturation at 96 ^{o}C, 10 s of annealing at 50 ^{o}C, and 4 min of elongation at 60 ^{o}C. Products were purified by subsequent 80 and 70 % ethanol precipitations. Final products were run on an ABI 310 DNA sequencer (Applied Biosystems). Forward and reverse strands were compared and aligned by Multiple sequence alignments generated with Clustal X 1.81 program (15).

CHAPTER IV

RESULTS

1. Collection of Specimens

Of the 1304 captured bats of 12 different species, six were frugivorous and 6 were insectivorous bats (Figure 2). Seventy-one percent belonged to *Pteropus* bats (932); of which 66% (857) were *P.lylei* (Table 1). *P.lylei* was captured from all sites except Ratcha Buri (RB) and Surat Thani (SR) (Figure 2). Seventeen field trips were made to collect samples from 15 different sites; one site in Chon Buri (CB3) was revisited one year later (February 10-12, 2003 and February 18-20, 2004). 1,054 blood specimens were obtained from a total of 1,304 bats. The remaining could not be obtained due to the small size of bats. A total of 142 pools each were collected from 1,286 saliva and 1,282 urine specimens, respectively.

Table 1. Assay of ELISA, saliva-PCR and urine-PCR for Nipah virus from 12 bat species, Thailand, 2002-2004

Specie	Total bat	ELISA		PCR-saliva *		PCR-urine*	
		No.analyzed	No.positive (%) †	No.analyzed	No. pool positive/total	No.analyzed	No. pool positive/total
Frugivorous							
Cynopterus sphinx	34	10	0	34	0/5	34	0/5
Eonycterus spelaea	64	54	0	64	0/7	64	0/7
Pteropus hypomelanus	36	26	4(15.4)	36	0/6	35	0/6
Pteropus lylei	857	813	76(9.3)	845	1/87	845	6/87
Pteropus vampyrus	39	39	1(2.6)	39	0/4	39	0/4
Insectivorous							
Emballonura monticola	14	12	0	14	0/2	14	0/2
Hipposideros armiger	88	6	0	88	0/10	88	0/10
Hipposideros larvatus	95	74	1(1.3)	94	1/10	91	0/10
Megaduma Spasma	13	0	0	13	0/2	13	0/2
Rousettus leschenault	11	4	0	6	0/3	6	0/3
Scotophilus heathi	3	3	0	3	0/1	3	0/1
Tadarida plicata	50	13	0	50	0/5	50	0/5
Total	1304	1054	82(7.8)	1286	2/142	1282	6/142

† ELISA Positve: titer ≥ 400

* 10 individual samples (saliva or urine) from the same species, colony and the time of capture were saved into the same pool.

2. Serologic testing for NV IgG antibodies

Of the 1054 sera tested, 82 (7.8%) were NV IgG antibody positive at titers 1:400 or higher (Table 1). NV antibodies were demonstrated among 4 bat species (3 frugivorous; *P. hypomelanus*, *P. lylei*, *P. vampyrus* and one insectivorous, *Hipposideros lavatus*). *P.lylei* was found to have higher serum antibody titers than other species (9 at 1:6400, 29 at 1:1600) (Table 2). NV antibodies were more commonly found in *P. hypomelanus* (4 of 26, 15.4%) (Table 1). As many as 21.4% of antibody positive bats were from Pra Chin Buri province, whereas 11% each were from Chon Buri and Cha Choeng Sao, the nearby provinces in the eastern part of Thailand (Table 3). A lesser prevalence rate was found in Bangkok (6.5%), Ayutthaya (4.4%), and Surat Thani (4.1%). Based on analysis of bat colony sites, a similar prevalence was found for *P.lylei* at Pra Chin Buri (21.4%), and for *P.hypomelanus* at Surat Thani-site 2 (21%) (Figure 5). No bats from Sing Buri, Ratcha Buri and Ra Yong provinces were found antibody-positive, however, these data might be inconclusive due to the small number size.

Table 2. Titers of ELISA positive bats sera

Species	No.analyzed	No.positive (%)	ELISA titer		
			1:400	1:1600	1:6400
Hipposideros larvatus	74	1(1.3)	1	0	0
Pteropus hypomelanus	26	4(15.4)	3	1	0
Pteropus lylei	813	76(9.3)	38	29	9
Pteropus vampyrus	39	1(2.6)	1	0	0

Table 3. Result of ELISA assay for antibody to NV in serum, PCR for NV RNA in saliva and urine of bats surveyed in 9 provinces, Thailand, 2002-2004

Province ¶	No.	ELISA		PCR-saliva*		PCR-urine*	
		No.analyzed	No.positive (%) †	No.analyzed	No. pool positive/total	No.analyzed	No. pool positive/total
RB	194	29	0	194	0/23	194	0/23
CB	396	381	42(11.0)	389	2 /43	386	4 /43
SB	99	63	0	93	0/11	92	0/11
SR	121	121	5(4.1)	121	0/14	121	0/14
AY	245	227	10(4.4)	243	0/26	243	0/26
CC	110	108	12(11.1)	110	0/11	110	1/11
PC	28	28	6(21.4)	28	0/3	28	0/3
BK	108	107	7(6.5)	108	0/11	108	1/11
RY	3	1	0	0	0/0	0	0/0
Total	1304	1054	82(7.8)	1286	2/142	1282	6/142

¶ Province:CB = Chon Buri, SB = Sing Buri, AY = Ayutthaya, CC = Cha Choeng Sao, RY = Ra Yong, PC = Pra Chin Buri,RB = Ratcha Buri, SR = Surat Thani, BK = Bangkok

† ELISA Positve: titer ≥ 400

* 10 individual samples (saliva or urine) from the same species, colony and the time of capture were saved into the same pool.

Year	ELISA result (no.positive/no.bats analyzed)											
2002			CB1*(Es)0/28 **CB1(Hl)1/38** CB1(Ph)0/0 CB1(Rl)0/0	SB(Es)0/26 SB(Pl)0/37 SB(Rl)0/0		SR1(Em)0/12 SR1(Hl)0/36		CB2† (Ph)0/7 **AY1(Pl)6/155** AY1(Sh)0/3		**SR2(Ph)4/19** SR2(Rl)0/4	**AY2(Pl)4/69**	
2003	**CC(Pl)12/108**	**CB3(Pl)12/152** RY(Pl)0/1		RB1(Cs)0/0 RB1(Ha)0/0 RB1(Ms)0/0 **PC(Pl)6/28**			RB2(Cs)0/3 RB2(Ha)0/2 RB2(Hl)0/0		RB3(Cs)0/7 RB3(Ha)0/4 RB3(Tp)0/13 **BK(Pl)7/107**	**SR3(Pv)1/39**		
2004		**CB3(Pl)29/155**										
Month	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Year	PCR result (pool positive of either saliva (s) or urine (u) source/total pools of saliva or urine)											
2002			CB1(Es)0/3 **CB1(Hl)1s/5** CB1(Ph)0/1 CB1(Rl)0/1	SB(Es)0/4 SB(Pl)0/6 SB(Rl)0/1		SR1(Em)0/2 SR1(Hl)0/4		CB2(Ph)0/2 AY1(Pl)0/17 AY1(Sh)0/1		SR2(Ph)0/3 SR2(Rl)0/1	AY2(Pl)0/8	
2003	**CC(Pl)1u/11**	CB3(Pl)0/16 RY(Pl)0/0		PC(Pl)0/3 RB1(Cs)0/1 RB1(Ha)0/4 RB1(Ms)0/2			RB2(Cs)0/2 RB2(Ha)0/5 RB2(Hl)0/1		**BK(Pl)1u/11** RB3(Cs)0/2 RB3(Ha)0/1 RB3(Tp)0/5	SR3(Pv)0/4		
2004		**CB3(Pl)1s/15 and 4u/15**										

Figure 5. Mapping of bat sample collection and Nipah virus detection results during Mar 2002-Feb 2004.

Species analyzed; Cs=Cynopterus sphinx, Em=*Emballonura monticola*,Es=*Eonycterus spelaea*, Ha=*Hipposideros armiger*, Hl=*Hipposideros lavatus*, Ms=*Megaduma spasma*, Ph=*Pteropus hypomelanus*, Pl=*Pteropus lylei*, Pv=*Pteropus vampyrus*, Rs=*Rousettus leschenault*, Sh=*Scotophilus heathi*, Tp=*Tadarida plicata* . Province: CB=Chon Buri, SB=Sing Buri, AY=Ayutthaya, CC=Cha Choeng Sao, RY=Ra Yong, PC=Pra Chin Buri,RB=Ratcha Buri, SR=Surat Thani, BK= Bangkok. * Number indicated site number in survey. † CB2 located in the same island as CB1 but different location.

3. Duplex nRT-PCR: Optimization

The IC amount and IC primer concentration were varied in the process of optimization. Higher concentration of IC RNA interfered with NV RNA amplification (Figure 6) whereas lesser quantity yielded an unreliable result (Figure 7). The appropriate IC RNA amount was 2,000 molecules per RT-PCR reaction (Figure 8). The optimized NV primer concentration was 0.6 uM. Two IC primer concentrations (0.2 and 0.4 uM) were compared, 0.2 uM was chosen for duplex reaction based on the band intensity. The lower IC band intensity shown in 0.2 uM (Figure 8, lane 1) thus, exerting less competitive effect as compared to 0.4 uM IC concentration (Figure 8, lane 2).

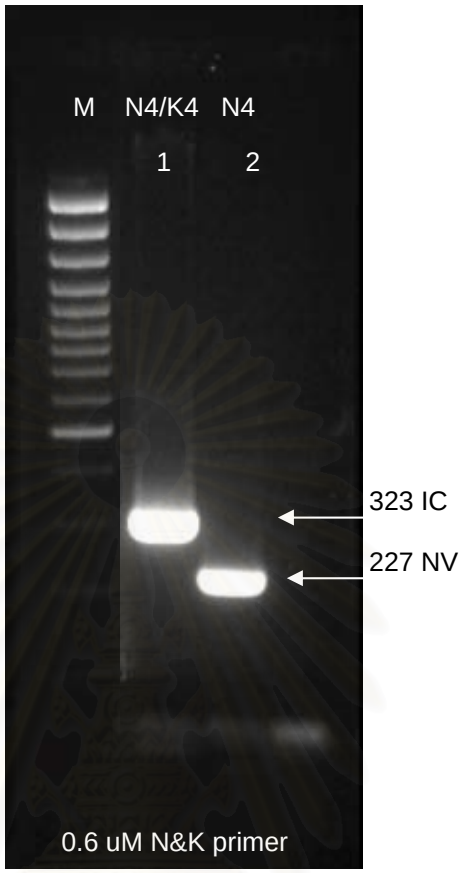


Figure 6. Optimization of IC concentration for duplex nRT-PCR. 4000 molecules of IC RNA were co-amplified in duplex reaction containing NV RNA at dilution $1:10^4$ (lane 1). Only NV RNA at the same dilution was amplified in nRT-PCR (lane2). NV primer concentration was 0.6 uM in both reactions. The upper band (323 bp) was the internal control band. The lower band (227 bp) was the Nipah virus-specific band. M represented 100 bp ladder markers.

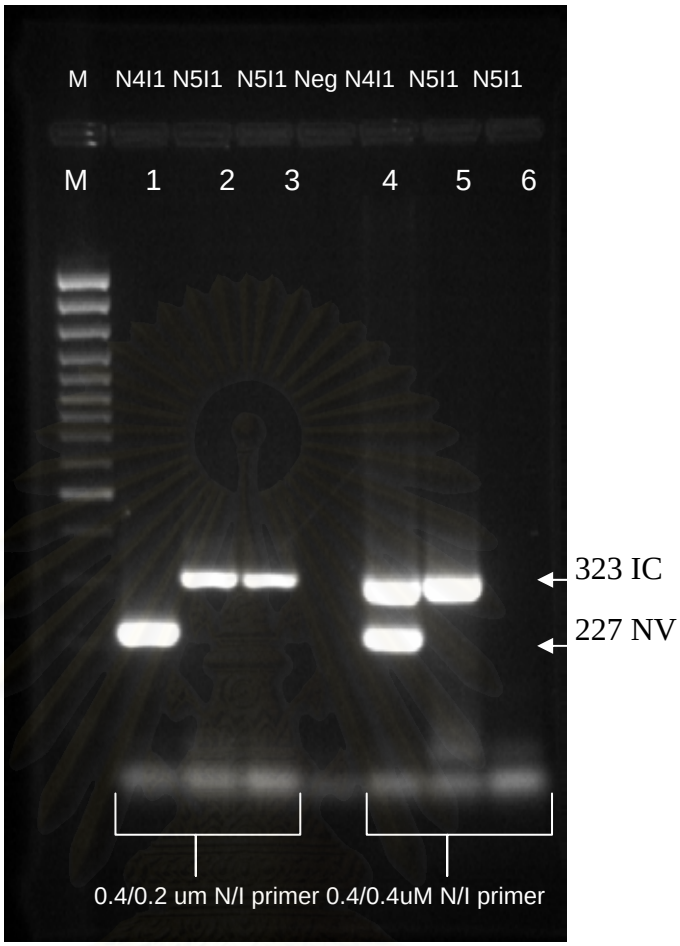


Figure 7. Optimization of IC concentration for duplex nRT-PCR. 1000 molecules of IC RNA were co-amplified in duplex reaction containing NV RNA at dilution $1:10^4$ (lanes 1 and 4) and $1:10^5$ (lanes 2-3 and 5-6). Lanes 1-3 contained 0.2 uM of IC RNA and lanes 4-6 contained 0.4 uM IC primer concentration. NV primers concentration was 0.4 uM in all reactions. The upper band (323 bp) was the internal control band. The lower band (227 bp) was the Nipah virus-specific band. M represented 100 bp ladder markers. Negative was duplex nRT-PCR without NV RNA template.

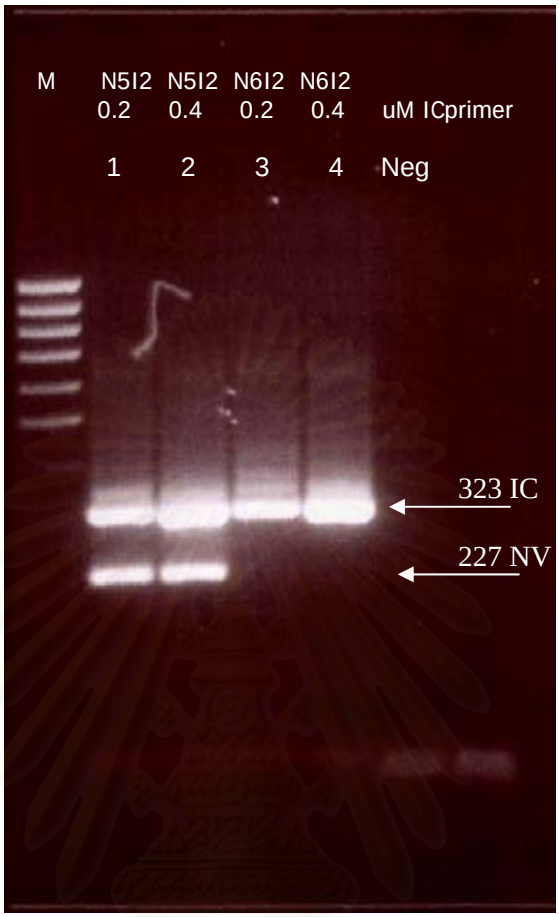


Figure 8. Optimization of IC concentration for duplex nRT-PCR. 2000 molecules of IC RNA were co-amplified in duplex reaction containing NV RNA at dilution $1:10^5$ (lanes 1-2) and $1:10^6$ (lanes 3-4). Lanes 1 and 3 contained 0.2 uM of IC primer and lanes 2 and 4 contained 0.4 uM IC primer concentration. NV primers concentration was 0.6 uM in all reactions. The upper band (323 bp) was the internal control band. The lower band (227 bp) was the Nipah virus-specific band. M represented 100 bp ladder markers. Negative was duplex nRT-PCR without NV RNA template.

4. Duplex nRT-PCR: assessment of limit of detection

To ensure the limit of detection of duplex nRT-PCR, comparison was made between duplex and uncoupled RT-PCR using extracted NV RNA from 10^{-4}-10^{-6} (Figure 9). Two ul of water was added in standard RT-PCR instead of IC RNA in duplex RT-PCR. The limit of detection of the duplex system was not notably altered by incorporation of the IC RNA. A similar lowest detection limit (at $1:10^5$, 0.37 pg/ul total RNA) could be obtained in both duplex and uncoupled systems (lanes 3 and 6 in figure 9, respectively). Lesser density of a 227 bp band in lanes 2 and 3 was found as compared to lanes 5 and 6, suggesting some degree of interference from IC RNA.

