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Serological evidence of human infection by bat orthoreovirus in Singapore

Running title: Human exposure to bat orthoreovirus, Singapore

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Abstract

To determine whether *Pteropine orthoreovirus* (PRV) exposure has occurred in Singapore, we tested 856 individuals from an existing serum panel collected from 2005-2013. After an initial screen with luciferase immunoprecipitation system (LIPS) and secondary confirmation with virus neutralization test, we identified at least seven individuals with specific antibodies against PRV in both assays. Our

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findings confirm that PRV spillover into human populations is relatively common in this region of the world.

Keywords

Bat, orthoreovirus, spillover, luciferase immunoprecipitation system, virus neutralization test

Conflict of interest: None

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Introduction

The family *Reoviridae* is a diverse group of non-enveloped, segmented, double-stranded RNA viruses. *Pteropine orthoreovirus* (PRV) is a group of fusogenic viruses in the genus *Orthoreovirus* with a ten-segment genome. PRV1NB, (alternatively known as Nelson Bay virus) was first isolated in 1968 from Australian bats (*Pteropus poliocephalus*) and then almost 40 years later PRV3M (alternatively known as Melaka virus) was isolated from humans with acute respiratory illness [1-3]. Subsequently, evidence of different PRV infection has been observed in other Southeast Asian countries in both humans and bats, with a seroprevalence of 4.4-13% in specific populations [4-7]. Numerous reports of PRV exposure across the region suggest continuous spillover events are occurring [8].

The objective of this study was to determine whether PRV-related spillover events had occurred in Singapore as, to our knowledge, no cases have been reported to date. In this study, we adopted the Luciferase Immunoprecipitation System (LIPS) as a primary serological screening platform. LIPS is a very convenient system that utilizes crude lysate and minimal sera volumes to screen for antibodies against specific targets [9]. This method has several key advantages over conventional ELISA: 1) it does not require purified antigen; 2) it can be established within a short period of time; and 3) it is highly sensitive. We developed LIPS assays for two different PRV strains as for rapid serological screening of human samples, followed by confirmation with virus neutralization test.

Materials and Methods

Serum samples included in this study were from the Early Dengue Infection and Outcome (EDEN) Study, a longitudinal study conducted in Singapore from 2005-2013. Adult (>21 years) patients were enrolled upon presentation of acute onset of fever ($\geq 38^{\circ}\text{C}$ for less than 72 h) with no rhinitis or clinically obvious alternative diagnoses. The study has previously reported 11% of its participants to be positive for acute dengue virus infection [10]. It should be emphasized that selection of this serum collection was based on availability and the goal of the current study is to determine sero-prevalence of PRV antibodies in Singapore population, and is not to determine etiology of the febrile illness of the patient cohort.

Adopting the LIPS platform, we generated Ruc-PRV antigen fusion constructs as previously described [9]. The cell adhesion protein, S1 or Sigma C, and the major outer capsid protein, S4 or Sigma 2, were selected from two phylogenetically distant PRV strains, PRV1NB and PRV3M. While S4 is the more conserved protein across the different PRV strains, S1 differs most amongst the strains and is likely responsible for neutralization, thereby it can be used to distinguish across the strains [11]. Both S1 and S4 genes were individually cloned downstream of the Renilla luciferase gene in the pREN2 vector [9]. The recombinant plasmid DNA was then transfected into COS-1 cells, where crude cell lysate containing antigen fusion constructs was subsequently harvested 48 h post-transfection. Protein expression and base line luciferase readout slightly differed across the four constructs, but were not significantly different (Figure 1a & 1b). For sensitivity assessment, lysates were incubated with hyperimmune PRV3M and PRV1NB sera. As the more sensitive protein, the S1 gene construct was selected for the cohort screening (Figure 1c).

To detect antibody responses to all presently known PRVs, our initial screening was conducted with a mixture of both S1 LIPS constructs following published methods [9]. Briefly, serum samples were diluted at 1:10 in assay buffer B (20 mM Tris, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 1% Triton X-100, 100 µg/ml BSA). 10 µl of the diluted sera was mixed with 90 µl of buffer A (20 mM Tris, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 1% Triton X-100), and 1x10⁶ Light Units (LU) of each Ruc-PRV construct. Pierce Protein A/G UltraLink Resin beads (ThermoFisher Scientific) were added to the sera-lysate mixture and incubated at

4°C, with rotation, for 2 h. The mixture was then transferred to a 96-well filter plate and washing steps were performed using a vacuum plate washer. After the final wash, coelenterazine substrate mix (Promega) was added and luminescence was measured using on a Cytation 5 imaging reader (BioTek Instruments). Each screened plate included serum-free Flag as positive control to address protein expression of the constructs, and diluent as negative control to address background luminescence.

