Original Article

RT-PCR Survey of Emerging Paramyxoviruses in Cave-dwelling Bats

Lerdchai Chintapitasakul[1*] Nimit Choengern[2] Sara Bumrungsri[3] Mutita Chalamat[2] Lamul Molee[1] Sujira Parchariyanon[1] Somchuan Ratanamungklanon[2] Kritaya Kongsuwan[4]

Abstract

Bats are the reservoir hosts for several Paramyxoviruses including two serious zoonotic viruses, Hendra virus and Nipah virus which are responsible for fatal infections in animals and humans. These two viruses are sufficiently different from previously described Paramyxoviruses and are included in a new genus, Henipavirus. We report here a survey of cave-dwelling, insectivorous bats in Thailand for the presence of henipaviruses. Pooled urine samples were collected in nine caves inhabited by six different bat species in the northern (Chiangmai and Nakornsawan) and southern (Songkla and Satoon) provinces of Thailand. A reverse transcription PCR (RT-PCR) assay using henipavirus-specific primers derived from the conserved region of the RNA polymerase (L) gene was used to detect known and unknown viruses in this genus. Samples from seven out of nine caves surveyed tested positive by RT-PCR. Nucleotide sequences of the PCR bands revealed the presence of diverse strains (three clusters and seven divergent genotypes) of previously uncharacterised paramyxovirus(es). Phylogenetic analysis based on the deduced L protein sequence revealed close correlations between the positive samples and the recently described but unclassified paramyxoviruses: Beilong virus and J-virus. This is the first report on the prevalence of paramyxovirus variants in cave-dwelling bats and highlights the importance of further epidemiological surveillance in bats.

Keywords: bats, natural reservoir, paramyxovirus, RT-PCR, Thailand, zoonoses

[1] *National Institute of Animal Health, Department of Livestock Development, Bangkok 10900 Thailand*
[2] *Eastern Veterinary Research and Diagnosis Centre, Baan Boung, Chonburi 20220 Thailand*
[3] *Department of Biology, Faculty of Science, Prince of Songkla University, Hadyai, Songkla 90110 Thailand*
[4] *CSIRO Livestock Industries, Queensland Bioscience Precinct, St Lucia QLD 4067 Australia.*
***Corresponding author: E-mail:** lerdchai@gmail.com*

บทคัดย่อ

การสำรวจหาไวรัสอุบัติใหม่-พารามิกโซไวรัส จากค้างคาวถ้ำ ด้วยวิธีปฏิกิริยาลูกโซ่พอลิเมอเรสแบบย้อนกลับ

*เลิศชัย จินตพิทักษ์สกุล [1 *] นิมิตร เชื้อเงิน [2] สาระ บำรุงศรี [3] มุทิตะ ชลามาตย์ [2] ละมูล โม้ลี [1] สุจิรา ปาจริยานนท์ [1] สมชวน รัตนมังคลานนท์ [2] กฤตยา กองสุวรรณ [4]*