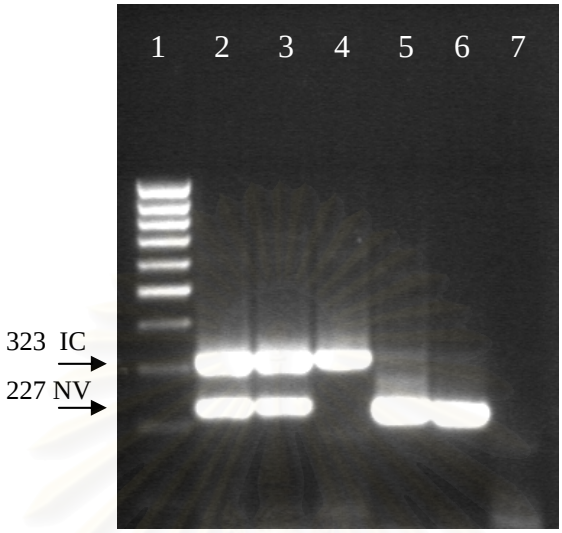


Figure 9. Determination of the limit of detection of amplification by duplex nested RT-PCR (lanes 2-4) and uncoupled RT-PCR (lane5-7). Serial 10 fold dilution of a NV RNA was made to a dilution of 10^6 (0.037 pg/ul total RNA). The upper band (323 bp) was the internal control band. The lower band (227 bp) was the Nipah virus-specific band. Lanes 2-4 and 5-7 were samples at the dilutions of 10^4 - 10^6, respectively; lane1, 100 bp ladder marker.

5. Duplex nRT-PCR: assessment of specificity

The specificity of duplex nRT-PCR for NV was determined as tested among viral encephalitis specimens of different etiologies (figure 10). All ten reactions were valid duplex nRT-PCR; IC bands were shown in all reactions. From these results it could be concluded that there was no cross reaction in duplex nRT-PCR system. Negative results were found when assessed with 5 documented clinical specimens containing enterovirus, Epstein-Barr virus (EBV), cytomegalovirus (CMV), varicella-zoster virus (VZV) and herpes simplex virus (HSV).

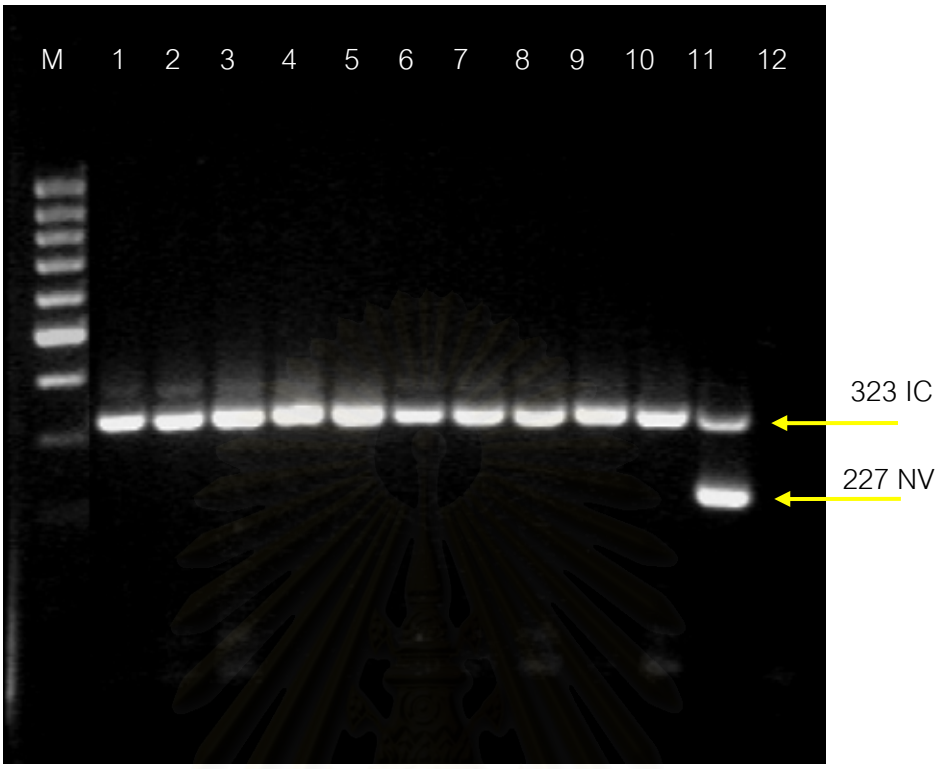


Figure 10. Results of the duplex nRt-PCR test on known viral positive clinical specimens other than NV. Lanes 1; enterovirus positive CSF, lane 2; EBV positive plasma, lane 3; CMV positive CSF, lane 4; VZV positive CSF, lane 5; HSV positive CSF, lane 6; CMV positive plasma, lane 7; EBV positive plasma, lane 8; VZV positive CSF, lane 9; enterovirus positive CSF, lane 10; HSV positive CSF, lane 11; NV positive control, lane 12; negative control. The upper band (323 bp) was the internal control band. The lower band (227 bp) was the Nipah virus-specific band. M represented 100 bp ladder markers.

6. Detection of NV RNA in Bats

Of 1,286 saliva and 1,282 urine specimens, 142 pools of each were obtained. They were analyzed by duplex nRT-PCR (Figure 11). One saliva pool of *H. lavatus* from Chon Buri province- site 1 (CB1) and another pool of *P. lylei* from Chon Buri- site 3 (CB3) samples were duplex nRT-PCR positive (Table 1-2). All 6 positive duplex nRT-PCR urine pools were collected from *P.lylei* captured from 3 different sites, 1 from Cha Choeng Sao, 1 from Bangkok and the other 4 from Chon Buri-site 3 (Table 1-2). An inhibitory reaction (for example, lane 9 in Figure 11) resulting in an absence of the IC band, was found in reactions performed with extracted RNA from 13 saliva and 9 urine pools. Dilution of extracted RNA with water to 1:5 dilutions alleviated this problem (Figure 12). IC band was visualized after dilution with water, confirming that these specimens contained inhibitors and this internal control system was helpful in assuring the quality of the test.

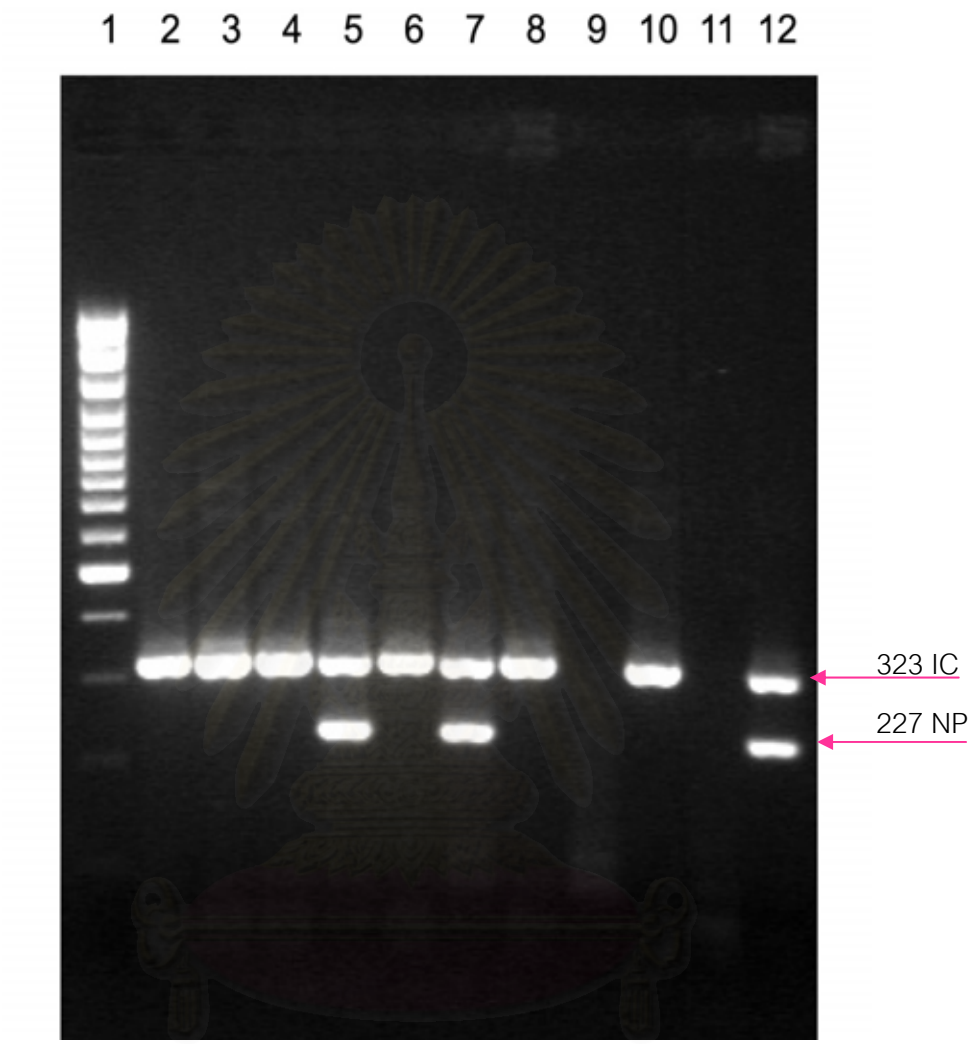


Figure 11. Duplex nRT-PCR of bat samples. The upper band (323 bp) was the internal control band. The lower band (227 bp) was the Nipah virus-specific band. Lanes 2-5 were saliva samples and 6-10 were urine samples. Lanes: 1, 100 bp ladder marker; 2-4, 6, 8, 10 were Nipah virus-negative, lane 5, 7 were Nipah virus-positive, lane 9 had enzyme inhibitors, and lanes 11 and 12 were negative and positive controls.

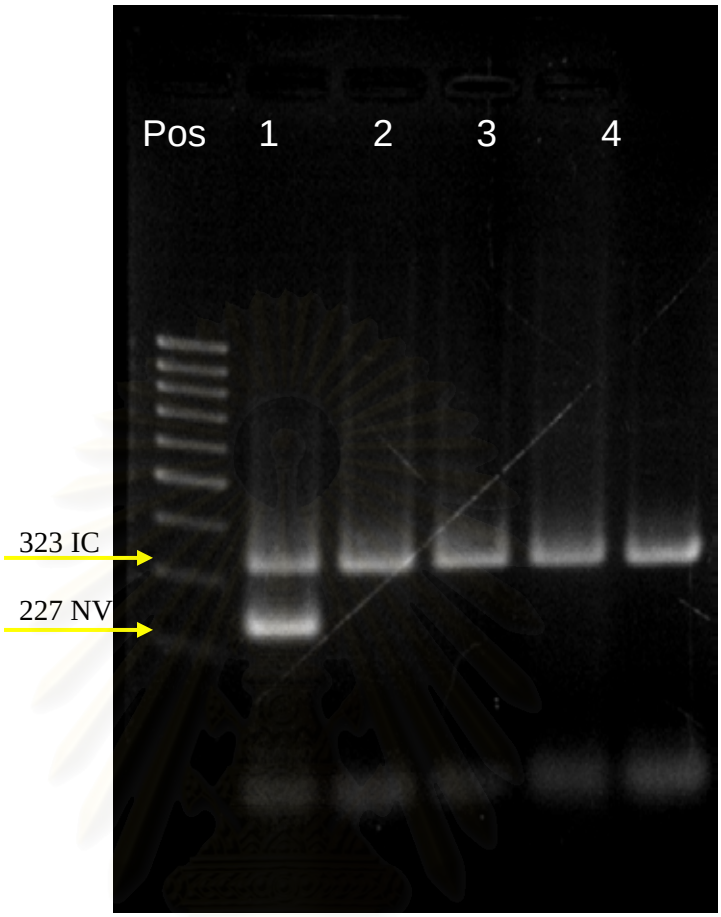


Figure 12. Duplex nRT-PCR of 1:5 diluted templates. The RNA templates from IC negative samples were further diluted to 1:5 and re-amplified. Lanes 1-4 were saliva pool no. 23, 49, urine pool no. 1, 24, respectively. Pos was NV positive control. The upper band (323 bp) was the internal control band. The lower band (227 bp) was the Nipah virus-specific band. M was 100 bp ladder markers.

7. Characterization of NV RNA in Bats

Nucleotide (nt) sequences were determined by re-amplifying the positive nRT-PCR products, without IC RNA and primers, obtained from positive pools [2 saliva pools (SP) and 6 urine pools (UP)]. The 181-nt sequences of the N gene obtained from 1 saliva pool of H.larvatus was identical to those reported from Malaysia (accession number NC_002728, AJ564621-23, AF212302, AF376747, and AY029767-8) (Figure 13). The sequences of 1 saliva pool from P.lylei and 6 urine pools from P.lylei were identical to those reported from Bangladesh (AY988601) (Figure 14) with 13 divergent nucleotides (92% identity) from Malaysia. The nucleotide changes at positions 1397, 1407 and 1481 resulted in amino acid substitutions (with 94% identity to Malaysia, 56 of 59) from isoleucine to valine, glycine to glutamic acid and asparagine to aspartic acid at codons 429, 432 and 457 of N protein, respectively (Figure 15). Nine divergent nucleotides among Thai, Bangladesh and Cambodia (AY858110) did not result to amino acid differences (Figure 15-16).

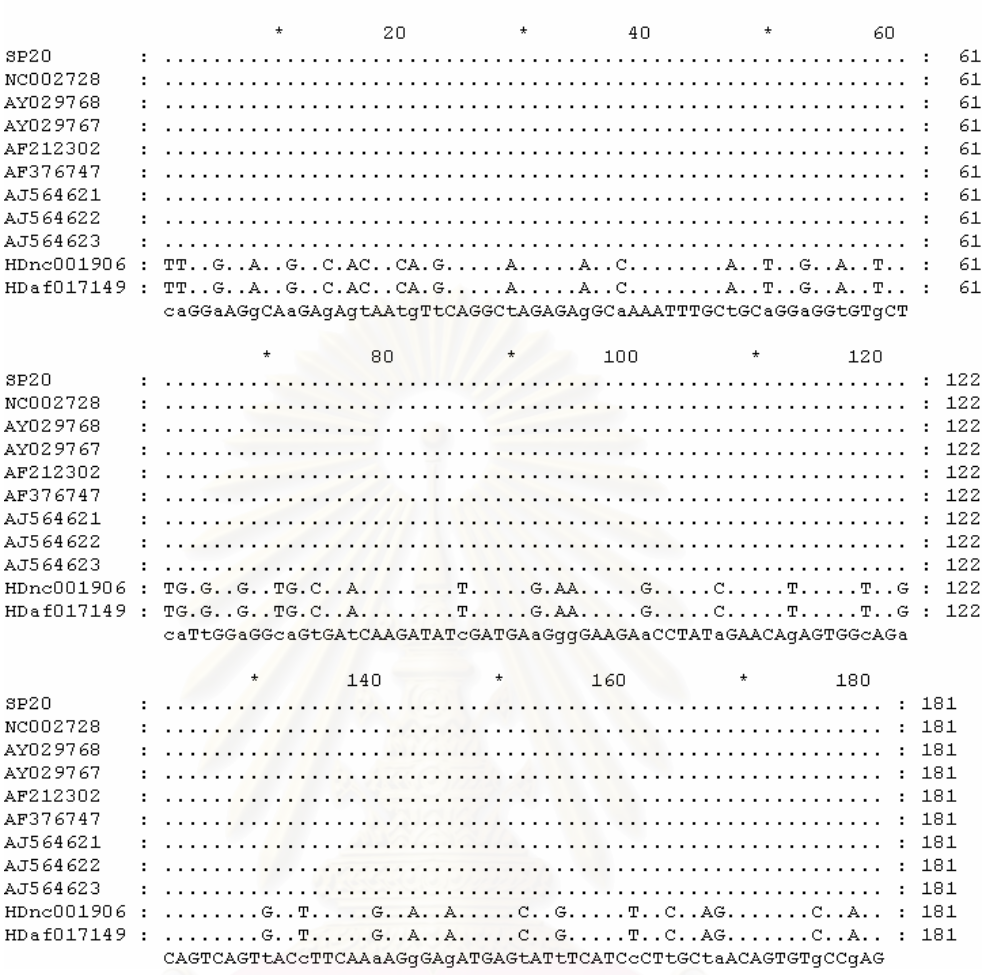


Figure 13. Comparison of the 181 nucleotides (nt) of nucleoprotein gene of NV strains from Malaysia (GenBank accession no. NC_002728, AY029767-68, AF212302, AF376747, AJ56421-23) and Hendra virus (GenBank accession no. NC001906 and AF017149) and Thai sample (SP20). The alignment showed the consensus sequence among these groups and indicated positions that differed in the N gene. Numbers corresponded to nt positions in the studied NV N gene (position 1314-1494 of GenBank accession no. NC_002728); stars marked lengths of 10 amino acids; dots indicated identical amino acids.

