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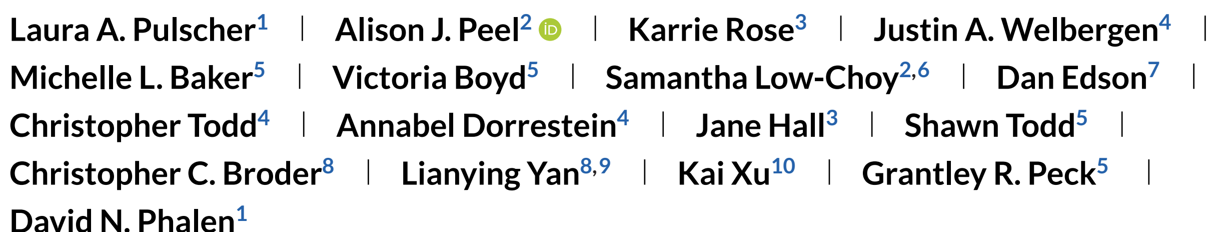
ORIGINAL ARTICLE

# Serological evidence of a pararubulavirus and a betacoronavirus in the geographically isolated Christmas Island flying-fox (*Pteropus natalis*)



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## Abstract

Due to their geographical isolation and small populations, insular bats may not be able to maintain acute immunizing viruses that rely on a large population for viral maintenance. Instead, endemic transmission may rely on viruses establishing persistent infections within hosts or inducing only short-lived neutralizing immunity. Therefore, studies on insular populations are valuable for developing broader understanding of viral maintenance in bats. The Christmas Island flying-fox (CIFF; *Pteropus natalis*) is endemic on Christmas Island, a remote Australian territory, and is an ideal model species to understand viral maintenance in small, geographically isolated bat populations. Serum or plasma ($n$ = 190), oral swabs ($n$ = 199), faeces ($n$ = 31), urine ($n$ = 32) and urine swabs ($n$ = 25) were collected from 228 CIFFs. Samples were tested using multiplex serological and molecular assays, and attempts at virus isolation to determine the presence of paramyxoviruses, betacoronaviruses and Australian bat lyssavirus. Analysis of

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serological data provides evidence that the species is maintaining a pararubulavirus and a betacoronavirus. There was little serological evidence supporting the presence of active circulation of the other viruses assessed in the present study. No viral nucleic acid was detected and no viruses were isolated. Age-seropositivity results support the hypothesis that geographically isolated bat populations can maintain some paramyxoviruses and coronaviruses. Further studies are required to elucidate infection dynamics and characterize viruses in the CIFF. Lastly, apparent absence of some pathogens could have implications for the conservation of the CIFF if a novel disease were introduced into the population through human carriage or an invasive species. Adopting increased biosecurity protocols for ships porting on Christmas Island and for researchers and bat carers working with flying-foxes are recommended to decrease the risk of pathogen introduction and contribute to the health and conservation of the species.

**KEYWORDS**
bat, betacoronavirus, insular populations, pararubulavirus, Pteropodidae, viral maintenance

## 1 | INTRODUCTION

Bats are hosts for coronaviruses, filoviruses, lyssaviruses and paramyxoviruses, among others (Brook & Dobson, 2015; Calisher et al., 2006). The strategies for viral maintenance in bat populations are not completely understood, and it is likely that maintenance strategies may differ among bat hosts and viruses. For example, it has been suggested that some viruses are maintained through episodic infection of local populations within a metapopulation structure (Plowright et al., 2011, 2016). Alternatively, some viruses are potentially maintained in bat populations through rapidly waning immunity, or persistent infections that are latent and recrudesce (Glennon et al., 2019; Jeong et al., 2017; Peel et al., 2012; Plowright et al., 2016; Wang et al., 2013). Based on either of these scenarios, it can be predicted that some viruses would fade out from a bat population falling below a certain population threshold, while other viruses may maintain themselves in small, isolated populations (Peel et al., 2012).

Bat populations that are small, geographically isolated, or have experienced multiple population bottlenecks may be expected to have decreased viral diversity compared to populations with larger geographic ranges and increased population genetic structure (Turmelle & Olival, 2009). However, few studies have tested this hypothesis in geographically isolated populations of bats, particularly on small, remote islands where bat species diversity is also low and there is no migration. These studies demonstrate that some populations, but not all, can maintain coronaviruses, henipaviruses and lyssaviruses and the prevalence of these viruses is lower than what has been reported in bats on larger, less isolated islands or continents (Joffrin et al., 2020; Mélade et al., 2016; Peel et al., 2012). Most of these studies have focused on one viral family; therefore, further studies on a range of viruses in small, isolated bat populations would provide additional insights into viral maintenance in bat populations.