Of the 856 samples, 40 positives, as determined by exceeding an intensity of 5 standard deviations (SD) above the geometric mean (Figure 2), were chosen for further analysis. In the secondary LIPS assay, PRV1NB and PRV3M constructs were deconvoluted and samples below the threshold aforementioned were excluded. The secondary LIPS assay yielded 12 positive individuals (Table 1).

PRV1NB and PRV3M virus neutralization tests (VNT) were performed on the sera samples from the 12 LIPS positive individuals. Briefly, serum samples were diluted two-fold from 1:10 to 1:1280 and mixed with 200 TCID₅₀ of virus in an equivalent volume of DMEM. The serum-virus mixture was incubated at 37°C for 30 min, and then added to Vero cells. After 1 h incubation at 37°C, the inoculum was removed and cells were washed twice with serum-free DMEM. Fresh DMEM containing 2% FCS was added and cells were incubated at 37°C for 3 days. Each VNT plate included incubation of hyperimmune PRV1NB and PRV3M monkey serum with virus as positive control, and incubation only in the presence of virus as negative control.

Results and Discussion

Of the 856 individuals screened, 12 individuals had high light units and 7 contained neutralizing antibodies against PRV3M (Table 1). One individual had high neutralizing titers to PRV3M at 1:1280 and low neutralizing to PRV1NB, suggesting low levels of cross-neutralization.

Access to the EDEN febrile cohort provided an opportunity to serologically detect PRV3M in Singapore, albeit in a select population. Through iterative testing, 12 individuals were LIPS positive of which only 7 were positive for neutralizing antibodies, suggesting LIPS' sensitivity to detect closely related PRVs. It is important to note that through the pulldown with Protein A/G beads, all antibodies – not just neutralizing antibodies – with binding properties to Protein A/G beads were targeted; hence high LIPS read out was not necessarily indicative of neutralizing properties. The high LIPS reading, but non-neutralizing, samples may also indicate exposure to other PRV(s) related, but different from PRV3M in the region. These results indicate that spillover event(s) may have taken place as early as 2006, a logical finding, given the geographic proximity of Singapore to Malaysia, where PRV3M was first isolated from humans in 2006. Our findings may be an underrepresentation of the PRV3M seropositivity in Singapore as this study only used sera from a selected patient cohort (EDEN).

We demonstrate that LIPS is an appropriate tool for rapid detection of antibodies with high level of sensitivity. Although ELISAs remain to be the method of choice in serology, LIPS provides a rapid alternative that negates the

need for protein purification. This is especially crucial for pathogens that do not have well-established serologic assays, as for most recently emerged pathogens. Additionally, as the LIPS assay does not require a secondary antibody for detection, the same assay can be used for surveillance of zoonotic infections in both human and animal samples.

Our study corroborates findings from previous serological and molecular surveillance conducted in Malaysia and Vietnam revealing a PRV exposure rate of 3-11% in human populations in this region. It is unclear that whether such exposure was all of a direct spillover from bats to humans, as suspected for the PRV3M human cases [2], or from an intermediate host(s) yet to be identified. As our LIPS platform is equally applicable to serum of different animal species origin, it is ideally suited for future sero-epidemiological studies of wild and livestock animals in this region.

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Figures

Figure 1a: Protein expression for Flag-tagged PRV LIPS constructs. Flag expression for the four PRV LIPS constructs (PRV3M_S1, PRV3M_S4, PRV1NB_S1 and PRV1NB_S4) along with empty pREN2 vector was measured.

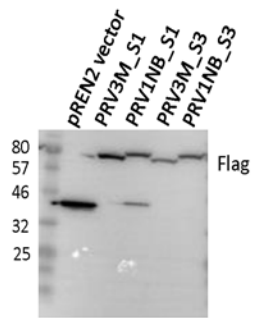


Figure 1b: Baseline luciferase measurement. Luciferase was measured for the four PRV LIPS constructs (PRV3M_S1, PRV3M_S4, PRV1NB_S1 and PRV1NB_S4) along with empty pREN2 vector.