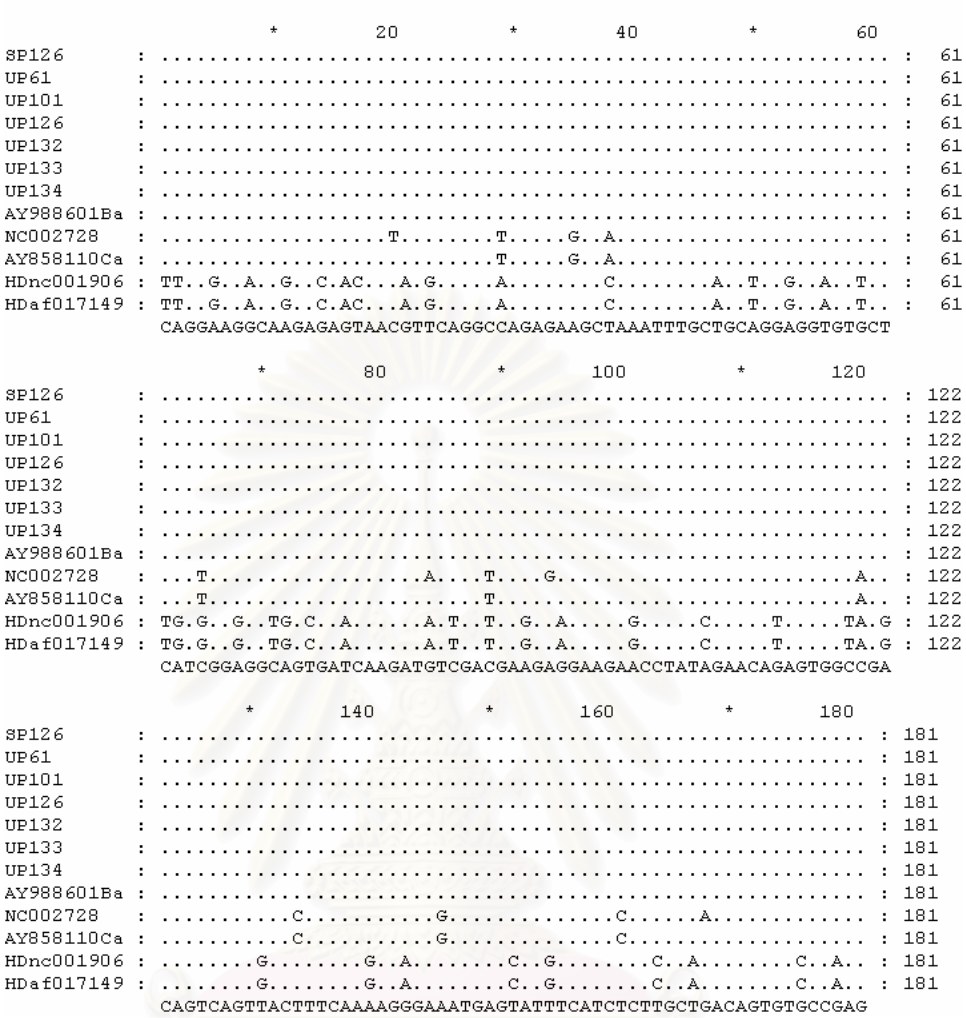


Figure 14. Comparison of the 181 nucleotides (nt) of nucleoprotein gene of NV strains from Bangladesh (GenBank accession no. AY988601), Malaysia (GenBank accession no. NC_002728) and Cambodia (GenBank accession no. AY858110) and Hendra virus (GenBank accession no. NC001906 and AF017149) and Thai samples (SP126, UP61, UP101, UP126, UP132, UP133 and UP134). The alignment shows the consensus sequence among these group and indicates positions that differ in the N gene. Numbers correspond to nt positions in the studied NV N gene (position 1314-1494 of GenBank accession no. NC_002728) ; stars marks lengths of 10 amino acids; dots indicate identical amino acids.

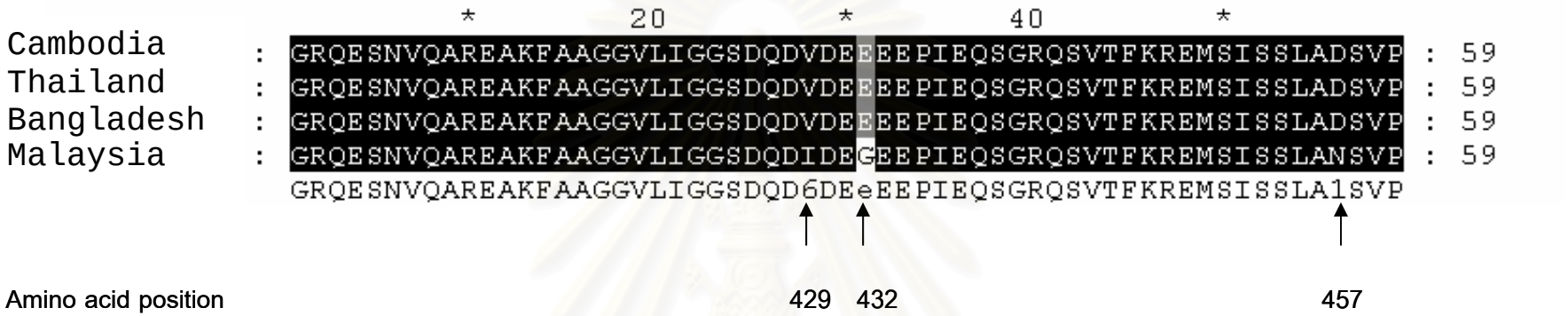


Figure 15. Comparison of the 59 amino acid of N proteins of Nipah virus strains from Thailand (PU134), Cambodia (GenBank accession no. AY858110), Bangladesh (GenBank accession no. AY988601) and Malaysia (GenBank accession no. NC_002728). Arrow indicated positions that differed in the N proteins. Amino acid positions in the NV N protein according to GenBank were indicated under the arrow.

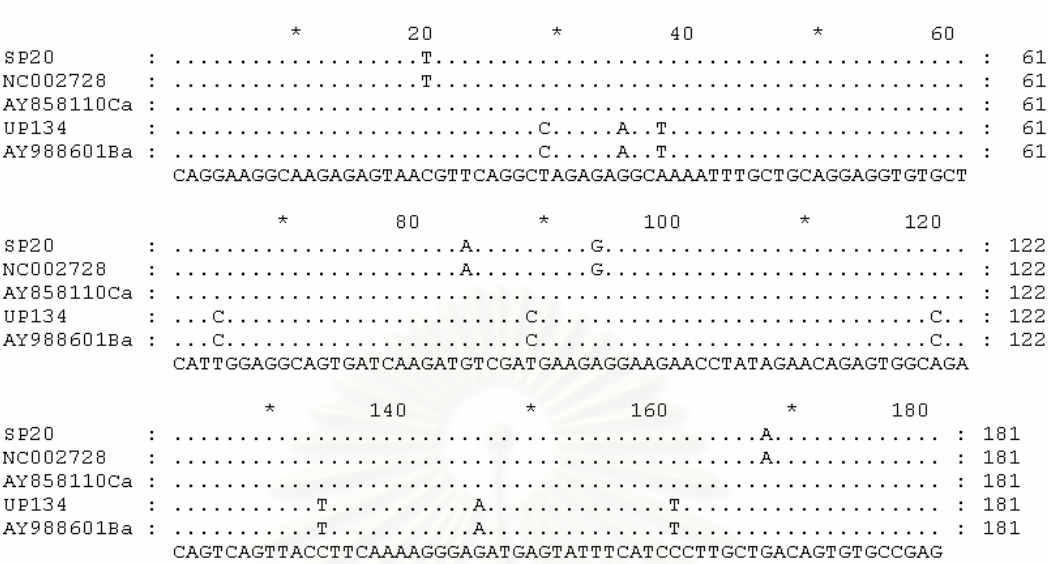


Figure 16. Comparison of the 181 nucleotides (nt) of nucleoprotein gene of NV strains from Malaysia (GenBank accession no. NC_002728), Cambodia (GenBank accession no. AY858110) and Bangladesh (GenBank accession no. AY988601) and 2 samples from Thailand (SP20 and UP134). The alignment showed the consensus sequence among these groups and indicated positions that differed in the N gene. Numbers corresponded to nt positions in the studied NV N gene (position 1314-1494 of GenBank accession no. NC_002728); stars marked lengths of 10 amino acids; dots indicated identical amino acids.

8. Determination of prevalence of NV infection in bats according to geographical regions

Analysis of the result of bat survey at 15 sites from 9 provinces in combination with both PCR and ELISA results suggested that 3 of 9 provinces, Chon buri, Cha Choeng Sao and Bangkok were prevalent areas for NV transmission (both antibody and RNA could be demonstrated) (Table 3). Samples from Surat Thani, Ayuttaya and Prachin Buri province showed only antibody positive. However, it was likely that bats in these 3 provinces, at the time of specimen collection, had antibody fully developed, thus eliminating virus from their secretions. Bats in other 3 provinces, Ratchaburi, Sing Buri and Rayong did not have evidence of NV infection (Figure 17).

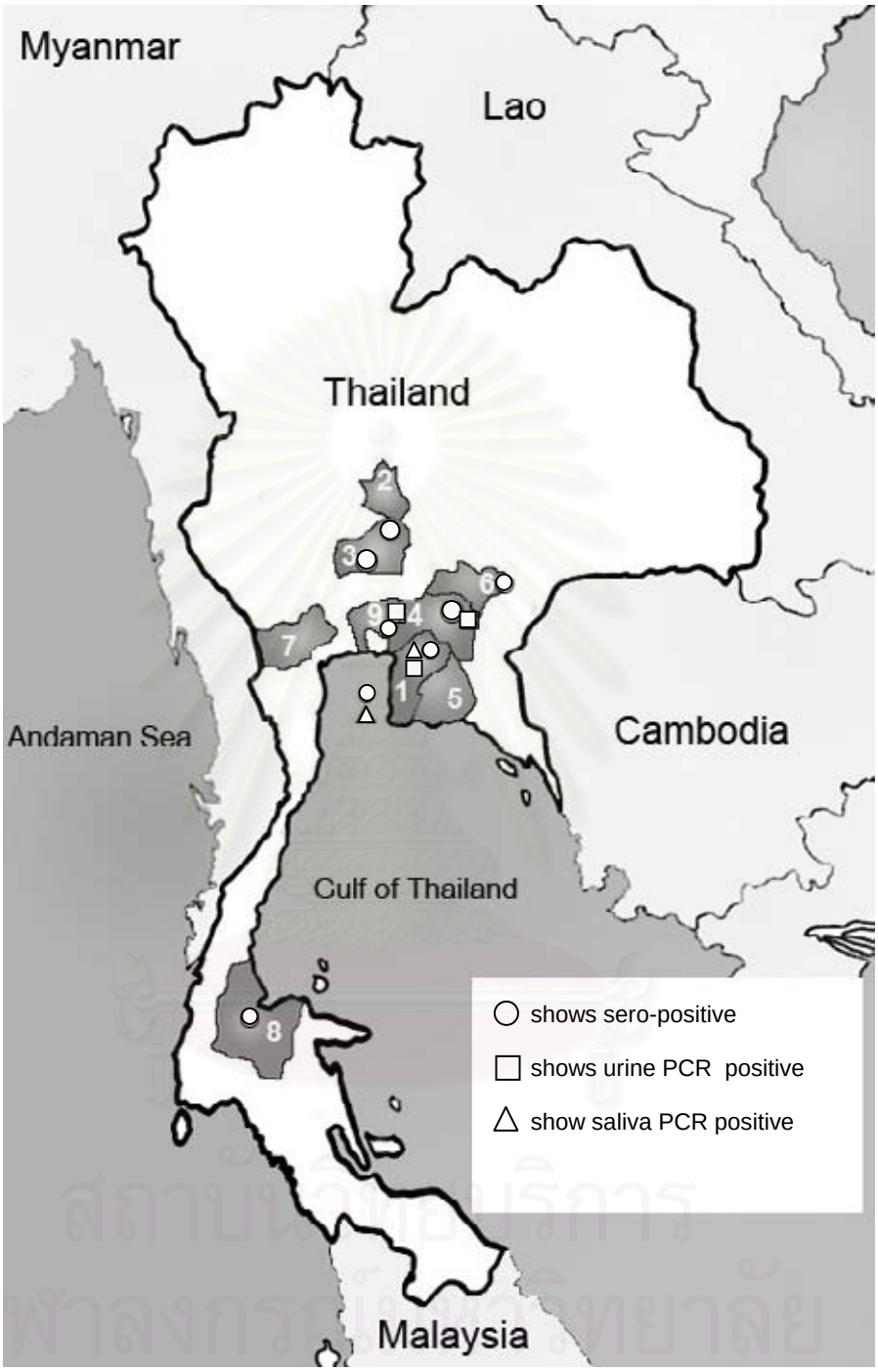


Figure 17. Map showing location of NV infection in bats in 9 studied provinces. 1.Chon Buri (CB); 2 Sing Buri (SB); 3. Ayutthaya (AY); 4. Cha Choeng Sao (CC); 5. Ra Yong (RY); 6. Pra Chin Buri (PC); 7. Ratcha Buri (RB); 8. Surat Thani (SR) and 9. Bangkok (BK). Circle represented the area of NV antibody positive bats. Square represented the area where PCR was positive in bat urine. Triangle represented the area where PCR was positive in saliva.

9. Determination of the preponderance of bat species in terms of susceptibility to NV infection

Twelve species of bats were studied. Six fruit bats were 3 Pteropus species (*P.hypomelanus, P.lylei* and *P.vampyrus)* and the rest were *Cynopterus shinx, Eonycteris spelaea* and *Rousettus leschenaulti*. The other 6 were insectivorous bats (*Emballonura monticola, Hipposideros armiger, H. larvatus, Megaderma Spasma, Scotophilus heathi and Tadarida plicata*). Higher number of PCR and ELISA positive results was found in *P.lylei* (Table 2). However, these may be due to a bias in species collection. Both *P.hypomelanus* and *P.vampyrus* showed IgG antibody positive by indirect ELISA but there was no detectable NV RNA in their secretions, saliva or urine. Evidences of NV infection were found in all 3 Ptropus species in this study. An evidence of NV infection in insectivorous bats was found only in *H. larvatus*, positive results were found in both PCR and antibody assay.

10. Demonstration of NV RNA in urine and saliva of infected bats

Higher number of NV RNA positive specimens by duplex nRT-PCR was found in urine than in saliva sources, 6 of 142 urine pools versus 2 of 142 saliva pools. Sequences of 181 nucleotides were identical among all positive urine specimens. Sequences of virus in 2 saliva specimens were different; one was similar to urine origin.

11. Analysis of correlation between seasonal variation, sero-prevalence and excretion data

There seemed no seasonal variation when antibody could be demonstrated (Figure 2). Although the results were all negative in specimens collected between May and July, the numbers analyzed might not be sufficient. Regarding the timing of excretion of virus in saliva and urine, almost all of RNA positive pools were came from samples collected during the first 4 months of the year (Figure 2). This was similar in all provinces except in Bangkok where RNA-positive samples were collected in September. Nevertheless, this finding remained inconclusive since specimens were not collected at the same site throughout the year.

CHAPTER V

SUMMARY AND DISCUSSION

1. Summary

From March 2002 through February 2004, seventeen trips were made to 15 sites in 9 provinces of central, eastern and southern Thailand (Figure 2). Bats were caught and blood samples were collected. Of 12 different bat species collected, six were frugivorous and 6 were insectivorous (Figure 2). Seventy-one percent of 1,304 samples were from *Pteropus* bats (932), of which 66% (857) were from *P.lylei* (Table 1).

IgG antibodies to NV were assayed by indirect ELISA at Chulalongkorn University Hospital, using a protocol developed by the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia. Serum samples were heated at 56^{o}C and then were titrated at 4 dilutions (1:100, 1:400, 1:1,600 and 1:6,400). Of the 1,054 sera tested, 82 serum samples (7.8%) from 4 different species, *P.hypomelanus* (n = 4), *P.lylei*, (n = 76), *P.vampyrus* (n = 1), *Hipposideros larvatus* (n = 1) were NV IgG antibody positive (titer of 1:400 or higher) with 43 at a titer of 1:400; 30 at 1:1600 and 9 at 1:6400. *P.lylei* had higher antibody titers than other species (9 of 76 at 1:6,400, 29 of 76 at 1:1,600) (Table 2).

A total of 142 pools each were collected from 1,286 saliva and 1,282 urine specimens, respectively. The pooled specimens were frozen to -70^{o}C until analysis. Total RNA was extracted from saliva and urine according to manufacturer's

protocol. NV nucleoprotein (N)-specific primers used for reverse transcription and first round PCR was as follows: NP1F, 5’ CTT GAG CCT ATG TAT TTC AGA C 3’; NP1R, 5’ GCT TTT GCA GCC AGT CTT G 3’. The internal primers for nested PCR were previously described (14). This allowed an IC and NV products to be visualized as the upper (323 bp) and lower bands (227 bp), respectively. Single-step RT-PCR was performed using the One Step RT-PCR kit followed by nested PCR. The PCR product was sized by gel electrophoresis in 2 % agarose. Only samples showing both the 323-bp IC and 227-bp NV specific bands, or only a NV-specific band, were considered positive; those showing only the IC band were considered negative; those showing no band were tested again and judged to contain enzyme inhibitors, if no band was shown on repetition. All samples with positive results were tested again without the positive control and sequence of the amplified product was determined using internal primers.

