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Pteropine orthoreovirus Infection Among Out-Patients With Acute Upper Respiratory Tract Infection in Malaysia

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This study aims to assess the incidence rate of *Pteropine orthreovirus* (PRV) infection in patients with acute upper respiratory tract infection (URTI) in a suburban setting in Malaysia, where bats are known to be present in the neighborhood. Using molecular detection of PRVs directly from oropharyngeal swabs, our study demonstrates that PRV is among one of the common causative agents of acute URTI with cough and sore throat as the commonest presenting clinical features. Phylogenetic analysis on partial major outer and inner capsid proteins shows that these PRV strains are closely related to Melaka and Kampar viruses previously isolated in Malaysia. Further study is required to determine the public health significance of PRV infection in Southeast Asia, especially in cases where co-infection with other pathogens may potentially lead to different clinical outcomes. ***J. Med. Virol. 87:2149–2153, 2015***. © 2015 Wiley Periodicals, Inc.

KEY WORDS: *Pteropine orthoreovirus*; Melaka virus; Nelson Bay virus; Kampar virus; Pulau virus

INTRODUCTION

The first *Pteropine orthoreovirus* (PRV), previously named Nelson Bay virus, was isolated from flying foxes in Australia in 1970 [Gard and Compans, 1970]. Subsequent isolation of PRVs were made from bats and patients first in Malaysia [Pritchard et al., 2005; Chua et al., 2007, 2008, 2011], followed by isolation from patients after visiting Bali, Indonesia which is geographically near Malaysia [Wong et al., 2012; Yamanaka et al., 2014; Lorusso et al., 2015] and from different species of bats in China [Du et al., 2010; Hu et al., 2014]. Clinical presentation of the patients ranged from acute respiratory syndrome to mild influenza-like illness. Up until now, all these studies were conducted on individual patients. Here, we report a prevalence study by molecular detection of PRV from oropharyngeal swabs of out-patients suffering from acute respiratory tract infections in Rembau, Malaysia. Phylogenetic analysis using partial gene sequences indicated that the PRVs detected in this study have great genetic relatedness to each other as well as to PRVs previously isolated in Malaysia.

MATERIALS AND METHODS

Sample Collection

A total of 200 oropharyngeal swabs samples was collected from patients aged 12 years old and above with acute upper respiratory tract infection (URTI) seen in Rembau Health Clinic from May to September 2012. Rembau is a small dispersedly populated district in the Negeri Sembilan state of Malaysia. Fruit trees are commonly planted in the vicinity of houses for both shade and fruits, and fruit bats (*Cynopterus* and *Eonycteris* spp.) are often sighted flying in the district at nights by local residents.

Patients presenting with an acute onset (<5 days) of subjective fever, cough or sore throat without any known cause were recruited. Informed consents were taken from all the patients. This study had obtained research and ethical approval (Ref. No. 4.10/JCM-54/2012[BMSc]) from IMU Joint Committee of the

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TABLE I. Epidemiological Details of the Specimens With and Clinical Features of the Patients