ค้างคาวเป็นพาหะของพารามิกโซไวรัสหลายตัว รวมทั้ง เฮนดราไวรัส และ นิปาห์ไวรัส โดยไวรัสทั้งสองชนิดนี้สามารถก่อให้เกิดโรคจนเป็นสาเหตุการตายของมนุษย์และสัตว์เลี้ยงลูกด้วยนม เนื่องจากไวรัสทั้งสองชนิดมีลักษณะที่แตกต่างจากพารามิกโซไวรัสตัวอื่นที่พบก่อนหน้านี้จึงถูกจำแนกออกมาเป็นสกุลใหม่ คือ เฮนิปาไวรัส คณะผู้วิจัยได้รายงานผลการสำรวจในค้างคาวถ้ำกินแมลงในประเทศไทยเพื่อหาเฮนิปาไวรัส โดยเก็บตัวอย่างปัสสาวะจากค้างคาว ๖ สายพันธุ์ ในถ้ำ ๙ แห่ง ตามพื้นที่ภาคเหนือและภาคใต้ (เชียงใหม่ นครสวรรค์ สงขลา และสตูล) การตรวจหาไวรัสใช้วิธีปฏิกิริยาลูกโซ่พอลิเมอเรสแบบย้อนกลับ โดยใช้ไพรเมอร์ที่จำเพาะต่ออาร์เอ็นเอพอลิเมอเรสยีนของเฮนิปาไวรัสมาเป็นไพเมอร์หาไวรัส พบว่าตัวอย่างปัสสาวะค้างคาวจากถ้ำ ๗ แห่ง (จาก ๙ ถ้ำ) ให้ผลบวกต่อการตรวจหาด้วยวิธีดังกล่าว เมื่อนำดีเอ็นเอสายใหม่ที่ได้จากขบวนการปฏิกริยาลูกโซ่มาหาลำดับเบส พบว่า เป็นสายลำดับเบสของพารามิกโซไวรัสชนิดที่ยังไม่เคยศึกษาคุณลักษณะมาก่อน สามารถแบ่งความแตกต่างทางลักษณะพันธุกรรมได้ ๓ กลุ่ม และ ๗ แบบพันธุธรรม เมื่อนำลำดับแอลโปรตีนมาของตัวอย่างที่ให้ผลบวกและมีความสัมพันธ์ใกล้ชิดกันมาวิเคราะห์หาสายสัมพันธ์พบว่า พารามิกโซไวรัสดังกล่าวมีความสัมพันธ์กับเบลองไวรัสและเจไวรัส การศึกษานี้เป็นการรายงานครั้งแรกของการสำรวจพบพารามิกโซไวรัสชนิดต่าง ๆ ในค้างคาวถ้ำ และ ยังเป็นจุดสำคัญที่นำไปสู่การสำรวจทางระบาดวิทยาในค้างคาวต่อไป

คำสำคัญ: ค้างคาว แหล่งรังโรคในธรรมชาติ พารามิกโซไวรัส ปฏิกิริยาลูกโซ่พอลิเมอเรสแบบย้อนกลับ ประเทศไทย โรคสัตว์สู่คน

[1] *สถาบันสุขภาพสัตว์แห่งชาติ กรมปศุสัตว์ กรุงเทพ 10900*

[2] *ศูนย์วิจัยและพัฒนาการสัตวแพทย์ภาคตะวันออก บ้านบึง ชลบุรี 20220*

[3] *ภาควิชาชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยสงขลานครินทร์ หาดใหญ่ สงขลา 90110*

[4] *CSIRO Livestock Industries, Queensland Bioscience Precinct, St Lucia QLD 4067 Australia*

***ผู้รับผิดชอบบทความ E-mail:** lerdchai@gmail.com*

Introduction

Approximately 70% of emerging infectious diseases (EIDs) are zoonotic (diseases that normally exist in animals but can infect humans) in origin. Southeast Asia is one of the hotspots of zoonotic diseases due to its high wildlife biodiversity, dense human population and its close contact between livestock and humans (Coker et al., 2011). This is best exemplified in the recent viral disease outbreaks in one or more countries in the region. An example of these outbreaks was the Nipah virus in Malaysia in 1998 (Chua et al., 2000). Virological and serological evidence supports the hypothesis that the fruit bat (flying fox), especially members of the genus *Pteropus*, is the reservoir host for the Nipah virus (Johara et al., 2001; Chua et al., 2002). Subsequently studies have shown that bats are an important host species for emerging viruses and play a significant role in disease transmission. To date more than 60 different viruses have been detected in and/or isolated from bats of diverse species (Calisher et al., 2006; Wong et al., 2007).

Paramyxoviruses are enveloped, non-segmented, negative-stranded RNA viruses that are divided into two subfamilies, Paramyxovirinae and Pneumovirinae. Viruses within the subfamily, Paramyxovirinae, have been associated with a number of emerging diseases in humans and various animals in the last decade and have been classified into five genera: Respirovirus, Morbillivirus, Rubulavirus, Avulavirus and Henipavirus (Mayo, 2002). Besides the five genera, Paramyxovirinae members also include several of the unclassified viruses recently isolated from a diverse range of animal species including Fer-de-lance virus (from a

Swiss snake- Clark et al., 1979), Tupaia paramyxovirus (from a tree shrew-Tidona et al., 1999), Mossman virus (Wang et al., 2003) and J-virus (Jack et al., 2005; Wang et al., 2005). Both Mossman virus and J-virus were isolated from pooled organs of native rodents trapped in Queensland, Australia. Narina virus was isolated from the pooled organ of a forest rodent species trapped in Eastern Trinidad (Tikasingh et al., 1966). Members of the genus Paramyxovirinae also include recently-isolated and unclassified viruses from cell cultures, Salem virus (from equine mononuclear cells-Renshaw et al., 2000), and Beilong virus (from human kidney mesangial cell line-Li et al., 2006; Wang et al., 2006). While the disease-causing potential of these unclassified paramyxoviruses remains unclear, it is clear that there is great genetic diversity within the subfamily Paramyxovirinae.