The sensitivity of the duplex system was not notably altered by incorporation of the IC RNA. One saliva pool of *H.larvatu*s from Chon Buri province- site 1 and another pool of *P. lylei* from Chon Buri- site 3 samples were duplex nRT-PCR positive. All 6 positive duplex nRT-PCR urine pools were collected from *P. lylei* captured from 3 different sites; 1 from Cha Choeng Sao, 1 from Bangkok and another 4 from Chon Buri-site 3. The 181-nt sequences of the N gene obtained from 1 saliva pool of *H. larvatu*s was identical to those reported from Malaysia (accession number NC_002728, AY029767-68, AF212302, AF376747, AJ56421-23). The sequences of 1 saliva pool from *P. lylei* and 6 urine pools from *P. lylei* were identical to those reported from Bangladesh (AY988601) with 13 divergent nucleotides (92% identity) from Malaysia. The nucleotide changes at

positions 1397, 1407 and 1481 resulted in amino acid substitutions (with 94% identity to Malaysia, 56 of 59) from isoleucine to valine, glycine to glutamic acid and asparagine to aspartic acid at codons 429, 432 and 457 of N protein, respectively. Nine divergent nucleotides among Thai, Bangladesh and Cambodia (AY858110) did not result to amino acid differences.

2. Discussion

Our study has demonstrated the evidence of NV infection in 4 bat species in Thailand, 3 of fruit bats (*P. hypomelanus*, *P. lylei*, *P. vampyrus*) and 1 insectivorous bats (*H. larvatus*) by serological and PCR methods. Three types of specimens, blood, saliva and urine, were collected from each individual bat and analyzed. This method of collection was labor-intensive (7), but it was harmless to bats and knowledge of both serology and NV secretion could be obtained. These data were necessary for effective surveillance and management (44). Moreover, it was useful not only to prevent the spread of the disease, but also to protect domestic animal industries as occurred in Malaysian incident.

This study reported the evidence of NV infection in Thai frugivorous and insectivorous bats as evidenced by demonstration of IgG antibodies to NV in serum samples and NV RNA in urine and saliva. Antibodies against NV could be detected in *P. hypomelanus*, *P. vampyrus*, *P. lylei* and *H. larvatus*. NV infections in the first 2 species were similar to that reported in Malaysia (24). *P. lylei* was the only bat species found NV-infected among 14 species tested in Cambodia (25). An earlier report demonstrated a correlation between ELISA and neutralization tests with 87% and 99% sensitivity and specificity, respectively (25). The methodology and criteria used in our study strictly followed an established CDC (US) protocol. Furthermore, we were able to demonstrate viral RNA in urine and saliva specimens, supporting evidence of NV infection as suggested by ELISA test. These data support our ELISA results as a first line screening tool for investigations of NV infection in countries where there is no BSL-4 facility for performance of neutralization assays. Eighty-two

bats (7.8%) from 4 different species, *P. hypomelanus, P. lylei, P. vampyrus* and *H. larvatus* were found antibody positive to NV. This differed from a Cambodian study in which only *P. lylei* (11.5%) were found ELISA positive at low titers (≥1/10), confirmed by a neutralization assay (10). There was no evidence of NV infection in 5 species of Indonesian fruit bats (33).

In Peninsular Malaysia, where a major outbreak had occurred, neutralizing antibody to NV was demonstrated in 5 bat species: 4 species of fruit bats, *C.brachyotis, E.spelaea, P.hypomelanus, P.vampyrus* and 1 insectivorous, *S.kuhlii* (24). The highest seroprevalence in our study was in *P.hypomelanus*, in accord with the result in Malaysian bats. These bats were from Surat Thani province at the south border to Malaysia. Alternatively, the highest ELISA titers were found in *P.lylei* (>1:6400). The finding of unusually high antibody titers from *P.lylei* suggests that NV circulates mainly in this bat species in Thailand and Cambodia (25). Based on the presence of NV antibody in the population of 4 bats species in 6 of 9 provinces of Thailand, we believe that NV infection is widespread in both fruit and insectivorous bats, but mainly prevalent in the former as previously reported in Malaysia and Cambodia (24, 25).

During our 2 year survey, bats were sampled in 9 provinces, in southern (n = 1), central (n = 3), eastern (n = 4) and western (n = 1) parts of the country. Areas in the eastern part of the country showed a more widespread infection than others (21.4 %, 11.1% and 11.0 % in Pra Chin Buri, Chon Buri and Cha Choeng Sao, respectively, versus 6.5%, 4.4% and 0% in the central provinces of Bangkok, Ayutthaya, and Sing Buri, respectively). Interestingly, there was no evidence of

infection in Sing Buri, which is adjacent to Ayutthaya (Figure 5). A more careful and thorough survey should be conducted in such highly populated regions, for a better epidemiological understanding of NV.

Although serum neutralization tests were not conducted, NV RNA was demonstrated in saliva and urine from *P. lylei* and saliva of *H. larvatus*. Given the fact that determination of PCR positivity by naked eye observations for the presence of a 227 bp fragment may not be the most sensitive method (our detection limit is 0.37 pg total RNA/ul), therefore, some low positive samples might be missed. Increasing the volume of sample tested by using a plastic sheet method in collection of urine may overcome such problems (7).

An internal control system was included in our RT-PCR method to avoid false-negative results in each individual reaction, due to the presence of enzyme inhibitors. A low number of plasmid IC RNA was added directly to the PCR master mix as an internal control. This system has been successfully used to check for possible PCR inhibitors from bat saliva and may be superior to ribosomal (r) RNA system based on the fact that the amount of rRNA was expected to be high in clinical samples. Thus, low grade RNA loss or enzyme inactivation could be missed and false negative results could be possible (14). We have shown that the sensitivity of our PCR assay was not affected by the incorporation of IC RNA, although a slight reduction in band density was observed. However, this did not have observed effects on the detection limit. This minor reduction is probably due to the result of competition for reactants in the duplex reaction.

The isolation of purified NV RNA for subsequent amplification was essential for obtaining valid and accurate results in the nucleic acid amplification assay. The isolation method utilized in the present study with the nRT-PCR system was based on capture of nucleic acid present in the specimens using silica particles in the presence of guanidine thiocyanate (45). Some of the most valuable advantages of this method are the versatility of clinical samples that can be used (volume ≤2 ml), the stability of RNA by inactivation of nuclease, the high extraction efficiency and the binding of RNA to a solid phase, which reduces cross-contamination by aerosols (46). A pool size of 10 collection tubes containing 1 ml of lysis buffer was chosen. This enabled similar RNA concentrations to 1 ml extractions due to the use of an equal volume of elution buffer. By using this pooling system, the cost for detection was reduced 10 times whereas the sensitivity would not be compromised. This system may be appropriate for screening of virus infection in a large scale epidemiological survey.

Southern blot analysis is also useful for PCR confirmation; however, sensitivity may not be markedly improved as previously reported in the case of rabies (47). We used a nested PCR method because less RNA amount was required initially and because of a shorter turnaround time. Confirmation was achieved by direct sequencing of amplified products. Taken together, our current ELISA and PCR data are sufficient to conclude that Thai bats were naturally infected with NV. Higher numbers of PCR positive samples found in *P. lylei* may be due to a bias in species collection. Alternatively, in the serologic study, *P. lylei* may be the most prevalent infected species. Sequence analysis of the short 181 nt sequence suggests there are at least 2 strains of NV circulating in Thai bats. A larger size of sequence data is

required to confirm this hypothesis. The finding of NV RNA in saliva of *H. larvatus*, may indicate insectivorous bat as another reservoir or this may be only an accidental spillage.

Most of the previous information about NV infection in bat populations was obtained from serological data (10, 13, 24, and 33) and virus isolation from urine or partially eaten fruit (9). A novel approach for collecting urine from fruit bats for isolation of infectious agents using plastic sheets has been developed (7). Evidence of active lyssavirus infection in bats has been shown by demonstration of virus RNA in oropharyngeal secretions as collected by using cotton swabs (14). In our study, saliva and urine were collected from individual bat by using a similar swab technique. These specimens were kept in lysis buffer containing guanidine thiocyanate which served as an RNA preservative and limited safety concerns. Of 12 bat species captured in this study, two had saliva and urine NV RNA positive; *P. lylei* (positive in both saliva and urine) and *H.lavatus* (in saliva). Higher numbers of PCR positive samples found in *P.lylei* may be due to a bias in species collection. Alternatively, *P. lylei* may be the most prevalent species. Previous reports showed that NV could be isolated from urine of *P. hypomelanus* on Tioman Island, Malaysia (9). We failed to demonstrate NV RNA in urine or saliva of *P. hypomelanus* although the highest seroprevalence was shown in this species. The discrepancy may be explained by the low virus excretion in saliva and urine of these infected bats or the inability to sampling during the active infection phase. Additionally, NV shedding from *P. hypomelanus* may be intermittent in nature (9).

Our results underscore the significance of surveillance for emerging infectious diseases among wildlife reservoirs. Outbreaks in Malaysia and in Bangladesh provide examples of NV transmission among wildlife, domestic animals and humans. The rather low detection of viral RNA (or infected virus) in secretions in this study does not necessarily indicate that NV is uncommon in Thailand. Mechanisms and what behaviors of bats, such as during pregnancy and birth process, that are responsible for a massive wide spread of infection among themselves and whether there is a seasonal preference for NV infection in bats should be studied. We believe that NV infection is prevalent in Thai fruit bats as previously reported in Malaysia and Cambodia (24, 25). Country-wide surveillance is needed to understand the epidemiology of NV infection in Thailand as it may relate to host, season, and geographical attributes.

3. Suggestions

Host management strategies for natural hosts seek to minimize the opportunity for effective contact between natural host and the spillover host. Complete knowledge of the ecology of the agents (the maintenance and transmission of infection, the natural and domestic host range, and the nature of the host-agent relationship) and the factors associated with emergence (habitat loss, land use change and demographic shifts) precludes a more targeted approach at this time. Strategies for intermediate hosts emphasize surveillance, detection and emergency response capabilities. Strategies for human hosts promote awareness, minimization of contact, use of personal protective equipment.

It is notable that neither the indiscriminate nor targeted killing of bats is contemplated as an effective host management strategy. Certainly such a strategy with nomadic species such as flying foxes is biologically flawed, and as argued earlier, may cause a net influx of bats to the resultant niche vacuum. The force movement of roosting flying foxes (flying foxes communally roost in trees, frequently in groups of thousands) by sustained noise, smoke, and flags has been attempted with mixed success. Permanent removal of flying fox roosts can only be guaranteed by the removal of the roost trees.

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APPENDICES

APPENDIX A

Chiroptera (48)

The clade Chiroptera includes two extant clades, *Megachiroptera* (Old World Fruit Bats) and *Microchiroptera* (echolocating bats). In addition, *Chiroptera* includes at least four extinct clades that are most closely related to *Microchiroptera*. There are over nine hundred extant species of bats. Bats vary greatly in size. The smallest bat, *Craseonycteris thonglongyai* (*Microchiroptera*), weighs less than 2 g and has a wingspan of 12-13 cm, while the largest bats, those of the genus *Pteropus* (*Megachiroptera*), weigh up to 1.5 kg and may have a wing span over 2m.

Bats are unique among mammals as they are the only group to have evolved true powered flight. Some other mammals such as "flying" squirrels and "flying" lemurs can glide through the air for long distances, but they are not capable of sustained flight. In contrast, bats can propel themselves with their wings, gaining and loosing altitude and flying for long periods.

Bats are nocturnal and usually spend the daylight hours roosting in caves, rock crevices, trees, or manmade structures such as houses and/or bridges. Some bats are solitary, while others are found in colonies that may include over a million individuals.

Activity begins around dusk, when bats leave the day roost and start feeding. The clade Chiroptera includes species with very diverse food preferences, including bats that eat either meat, insects, fish, fruit, nectar, or a variety of food types. Only three species of bats actually feed on blood *Desmodontinae*). Many bats remain at their feeding sites until just before dawn when they return to the day roost.

Flight and Echolocation top

Among living vertebrates, true flight is unique to bats and birds. Unlike most birds, however, bats are able to fly at relatively low speeds with extreme maneuverability. The wing is a thin, fleshy membrane supported near its leading edge by the greatly elongated bones of the forelimb and second finger, and toward the tip and rear by the even more attenuated third, fourth, and fifth fingers. It is attached along the midline of the trunk and outward-directed legs, and in various species it extends between legs and tail. Only the first finger, or thumb, is free, and in most bats it alone is clawed, together with the toes. This structure enables bats to vary dramatically the convexity of the wings and thus their aerodynamic lift.

All microbats navigate—and most insectivorous species also target their prey—by echolocation. This is the pulsed emission of high-frequency sounds that are reflected back as echos to a bat's ears from surrounding surfaces, indicating the position, relative distance, and even the character of objects in its environment. In this sense microbats "see" acoustically. This is the basis for their ability to navigate in total darkness. The physical properties of the emitted sounds vary in characteristic ways among species. The sound pulses are generated in the larynx, and in different species are emitted either from the mouth or nostrils.

By contrast, megabats use vision rather than acoustics for orientation. Only one genus (*Rousettus* of Africa, the Middle East, and Asia) has evolved an echolocation mechanism, involving the emission of audible "clicks," and it is used only when the bats fly in darkness. The eyes of megabats are also relatively larger than those of microbats. No bat is blind, however, and even echolocating microbats may use gross visual landmarks for homing during flight.

Behavior

With a few exceptions, such as *Saccopteryx* of the western hemisphere and the African Lavia, all microbats are nocturnal. During the day they may rest in a variety of roosting places, such as caves, crevices, hollow trees, foliage, hiding places beneath rocks or bark, and in buildings. They may even roost in exposed situations; certain larger megabats hang upside down in enormous aggregations from tree branches, like so many gourds. Nocturnality gives bats many advantages, such as greatly reduced competition for insects and other food items, substantial freedom from attack, and protection from overheating and dehydration, to which bats are especially liable because of their enormous skin area relative to their size.

A few species of bats live solitarily, such as *Lasiurus cinereus* of North America, but most are gregarious. Aggregation during the day may vary from small groups consisting of a single male and a dozen or more females to enormous assemblages numbering many thousands or even millions of individuals (for example, *Tadarida brasiliensis* of the southwestern U.S.). Aggregations of members of specific species may show seasonal variation and sexual segregation in varying combinations. Mixed-species associations of a casual sort are common among bats using protected shelters such as caves.

Certain species of Temperate Zone bats are migratory to some degree, and movements of nearly 1600 km (about 1000 mi) between summer and winter quarters have been recorded in *Tadarida brasiliensis.* Others may fly only a few or up to 40 km (about 25 mi) or more daily between roosting and feeding sites, but the majority forage within more restricted home ranges.

Most bats are insectivorous and are able to hawk their prey on the wing or to seek out stationary insects on foliage or other surfaces. Most megabats, and many species of leaf-nosed bats of tropical America (so named for the remarkable folds of skin projecting upward from the nose), are fruit eaters. Still others in both groups consume flower parts or extract the nectar from flowering plants by means of greatly elongated tongues, aiding cross-pollination of the plants in the process. Some of the larger leaf-nosed bats as well as members of the Old World family *Megadermatidae* are carnivorous or omnivorous; they attack small amphibians, lizards, birds, mice, and even other bats, in addition to consuming insects and fruit. Closely related to the leaf-nosed bats are the three genera of true vampires of the American Tropics (*Desmodus, Diphylla,* and *Diaemus*), which subsist entirely on blood freshly drawn from small wounds inflicted on mostly warm-blooded prey such as fowl, cattle, horses, swine, and occasionally human beings. At least three species of bats supplement their diets with small fish, which are caught as the flying bats drag their enlarged feet and claws just beneath the water surface.

Life Cycle

The gestation periods of bats are relatively long, ranging from about 44 days to 8 months in various species. Few produce more than a single offspring each year, and the young tend to mature slowly. The reproductive cycles of most species follow general mammalian patterns, but exceptions exist. Most interesting are Temperate Zone species such as *Myotis, Rhinolophus,* and *Eptesicus*, which hibernate during the winter months. Copulation occurs prior to hibernation, and sperm are retained within the females throughout the hibernation period. Fertilization occurs

when the ovum is released from the ovary after the bats arouse in the spring. This is called delayed fertilization. A variant of this pattern is observed in the European genus *Miniopterus*, in which copulation, ovulation, and early development of the embryo all occur in normal sequence immediately prior to hibernation, but the embryo experiences developmental arrest before uterine attachment. It remains free and undeveloped until after the torpid mother resumes normal metabolism in spring. This is called delayed implantation.

The abundance of bats despite their low individual reproductive performances is attributable not only to the survival value of their habits but also to their remarkable longevity. Some larger species of megabats (*Pteropus*) and the smaller vampire bat (*Desmodus*) have survived in zoos for 20 years. Among various species of microbats banded and released in the wild, many have been recaptured after years of freedom. The record is a specimen of *Myotis lucifugus* recovered 31 years after it was initially marked and released in New England.