An example of a geographically isolated bat species is the critically endangered Christmas Island flying-fox (CIFF; *Pteropus natalis*) which is confined to Christmas Island, a small 135 km$^2$ island approximately 380 km south of Java, Indonesia and 1500 km off the coast of Western Australia. The CIFF population has likely been separated from other bat populations for tens of thousands of years and is presumed to have been established by a small number of individuals (Phalen et al., 2017). Furthermore, the CIFF currently persists as a small population, ranging from 1000 to 6000 individuals, which has likely undergone multiple population bottlenecks (James et al., 2007; Tidemann, 1985; Todd, 2020). Given its presumed history of a small founder population, a relatively small extant population and a plausible history of fluctuations in population size, it seems likely that viral diversity in this species would be low. Another factor expected to limit the viral diversity in the CIFF would be its limited exposure to other populations or species of bats. For example, only one distantly related bat, the Christmas Island pipistrelle (*Pipistrellus murrayi*), was historically present on the island and is now thought to be extinct (Lumsden et al., 2017). Occasionally, vagrant bat species have been spotted on the island but are not known to have established colonies. To date, only a single cross-sectional study on viral prevalence and diversity in the CIFF has been undertaken. In this study, Vidgen et al. (2015) detected novel paramyxoviruses in 3 of 28 urine samples collected from individual bats, using a degenerative primer set specific for the L gene in the paramyxovirus genera respirovirus, morbillivirus and henipavirus. While sample sizes were small, this study provided evidence that paramyxoviruses are present and appear to be maintained in the CIFF population, but at a lower prevalence compared to Australian mainland flying-foxes (Vidgen et al., 2015). Given that paramyxoviruses have been identified, we predicted that other viruses would also be circulating in this small geographically isolated population.

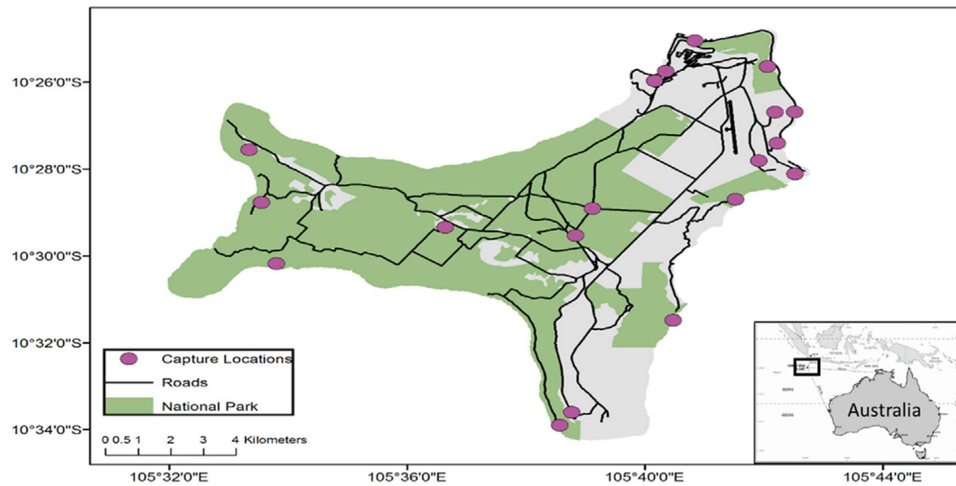


**FIGURE 1** Map indicating Christmas Island flying-fox (*Pteropus natalis*) capture locations. Inset shows location of Christmas Island relative to mainland Australia and the nearby island of Java, Indonesia

The objective of this study was to determine if multiple viral families could be maintained in small, geographically isolated bat populations, using the CIFF as a model species. To this end, serological and molecular techniques were used to screen CIFFs for paramyxoviruses, including Hendra virus (HeV), Nipah virus (NiV), Cedar virus (CedV), Menangle virus (MenV) and Tioman virus (TioV), betacoronaviruses including severe acute respiratory syndrome coronavirus (SARS-CoV-1) and Middle East respiratory syndrome coronavirus (MERS-CoV), and a rabies-related virus, Australian bat lyssavirus (ABLV), or related viruses which may be cross-reactive.