Pteropus bats, commonly known as fruit bats or flying foxes, are widely distributed in the tropical regions of Asia, Australia and Africa. The discovery that they are natural reservoirs for henipaviruses and that Nipah infections (in Bangladesh) can be transmitted directly from human to human (Luby et al., 2009) make it very important to continue monitoring for these diseases. This is especially significant in countries where intense depletion and fragmentation of the natural habitats of bats is occurring. In Thailand, survey studies (Wacharapluesadee et al., 2005; 2010) conducted mainly in the central part of the country confirmed that Pteropid fruit bats were the primary reservoir for Nipah virus in Thailand. In addition, viral RNA could be extracted from urine and saliva of P. *lylei* and a microbat, Horsfield's roundleaf bat (Hipposideros larvatus) (Wacharaplueasdee et al., 2005).

While many research efforts focused on fruit bats as they are now recognised hosts of many of the most virulent diseases globally, smaller insectivorous bats (also known as microbats) played a subordinate role and investigations involving microbats and infectious agents were largely limited to rabies (e.g. Favi et al., 2002). However insectivorous bats are important for ecological and economic reasons through natural pest control as they are voracious predators of nocturnal insects, including many crops and forest pests. In the present study, we attempt to fill this knowledge gap and investigate the presence of viral pathogens and, in particular, members of the genus Henipavirus in cave-dwelling, insectivorous bats by reverse transcription PCR (RT-PCR) assays.

Materials and Methods

Experimental design: In order to provide a certain chance of pathogen detection, we collected pooled urine samples rather than individual specimens. The approach also excluded bat handling which is labour-extensive and minimised disturbing bat habitats which is important from the welfare and conservation perspectives. The field studies took place in 2010 between August and November to avoid the annual bat cycle of sexual activity and birth (usually between May and July). They were conducted in 4 provinces in southern and northern Thailand (Table 1). Sites (caves) were chosen as locations of roosting sites in large colonies. Nine roosting sites were chosen in this study, each consisting of different or a mixture of bat species (Table 1). The first trip in August 2010 was in Hadyai-Songkla and Satoon provinces and the second trip in October 2010, five caves in the provinces of Chiangmai and Nakornsawan were surveyed (Table 1).

Sample collection: Urine was sampled using clean plastic sheets spread on the cave floor. Plastic sheets (70x72 cm) were placed near spots where previous droppings were observed in the evening prior to the sample collection day. The location of each sheet, cave name and number as well as GPS location and site elevation were recorded. Fresh urine droppings were collected early in the morning of the following day to avoid evaporation. Syringes and sterile cotton swabs were used to collect or soak up the urine. The samples were immediately mixed with an equal volume of buffer RLT (β-ME added) (Qiagen, Santa Clara, CA). Bat classification was performed by the Prince of Songkla bat team using standard morphological characters, body measurements and ultrasonic echolocation calls recorded by the bat-detector.