Ecology

Insect-eating bats can be considered generally helpful to humans, and many bat species play an important role in plant pollination and seed dispersal. Because of their size and numbers, the larger fruit bats of the Old World can be an economic menace when they invade fruit orchards, but perhaps the greatest adverse effect of bats is the transmission of disease, especially rabies, to domesticated animals. This is a particular problem in the American Tropics, where local outbreaks of rabies among cattle, stemming from the bites of infected vampire bats, have decimated whole herds. Bats other than vampires can also contract and transmit

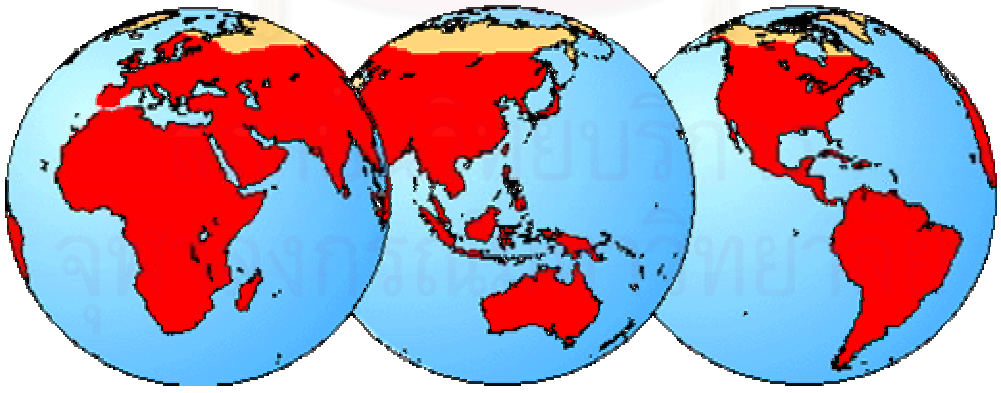
rabies; most species of bats inhabiting the U.S. and Canada have been reported at one time and place or another to be infected. The prevalence of infection, however, has been low, outbreaks seldom, and unprovoked attacks on humans rare.

Many bats have low tolerance for certain insecticides such as DDT, which they can concentrate through the food chain. Some populations in the U.S. may have been adversely affected, inasmuch as three species on the list of endangered species have suffered from the agricultural use of pesticides.

Geographic Distribution

Bats are found throughout the world in tropical and temperate habitats. They are missing only from polar regions and from some isolated islands. Although bats are relatively common in temperate regions, they reach their greatest diversity in tropical forests.

The geographical distribution of bats is shown in red.



Distribution from Hill and Smith (1984).

Classification of Bats

Classification from Simmons and Geisler (1998),modified following suggestions of Kirsch et al.(1998).

Order *Chiroptera*

Megachiropteramorpha (unranked name)

Suborder *Megachiroptera*

Family *Pteropodidae*

Microchiropteramorpha (unranked name)

†Family *Icaronycteridae*

†Family *Archaeonycteridae*

Microchiropteraformes (unranked name)

†Family *Palaeochiropterygidae*

†Family *Hassianycteridae*

Suborder *Microchiroptera*

Superfamily *Emballonuroidea*

Family *Emballonuridae*

Subfamily *Taphozoinae*

Subfamily *Emballonurinae*

Infraorder *Yinochiroptera*

Superfamily *Rhinopomatoidea*

Family *Craseonycteridae*

Family *Rhinopomatidae*

Superfamily *Rhinolophoidea*

Family *Nycteridae*

Family *Megadermatidae*

Family *Rhinolophidae*

Subfamily *Rhinolophinae*

Subfamily *Hipposiderinae*

Infraorder *Yangochiroptera*

Superfamily *Noctilionoidea*

Family *Mystacinidae*

Family *Phyllostomidae*

Family *Mormoopidae*

Family *Noctilionidae*

Superfamily *Nataloidea*

Family *Myzopodidae*

Family *Furipteridae*

Family *Thyropteridae*

Family *Natalidae*

Superfamily *Molossoidea*

Family *Antrozoidae*

Family *Molossidae*

Subfamily *Tomopeatinae*

Subfamily *Molossinae*

Superfamily *Vespertilionoidea*

Family *Vespertilionidae*

Subfamily *Vespertilioninae*

Subfamily *Miniopterinae*

Subfamily *Myotinae*

Subfamily *Murininae*

Subfamily *Kerivoulinae*

APPENDIX B

Family Pteropodidae (flying foxes and Old World fruit bats) (42)

Members of this family are the "flying foxes" and other fruit-eating bats of the Old World. Pteropodids are the only family of the Suborder Megachiroptera. They include around 166 living species placed in approximately 42 genera. Species of pteropodids can be found in tropical and subtropical regions of Africa, through southern and central Asia to Australia, including the Philippines and a number of Pacific islands. They are especially diverse in southeastern Asia and Indo-Australia.

Some pteropodids are big. Members of the largest species of the family, in the genus *Pteropus*, approach a kilogram in weight and have a wingspan of up to 1.7 meters. Most species, however, are small to medium in size.

Pteropodids are distinguished from other bats by a combination of the following characteristics:

- the second finger is relatively independent of the third finger and usually retains a claw (absent in all Microchiropterans);
- postorbital processes of skull well developed;
- bony palate elongated, extending well beyond the last upper molar;
- dental formula highly variable, but never more than 2 upper and 2 lower incisors on each side of the jaw, and the total number of teeth is usually small compared to microchiropterans;

- the premaxilla (the bone that bears the incisor teeth in the upper jaw) is well developed and lacks a palatal branch;
- ears are relatively simple, lacking specializations for echolocation (such as a tragus), and with a relatively small cochlea;
- the tail is usually small or absent; when present, it is not ensheathed in a tail membrane.

Many species are sexually dimorphic. Differences between the sexes include the larger body size of males (most species), males having larger canines (many species), and males with conspicuous skin glands (a few species). Sexual differences are extreme in one species, *Hypsignathus monstrosus*, in which males have very large pharangeal sacs that extend into the chest and a huge larynx (used in producing a loud "honk" that is part of a sexual display).

All pteropodids are frugivorous or nectarivorous. Their molar-like teeth are simple in structure and often reduced in size compared to those of other bats, a tendency that is carried to an extreme in nectar-feeding species.

Members of this family are usually strong fliers, but they lack the specializations of wings and shoulders seen in many microchiropterans, and their flight style is relatively simple.

Pteropodids also differ from other bats in that most use sight, rather than echolocation, as a means of navigation. Their eyes are large and they see very well. Echolocation is known in at least one pteropodid, but it differs in a number of

ways from that of other bats and is probably independently evolved. Pteropodids also rely heavily on their sense of smell to help them locate fruit.

Pteropodids are important pollinators and dispersers of tropical trees, many of which are adapted to attract bats and make their pollination/dispersal activities more efficient. In some instances pteropodids may also cause significant damage to orchards. The larger species are sometimes hunted for their meat.

APPENDIX C

Bats in Thailand (27)

No.	Scientific name	Thai name
	FAMILY PTEROPODIDAE	
1	*Pteropus hypomelanus*	ค้างคาวแม่ไก่เกาะ
2	*P. intermedius*	ค้างคาวแม่ไก่นครสวรรค์
3	*P. vampyrus*	ค้างคาวแม่ไก่ป่าฝน
4	*P. lylei*	ค้างคาวแม่ไก่ภาคกลาง
5	*Rousettus leschenaulti*	ค้างคาวบัวฟันรี
6	*R. amplexicaudatus*	ค้างคาวบัวฟันกลม
7	*Cynopterus brachyotis*	ค้างคาวขอบหูขาวเล็ก
8	*C. sphinx*	ค้างคาวขอบหูขาวกลาง
9	*C. horsfieldi*	ค้างคาวขอบหูขาวใหญ่
10	*Megaerops ecaudatus*	ค้างคาวขอบหูดำใต้
11	*M. niphanae*	ค้างคาวขอบหูดำเหนือ
12	*Dyacopterus spadiceus*	ค้างคาวดายัค
13	*Chironax melanocephalus*	ค้างคาวหัวดำ
14	*Sphaerias blanfordi*	ค้างคาวดอย
15	*Balionycteris maculata*	ค้างคาวปีกจุด
16	*Eonycteris spelaea*	ค้างคาวเล็บกุด
17	*Macroglossus minimus*	ค้างคาวหน้ายาวเล็ก
18	*M. sobrinus*	ค้างคาวหน้ายาวใหญ่
	FAMILY RHINOPOMATIDAE	
19	*Rhinopoma microphyllum*	ค้างคาวหางหนู
	FAMILY EMBALLONURIDAE	
20	*Emballonura monticola*	ค้างคาวหางโผล่
21	*Taphozous melanopogon*	ค้างคาวปีกถุงเครา
22	*T. longimanus*	ค้างคาวปีกถุงต่อมคาง
23	*T. theobaldi*	ค้างคาวปีกถุงใหญ่
24	*T. saccolaimus*	ค้างคาวปีกถุงปลอม
	FAMILY CRASEONYCTERIDAE	
25	*Craseonycteris thonglongyai*	ค้างคาวคุณกิตติ

No.	Scientific name	Thai name
FAMILY NYCTERIDAE		
26	*Nycteris tragata*	ค้างคาวหน้าร่อง
FAMILY MEGADERMATIDAE		
27	*Megaderma spasma*	ค้างคาวแวมไพร์แปลงเล็ก
28	*M. lyra*	ค้างคาวแวมไพร์แปลงใหญ่
FAMILY RHINOLOPHIDAE		
29	*Rhinolophus trifoliatus*	ค้างคาวมงกุฎสามใบพัด
30	*R. luctus*	ค้างคาวมงกุฎใหญ่
31	*R. paradoxolopghus*	ค้างคาวมงกุฎหูโตใหญ่
32	*R. marshalli*	ค้างคาวมงกุฎหูโตมาร์แชล
33	*R. macrotis*	ค้างคาวมงกุฎหูโตเล็ก
34	*R. coelophyllus*	ค้างคาวมงกุฎปลอมเล็ก
35	*R. shameli*	ค้างคาวมงกุฎปลอมใหญ่
36	*R. pearsoni*	ค้างคาวมงกุฎจมูกยาวเล็ก
37	*R. yunanensis*	ค้างคาวมงกุฎจมูกยาวใหญ่
38	*R. acuminatus*	ค้างคาวมงกุฎยอดสั้นใหญ่
39	*R. lepidus*	ค้างคาวมงกุฎจมูกแหลมเหนือ
40	*R. pusillus*	ค้างคาวมงกุฎเล็ก
41	*R. megaphyllus*	ค้างคาวมงกุฎเลียนมลายูเล็ก
42	*R. malayanus*	ค้างคาวมงกุฎมลายู
43	*R. stheno*	ค้างคาวมงกุฎเลียมมลายูหางสั้น
44	*R. thomasi*	ค้างคาวมงกุฎยอดสั้นเล็ก
45	*R. rouxi*	ค้างคาวมงกุฎอินเดีย
46	*R. affinis*	ค้างคาวมงกุฎเทาแดง
FAMILY HIPPOSIDERIDAE		
47	*Hipposideros bicolor*	ค้างคาวหน้ายักษ์เล็กสองสี
48	*H. pomona*	ค้างคาวหน้ายักษ์เล็ก
49	*H. ater*	ค้างคาวหน้ายักษ์เล็กดำ
50	*H. cineraceus*	ค้างคาวหน้ายักษ์สีจาง
51	*H. halophyllus*	ค้างคาวหน้ายักษ์จมูกปุ่ม
52	*H. galeritus*	ค้างคาวหน้ายักษ์สองหลืบ

No.	Scientific name	Thai name
53	*H. lylei*	ค้างคาวหน้ายักษ์กระบังหน้า
54	*H. armiger*	ค้างคาวหน้ายักษ์ทศกัณฐ์
55	*H. turpis*	ค้างคาวหน้ายักษ์กุมภกรรณ
56	*H. larvatus*	ค้างคาวหน้ายักษ์สามหลืบ
57	*H. diadema*	ค้างคาวหน้ายักษ์หนอนโค้ง
58	*H. lekaguli*	ค้างคาวหน้ายักษ์หมอบุญส่ง
59	*Aselliscus stoliczkanus*	ค้างคาวสามศร
60	*Coelops frithii*	ค้างคาวอ้ายแหว่งใหญ่
61	*C. robinsoni*	ค้างคาวอ้ายแหว่งเล็ก
	FAMILY VESPERTILIONIDAE	
62	*Myotis chinensis*	ค้างคาวหูหนูยักษ์
63	*M. altarium*	ค้างคาวหูหนูดอยอ่างขาง
64	*M. siligorensis*	ค้างคาวหูหนูตีนเล็กเขี้ยวสั้น
65	*M. rosseti*	ค้างคาวหูหนูมือตีนปุ่ม
66	*M. muricola*	ค้างคาวหูหนูตีนเล็กเขี้ยวยาว
67	*M. montivagus*	ค้างคาวหูหนูพม่า
68	*M. annectans*	ค้างคาวหูหนูหน้าขน
69	*M. horsfieldii*	ค้างคาวหูหนูตีนโตเล็ก
70	*M. hasseltii*	ค้างคาวหูหนูตีนโตใหญ่
71	*Scotomanes ornatus*	ค้างคาวดอยหลังลายขาว
72	*Scotophilus kuhlii*	ค้างคาวเพดานเล็ก
73	*S. heathi*	ค้างคาวเพดานใหญ่
74	*Eptesicus serotinus*	ค้างคาวท้องน้ำตาลใหญ่
75	*E. pachyotis*	ค้างคาวท้องน้ำตาลหูหนา
76	*E. demissus*	ค้างคาวท้องน้ำตาลสุราษฎร์
77	*Ia io*	ค้างคาวอีอาอีโอ
78	*Tylonycteris pachypus*	ค้างคาวไผ่หัวแบนเล็ก
79	*T. robustula*	ค้างคาวไผ่หัวแบนใหญ่
80	*Pipistrellus javanicus*	ค้างคาวลูกหนูบ้าน
81	*P. coromandra*	ค้างคาวลูกหนูอินเดีย
82	*P. circumdatus*	ค้างคาวลูกหนูสีทอง

No.	Scientific name	Thai name
83	*P. tenuis*	ค้างคาวลูกหนูจิ๋ว
84	*P. pulveratus*	ค้าวคาวลูกหนูถ้ำ
85	*P. cadornae*	ค้างคาวลูกหนูกรามหน้าบนเล็ก
86	*P. circumdatus*	ค้างคาวลูกหนูดำเหลือบ
87	*P. mimus*	Indian pigmy pistrelle
88	*Glischropus tylopus*	ค้างคาวมือปุ่ม
89	*Nyctalus noctula*	ค้างคาวกินแมลงนิ้วสั้น
90	*Hesperoptenus tickelli*	ค้างคาวฟันหน้าซ้อนใหญ่
91	*H.blandfordi*	ค้างคาวฟันหน้าซ้อนเล็ก
92	*Miniopterus schreibersi*	ค้างคาวปีกพับใหญ่
93	*M. magnater*	ค้างคาวปีกพับดำใหญ่
94	*M. medius*	ค้างคาวปีกพับกลาง
95	*M. pusillus*	ค้างคาวปีกพับเล็ก
96	*Murina leucogaster*	ค้างคาวจมูกหลอดท้องขาว
97	*M. aurata*	ค้างคาวจมูกหลอดเล็ก
98	*M. tubinaris*	ค้างคาวจมูกหลอดแดง
99	*M. huttoni*	ค้างคาวจมูกหลอดหูยาว
100	*M. cyclotis*	ค้างคาวจมูกหลอดหูสั้น
101	*Harpiocephalus harpia*	ค้างคาวปีกขนใต้
102	*H. mordax*	ค้างคาวปีกขนเหนือ
103	*Kerivoula whiteheadi*	ค้างคาวยอดกล้วยป่า
104	*K. picta*	ค้างคาวยอดกล้วยผีเสื้อ
105	*K. minuta*	ค้างคาวยอดกล้วยเล็ก
106	*K. papillosa*	ค้างคาวยอดกล้วยปีกปุ่ม
107	*K. hardwickei*	ค้างคาวยอดกล้วยปีกใส
108	*K. jagori*	Peter’s trumpet-eared bats
109	*Phoniscus atrox*	ค้างคาวฟันร่อง
FAMILY MOLOSSIDAE		
110	*Tadarida teniotis*	ค้างคาวปากย่นใหญ่
111	*T. plicata*	ค้างคาวปากย่น
112	*Cheiromeles torquatus*	ค้างคาวขุนช้าง

APPENDIX D

Pteropid bat population in central region of Thailand (49)

No.	Location (District/Province)	Number of bat population	Counting date
1	Phrom Buri/Sing Buri	2,609	Nov 1, 2000
2	Popraya/ Suphan Buri	1,920	Jul 13, 2002
3	Bang Pla Ma/ Suphan Buri	398	Jul 13, 2002
4	Pho Thong/ Ang-Thong	1,268	Apr 29, 2002
5	Bang Pa Han/ Ayutthaya	833	Oct 31, 2001
6	Bang Sai/ Ayutthaya	4,017	Apr 22, 2002
7	Bang Pa In/ Ayutthaya	650	Apr 21, 2002
8	Nong Khae/ Sara Buri	2,819	Jan 24, 2001
9	Ban Na/ Nakhorn Nayok	1,208	Sept 17, 2002
10	Ban Sang/ Pra Chin Buri	878	Feb 26, 2002
11	Muang/ Pra Chin Buri	2,599	Feb 26, 2002
12	Bang Khla/ Cha Choeng Sao	11,010	Jan 22, 2002
13	Ban Pho/ Cha Choeng Sao	1,152	Nov 30, 2003
14	Muang/ Chon Buri	959	Dec 25, 2001
15	Phanat Nikhom/ Chon Buri	3,517	Dec 24, 2001
16	Min Buri/ Bangkok	2,000	Aug 5, 2003
	Total	37,837	

APPENDIX E

Differentiation between Hendra (HeV) and Nipah (NV) virus

1. Biological properties of HeV and NV (50)

HeV grows to high titre in a range of cultured cells from diverse species and has been purified by rate zonal centrifugation in sucrose gradients. Virus structural proteins have been characterised by polyacrylamide gel electrophoresis and individual proteins identified using monospecific rabbit antisera generated against bacterially expressed HeV proteins. The protein profile resembles that of a typical member of the subfamily Paramyxovirinae, although the P protein is significantly larger than cognate proteins in the subfamily 6. Both cleaved (F1) and uncleaved (F0) forms of the fusion protein are present in approximately equivalent amounts in HeV generated in a range of cultured cells including Vero cells and from the allantoic fluid of infected chicken embryos. In contrast, the fusion protein in Vero cell-derived NV appears to be completely cleaved.