Specimen	Age	Sex	Occupation	Smoking	Fruit tree near house?	Contact with bats?	Ill family members?	Date of collection	No. days into fever	Temperature [°C]	Cough	Cough with phlegm	Sore throat	Cervical lymph node palpable	Throat injected	Tonsils enlarged
Rembau 1	31	Male	Mechanic	No	Yes	No	No	May 21	0	No record	No	No	Yes	No	Yes	Yes
Rembau 3	28	Male	Rubber tapper	Yes	Yes	No	No	May 21	10	37.0	Yes	No	Yes	No	Yes	No
Rembau 5	20	Female	Student	No	No	No	Yes	May 22	3	No record	No	No	Yes	No	Yes	Yes
Rembau 7	47	Female	Housewife	No	Yes	Yes	Yes	May 22	5	No record	Yes	Yes	Yes	No	Yes	Yes
Rembau12	27	Male	Businessman	Yes	Yes	No	No	May 22	1	39.4	Yes	Yes	Yes	No	No	No
Rembau 15	61	Male	Policeman (retired)	No	Yes	No	Yes	May 23	4	36.9	Yes	Yes	No	No	Yes	No
Rembau 34	47	Male	Medical staff	No	Yes	No	No	May 25	1	38.0	No	No	Yes	No	Yes	No
Rembau 36	13	Female	Student	No	Yes	No	No	May 25	2	37.0	No	No	Yes	No	Yes	No
Rembau 38	23	Male	Student	No	Yes	Yes	Yes	May 25	2	37.7	Yes	Yes	Yes	No	Yes	No
Rembau 45	18	Female	Cashier	No	Yes	No	Yes	May 28	2	36.9	Yes	No	No	No	No	No
Rembau 47	19	Male	Executive	Yes	No	Yes	Yes	May 29	0	No record	Yes	No	Yes	No	Yes	Yes
Rembau 52	38	Female	Teacher	No	Yes	No	Yes	May 29	2	36.5	Yes	Yes	Yes	No	No	No
Rembau 54	18	Male	Student	Yes	No	No	No	May 29	0	36.8	Yes	Yes	Yes	No	Yes	No
Rembau 56	21	Female	Waitress	No	No	No	No	May 30	0	37.0	Yes	Yes	Yes	No	Yes	No
Rembau 64	22	Male	Student	Yes	Yes	No	Yes	May 31	4	38.1	Yes	No	Yes	No	Yes	No
Rembau 76	30	Male	Fireman	No	Yes	No	No	Jun 11	7	37.2	Yes	No	Yes	No	Yes	No
Rembau 79	17	Male	Student	No	Yes	No	Yes	Jun 12	1	No record	Yes	Yes	Yes	No	No	No
Rembau 85	73	Male	Retiree	No	Yes	No	No	Jun 12	1	36.7	Yes	Yes	Yes	No	No	No
Rembau 97	26	Female	Teacher	No	Yes	No	No	Jun 14	5	37.8	Yes	Yes	Yes	No	No	No
Rembau 113	13	Male	Student	No	Yes	No	No	Jun 19	4	38.8	No	No	Yes	No	Yes	No
Rembau 114	16	Female	Student	No	No	No	No	Jun 19	1	37.1	Yes	No	Yes	Yes	Yes	No
Rembau 117	30	Male	Self-employed	Yes	Yes	No	Yes	Jun 19	3	36.2	Yes	Yes	Yes	Yes	Yes	No
Rembau 121	14	Female	Student	No	Yes	Yes	Yes	Jun 20	1	38.3	Yes	No	Yes	No	Yes	No
Rembau 125	50	Male	Civil servant	Yes	No	No	No	Jun 20	8	36.6	Yes	Yes	No	No	Yes	No
Rembau 126	38	Female	Cafeteria worker	No	No	No	No	Jun 20	3	36.9	Yes	Yes	Yes	No	Yes	No
Rembau 127	12	Female	Student	No	Yes	No	No	Jun 20	2	38.0	No	No	Yes	No	Yes	Yes
Rembau 130	24	Male	Welder	Yes	Yes	Yes	Yes	Jun 21	1	36.6	No	No	No	No	Yes	No
Rembau 135	60	Female	Cafeteria worker	No	No	No	No	Jun 22	2	37.2	Yes	Yes	Yes	No	Yes	No
Rembau 142	14	Female	Student	No	Yes	No	No	Jun 25	4	37.0	No	No	No	No	No	Yes
Rembau 143	66	Female	Mechanic	No	No	No	Yes	Jun 25	4	37.3	Yes	Yes	Yes	No	Yes	No
Rembau 146	15	Female	Student	No	Yes	No	No	Jun 25	2	37.0	Yes	Yes	Yes	No	No	No
Rembau 150	17	Female	Student	No	No	No	Yes	Jun 25	3	35.5	Yes	Yes	Yes	No	Yes	Yes

TABLE I. (*Continued*)

Specimen	Age	Sex	Occupation	Smoking	Fruit tree near house?	Contact with bats?	Ill family members?	Date of collection	No. days into fever	Temperature [°C]	Cough	Cough with phlegm	Sore throat	Cervical lymph node palpable	Throat injected	Tonsils enlarged
Rembau 155	14	Male	Student	No	Yes	No	No	Jun 26	2	38.0	Yes	No	No	No	Yes	No
Rembau 169	40	Male	Weed control	Yes	No	Yes	Yes	Jun 27	2	36.8	Yes	Yes	Yes	No	No	No

The six specimens for phylogenetic analysis are highlighted in gray.

Research and Ethics Committee and registered in National Medical Research Register (ID No. 12222). The collected swabs were immediately placed in Universal Transport Medium (Copan, Italia). The specimens were stored in −20.0°C and were transported in ice box with ice pack to International Medical University (IMU) for processing every 2 days.