## 2 | MATERIALS AND METHODS

### 2.1 | Sample collection

CIFFs were captured on Christmas Island annually between May and October from 2015 to 2018. The species is considered a single panmictic population since individuals readily cross the entire island for foraging and roosting (Todd, 2020). CIFFs were captured at 19 foraging or roost sites across the island, including 11 sites in the national park (Figure 1) with nylon mist nets or an aluminium angler's landing net with a 64 × 56 cm hoop dimension attached to an extendable pole. Upon capture, individual CIFFs were temporarily placed into pillowcases that had the bottom third lined with plastic to assist in collection of urine and faecal samples. Captured CIFFs were then anaesthetized with 2% isoflurane in 1 L/min oxygen (Isoflurane 100%, Zoetis Inc., Australia) via mask as previously described (Hall et al., 2014; Jonsson et al., 2004). Sex, body mass (g) and forearm length (mm) were recorded, and individuals were banded for individual identification as previously described (Todd et al., 2018). Using established methods, age was determined based on morphometric measurements, extent of tooth wear, and reproductive status/sexual characteristics (Todd et al., 2018). A variety of samples were collected from captured flying-foxes, including serum or plasma for antibody testing, and urine, faeces, and saliva for detection of viral nucleic acid. Approximately 0.5–1 mL of whole blood ($n$ = 190) was collected from the uropatagial vein and placed into BD Microtainer® lithium-heparin tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) or plain tubes and stored at 4°C overnight. Blood samples were centrifuged (Qik Spin, Edwards Group Pty Ltd, Narellan, NSW, Australia) for 10 min at 10,000 g, and plasma or serum was aliquoted into a sterile cryovial and frozen at −20°C until further analysis. Oral swabs ($n$ = 199) were collected and placed into RNAlater (Invitrogen, Carlsbad, California, USA) ($n$ = 148) or a lysis solution comprised a 1:1 volume of MagMax Lysis/Binding Solution Concentrate (Invitrogen, CA, USA) and 100% isopropanol ($n$ = 51), then stored at −20°C until further analysis. Opportunistically collected faeces ($n$ = 31), urine ($n$ = 19), urine swabs (swab of the urethra; $n$ = 25) and urine samples in viral transport medium [VTM $n$ = 13; comprised 10% bovine serum (Gibco, MD, USA) in phosphate buffered saline (Gibco, MD, USA) and double strength antibiotic/antimycotic solution (Gibco, MD, USA)] were collected from CIFF. Faecal material was placed into sterile cryovials on ice and frozen at −20°C within 6 h of collection until further analysis. From August 2015 to September 2017, urine ($n$ = 19) was aliquoted into sterile cryovials, immediately placed on ice and frozen at −20°C. From May to August 2018, urine was prioritized for virus isolation. When sufficient urine was present, approximately 150–200 $\mu$l was aliquoted into 500 $\mu$l VTM ($n$ = 13), chilled on ice for up to 4 h and frozen at −20°C. When insufficient urine was present for isolation, urine swabs ($n$ = 25) were collected, placed into a lysis solution comprised a 1:1 volume of MagMax Lysis/Binding Solution Concentrate and 100% isopropanol and stored at −20°C. Thirteen of the urine samples collected in VTM for virus isolation also had a duplicate urine swab sample. All samples were transported to Taronga Conservation Society Australia at −140°C in a liquid nitrogen dry shipper, and then stored at −80°C until processing and analysis.

CIFFs were captured under permits issued by Christmas Island National Park (Permit Nos. CINP_2015-16_1 & CINP_2018_2) and the Australian Government Environment Protection and Biodiversity Conservation Regulations 2000 license to access biological resources in

a Commonwealth area for non-commercial purposes (Permit No AU-COM2018-414). Samples were imported under permits granted by the Australian Government Department of Agriculture (Permit Nos. IP15007146 & IP1368078). Animal capture protocols and sample collection were approved by the Animal Care and Ethics Committee of Western Sydney University (Project Protocol Nos A11140 & A12791).