HeV displays a number of interesting biological properties. In addition to infecting a wide range of cells in vitro, the virus causes systemic infections in vivo in species as diverse as flying foxes, man, cats and horses, displaying a predilection for endothelial cells. During the NV outbreak in 1998–1999, infection and mortality occurred in humans, pigs, cats and dogs 7; 8 and 9; thus, NV is the second paramyxovirus to infect and cause disease in a wide variety of species. Autopsy studies following the 1999 outbreak demonstrated that NV produced a multi-organ vasculitis associated with infection of endothelial cells 10. HeV does not agglutinate

erythrocytes from a variety of sources, nor does it display neuraminidase activity when tested on fetuin, activities that characterize members of the Respirovirus and Rubulavirus genera 11. The ability of NV to agglutinate erythrocytes and display neuraminidase activity has not been investigated. However, the absence of both a putative sialic acid binding domain and six of seven aa residues known to be critical for neuraminidase activity in the NV G protein suggest that NV will also be unreactive in haemagglutination and neuraminidase assays. With the exception of antibody to NV 7, antibodies to a wide range of viruses within the Paramyxoviridae do not react with cells infected with HeV. In fact, antibodies to HeV and NV cross-neutralise 9 and 12 and antigen preparations from HeV-infected cells were initially used in the enzyme immunoassays that were developed to support the outbreak investigation in Malaysia 8. Sequencing of the genomes of HeV and NV and analysis of the deduced amino acid sequence of encoded proteins have attempted to identify the molecular basis of these unusual biological properties.

2. The genomes of HeV and NV and their encoded proteins. (50)

The complete genome sequence of HeV has been determined as well as greater than 99% of the NV genome 12 (Harcourt B.H., unpublished results). As with other viruses in the subfamily Paramyxovirinae, there are a total of six transcription units (figure 1) encoding six major structural proteins. They are nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), glycoprotein (G) or attachment protein, and large protein (L) or RNA polymerase, in the order 3&z.verts;-N-P-M-F-G-L-5.

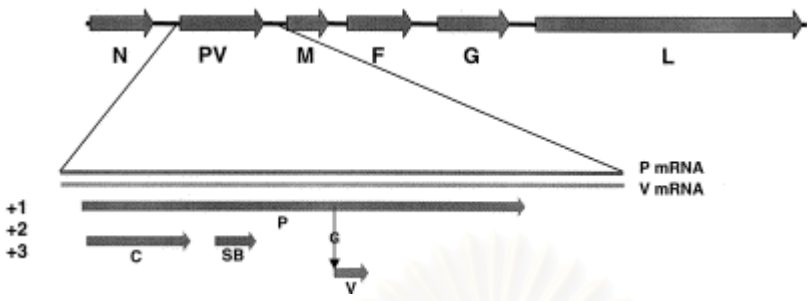


Figure1. Genome structure of HeV and the coding capacity of the P gene. The upper part represents the HeV genome (18.2 kb) and the location and order of the six major structural protein genes encoded by the anti-genome from 5&z.verts; (left) to 3&z.verts; end (right). The lower part contains the enlarged diagram showing the P gene coding strategy. The translation reading frames are shown at left as +1, +2 and +3. The P protein is translated from the +1 reading frame, whereas the C is derived from an internal translation initiation site in the +2 reading frame. The SB protein may also be derived from an internal initiation site in the +2 reading frame. The V-specific peptide is encoded in the +3 reading frame, and the mature V protein is a fusion protein consisting of the N-terminal part of the P protein and the V-specific peptide. The fusion junction is shown by the downward arrow corresponding to the G-insertion site in the V-specific mRNA.

Table 1. Comparison of the sequences of the N, P/C/V, M, F and G genes of HeV and NV.

Gene	Virus	Open reading frame length[a]	5′ non-translated % identity[b]	% nucleotide homology[b]	3′ non-translated length[a]	% nucleotide homology[b]	length[c]	% nucleotide homology[b]
N	Hendra	532	92.1	78.4	57	66.7	568	41.1
	Nipah	532			57		585	
P	Hendra	707	67.6	70.5	105	41.9	469	40.9
	Nipah	709			103		469	
V	Hendra	54	81.1	88.5				
	Nipah	51						
C	Hendra	166	83.2	85.0				
	Nipah	166						
M	Hendra	352	89.0	77.1	100	40.0	200	40.0
	Nipah	352			100		200	
F	Hendra	546	88.1	74.2	272	44.1	418	41.4
	Nipah	546			284		412	
G	Hendra	604	83.3	70.8	233	43.8	516	45.6
	Nipah	602			233		504	
L	Hendra	2244	86.8	75.0	153	54.0	67	58.2
	Nipah	2244			153		67	

Overall, the arrangement of genes on the genomes of HeV and NV is most closely related to that of the *Respirovirus* and *Morbillivirus* genera. However, several features make HeV and NV unique in the subfamily Paramyxovirinae. HeV has a genome size of 18.2 kb, which is much larger than the relatively uniform genome sizes of all other known members of the Paramyxovirinae, which vary between 15.1 and 15.9 kb. The NV genome is approximately 18.2 kb in size, although the final nucleotide number has not yet been determined. Both the HeV and NV genome sizes are closer to those of members of the family Filoviridae (18.9–19.1 kb). HeV and NV have longer untranslated regions, mostly at the 3&z.verts; end, in each of the six transcription units than have other members of the Paramyxovirinae . This feature has also been observed for filoviruses, including Ebola and Marburg viruses. Interestingly, although the sizes of the 3&z.verts; untranslated regions of HeV and NV are conserved, the sequence homology ranges from only 40 to 45% (table 1), which is much lower than that of the coding regions (70–85%). It is possible that these sequences form conserved secondary structures that could be important in mRNA stability and/or translatability. The P gene of HeV and NV encodes a P protein that is more than 100 amino acids (aa) longer than any other known P protein in the family. The HeV P gene , but not the NV P gene, also contains an open reading frame (ORF) encoding a small basic protein (SB) that has an unusually high pI.

3. Ultrastructural characteristics of Hendra virus and Nipah virus (51)

The ultrastructural characteristics of HeV and NV are consistent with other viruses in the order Mononegavirales, family Paramyxoviridae, subfamily Paramyxovirinae. However, some subtle differences exist between the two viruses

that may allow for their differentiation at the ultrastructural level. The major differences are summarised in table 2, table 3 and table 4. First, when examined by negative-contrast electron microscopy, HeV has a double-fringed appearance; this feature is prevalent in any sampled population. NV, on the other hand, is more difficult to observe and possesses a single fringe. Second, within syncytia generated in infected cell cultures there is a difference in the distribution of both cell nuclei and nucleocapsid aggregates. In NV-infected syncytia, nucleocapsids and nuclei are frequently located at the cell periphery, especially late in infection. In contrast, in HeV-infected syncytia, both structures tend to be either more centrally located or distributed randomly throughout the cytoplasm. These ultrastructural dissimilarities may be used to differentiate between the two viruses in infected cell cultures (figure 2).

Within the infected lungs of amplifier host animals, apparent differences in target cell type lead to differences in pathological outcomes. HeV replicates in the respiratory vascular endothelia, resulting in the partial destruction of the endothelium, which may lead to vascular leakage and pulmonary oedema in the equine lung. In contrast, NV replicates predominantly in the respiratory epithelia, which may lead to epithelial destruction and the release of virus into the porcine respiratory airways.

The unique ultrastucture of the two viruses and their varying antigenic activity (table 2, table 3 and table 4) observed in immunogold studies can be used to make a preliminary differential identification between the two zoonotic viruses.

Table 2. Ultrastructural characteristics of Hendra virus and Nipah virus in infected cell cultures.

Ultrastructural characteristics	Hendra virus	Nipah virus
Electronmicroscopy		
Virus shape	pleomorphic	pleomorphic
Virus diameter	variable > 40 nm	variable > 40 nm
Virus envelope	present	present
Fringe	predominantly double	predominantly single
Length of projections	15 ± 1 nm; 8 nm 1 (n = 20)	17 ± 1 nm (n = 20)
Diameter of nucleocapsids	18 ± 1 nm (n = 20)	19 ± 2 nm (n = 29)
Pitch of nucleocapsids	5 ± 1 nm (n = 20)	5 ± 0.4 nm (n = 15)
Site of viral egress	plasma membrane	plasma membrane
Nucleocapsid aggregates	cytoplasm	cytoplasm
Location of aggregates of nucleocapsids and nuclei	central	tend to be peripheral
Syncytia	present	present
Electron dense membrane regions	present	present
Intracellular tubules	present	present
Immunoelectron microscopy		
Anti-Nipah antibodies*	positive	positive
Anti-Hendra antibodies*	positive	positive

Table 3. Ultrastructural characteristics of Hendra virus and Nipah virus in the lungs of amplifier hosts.

Ultrastructural characteristics	Hendra virus	Nipah virus
Predominant site of replication	vascular endothelial cells	alveolar epithelial cells
Syncytia	present	present
Nucleocapsid aggregates	present	present
Prevalence of intracellular tubule-like structures	occasional	prevalent*
Contents of alveolar air spaces	'proteinacous' fluid	exfoliated alveolar (type 1) cells and neutrophils

Table 4. Immunoreactivity of Hendra and Nipah viral nucleocapsids in homologous and heterologous systems.

Antibody	Hendra virus-infected equine lungs	Nipah virus-infected porcine lungs
Anti-Hendra*	++++	++
Anti-Nipah**	++	++++

Reactivity was assessed from negative to ++++. * Rabbit antiserum; ** convalescent human serum.

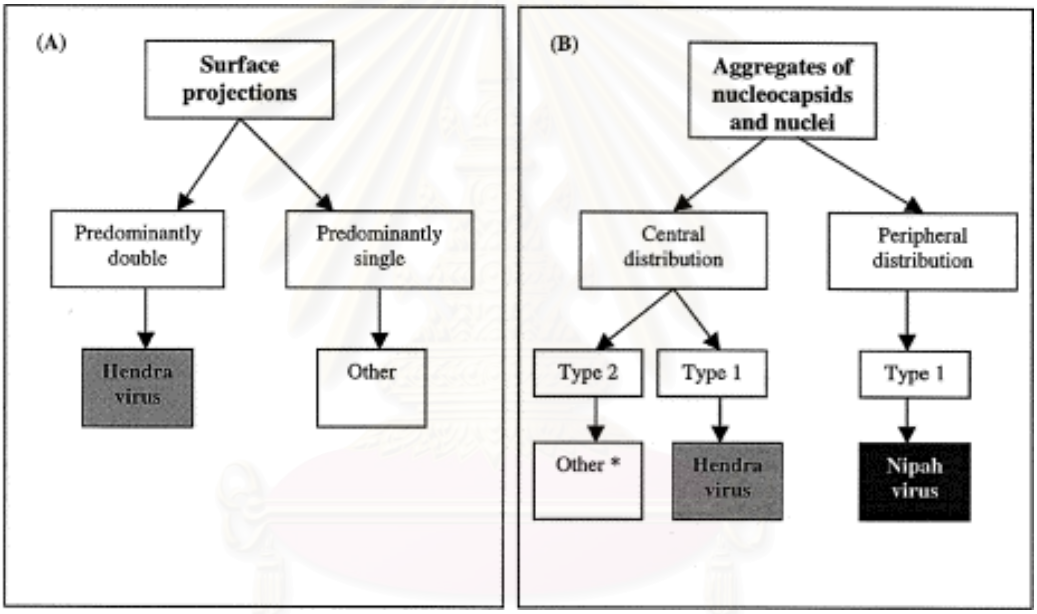


Figure 2. Flow charts for ultrastructural differentiation of Hendra virus from Nipah virus in infected cell cultures. Differentiation is based on the characteristics of surface projections (**A**) and ultrastructural characteristics of nucleocapsid aggregates (**B**). These flow charts are meant as a guide and are not intended for definitive identification. * The virus may be other than Nipah or Hendra virus, for example Menangle or Tioman viruses that have type 2 nucleocapsid inclusions.

5. Virus classification (50, 25)

Phylogenetic analysis of the nucleotide sequences of the N, P, C, M, F and G ORFs from representatives of the subfamily Paramyxovirinae showed that HeV and NV consistently formed a unique cluster which was more closely related to the morbilliviruses and the respiroviruses than to the rubulaviruses (figure 3). Based on these and other genomic finding there is now a suggestion that a new genus *Henipavirus* (*Hendra+Nipah*) be created to accommodate these new paramyxoviruses onder *Paramyxovirinae*.

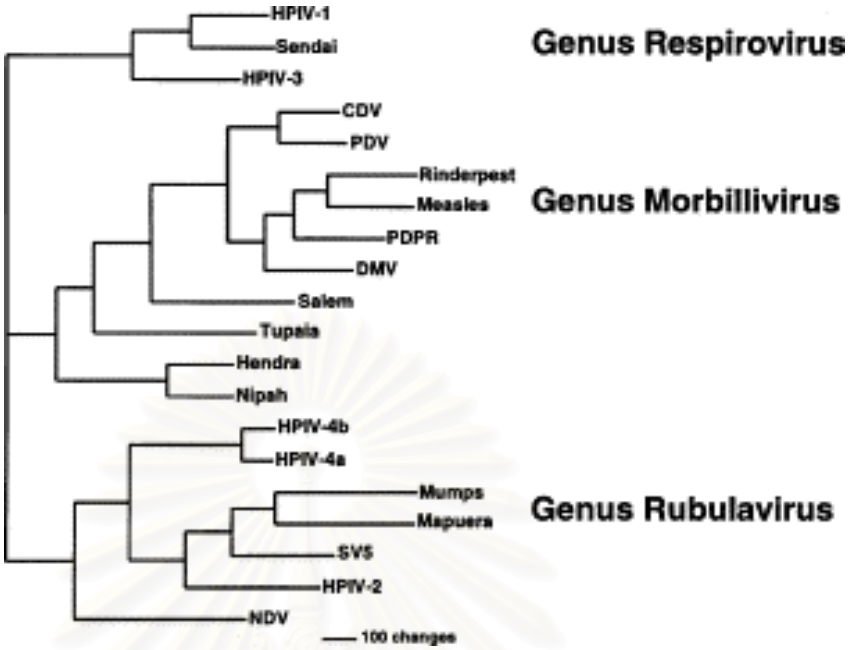


Figure 3. Phylogenetic analysis of the N genes from members of the subfamily Paramyxovirinae. The scale representing the number of nucleotide changes is shown at top left. Accession numbers used: canine distemper virus (CDV), AF014953; dolphin morbillivirus (DMV), X75961; Hendra virus, AF017149; human parainfluenza virus type 1 (HPIV-1), D01070; human parainfluenza virus type 2 (HPIV-2), M55320; human parainfluenza virus type 3 (HPIV-3), D10025; human parainfluenza virus type 4a (HPIV-4a), M32982; human parainfluenza virus type 4b (HPIV-4b), M32983; Mapuera virus, X85128; mumps virus, D86172; measles virus, K01711; Newcastle disease virus, AF064091; peste-des-petits-ruminants virus, (PPRV), X74443; phocid distemper virus, (PDV), X75717; rinderpest virus (RPV), X68311; Sendai virus, X00087; simian virus 5 (SV5), M81442; and Tupaia paramyxovirus, AF079780.