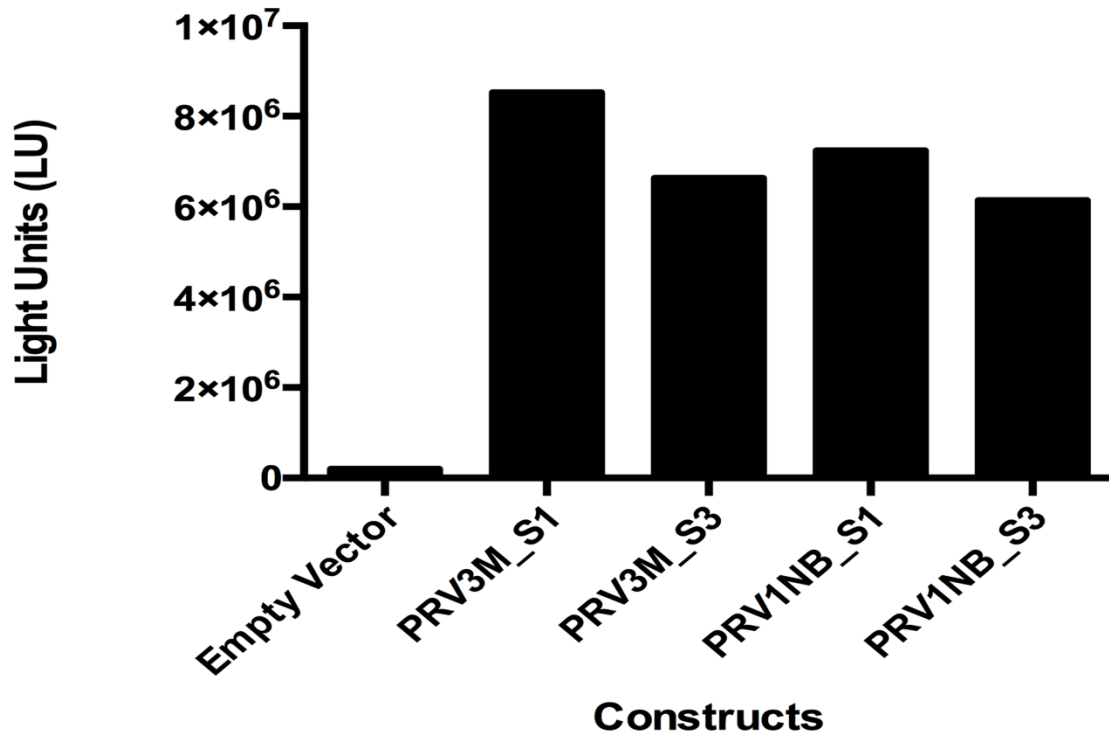


Figure 1c: Sensitivity assessment for PRV LIPS constructs. Four PRV LIPS constructs (PRV3M_S1, PRV3M_S4, PRV1NB_S1 and PRV1NB_S4) along with empty pREN2 vector were incubated with PRV negative and hyperimmune PRV3M and PRV1NB sera.