RT-PCR: Viral RNAs were extracted from ~200 µl of pooled urine sample with RNAeasy Mini Kit (Qiagen, Santa Clara, CA) according to the manufacturer's instructions. For semi-nested RT-PCR, the team followed the protocol of Tong et al. (2008) which targets the conserved region of the RNA polymerase (L) gene. Briefly, three sub-family specific primers were used for viral screening: two forward nested primers PAR-F1, 5'GAAGGITATTGTCAIAARNT NTGGAC3'; PAR-F2, 5'GTTGCTTCAATGGTTCAR GGNGAYAA3'; and a reverse primer PAR-R, 5'GCT GAAGTTACIGGITCICCDATRTTNC3'. Primers for detection of the subgroup Henipavirus were HEN-F1, 5'TCITTCTTTAGAACITTYGGNCAYCC3'; HEN-F2, 5'GCCATATTTTGTGGAATAATHATHAAYGG3'; and HEN-R, 5'CTCATTTTGTAIGTCATYTTNGC RAA3'. The SuperScript III One-Step RT-PCR kit (Invitrogen, Carlsbad, CA) was used for the semi-nested and one tube/one step RT-PCR. The PCR amplification was carried out in a 25 µl reaction mix containing 2 µl of extracted RNA, 100 pmol of each of forward and reverse primers and 12.5 µl of 2x QIAGEN OneStep RT-PCR Enzyme Mix. Water was added to obtain a final volume of 25 µl. The cycle conditions were 60^0C for 1 min and 50^0C for 30 min for RT, 94^0C for 2 min (for hot start) and then 40 cycles at 94^0C for 15 sec, 49^0C for 30 sec and 72^0C for 1 min (for PCR amplification), and a final extension at 72^0C for 5 min. Standard precautions were taken to avoid PCR contamination, and water controls which were included in all RT-PCR assays did not show false-positive results. Finally, PCR products (5 µl) were electrophoresed in 2.0% agarose gels in a standard TAE buffer and visualised by UV light after staining with ethidium bromide.

Sequencing: After the PCR screening, the amplicons of expected size (500 nt) were purified using Wizard DNA Clean-up kit (Promega Corporation, Madison, Wisconsin, USA) following the kit's instructions. The

recovered DNA was quantitated by running a small amount (about 20-50 ng) of the DNA on a TBE gel containing ethidium bromide along with a standard (Promega Bench Top 1 kb DNA ladder). The PCR products were subsequently cloned into the pGEMT-Easy Vector (Promega) and at least ten clones of each sample were sequenced. Sequencing was done on ABI 96-capillary DNA Analyzer (Applied Biosystems, Foster City, USA) with SP6 and T7 primers. Highly similar sequences (>98 % nucleotide identity) derived from the same sample were grouped and their majority consensus sequences were taken as representatives of the distinctive genotypes in these samples.

Phylogenic analysis: Nucleotide and protein alignments were achieved by CLUSTALW (version 1.83) and corrected manually by visual inspection. Phylogenetic analysis and genetic distances calculation were based on the partial RNA polymerase (L) protein sequence and was performed using MEGA version 5.05 (http://www.megasoftware.net). The tree was constructed using the neighbour-joining method, and evaluated statistically using 1000 bootstrap replicates.

Results

Pooled bat urine samples from nine caves representing six species of bats were found positive when pan-Paramyxovirinae and henipavirus specific L gene primers were used in the RT-PCR assays. Both sets of primers yield similar results, but the sensitivity of the less degenerate henipavirus was found to be higher than the pan-paramyxovirus primers (data not shown). Positive PCR products of the expected size of ~500 nt were detected in 18/100 samples tested with 18% virus burden (Table 1).

Table 1 Detection of paramyxoviruses in 9 caves in 4 provinces of Thailand

Cave	Location	Coordinates	Bat species	No. Sample		No. Genotypes
				tested	positive	
Sri son, Songkla	S	N 7°4'37.58", E 100°9'55.63"	Ha, Hd, Es	11	5	2
Ta nuam, Songkla	S	N 6°42'23.37", E 100°16'30.56"	Hl	3	2	1
Khao teap, Songkla	S	N 6°59'36.01", E 100°8'26.37"	Ha, Hc	20	1	1
Khao kao, Songkla	S	N 6°42'34.99", E 100°16'42.94"	Es	5	0	0
Phu pha phet, Satoon	S	N 7°7'39.15", E 99°59'45.42"	Es, Ts	2	0	0
Pha daeng, Chiangmai	N	N 19°30'11.24", E 99°8'50.44"	Ha, Hl and Ts	20	3	5
Chiang dao, Chiangmai	N	N 19°23'39.95", E 98°55'41.02"	Ha	15	1	2
Khao hai pyar, Chiangmai	N	N 18°02'45.50", E 98°32'38.75"	Hl, Ts	12	4	3
Khao nor, Nakornsawan	N	N 15°57'06.76", E 99°52'42.96"	Tp	12	2	2
Total				**100**	**18**	**16**