Molecular Detection

Viral nucleic acids were extracted from the specimens using QIAamp viral RNA mini kit according to manufacturer protocol, followed by reverse transcription following previously published protocol with random primers replacing Primer B [Attoui et al., 2001]. Nested PCR were performed using AmpONE TM Pfu DNA polymerase (GeneALL, Korea) with primers targeting the conserved viral sigma1/A gene (major outer capsid). Cultured PRV (Melaka virus) on Vero cells was used as positive control and non-template controls were included as negative control (refer Figs. S2 and S5 in Supplementary Data). Primer sequences used in this study are Sig1F 5′-GTGCCGTGTTCGACTTCTTTAC-3′ and Sig1R 5′-ACAACAGCATTCGACCCTAC-3′ for outer sequence and PRVSig1F2 5′-TGCTGATTGGAA CGCTGACT-3′ and PRVSig1R2 5′-CGGAAAAGGTTTGAGACGCC-3′ for internal sequence, respectively. The PCR condition was set at 95°C for 3 min, followed by 40 cycles of 95°C for 40 sec, 57°C for 40 sec, 72°C for 90 sec, and ends with 72°C for 6 min. The expected PCR product is 364 bp in length. Primers specific to influenza viruses (A and B) and coronavirus and nested PCR conditions were adapted from previous published studies [Coiras et al., 2003, 2004].

Sequencing and Phylogenetic Analysis

For phylogenetic analysis, nested PCR of sigma 2/B gene (minor outer capsid) was conducted using primers Sig2F 5′-GAACRCCCAAYTTCCACTCG-3′ and Sig2R 5′-TGTCTCRGCTRACCCTGTCC-3′ for outer sequence and PRVSig2F2 5′-GCTGTGTGGCTTCAGTCTCT-3′ and PRVSig2R2 5′-GGYARDCCYGCCATAATCGG-3′ for internal sequence, respectively. The PCR condition was similar to above except for the annealing temperature, which is at 55°C. The predicted 470-bp amplicons were purified and sequenced directly. Phylogenetic trees were constructed using neighbor-joining method using MEGA5 [Tamura et al., 2011].

Statistical Analysis

χ^2 test or Fisher's exact test, whichever applicable, was used to calculate the *P*-value for various signs and symptoms. Any sign and symptom that had *P*-value of less than 0.05 is considered statistically significant correlating to *Pteropine orthoreovirus* infection. Odd ratio and confident interval were calculated using SPSS18.1.

RESULTS

Of the 200 out-patients with URTI, PRV was detected in the oropharyngeal samples of 34. Among the positively identified patients, nine were co-infected with influenza A virus and one with coronavirus OC43. This study showed the incidence rate of 17% (34/200). It is interesting to note that this prevalence level is similar to that found from a previous serological surveillance on inhabitants in Tioman island, Malaysia that showed a sero-prevalence rate of 13% [Chua et al., 2007]. χ^2 test shows the significant clinical symptoms of patients with *Pteropine orthoreovirus* infection were cough and sore throat. The *P*-value for cough with phlegm is 0.047 (odds ratio 0.378, 95%CI 0.148–0.961) and for sore throat is 0.031 (odds ratio 2.726, 95%CI 1.067–6.967).

To determine the genetic relatedness of PRVs detected in this study with previously isolated PRVs, partial sequences of the sigma1/A and sigma 2/B genes from six selected specimens with high viral nucleic acid level were determined from the respective PCR products. The epidemiological details and the clinical features of the six patients randomly select for this analysis are given in Table I. As shown in Figure S1, sequence alignment of these regions indicates that although they are high related to each other and to the other known PRVs previously detected in Malaysia, they are not identical to each other. Furthermore, phylogenetic analyses based on these partial gene sequences revealed two important findings. First, all the PRV sequences detected in the six independent specimens are closely related, but not completely identical (see Table SI). Second, the PRVs detected in this study are more related to Malaysian/Indonesian PRVs than to other PRVs (92–99% and 97–98% similarity to sigma1/A and sigma2/B proteins of Melaka virus, respectively). In addition, comparing the phylogenetic topology (Fig. 1) of the two gene segments among the six different PRVs detected in this study, it is plausible to conclude that reassortment between PRVs occurs at a high frequency.

Finally, to confirm that the genetic sequences detected in patient specimens were a result of active infection, virus isolation was attempted on the six specimens using Vero cells. After 6–8 blind passages, syncytial cytopathic effect similar to those of other PRVs in Vero cells were observed (Fig. S2) and identity of PRV from each sample was further confirmed by PCR and sequencing to ascertain that the isolate has the same sequences as detected in the initial PCR fragments (data not shown).

