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SHORT COMMUNICATION

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Serological evidence of human infection by bat orthoreovirus in 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 and secondary confirmation with virus neutralization test, we identified at least seven individuals with specific antibodies against PRV in both assays. Our findings confirm that PRV spillover into human populations is relatively common in this region of the world.

KEYWORDS
bat, luciferase immunoprecipitation system (LIPS), orthoreovirus, spillover, virus neutralization test (VNT)

1 | INTRODUCTION

The family *Reoviridae* is a diverse group of nonenveloped, 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 policephalus*) 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 that 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 enzyme-linked immunosorbent assay (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 (VNT).

2 | 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

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enrolled upon presentation of acute onset of fever (≥38°C for less than 72 hours) 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 the seroprevalence 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 hours after transfection. Protein expression and baseline luciferase readout slightly differed across the four constructs but were not significantly different (Figure 1A and 1B). For sensitivity assessment, lysates were incubated with hyperimmune PRV3M and PRV1NB sera. As the

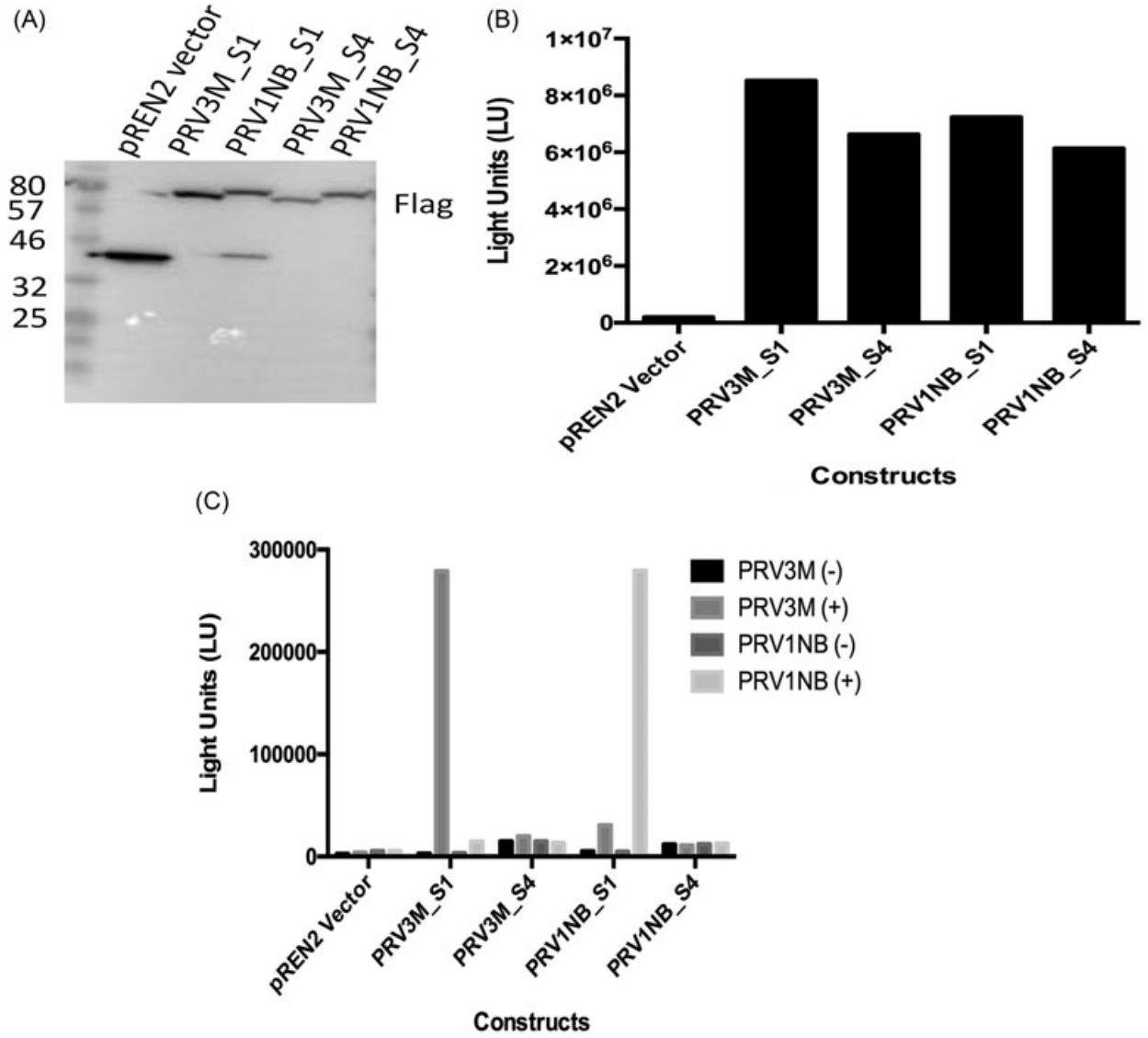

FIGURE 1 A, Protein expression for Flag-tagged PRV LIPS constructs. Flag expression for the four PRV LIPS constructs (PRV3M_S1, PRV3M_S4, PRV1NB_S1, and PRV1NB_S4) along with empty pREN2 vector was measured. B, Baseline luciferase measurement. Luciferase was measured for the four PRV LIPS constructs (PRV3M_S1, PRV3M_S4, PRV1NB_S1, and PRV1NB_S4) along with the empty pREN2 vector. C, Sensitivity assessment for PRV LIPS constructs. Four PRV LIPS constructs (PRV3M_S1, PRV3M_S4, PRV1NB_S1, and PRV1NB_S4) along with the empty pREN2 vector were incubated with PRV negative and hyperimmune PRV3M and PRV1NB sera. LIPS, Luciferase Immunoprecipitation System; PRV, *Pteropine orthoreovirus*