S: Southern province, N: Northern province

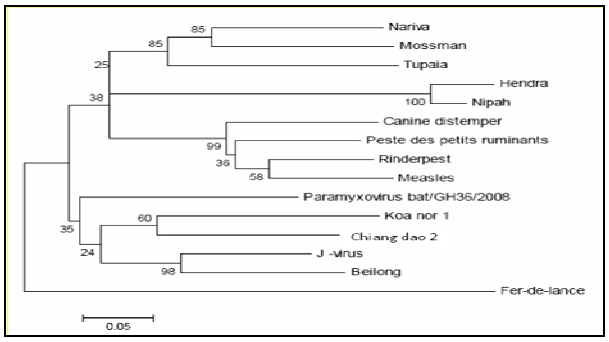
Bat species: Ha: *Hipposideros armiger*, Hd: *Hipposideros diadema*, Es: *Eonycteris spelaea*, Hl: *Hipposideros arvatus*, Hc: *Hipposideros cineraceus*, Ts: *Taphozous spp*, Tp: *Tadarida plicata*

Table 2 Genetic diversity of bat paramyoviruses. Pairwise comparison of the nucleotide sequences of L gene amplicon. Results are shown as ClustalW scores (pairwise alignment)

Genotype	Khao nor 1	Hai pyar 3	Ta nuam	Pha daeng 3	Pha daeng 4	Khoa teap	Chiang dao 1	Pha daeng 2	Sri son 2	Pha daeng 5	Khao nor 2	Sri son 1	Pha daeng 1	Hai pyar 1	Hai pyar 2	Chiang dao 2
Khao nor 1	100	100	98	97	82	82	82	82	82	77	75	76	76	75	74	75
Hai pyar 3		100	98	97	82	82	82	82	82	77	75	76	76	75	74	72
Ta nuam			100	98	82	82	82	82	81	77	76	75	76	75	74	72
Pha daeng 3				100	81	81	81	81	81	77	75	73	75	75	74	72
Pha daeng 4					100	99	84	85	84	75	76	73	71	75	74	72
Khoa teap						100	84	84	84	75	76	73	71	74	74	71
Chiang dao 1							100	98	95	75	77	74	75	76	73	76
Pha daeng 2								100	95	76	77	76	72	76	74	75
Sri son 2									100	76	76	76	75	76	75	75
Pha daeng 5										100	76	76	78	72	74	72
Khao nor 2											100	79	78	75	74	76
Sri son 1												100	77	74	75	74
Pha daeng 1													100	74	74	72
Hai pyar 1														100	78	73
Hai pyar 2															100	73
Chiang dao 2																100

Figure 1 Unrooted phylogenetic tree based on the partial L gene (502 nt) of 16 genotypes identified in the present study. The tree was generated from CLUSTALW nucleotide alignment and drawn using DRAWTREE (http://seqtool.sdsc.edu). Branch lengths represent relative genetic distances. Clusters are indicated by circles.

The positive samples were not restricted geographically as they were obtained from three caves in the south and four caves in the north (Table 1). Various genotypes were characterised by sequencing multiple plasmid DNA clones of each positive sample. The consensus sequences of the highly similar cloned sequences were regarded as the representative sequence of the distinctive genotypes. Overall, substantial genetic variability was detected within and between sites represented by the presence of 16 genotypes and their nucleotide comparison is shown in Table 2. The unrooted phylogenic tree drawn from ClustalW alignment of the 500 nt fragment of the L gene suggested the existence of three tight clusters. Cluster 1: Khao nor 1, Hai pyar 3, Ta nuam and Pha daeng 3 genotypes with ≥98% nt identity; cluster 2: Pha daeng 4 and Khao teap genotypes with 99% nt identity and cluster 3: Chiang dao 1, Pha daeng 2 and Sri son 2 with ≥95% nt identity. The remaining genotypes were very heterogeneous with a range of 71-78% nt identity with other genotypes (Table 2). Interestingly, there was no evidence of a differential geographical distribution (Table 2 and Fig 1). For example, Ta nuam (Fig 1) genotype (cluster 1) was more closely related to Khao nor 1 and Hai pyar 3 genotypes than to Khao teap (cluster 2) and Sri son 2 (cluster 3) genotypes in spite the fact that Ta nuam, Khao teap and Sri son caves, all located in Songkla province, are several hundred kilometres away from Khao nor and Khao hai pyar caves in the North (Fig 1).