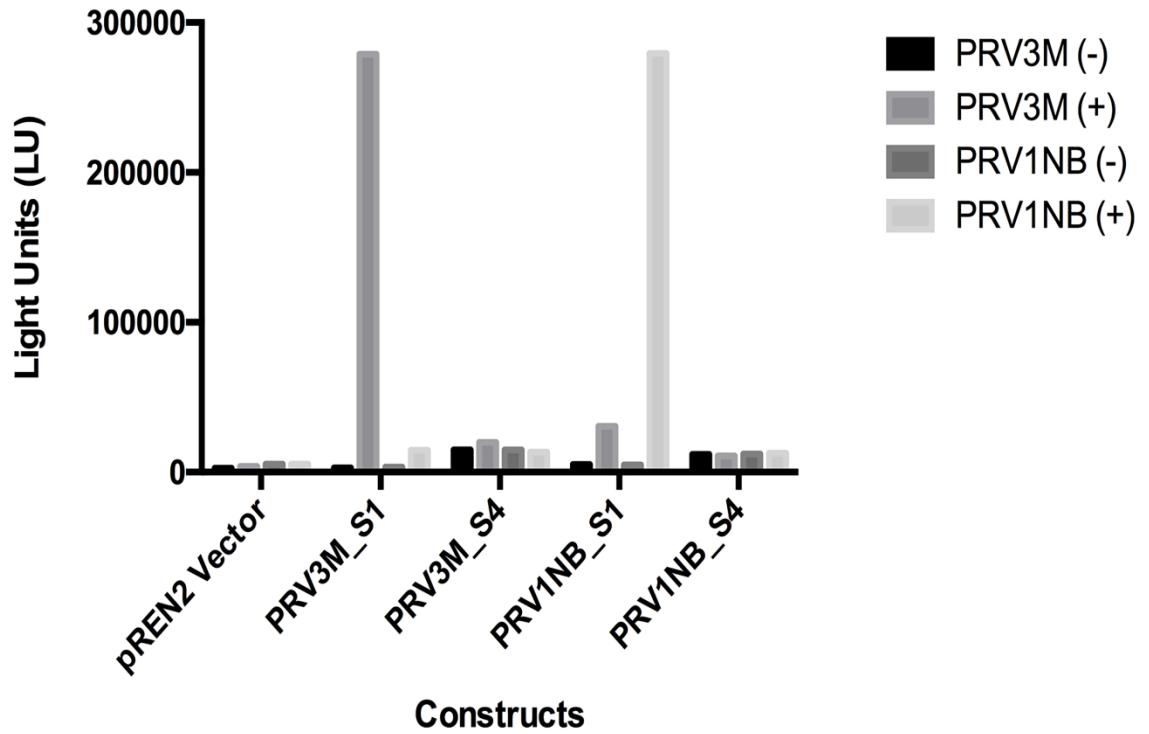


Figure 2: Initial LIPS screen of PRV where convalescent samples were screened for PRV1NB and PRV3M simultaneously by mixing the two constructs. Dashed line indicates threshold (5 SD above geometric mean).

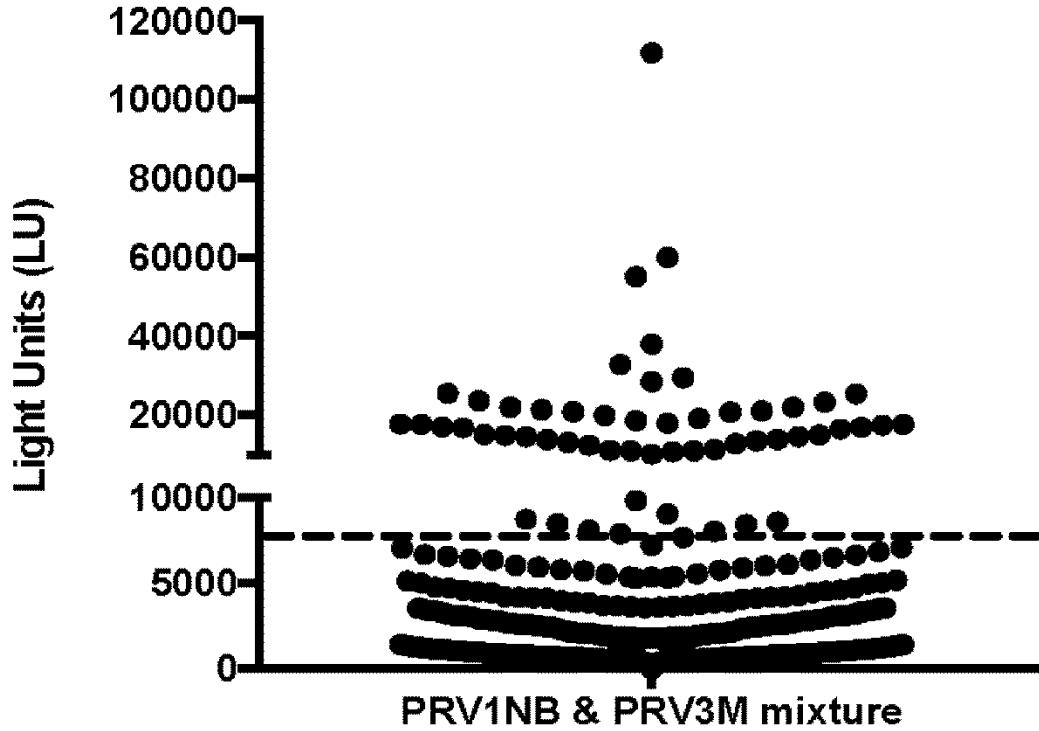


Table I: PRV serologic assay results

Year	Sample ID	PRV3M	PRV1NB	PRV3M	PRV1NB
		LIPS (LU)	LIPS (LU)	SNT	SNT
2006	4675	21489	115349	160	<10
2006	1043	54272	37322	<10	<10
2006	2363	26198	28214	<10	<10

2006	4658	5675	30548	80	<10
2006	5617	30626	30116	1280	40
2006	4669	16521	1360	160	<10
2007	1540	27095	23520	<10	<10
2007	2351	74447	14398	320	<10
2007	3601	51691	65493	<10	<10
2009	12022	41139	33011	<10	<10
2009	11266	71437	12678	20	<10
2009	7535	82350	7056	160	<10

Values in boldface indicate readouts above the threshold for LIPS or neutralizing titer for SNT. LIPS threshold was determined as 5SD above the geometric mean.