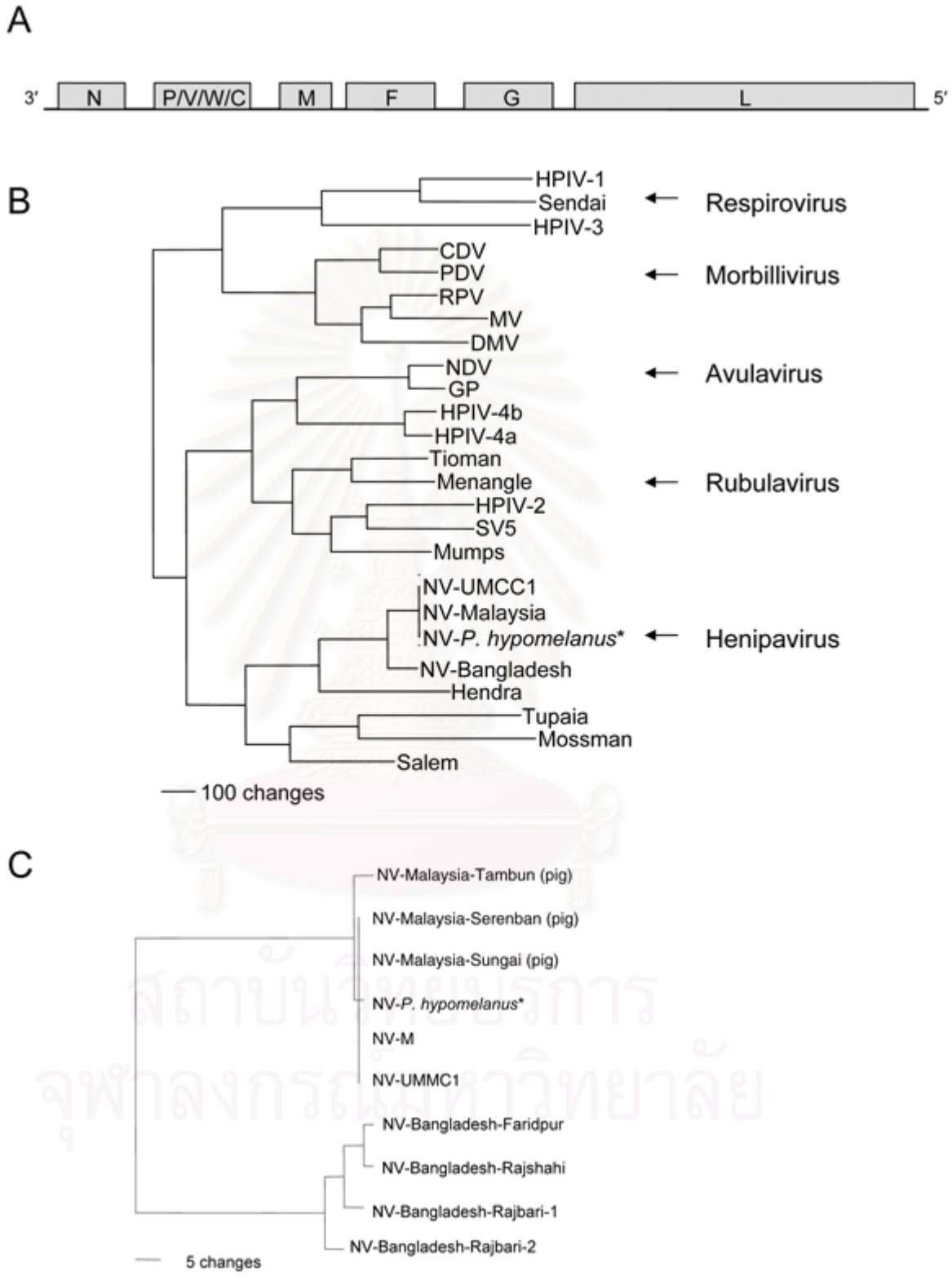

A
3′
N
P/V/W/C
M
F
G
L
5′
B
HPIV-1
Sendai
HPIV-3
CDV
PDV
RPV
MV
DMV
NDV
GP
HPIV-4b
HPIV-4a
Tioman
Menangle
HPIV-2
SV5
Mumps
NV-UMCC1
NV-Malaysia
NV-P. hypomelanus*
NV-Bangladesh
Hendra
Tupaia
Mossman
Salem
Respirovirus
Morbillivirus
Avulavirus
Rubulavirus
Henipavirus
100 changes
C
NV-Malaysia-Tambun (pig)
NV-Malaysia-Serenban (pig)
NV-Malaysia-Sungai (pig)
NV-P. hypomelanus*
NV-M
NV-UMMC1
NV-Bangladesh-Faridpur
NV-Bangladesh-Rajshahi
NV-Bangladesh-Rajbari-1
NV-Bangladesh-Rajbari-2
5 changes

Figure 4

Figure 4. Schematic representation of the genome of Nipah virus (NV). Negative-sense genomic RNA is shown in 3´ to 5´ orientation. Open reading frames (ORFs) are indicated by shaded boxes: N, nucleoprotein; P, phosphoprotein; M, matrix protein; F, fusion protein; G, attachment protein; L, polymerase protein. B) Phylogenetic analysis of the N ORFs from members of the subfamily *Paramyxovirinae*. Arrows identify the 5 genera. A phenogram of the N ORFs of members of this subfamily was created by using maximum parsimony analysis with PAUP 4.02 (Sinauer Associates, Sunderland, MA, USA). Abbreviations and accession numbers: HPIV-1, human parainfluenza virus, D01070; Sendai, X00087; HPIV-3, D10025; CDV, canine distemper virus, AF014953; PDV, phocine distemper virus, X75717; RPV, Rinderpest virus, X68311; MV, K01711; DMV, dolphin Morbillivirus, X75961; NDV, Newcastle disease virus, AF064091; GP, goose paramyxovirus, AF473851; HPIV-4b, M32983; HPIV-4a, M32982; Tioman, AF298895; Menangle, AF326114; HPIV-2, M55320; Simian virus 5 (SV5), M81442; Mumps, D86172; NV-UMCC1, AY029767; NV-Malaysia, AF212302; NV-P. hypomelanus,* AF376747; NV-Bangladesh; Hendra virus, AF017149; Tupaia paramyxovirus, AF079780; Mossman virus, AY286409; and Salem virus, AF237881. C) The phylogenetic relationship between the N gene sequences of the 4 human NV isolates from the Bangladesh outbreak in 2004 and the N gene sequences from pig and human NV isolates from Malaysia. Accession numbers for the pig isolates of NV are AJ627196, AJ564622, and AJ564621. *NV-*P. hypomelanus* is sequence from a virus isolated from *Pteropus hypomelanus*, the Island Flying Fox.

APPENDIX F

Clinical Features of Nipah Virus Encephalitis among Pig Farmers in Malaysia (18)

TABLE 1. CLINICAL FEATURES AT PRESENTATION IN PATIENTS WITH NIPAH VIRUS INFECTION.

FEATURE	NO. OF PATIENTS (%) (N=94)
Fever	91 (97)
Headache	61 (65)
Dizziness	34 (36)
Vomiting	25 (27)
Reduced level of consciousness*	20 (21)
Nonproductive cough	13 (14)
Myalgia	11 (12)
Focal neurologic signs	10 (11)
Cerebellar signs	3 (3)
Segmental myoclonus	3 (3)
Cerebellar signs and segmental myoclonus	2 (2)
Rotatory nystagmus	1 (1)
Dysphasia	1 (1)

*A score of 15 on the Glasgow Coma Scale was considered to indicate a normal level of consciousness.

TABLE 2. NEUROLOGIC CHARACTERISTICS OF PATIENTS DURING THE COURSE OF NIPAH VIRUS INFECTION.

CHARACTERISTIC	ALL PATIENTS (N=94)	PATIENTS WITH NORMAL LEVEL OF CONSCIOUSNESS (N=42)*	PATIENTS WITH REDUCED LEVEL OF CONSCIOUSNESS (N=52)*
		no. of patients (%)	
Absent or reduced reflexes	53 (56)	11 (26)	42 (81)
Abnormal pupils	49 (52)	1 (2)	48 (92)
Tachycardia (heart rate >120/min)	37 (39)	0	37 (71)
Hypertension (blood pressure >160/90 mm Hg)	36 (38)	3 (7)	33 (63)
Abnormal doll's-eye reflex	36 (38)	0	36 (69)
Segmental myoclonus			
Overall	30 (32)	1 (2)	29 (56)
Diaphragm	26 (28)	1 (2)	25 (48)
Arms	13 (14)	0	13 (25)
Legs	9 (10)	0	9 (17)
Anterior muscles of neck	8 (9)	0	8 (15)
Facial muscles	1 (1)	0	1 (2)
Meningism	26 (28)	9 (21)	17 (33)
Seizures	22 (23)	0	22 (42)
Nystagmus	15 (16)	1 (2)	14 (27)
Cerebellar signs	8 (9)	0	8 (15)
Bilateral ptosis	4 (4)	2 (5)	2 (4)
Bilateral postural tremor	3 (3)	0	3 (6)
Dysarthria	3 (3)	1 (2)	2 (4)
Dysphasia	2 (2)	1 (2)	1 (2)

*A score of 15 on the Glasgow Coma Scale was considered to indicate a normal level of consciousness.

TABLE 3. RESULTS OF CEREBROSPINAL FLUID EXAMINATION IN PATIENTS WITH NIPAH VIRUS INFECTION.

EXAMINATION	NO. OF PATIENTS	DAY OF ILLNESS*	WHITE-CELL COUNT*	PROTEIN*	GLUCOSE*	PRESSURE*	PATIENTS WITH ABNORMAL RESULTS		
							TOTAL	ELEVATED PROTEIN LEVELS ONLY	ELEVATED WHITE-CELL COUNTS AND PROTEIN LEVELS
			cells/mm³	g/liter	mmol/liter	cm of water	no./total no. (%)		
First	92	5.2 (2–24)	41.2 (0–842)	0.69 (0.12–2.15)	3.8 (2.0–5.5)	17.4 (3–58)	69/92 (75)	42/69 (61)	27/69 (39)
Second	31	12.1 (4–38)	59.2 (0–720)	0.90 (0.24–5.80)	3.3 (2.0–4.5)	16.1 (8–25)	24/31 (77)	13/24 (54)	11/24 (46)

*Mean values are shown, with the range in parentheses.

TABLE 4. FACTORS ASSOCIATED WITH THE PROGNOSIS OF NIPAH VIRUS INFECTION.

FACTOR	DEATH (N=30)	SURVIVAL (N=64)	P VALUE
Mean age — yr	40.9	35.2	0.02
Vomiting — no. (%)	12 (40)	13 (20)	0.04
Mean lowest Glasgow Coma scores	6.8	12.8	0.005
Segmental myoclonus — no. (%)	20 (67)	10 (16)	<0.001
Abnormal doll's-eye reflex — no. (%)	26 (87)	10 (16)	<0.001
Abnormal pupils — no. (%)	29 (97)	20 (31)	<0.001
Hypertension — no. (%)	23 (77)	14 (22)	<0.001
Tachycardia — no. (%)	28 (93)	8 (12)	<0.001
Absent or reduced reflexes — no. (%)	22 (73)	31 (48)	0.02
Seizures — no. (%)	12 (40)	10 (16)	0.01
Mean aspartate aminotransferase level at admission — U/liter	87	34.4	0.001
Mean alanine aminotransferase level at admission — U/liter	94.2	53.6	0.006
Mean platelet count at admission — per mm^3	151,000	197,000	0.005

APPENDIX G

Standard Operating Procedure (SOP)

NIPAH Bat lgG Procotol (Cell Lysate) (52)

1. Safety: The material to be tested for the presence of Nipah lgG is potentially contaminated with viable Nipah or agents for which a differential determination is being sought. The antigen and control antisera used in the performance of this assay have been treated to kill any Nipah that may have been in them at the time of production. Within the limits of our ability to detect viable virus these products are safe. Accordingly, caution should be exercised in handling all materials associated with this test. If hazard is high, should be conducted in an appropriate containment environment. Animal sera imported from overseas must be irradiated to meet USDA import permit requirements. Good laboratory practices at the P2 level should be used at minimum.

2. Background: Samples submitted for serological testing may include sera from a variety of species. This protocol describes a serological test for the detection of lgG in bats.

3. Protocol.

a. The basic approach is that of an lgG assay in which Nipah antigen, prepared as an extract of infected cells, is applied onto the solid phase of a microtiter plate.

b. Nipah specific lgG in the sera to be testes as well as the positive and negative controls are allowed the opportunity to bind to the antigen. After washing, recombinant protein G/Protein A conjugated to HRPO is applied and allowed to bind.

c. This, in turn, is followed with ABTS substrate.

d. ABTS, in the presence of the enzyme HRPO, is converted from a colorless liquid to an intense green color with maximum light adsorption at 414 nm.

e. The amount of color developed is proportional to the amount of lgG which bound to the antigen. We adjust for sera which are sticky or have antibodies to the cell system used to make the antigen by using a mock antigen. The OD values of the mock antigen are subtracted from those of positive antigen to give a "net" positive or adjusted OD value.

f. This assay is a departure from the basic immunocapture lgG assay which we in the RDX group used for long period of time. The reason for our use of that assay was a practical means of placing adequate amounts of antigen onto the solid phase of a microtiter plate. In Mid – 1987 we discovered that we could get adequate antigen from a simple extraction of infected cells. This allows a practical lgG ELISA test with minimal effort. It also reduces the amount of steps in the basic lgG assay. Therefore, we are cautiously making a move to this form of assay to determine its relative merit.

g. Determination of positive (cut – off value).

i. A panel of 5 – 6 normal sera are run each time the assay is used. The mean and standard deviation of the value of the adjusted ODs are accumulated and to calculate a value equal to the mean plus 3 standard deviations. This represents the cutoff value for the assay. Sera having adjusted OD values above this cut -off are considered “positive “; the values are also used determine the point at which the extinction limit is reached when titrations are performed.

ii. Several words of caution. In performing lgG tests, sera which are specific for a particular agent rarely give values which are “marginal”. Marginal values may represent very early post – infection sera; sera which are positive for some related, cross – reacting virus; or noise in the system. In interpretation of lgG assays, one should be conservative in assigning positive meaning to sera with other than moderate to high adjusted OD values. Most of the lgG assays that we use this sort of antigen in have practical lgG cutoffs of 0.2 for each adjusted OD value in assigning titers, that is, if the adjusted OD value for the 1:100 dilution is 0.25, we would call that dilution positive and count that dilution positive. Similarly, if the adjusted OD of the 1:400 were above 0.2, we would also count that dilution as positive, and so on for the subsequent dilutions. We would assign a titer to the serum based on the last dilution

which was counted as positive. We have routinely considered sera with titers less than 1:400 as not positive. We also use the sum of the ODs through the dilution series as a measure of reactivity. In lgG assays, we have generally used adjusted OD sums of 0.95 as the cutoff for consideration of positivity.

4. Methods:

a. PBS: Phosphate buffered saline (0.01 M, pH 7.4). Use Sigma 1 liter packs.

b.Wash buffer: PBS with Tween -20 (0.1% M, pH 7.4), with or without thimerosol.

c. Serum Diluent: Wash buffer with 5% Skim Milk, pH 7.4.

d. Plates: PVC, Falcon Cat. No. 35391.

e. Antigens:

i. Cell lysates: Coat the plate with lysate in PBS with thimerosal @1:2000. Coat second half of plate with a control antigen (normalcontrol antigen), same dilutions. Allow to adsorb overnight at 4 °C.

(1) Gamma – irradiated lysate of Vero cells infected with Nipah virus (Lot No. SPR612).

(2) Lysate from normal E6 cells (Lot No. SPR527)

f. Sera: Test Sera: test at 1:100→ (4 – fold) or screen in duplicate at 1:100 in both positive and negative wells.

i. Positive Sera: For now: Since we don't have large volumes of serum from bats, we can use either the positive HMAF,

Human, or rabbit sera that we have.

ii. Negative Panel:

g. Conjugate: Protein G/Protein A conjugated to HRPO (Pierce, No. 32490, Lot No. XXX, diluted 1:1500 in SerDil) is then added.

h. Substeate: ABTS (Kirkegaard and Perry, Cat Nos. 506500).

Combined 1:1.

5. Flow chart. (All volumes =100 microliters)

Coat plate with Nipah antigen, 1:2000 (Lot No. SPR612)

(Control Ag, Lot No.SPR527 Normal E6 Cell Lysate, same Dilution)

PBS, pH 7.4, overnight, 4 °C

↓ Wash 3X

Add Test Sera

1:100 - -> (4 – fold, down plate, or screen at 1:100 in duplicate)

(Positive Serum and Neg control panel)

incubate 60 min 37° C

↓ Wash 3X

Add Protein A/Protein G conjugate (Pierce) at 1:1500 in SerDil

(Cat. No.32490, Lot No. XXXX)

incubate 60 min at 37 °C

↓ Wash 3X

Add substrate

Incubate 30 min at 37 °C.

↓

Read at 410 nm

↓

Record and analyzed results using Excel program

RDX Recipe: Master Plate Diluent: 5% Skim Milk in PBS, 0.5 % Triton X100

1. **Safety :** Don't drop any heavy bottles on yourself. The chemicals used in this recipe are non – toxic. Precaution with acids bases used to adjust pH is, of course, necessary.
2. **Background and Purpose:** This skim milk –PSB is used as a serum diluent in some of our ELISA assays. This formulation is used for the preparation of specimens for Hanta rodent testing and contains additional detergent to aid in inactivation of the virus and is used in conjunction with heat inactivation (56 °C, 30 minutes).
3. **Recipe**

Recipe : 5% Skim Milk in PBS, pH 7.4, for each liter

Sigma PBS, Sigma 1 liter packs,Cat. No. 1000 – 3	
(or use local formulation for PBS……. ……..	1 pkg
DdHOH……..	800 pkg
Skim Milk Powder, DIFCO…….	50 pkg
Merthiolate (Thimersol)……..	10 ml
2 M NaOH, adjust pH to…….	7.4
Triton X 100…….	5.0 ml
ddHOH, q.s. to……	1000 ml

4. **Preparation:** Prepare as above, generally in multi – liter quantities and freeze in useable quantities such as 100 or 250 ml bottles.
5. **Storage.** Stoee at 4 °C and use within 1 -2 days or store frozen at > 20° C.