## 2.2 | Sample processing and analysis

### 2.2.1 | RNA extraction

Faecal samples were resuspended in 500 $\mu$l PBS with 1% antibiotic/antimycotic and 0.1% BSA and vortexed for 10 s, centrifuged at 13,000 $\times g$ for 10 min and then the supernatant was aliquoted into a new cryovial for RNA extraction. Viral RNA was extracted from urine, urine swabs, oral swabs and faeces with the QIAamp Viral RNA Mini Kit (Qiagen, Victoria, Australia). An aliquot of faecal RNA extract was set aside for coronavirus testing at the School of Veterinary Science, University of Sydney. All other samples (RNA extracts, serum or plasma and urine in VTM) were transported to the CSIRO Australian Centre for Disease Preparedness (ACDP) for analysis.

## 2.3 | Serological analysis

### 2.3.1 | Serum and plasma testing

Serum or plasma was screened for antibodies against the paramyxoviruses (CedV, HeV, NiV, MenV and TioV), the betacoronaviruses (SARS-CoV-1 and MERS-CoV), and a lyssavirus, ABLV, at the CSIRO ACDP using multiplex microsphere-based immunoassays (MMIA) (Luminex Corporation, Austin, TX, USA) as described previously (Boardman et al., 2020a, 2020b; Bossart et al., 2007; Boyd et al., 2015; Burroughs et al., 2016; Dovih et al., 2019; Edson et al., 2019; Laing et al., 2018; Peel et al., 2012, 2013; Prada et al., 2019a, 2019b; Schulz et al., 2020). Briefly, a recombinant, soluble, tetrameric MenV haemagglutinin-neuraminidase (sHN) attachment glycoprotein was constructed and prepared as previously devised for other henipavirus G glycoproteins (Cheliout Da Silva et al., 2021) (Supplementary Text S1); soluble, tetrameric, receptor-binding protein (RBP) glycoproteins of HeV, NiV, and CedV (sG) (Laing et al., 2019; Schulz et al., 2020; Yan et al., 2021), nucleocapsid protein (N) of TioV (Petraityte et al., 2009) and lyssaviruses (Prada et al., 2019; Rahmadane et al., 2017), and SARS-CoV-1 N and MERS-CoV N (Yu et al., 2008) were produced as previously described. For the present study, it is important to recognize that polyclonal antibodies that are specific for the viral envelope glycoproteins associated with attachment or cell entry (e.g. the sG glycoproteins of henipaviruses) elicited by virus infection are expected to bind more specifically to those specific viral antigens, or closely related viruses, because of a lower degree of protein sequence identity between virus species. Whereas the more protein sequence conserved nucleoproteins (N) employed here for TioV, SARS-CoV-1 and MERS-CoV would be expected to demonstrate more cross-reactive antibody binding against a larger suite of viruses within the same genus (i.e. Laing et al., 2021). Here, we were limited in our abilities to incorporate the viral glycoproteins from betacoronaviruses (SARS-CoV-1 and MERS-CoV), TioV and ABLV and chose to use the N protein for those viral genera examined.

### 2.3.2 | Serological MMIA assay

Assay proteins were coupled to a predetermined number of carboxylated magnetic microsphere bead sets, MagPlex® (Luminex Corporation, Austin, TX, USA). Each of the eight protein-coupled microsphere bead sets were mixed for the multiplex assay and added to individual wells of a 96-well plate. Serum or plasma samples were heat treated at 56°C for 30 min to inactivate complement, diluted 1:50 in PBSA and 100 $\mu$l was added to wells with the microsphere bead sets and incubated for 30 min. Liquid was removed and biotinylated Protein A (1:500) (Pierce, Thermo Fisher Scientific, Waltham, MA, USA) and biotinylated Protein G (1:250) (Pierce, Thermo Fisher Scientific, Waltham, MA, USA) were added to wells and incubated for 30 min at room temperature. Liquid was removed and 100 $\mu$l of streptavidin-phycoerythrin (1:1000) (Qiagen Pty Ltd, Australia) was added and incubated for 30 min at room temperature. After incubation, samples were analysed for antigen-bound IgG, expressed as a median florescence intensity (MFI) using a Bio-Plex 200 HTF multiplexing system with Bio-Plex Manager Software version 6.2 (Bio-Rad Laboratories, Hercules, CA, USA), with settings set for 100 beads per region and calibrated on the high RP1 target setting.