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 an assay buffer B (20 mM Tris, pH 7.5; 150 mM NaCl; 5 mM $MgCl_2$; 1% Triton X-100; 100 µg/mL BSA). Ten microliters of the diluted sera were mixed with 90 µL of buffer A (20 mM Tris, pH 7.5; 150 mM NaCl; 5 mM $MgCl_2$; 1% Triton X-100), and 1×10^6 LU of each Ruc-PRV construct. Pierce Protein A/G UltraLink Resin beads (Thermo Fisher Scientific, Waltham, MA) were added to the sera-lysate mixture and incubated at 4°C, with rotation, for 2 hours. 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, Madison WI) was added and luminescence was measured using on a Cytation 5 imaging reader (BioTek Instruments, Winooski, VT). Each screened plate included serum-free Flag as a positive control to address the protein expression of the constructs, and diluent as a negative control to address background luminescence.

Of the 856 samples, 40 positives, as determined by exceeding an intensity of 5 standard deviations 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 VNTs 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 $TCID_{50}$ of virus in an equivalent volume of DMEM. The serum-virus mixture was incubated at 37°C for 30 minutes, and then added to Vero cells. After 1 hour 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 the virus as the positive control, and incubation only in the presence of virus as the negative control.

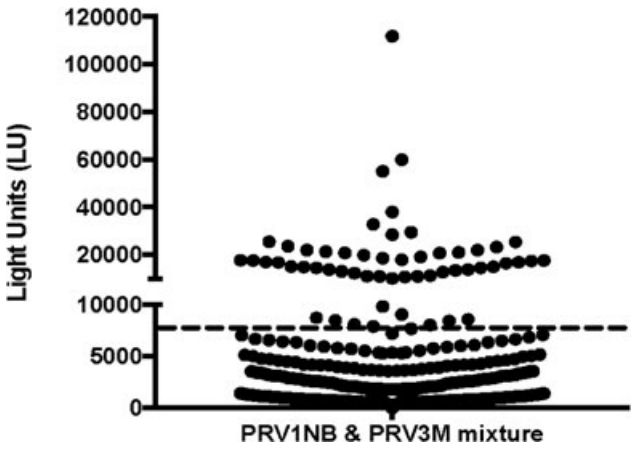

FIGURE 2 Initial LIPS screen of PRV where convalescent samples were screened for PRV1NB and PRV3M simultaneously by mixing the two constructs. The dashed line indicates threshold (5 SD above geometric mean). LIPS, Luciferase Immunoprecipitation System; PRV, *Pteropine orthoreovirus*

TABLE 1 PRV serologic assay results

Year	Sample ID	PRV3M LIPS, LU	PRV1NB LIPS, LU	PRV3M VNT	PRV1NB VNT
2006	4675	**21 489**	**115 349**	**160**	<10
2006	1043	**54 272**	**37 322**	<10	<10
2006	2363	**26 198**	**28 214**	<10	<10
2006	4658	5675	**30 548**	**80**	<10
2006	5617	**30 626**	**30 116**	**1280**	**40**
2006	4669	**16 521**	1360	**160**	<10
2007	1540	**27 095**	**23 520**	<10	<10
2007	2351	**74 447**	14 398	**320**	<10
2007	3601	**51 691**	**65 493**	<10	<10
2009	12 022	**41 139**	**33 011**	<10	<10
2009	11 266	**71 437**	12 678	**20**	<10
2009	7535	**82 350**	7056	**160**	<10

Abbreviations: LIPS, Luciferase Immunoprecipitation System; LU, light units; PRV, *Pteropine orthoreovirus*; SD, standard deviations; VNT, virus neutralization test.

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

3 | 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 readout 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 a 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. In addition, 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 from 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 seroepidemiological studies of wild and livestock animals in this region.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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