Virus	Refseq protein/Genbank accession no.	% Similarity to	
		Kao nor 1	Chiang dao 2
Beilong	YP_512254	70.8	75.7
J-virus	YP_3380885	70.8	75.1
Paramyxovirus/bat/GH36/2008	FJ609192	73	68.4
Peste-des-petits -ruminants	YP_133828	70.1	69.5
Measles	NP_056924	68.3	68.9
Canine distemper	NP_047207	66.4	68.3
Rinderpest	YP_087126	65.8	67.7
Tupaia	NP_054697	66.4	67
Mossman	NP_958055	65.2	68.9
Nariva	ACL97360	65.2	68.3
Nipah	NP_112028	61.4	62.7
Hendra	NP_047113	60.8	60.8
Fer-de-lance	NP_899661	58.3	62.1

Fig. 2 Phylogenetic relationship of Kao nor 1 and Chiang dao 2 genotypes with other 13 relevant paramyxoviruses based on a fragment of RNA-dependent RNA polymerase region. Percent similarity was obtained from simple alignment of the 15 sequences using ClustalW with default gap penalties and Gonnet correction values. Tree constructed using neighbour-joining method with 1000 bootstrap resamplings. Branch lengths represent relative genetic distances. The program chose Fer-de-lance virus as the out group.

The amino acid sequences of the 500 nt fragment of the L gene from two representative genotypes, Khao nor 1 and Chiang dao 2, were blasted against GenBank and refseq protein databases. These sequences of these two genotypes were relatively distantly related (75% nt and 78% amino acid identity) and could represent two unique virus species in bats. The deduced proteins of Khoa nor 1 and Chiang dao 2 genotypes shared an average of 66.3% (ranging from 58.3 to 70.8%) and 67.9% (ranging from 62.1 to 75.7%) amino acid identity, respectively, to their homologues of relevant animal Paramyxovirinae members (Table in Fig 2). Neighbour-joining phylogenetic tree of paramyxovirus partial polymerase protein sequence data (corresponding to the pan-paramyxovirus fragment) (Fig 2) suggested that Khoa nor 1 and Chiang dao 2 were phylogenetically close to the recently characterised but unclassified Beilong virus and J-virus than to Hendra and Nipah viruses (Fig 2).

Discussion

In this study we report the widespread presence of new paramyxovirus(es), closely related to members of unclassified, larger genome viruses, the Beilong virus (Li et al., 2006) and J-virus (Jack et al., 2005) lineage in Thai bats. While the disease-causing potential of Beilong and J-viruses is unclear, they were isolated from rodent and human cells, respectively. Together the data suggest that potential opportunities exist for cross-species transmission (or spilled over from bats) of the new paramyxovirus to other mammals and underlie the necessity to conduct further surveillance investigation in bats.

Preliminary phylogenetic analysis of the L gene sequence obtained from urine specimens indicated great genetic diversity of paramyxoviruses in Thai bats. For example, here we observed the existence of at least three genetic clusters with >95% nt identity and the presence of seven other divergent genotypes with a range of 71-77% nt identity. In the study we sampled bats from only a very small subset (nine caves) of the several hundreds of bat caves in Thailand, therefore, it is possible that a larger study covering more caves to include additional bat species may reveal hitherto undetected varieties of paramyxoviruses. Based on the available data, our work supports the enormous diversity of the paramyxovirus genomes in bats and the hypothesis that paramyxoviruses may have co-evolved with their bat hosts. Future studies will include characterisation of complete genome sequences of some of our isolates to identify a number of unique viruses and their degree of diversity. In any case, this is the first report that provides evidence of new paramyxovirus(es) closely related to Beilong and J-viruses circulating in Thai bats. The possible ongoing evolution of the paramyxoviruses in bats may have implications for the emergence of new viruses into new hosts and for future disease risk management.