## 2.4 | Multiplex X-TAG and PCR analysis

To detect viral nucleic acid, RNA from urine and urine swabs was tested for five known paramyxoviruses (CeV, HeV, NiV, MenV and TioV) with a multiplex bead assay ('X-TAG assay'), using primers targeting each of the five paramyxoviruses, as previously described (Boyd et al., 2015). In addition, generic primers for henipaviruses were included to detect known and unknown HeV and NiV virus isolates (Boyd et al., 2015; Foord et al., 2013). Briefly, target specific primer extensions for each paramyxovirus were designed and hybridized to X-TAG beads. Hybridized microspheres were then analysed with Bio-Plex Manager Software and determined for MFI. To confirm the presence or absence of HeV and NiV in the population, RNA from urine and urine swabs, oral swabs and faeces was also screened using a real-time PCR (qRT-PCR) assay targeting the N gene, as previously described (Feldman et al., 2009). Samples with a cycle threshold (Ct) $\leq$ 40 were considered positive.

To detect viral nucleic acid of known and novel coronaviruses, faecal RNA extracts were screened for coronaviruses at the Sydney School of Veterinary Science, University of Sydney using a pan-coronavirus degenerate primer-based reverse transcriptase PCR (RT-PCR) (Vijgen et al., 2008) and a semi-nested RT-PCR targeting group 1

coronaviruses in bats (Poon & Peiris, 2008) with the addition of an annealing touch-down procedure. Two faecal RNA extracts were pooled ($n$ = 15 pools) for each PCR reaction. Reactions were made up with SuperScript™ IV One-Step RT-PCR System (Invitrogen, Carlsbad, CA, USA) and 0.5 $\mu$M primers for both assays in a final volume of 20 $\mu$l. Cycle conditions for the pan-coronavirus RT-PCR and outer reaction of the semi-nested RT-PCR were 50°C for 10 min followed by 98°C for 2 min and then 50 cycles of 98°C for 10 s, followed by touch-down from 56°C to 48°C for 20 s, then 72°C for 30 s. Cycle conditions for the inner reaction of the semi-nested RT-PCR were 95°C for 3 min and then 40 cycles of 98°C for 10 s, followed by touch-down from 56°C to 49°C for 20 s, then 72°C for 30 s, followed by 72°C for 2 min.

### 2.5 | Virus isolation

Virus isolation was attempted on urine samples collected in VTM ($n$ = 13), as previously described (Barr et al., 2012). Briefly, samples were thawed at room temperature and centrifuged at 9800 × $g$ for 3 min. Urine in VTM (150–200 $\mu$l of urine in 500 $\mu$l VTM) was added to 4 ml of cell culture medium (Dulbecco's modified Eagle's medium nutrient mixture Ham's F-12 supplemented with 200 U penicillin/ml, 200 $\mu$g streptomycin/ml, 0.5 mg amphotericin B/ml and 10% FBS). Diluted urine-cell culture mixture was then centrifuged at 2377 × $g$ for 5 min before 2 ml of supernatant was added to both Vero and *P. alecto* kidney (PaKi) cell (Crameri et al., 2009) monolayers in 75-cm tissue culture flasks. The flasks were then incubated at 37°C for 1 h with rocking. Twelve millilitres of cell culture medium were added to the flasks and incubated for 7 days at 37°C and observed daily for toxicity, contamination or viral cytopathic effects (CPEs). Isolation supernatant was passaged twice more to assess CPEs.

### 2.6 | Statistical analysis

To determine the threshold values to categorize bats as seronegative or seropositive is challenging as the MMIA and X-TAG assays have not been validated for all viruses in flying-foxes due to the lack of species-specific positive or negative controls (Bossart et al., 2007; Boyd et al., 2015; Peel et al., 2013). In the absence of sera from naïve captive-bred or PCR-confirmed CIFFs, Bayesian mixture models have been employed across multiple studies, providing a robust analytical method to determine threshold values for 'natural' groupings of seronegative and seropositive individuals (Peel et al., 2013). This method has been previously used for various bat species (Boardman et al., 2020a, 2020b; Boyd et al., 2015; Burroughs et al., 2016; Edson et al., 2019; Laing et al., 2018; Peel et al., 2013) and domestic mammals (Chowdhury et al., 2014).