RDX Recipe: (SERUM DILUENT) 5% Skim Milk in PBS

1. Satety: Don't drop any heavy bottles on yourself. The chemicals used in this recipe are nontoxic. Precaution with acids and bases used to adjust pH is, of course, necessary.

2. Background and Purpose: This skim milk – PBS is used as a serum diluent in some of our ELISA assays.

3. Recipe :

5% Skim Milk in PBS, pH 7.4, for each liter :

Sigma PBS, Sigma 1 liter packs (or PBS from Scratch),		
Cat. No. 1000-3	1	btl
ddHOH	800	ml
Skim Milk Pwoder, DIFCO	50	g
Merthiolate (Thimersol 1% solkution)	10	ml
2 M NaOH, adjust pH to	7.4	
Tween – 20	1.0	ml
ddHOH, q. s. to	1000	ml

4. Preparation : Preparation as above, generally in multi – liter quantities and freeze in useable quantities such as 200 or 500 ml bottles.

5. Storage. Use within 1 – 2 days or store frozen at > 20 C.

RDX Recipe: PBS Wash Buffer, pH 7.4

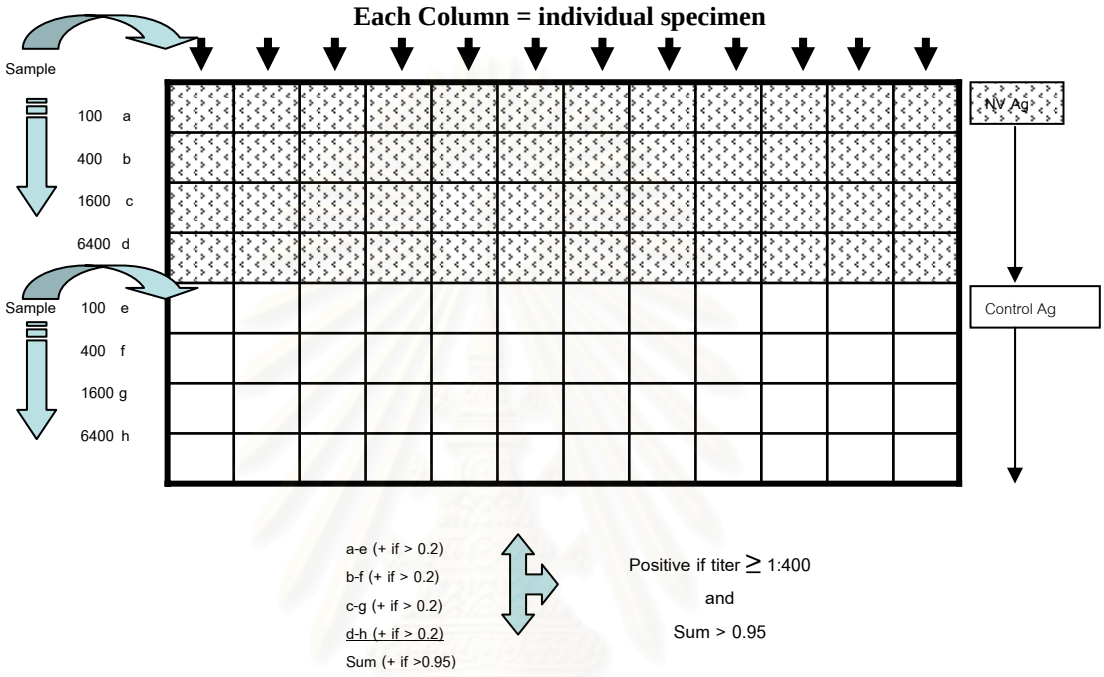
1. **Safety:** Don't drop any heavy flasks on your toes.
2. **Background and Purpose:** This PBS, made small 1 liter containers for convenience sake, is used a wash for alf our ELISA assays that we perform in the Virology lab.
3. **Recipes**

Recipe : PBS, pH 7.4, for each liter ;

Sigma PBS, Sigma 1 liter packs, Cat. No.1000 – 3……	1	btl
ddHOH……..	800	ml
Merthiolate (Thimersal), 1% Stock Solution……	10	ml
Tween 20……..	1	ml
DdHOH,q. s. to……	1000	ml

4. **Preparation:** Prepare as above in 1 liter quantities.
5. **Storage.** Use within 5 days. Discard if becomes cloudy or if precipitates form.

ELISA Schematic



APPENDIX H

Publication

Decision Letter

From: eidchief@cdc.gov

To: spwa02@yahoo.com, th-cu@usa.net

Cc:

Subject: Disposition for Manuscript # EID-05-0613.R1

Body: 15-Jul-2005

Dear Miss Supaporn Wacharapluesadee:

We are pleased to inform you that your article "Survey for Nipah virus infection among bats in Thailand" has been accepted for publication in Emerging Infectious Diseases. The article will undergo substantive editing for length, grammatical correctness, and journal style. The galleys will be sent to you for approval.

Thank you for sending us your article. We look forward to working with you in the months to come.

Dr. D. P. Drotman
Editor-in-Chief
Emerging Infectious Diseases
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404-371-5449 (fax)

http://mc.manuscriptcentral.com/eid

Date Sent: 15-Jul-2005

Bat Nipah Virus, Thailand

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Surveillance for Nipah virus (NV) was conducted in Thailand's bat population. Immunoglobulin G antibodies to NV were detected with enzyme immunoassay in 82 of 1,304 bats. NV RNA was found in bat saliva and urine. These data suggest the persistence of NV infection in Thai bats.

Nipah virus (NV) caused a major outbreak in swine and humans in Malaysia from September 1998 to April 1999 that led to 265 human cases with 105 deaths and the culling of >1 million swine (1). The genesis of the outbreak was suggested to be associated with bats (2,3). NV and Hendra virus (HV) are members of the *Paramyxoviridae* family in the genus *Henipavirus* (4). A seroepidemiologic study in Malaysia implicated 4 fruit bat species, *Pteropus hypomelanus*, *P. vampyrus*, *Cynopterus brachyotis*, *Eonycteris spelaea*, and an insectivorous bat, *Scotophilus kuhlii* (2). NV was also identified and isolated from bat urine samples of *P. hypomelanus* (5). Unlike NV's first appearance in Malaysia, in outbreaks in Bangladesh, infection may have been contracted by eating fruits contaminated with bat saliva, and transmitted from person to person (6). Antibodies to NV antigen were detected in 2 *P. giganteus* adult females from Bangladesh (6). Recently, antibodies to NV and virus isolation were successfully demonstrated in *P. lylei* from Cambodia (7).

Thailand is bordered by Malaysia to the south and Cambodia to the southeast. No NV infections in humans have been reported in Thailand. Surveillance in swine by enzyme-linked immunosorbent assay (ELISA) showed negative results (8). Estimates suggest ≈112 bat species in Thailand; 18 are fruit bats and 94 are insectivorous bats (9). Given that NV has caused several outbreaks in the region, obtaining baseline data for surveillance and planning for future public health assessment of its impact are essential.

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The Study

From March 2002 to February 2004, a total of 17 trips were made to 15 sites in 9 provinces in central, eastern, and southern Thailand (Figure). Bats were caught and blood samples were collected as previously described (10). Of 12 bat species collected, 6 were frugivorous and 6 were insectivorous (Figure). Seventy-one percent (932) of 1,304 samples were from *Pteropus* bats and 66% (857) were from *P. lylei*. Saliva and urine were obtained by swabbing and stored in tubes with 1.0 mL of NucliSens lysis buffer containing guanidine thiocyanate (bioMérieux, Boxtel, the Netherlands) for transporting. Liquid from ≈10 individual samples from the same species, colony, and time of capture was saved into the same pool. A total of 142 pools each were collected from 1,286 saliva and 1,282 urine specimens. The pooled specimens were frozen at −70°C until analysis.

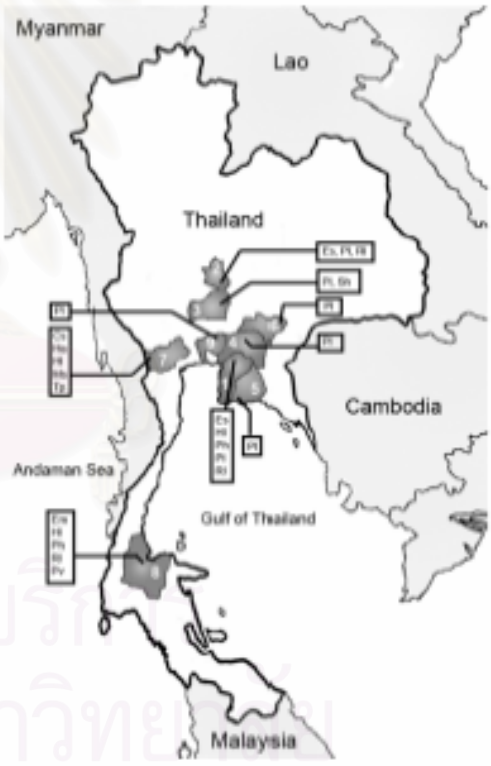

Figure. Locations in Thailand where bats have been captured. 1 = Chon Buri, 2 = Sing Buri, 3 = Ayutthaya, 4 = Cha Choeng Sao, 5 = Ra Yong, 6 = Pra Chin Buri, 7 = Ratcha Buri, 8 = Surat Thani, 9 = Bangkok. Species analyzed: Cs = *Cynopterus sphinx*, Em=*Embalonura monticola*, Es = *Eonycteris spelaea*, Ha = *Hipposideros armiger*, Hl = *Hipposideros larvatus*, Ms = *Megaderma spasma*, Ph = *Pteropus hypomelanus*, Pl = *P. lylei*, Pv = *P. vampyrus*, Rs = *Rousettus leschenaulti*, Sh = *Scotophilus heathi*, Tp = *Tadarida plicata*.

Immunoglobulin G (IgG) antibodies to NV were assayed by indirect ELISA at Chulalongkorn University Hospital, with a protocol developed by the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia. Serum samples were heated to 56°C and titrated at 4 dilutions (1:100, 1:400, 1:1,600, and 1:6,400). Of the 1,054 serum specimens tested, 82 (7.8%) from 4 species—*P. hypomelanus* (n = 4), *P. lylei,* (n = 76), *P. vampyrus* (n = 1), and *Hipposideros larvatus* (n = 1)—were NV IgG antibody–positive (titer ≥1:400) with 43 at a titer of 1:400; 30 at 1:1,600, and 9 at 1:6,400. *P. lylei* contained higher serum antibody titers than other species (9 of 76 at 1:6,400, 29 of 76 at 1:1,600) (Table).

Total RNA was extracted from saliva and urine according to manufacturer's protocol. A RNA plasmid was introduced as an internal control RNA in the duplex reverse transcription–polymerase chain reaction (RT-PCR) as previously described (11). NV nucleoprotein (N)-specific primers used for reverse transcription and first-round PCR were: NP1F, 5′ CTT GAG CCT ATG TAT TTC AGA C 3′; NP1R, 5′ GCT TTT GCA GCC AGT CTT G 3′. The internal primers for nested PCR were previously described (1). This process allowed an internal control to be visualized as the upper (323 bp) bands and NV product as lower bands (227 bp). Single-step RT-PCR was performed by using the One Step RT-PCR kit (Qiagen Inc., Valencia, CA, USA) followed by nested PCR. The PCR product was sized by gel electrophoresis in 2% agarose. Only samples showing both the 323-bp internal control and 227-bp NV-specific bands, or only a NV-specific band, were considered positive; those showing only the internal control band were considered negative. Those showing no band were tested again and judged to contain enzyme inhibitors if no band was shown on repetition. All samples with positive results were tested again without the positive control, and the sequence of amplified product was determined by using internal primer.

The sensitivity of the duplex system is not notably altered by incorporation of the internal control RNA (data not shown). Samples from a saliva pool of *H. larvatus* from site 1 in Chon Buri Province and another pool of *P. lylei* from site 3 in Chon Buri Province were duplex nRT-PCR positive. All 6 positive duplex nRT-PCR urine pools were collected from *P. lylei* captured from 3 different sites, 1 from Cha Choeng Sao, 1 from Bangkok, and 4 from site 3 in Chon Buri. The 181-nucleotide (nt) sequences of the N gene obtained from 1 saliva pool of *H. larvatus* was identical to those reported from Malaysia (accession no. NC_002728). The sequences of 1 saliva pool from *P. lylei* and 6 urine pools from *P. lylei* were identical to those reported from Bangladesh (AY988601) with 13 divergent nt (92% identity) from Malaysia. The nucleotide changes at positions 1397, 1407, and 1481 resulted in amino acid substitutions (with 94% identity to Malaysia, 56 of 59) from isoleucine to valine, glycine to glutamic acid, and asparagine to aspartic acid at codons 429, 432, and 457 of N protein, respectively. Nine divergent nucleotides among Thai, Bangladesh, and Cambodia (AY858110) did not result in amino acid differences.

Conclusions

This study reports the evidence of NV infection in Thai frugivorous and insectivorous bats demonstrated by IgG antibodies to NV in serum samples and NV RNA in urine and saliva. Antibodies against NV were detected in *P. hypomelanus, P. vampyrus, P. lylei,* and *H. larvatus*. NV

Table. ELISA, PCR saliva, and PCR urine results for Nipah virus from 12 bat species, Thailand, 2002–2004*

Species	Total bats	ELISA		PCR saliva‡		PCR urine‡	
		No. analyzed	No. positive (%) †	No. analyzed	No. pool positive/total	No. analyzed	No. pool positive/total
Frugivorous							
Cynopterus sphinx	34	10	0	34	0/5	34	0/5
Eonycteris spelaea	64	54	0	64	0/7	64	0/7
Pteropus hypomelanus	36	26	4 (15.4)	36	0/6	35	0/6
P. lylei	857	813	76 (9.3)	845	1/87	845	6/87
P. vampyrus	39	38	1 (2.6)	39	0/4	39	0/4
Rousettus leschenaulti	11	4	0	6	0/3	6	0/3
Insectivorous							
Emballonura monticola	14	12	0	14	0/2	14	0/2
Hipposideros armiger	88	6	0	88	0/10	88	0/10
H. larvatus	95	74	1 (1.3)	94	1/10	91	0/10
Megaderma spasma	13	0	0	13	0/2	13	0/2
Scotophilus heathi	3	3	0	3	0/1	3	0/1
Tadarida plicata	50	13	0	50	0/5	50	0/5
Total	1,304	1,054	82 (7.8)	1,286	2/142	1,282	6/142

*ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction.
†ELISA positive: titer ≥1:400.
‡10 individual samples (saliva or urine) from the same species, colony, and the time of capture were saved into the same pool.

infections in the first 2 species were similar to those reported in Malaysia (2). *P. lylei* was the only bat species found NV-infected among 14 species tested in Cambodia (7). An earlier report demonstrated a correlation between ELISA and neutralization tests with 87% sensitivity and 99% specificity (7). These data support our ELISA results as a firstline screening tool to investigate NV infection in countries that do not have a BSL-4 facility in which to perform neutralization assays. The finding of unusually high antibody titers from *P. lylei* suggests that NV circulates mainly in this bat species in Thailand and Cambodia (7).

Although serum neutralization tests were not conducted, NV RNA was demonstrated in saliva and urine from *P. lylei* and saliva of *H. larvatus*. Determining PCR positivity by naked eye observations for the presence of a 227-bp fragment is not likely the most sensitive method (our detection limit is 0.37 pg total RNA/μL); therefore, some low-positive samples might be missed. Increasing the volume of sample tested by using a plastic sheet method in urine collection may overcome such problems (12).

Southern blot analysis is also useful for PCR confirmation; however, sensitivity may not be markedly improved as previously reported in the case of rabies (13). We used a nested PCR method because less RNA was required initially and because of a shorter turnaround time. Confirmation was achieved by direct sequencing of amplified products. Taken together, our current ELISA and PCR data are sufficient to conclude that Thai bats were naturally infected with NV. Higher numbers of PCR-positive samples in *P. lylei* may be due to a bias in species collection. Alternatively, in the serologic study, *P. lylei* may be the most prevalent infected species. Sequence analysis of the short 181-nt sequence suggests that ≥2 strains of NV are circulating in Thai bats. More sequence data are required to confirm this hypothesis. Finding NV RNA in saliva of *H. larvatus*, may indicate the insectivorous bat as another reservoir or this may be only an accidental spillage.

We believe that NV infection is prevalent in Thai fruit bats as previously reported in Malaysia and Cambodia (2,7). Countrywide surveillance is needed to clarify the epidemiology of NV infection in Thailand as it relates to host, seasonal, and geographic attributes.

Acknowledgments

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Ms Wacharapluesadee is head of the Molecular Biology Laboratory for Neurological Diseases, Chulalongkorn University Hospital. Her areas of interest are the diagnosis, pathogenesis, and pathophysiology of viral encephalitis.

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