It is interesting to note that the extent of genetic variation within sites was higher in the northern provinces than the southern provinces although the reason for the differences remains unclear. However, it is noteworthy that the caves in Songkla and Satoon were not usually frequented by people, but the caves in Chiangmai, especially Pha daeng cave, where 5 different genotypes were found, (Table 1) is a tourist destination and is visited by both locals and travellers. It may be that the pressure on the bat populations in Pha daeng cave has increased significantly with human interference and changing local habitats, and this in turn has resulted in an increase in the mutation and/or recombination rates of the viral populations. Alternatively, the high level of genetic variability within the site may also be due to some relationship with the number of species, or a high level of mixing of bats between the different species.

The results did not indicate a significant relationship between geographically close populations. Instead, the results indicated that the virus is present over a large geographical area and is consistent with the broad geographical range of species of bats included in the present study. The main species investigated: *Hipposideros armiger*, *H. larvatus* and *Tadarida plicata* all have a broad geographical range which is known to include the south of China, India, much of South-east Asia, the Philippines and Indonesia. Hence, it is possible that the distribution of the new viruses may be even greater than that documented here. The result is consistent with the recent study of long-distance bat movements using satellite transmitters that there is the potential to transfer viruses between regions and countries (Breed et al., 2010).

Although our sampling size and location were not exhaustive, the data gathered in this study were sufficient to demonstrate the moderate virus burden (18%). In Thailand previous work testing for neutralising antibodies against lyssavirus and Nipah virus infections found that the prevalence in Thai bats was 7.3% for lyssavirus (Lumlertdacha et al., 2005) and up to 12% for Nipah virus (in *P. lylei*) (Wacharapluesadee et al., 2010). In the present study there was some evidence of a positive correlation between the virus burden and colony size which were larger in caves where positive samples were found (data not shown). Despite using degenerate primers that could detect members of the henipavirus group, we did not detect Nipah virus RNA positive in our urine specimens. A possible seasonal variation in the Nipah virus concentration and infection in bats (Luby et al., 2009; Wacharapluesadee et al., 2010) may explain why in our study we did not detect any Nipah virus positives among insectivorous bats. Our survey was conducted from August to October and the shedding of this virus was observed from December to June with the highest peak of the virus detection occurring in May (Wacharapluesadee et al., 2010).

In conclusion, we identified new paramyxovirus(es) in various cave-dwelling bat populations in Thailand and suggest that, similar to the fruit bats, microbats may also act as a reservoir of emerging viruses that might pose a zoonotic threat to

animals and humans. The discoveries of genetically diverse strains of the virus in different bat species have implications for the virus evolution in bats and the continuing threat of new, emerging diseases.

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References

Breed, A.C., Field, H.E., Smith, C.S., Edmonston, J. and Meers, J. 2010. Bats without borders: Long-distance movements and implications for disease risk management. Eco Health. DOI: 10.1007/s10393-010-0332-z.

Calisher, C.H., Childs, J.E., Holmes, K.V. and Schountz. T. 2006. Bats: Important reservoir hosts of emerging viruses. Microbiol Rev. 19: 531-545.

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

Chua, K.B., Koh, C.L., Hooi, P.S., Wee, K.F., Khong, J.H., Chua, B.H., Chan, Y.P., Lim, M.E. and Lam, S.K. 2002. Isolation of Nipah virus from Malaysian island flying-foxes. Microbes Infect. 4: 145-151.

Clark, H.F., Lief, F.S., Lunger, P.D., Waters, D., Leloup, P., Foelsch, D.W. and Wyler, R.W. 1979. Fer De Lance Virus (Fdlv) - Probable paramyxovirus isolated from a reptile. J Gen Virol. 44: 405-418.

Coker, R.J., Hunter, B.M., Rudge, J.W., Liverani, M. and Hanvoravongchai, P. 2011. Health in southeast Asia 3 emerging infectious diseases in southeast Asia: Regional challenges to control. Lancet. 377: 599-609.

Favi, M., de Mattos, C.A., Young, V., Chala, E., Lopez, L.R. and de Mattos, C.C. 2002. First case of human rabies in Chile caused by an insectivorous bat virus variant. Emerg Infect Dis. 8: 79-81.

Jack, P.J., Boyle, D.B., Eaton, B.T. and Wang, L.F. 2005. The complete genome sequence of J-virus reveals a unique genome structure in the family Paramyxoviridae. J Virol. 79: 10690-10700.