MFI values for the MMIA and X-TAG assays were log-transformed (lnMFI) and plotted as histograms. Visually, histograms appeared to represent single distributions for most viruses. To assess this visual observation statistically, a mixture model was fit to the data to describe two sub-populations with different serological responses. Due to the positively skewed data, a model comprising a mixture of symmetric Gaussians (Peel et al., 2013) was extended to a mixture of asymmetric distributions, a shifted-Gompertz/Gompertz mixture following the approach used by Edson et al. (2019). Credible intervals around the upper percentile were then used as a serological threshold and were based on the best estimates (posterior mean) of parameters for the seronegative subpopulation (Low-Choy et al., 2021). Only a small proportion of individuals estimated to be seronegative exceed this threshold. Computation was undertaken in R version 3.6.0 using the Rcpp package (Eddelbuettel & Balamuta, 2018). Briefly, Maximum Likelihood Estimation (MLE) was used to fit a single Gompertz distribution, then the Metropolis-Hastings algorithm was used to fit a Bayesian Gompertz mixture (suggested in Edson et al., 2019 and detailed in Low-Choy et al., 2021), the latter also using MLE to estimate starting values. Following standard practices for evaluating convergence of Markov Chain Monte Carlo methods (Plummer et al., 2006), a visual assessment of trace plots indicated that the simulations had not converged, even after 800,000 iterations, suggesting that the two-component distribution did not fit the data. Examination of the posterior proportion of seronegative individuals [referred to as lambda ($\lambda$) in Low-Choy et al., 2021] showed that zero was the most plausible value, suggesting only one distribution was identified for each viral assay.

Due to the lack of a clear bimodal distribution of lnMFI values, we used two methods to establish thresholds, which was consistent with other studies assessing seropositivity in populations with unimodal serological distributions (De Nys et al., 2018; Dovih et al., 2019). The first method fit a one-component shifted-Gompertz model, using MLE for computation (Edson et al., 2019; Low-Choy et al., 2021). Thresholds were estimated as the 95th percentile of the shifted-Gompertz distribution (Supporting Figures S2 and S3). Bootstrapping with 1000 resamples estimated dataset-based uncertainty for these thresholds for these one-component models, by resampling the original dataset (Low-Choy et al., 2021) (Supporting Figures S2 and S3). As a summary measure of fit, bootstrap $P$ values (Bp) reflect the chance of the model outputs across resamples, specifically the proportion of resamples where the estimated threshold or Gompertz parameter was exceeded by the estimate obtained from analysing the original data.

The second method employed a previously used principle that was based on taking a value of three times the mean MFI of negative control samples (Breed et al., 2010; Prada et al., 2019a, 2019b). Mean negative control MFI values were 294 (range 68–763) and 350 (range 303–414) for the MMIA and X-TAG assays, respectively. Therefore, a threshold roughly three times these values (1000 MFI) was established for both the MMIA and X-TAG assays. This same threshold method has been used in previous studies where the same assays were conducted at the CSIRO, ACDP, with sera from other bat species, including multiple Australian insectivorous bat species (Prada et al., 2019a, 2019b) and the grey-headed flying-fox (*P. poliocephalus*) (Boardman et al., 2020a, 2020b; Burroughs et al., 2016). Samples were defined as positive or cross-reactive if they exceeded both the MFI threshold of 1000 and the threshold estimated from the 95th percentile of the shifted-Gompertz distribution. Binomial proportional confidence intervals were calculated around the proportion of positive individuals.

**TABLE 1** Details of sample types and numbers collected from the Christmas Island flying-fox (*Pteropus natalis*), storage conditions and assays

| | | | | Viral nucleic acid detection | | |
|---|---|---|---|---|---|---|
| **Sample type** | **Storage** | **Serology** | **Viral isolation** | **Multiplex X-TAG assay** | **Hendra and Nipah virus qRT-PCR** | **Pan-coronavirus RT-PCR** |
| Serum or plasma ($n = 190$) | Frozen | 190 | | | | |
| Faeces ($n = 31$) | Frozen | | | | 31 | 31 |
| Oral swabs ($n = 199$) | RNAlater | | | | 148 | |
| | Lysis solution | | | | 51 | |
| Urine ($n = 32$) | Frozen | | | 19 | 19 | |
| | Viral transport media | | 13 | | | |
| Urine swabs ($n = 25$*) | Lysis solution | | | 25 | 25 | |

*Of the $n = 25$ urine swabs, 13 were collected as duplicates, with approximately 150 $\mu$l of urine added to VTM and the remaining urine swabed off of Christmas Island flying-foxes and placed into lysis solution.