DISCUSSION

To our knowledge, this is the first molecular surveillance study of PRV infection among URTI patients. This study demonstrated that PRV infection is more common than expected, at least in this region of Malaysia. The new finding should be taken into consideration in future diagnosis and treatment of patients with URTI in bat-populated areas in

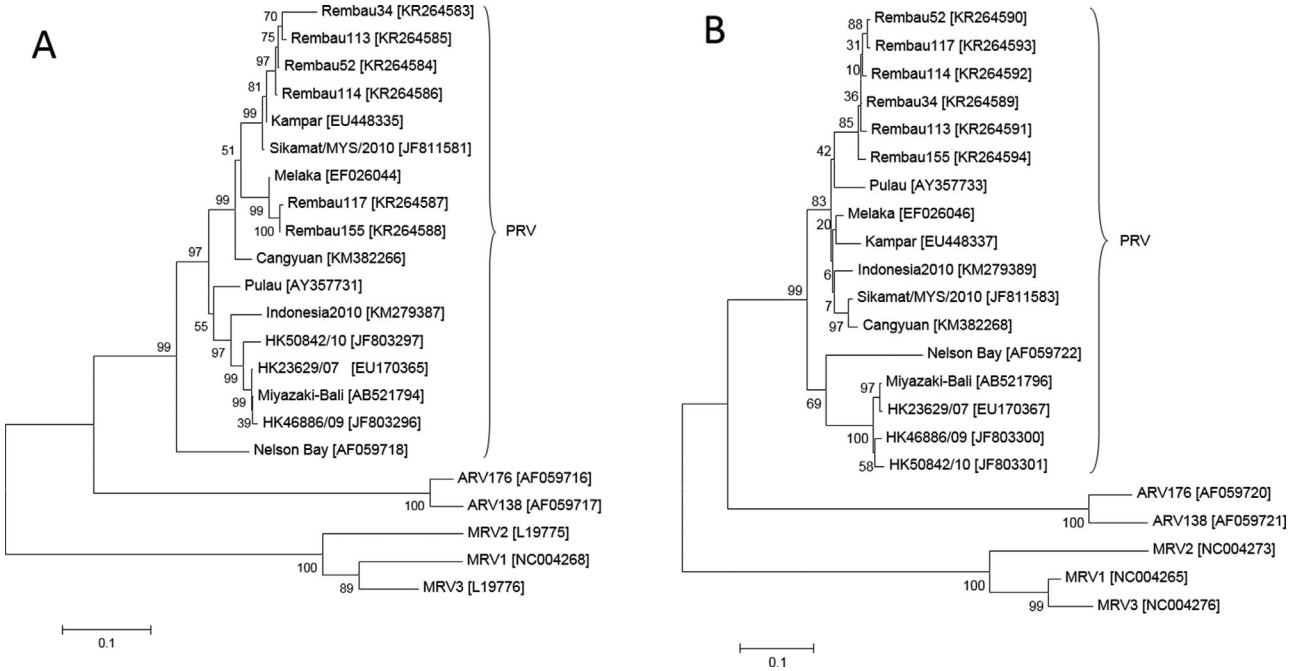

Fig. 1. Neighbor joining trees based on partial gene sequences of sigma1/A (**A**) and sigma 2/B (**B**). Numbers at nodes indicate levels of bootstrap support calculated from 1,000 trees. Scale bar indicates amino acid residue substitution per site. GenBank accession number for each sequence is provided in bracket. ARV, avian orthoreovirus; MRV, mammalian orthoreovirus; PRV, *Pteropine orthoreovirus*.

Malaysia and neighboring Southeast Asian nations. On reviewing patients' histories, approximately 90% of the patients did not reveal history of direct contacts with bats and, therefore, the mode of transmission of PRV is yet to be determined. It is worth to note that human-to-human transmission of PRV has been observed previously in at least two independent studies [Chua et al., 2007, 2008]. In this context, it will also be important to determine in future studies whether similar incident rate of PRV infection is also present in URTI patients in other regions or states in Malaysia where no bats or different bats are in circulation.

When using PCR to evaluate prevalence of infection, it is extremely important to eliminate laboratory contamination in the process. Although we cannot be 100% sure that the 17% infection rate in the current cohort is absolute accurate, we are confident that laboratory contamination was not a major issue in our analysis based on the following. First, as shown in Figure S1, the sequences from different samples were not identical (as one would expect from laboratory contamination). Second, the positive samples were randomly distributed, rather than clustered (e.g., for the first 20 samples, #1, 3, 5, 7, 12, and 15 were positive and the rest were negative). Third, negative control swabs samples consistently showed negative results in the same PCR reactions (data not shown).

One of the shortcomings of the current study is the lack of seroprevalence analysis due to the lack of matching serum samples from these patients. Although a previously published study conducted for residents of the Tioman Island demonstrated a seroprevalence of 13% [Chua et al., 2007], close to the 17% of PCR-positive rate in this study, it will be more informative to conduct parallel serological and molecular surveillance in future studies.

Finally, the detection of PRV in URTI patients at such a high rate is a significant discovery. However, our current study was not designed to establish a causative relationship between PRVs and URTI in these patients. This could be partially addressed in future studies by including a cohort from the same locality without URTI syndrome.

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SUPPORTING INFORMATION

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