Johara, M., Field, H., Rasdi, A., Morrissy, C. vander Heide, B., Rota, P., Azri, A., White, J., Daniels, P. Jamaluddin, A. and Ksiazek, T. 2001. Serological evidence of infection with Nipah virus in bats (order Chiroptera) in Peninsular Malaysia. Emerg Infect Dis. 7: 439-441.

Li, Z., Yu, M., Zhang, H., Magoffin, D.E., Jack, P.J., Hyatt, A., Wang, H.Y. and Wang, L.F. 2006. Beilong virus, a novel paramyxovirus with the largest genome of non-segmented negative-stranded RNA viruses. Virol. 346: 219-228.

Luby, S.P., Hossain, M.J., Gurley, E.S., Ahmed, B.N., Banu, S., Khan, S.U., Homaira, N., Rota, P.A., Rollin, P.E., Comer, J.A., Kenah, E., Ksiazek, T.G. and Rahman, M. 2009. Recurrent zoonotic transmission of Nipah virus into humans, Bangladesh, 2001-2007. Emerg Infect Dis. 15: 1229-1235.

Lumlertdacha, B., Boongird, K., Wanghongsa, S., Wacharapluesadee, S., Chanhome, L., Khawplod, P., Hemachudha, T., Kuzmin, I. and Rupprecht, C.E. 2005. Survey for bat lyssaviruses, Thailand. Emerg Infect Dis. 11: 232-236.

Mayo, M.A. 2002. Virus taxonomy-Houston 2002. Arch Virol. 147: 1071-1076.

Renshaw, R.W., Glaser, A.L., Van Campen, H., Weiland, F. and Dubovi, E.J. 2000. Identification and phylogenetic comparison of Salem virus, a novel paramyxovirus of horses. Virol. 270: 417-429.

Tidona, C.A., Kurz, H.W., Gelderblom, H.R. and Darai, G. 1999. Isolation and molecular characterization of a novel cytopathogenic paramyxovirus from tree shrews. Virol. 258: 425-434.

Tikasingh, E.S., Jonkers, A.H., Spence, L. and Aitken, T.H. 1966. Nariva virus, a hitherto undescribed agent isolated from the Trinidadian rat, *Zygodontomys b. brevicauda* (J.A. Allen & Chapman). Am J Trop Med Hyg. 15: 235-238.

Tong, S., Chern, S.W., Li, Y., Pallansch, M.A. and Anderson, L.J. 2008. Sensitive and broadly reactive reverse transcription-PCR assays to detect novel paramyxoviruses. J Clin Microbiol. 46: 2652-2658.

Wacharapluesadee, S., Boongird, K., Wanghongsa, S., Ratanasetyuth, N., Supavonwong, P., Saengsen, D., Gongal, G.N. and Hemachudha, T. 2010. A longitudinal study of the prevalence of Nipah virus in *Pteropus lylei* bats in Thailand: evidence for seasonal preference in disease transmission. Vector Borne Zoonotic Dis. 10: 183-190.

Wacharapluesadee, S., Lumlertdacha, B., Boongird, K., Wanghongsa, S., Chanhome, L., Rollin, P., Stockton, P., Rupprecht, C.E., Ksiazek, T.G. and Hemachudha, T. 2005. Bat Nipah virus, Thailand. Emerg Infect Dis. 11: 1949-1951.

Wang, L.F., Jack, P.J.M., Boyle, D.B. and Eaton, B.T. 2005. The complete genome sequence of J virus reveals a unique genome structure in the family Paramyxoviridae. J Virol. 79: 10690-10700.

Wang, L.F., Li, Z., Yu, M., Zhang, H., Magoffin, D.E., Jack, P.J.M., Hyatt, A. and Wang, H.Y. 2006. Beilong virus, a novel paramyxovirus with the largest genome of non-segmented negative-

stranded RNA viruses. Virol. 346: 219-228.
Wang, L.F., Miller, P.J., Boyle, D.B. and Eaton, B.T. 2003. Full-length genome sequence of Mossman virus, a novel paramyxovirus isolated from rodents in Australia. Virol. 317: 330-344.
Wong, S., Lau, S., Woo, P. and Yuen, K.Y. 2007. Bats as a continuing source of emerging infections in humans. Rev Med Virol. 17: 67-91.