## 3 | RESULTS

A total of 228 CIFFs were captured and sampled over the 4-year study period. Of the CIFFs collected, 40% ($n$ = 91) were female and 60% ($n$ = 137) were male. Sixty-five percent ($n$ = 148) of captured CIFFs were juveniles (< 1 year), 7% ($n$ = 15) were sub-adults (1–2 years) and 29% ($n$ = 65) were adults (> 2 years). Serum or plasma was collected from 190 CIFF, faeces from 31 CIFF, oral swabs from 199 CIFF and urine (including urine neat, urine swabs and urine in VTM) from 44 CIFF (Table 1). Eleven CIFF had all samples (serum or plasma, faeces, oral swabs and urine) available for testing.

### 3.1 | MMIA analysis

Simulations from the two-component mixture models fit to the data did not converge to a posterior distribution, indicating that a single-component distribution was sufficient to describe the data for each virus. As a result, all assays were fit using a one-component shifted-Gompertz model (Figure 2) with bootstrapping to estimate standard error of the Gompertz parameters and the threshold (Supporting Figure S2). Based on the ($x$-axis) alignment of the single distribution with MFI values for negative control samples, it was generally assumed that the distribution for each virus represented seronegative individuals and a distribution of seropositive individuals was absent. However, visual inspection of the fitted one-component models suggested that lnMFI values against TioV and, to a lesser degree, SARS-CoV-1 had a broader distribution with a number of high lnMFI readings that did not fit the one-component model as well as the other virus types (Figure 2e and f). This finding is further supported by the consistently under-predicted peak of the shifted-Gompertz distribution for most viral families (Figure 2). Additionally, TioV was the only virus where the threshold established by the shifted-Gompertz model fit was higher than the rule-of-thumb threshold (1000 MFI) indicating a much broader spread.

Based on the MFI threshold of 1000 (lnMFI value of 6.9) and the threshold determined by the shifted-Gompertz model fit (Supporting

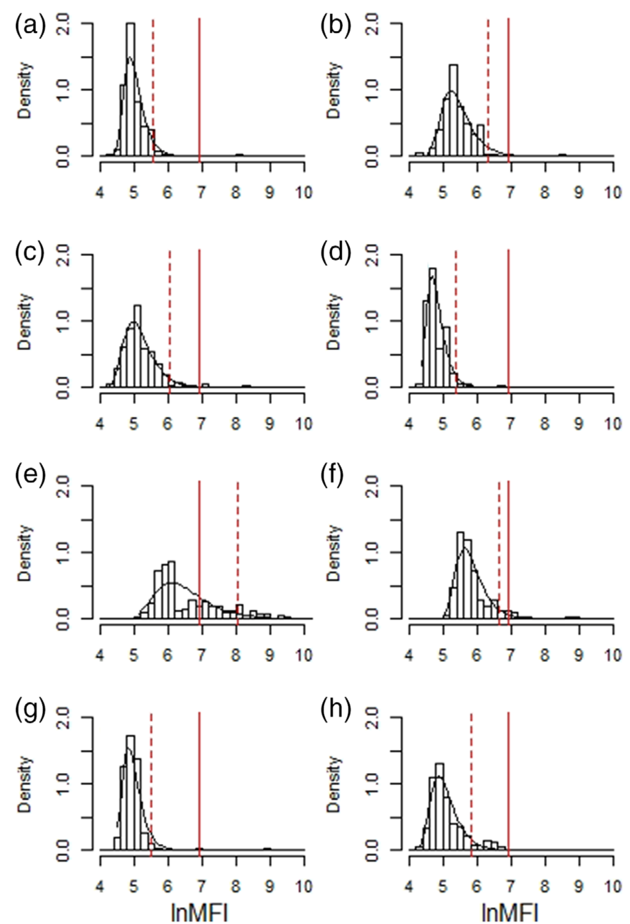


**FIGURE 2** Histogram of data overlaid with density of the fitted one-component shifted-Gompertz model for bound antibodies to (a) Cedar virus, (b) Hendra virus, (c) Menangle virus, (d) Nipah virus, (e) Tioman virus, (f) severe acute respiratory syndrome coronavirus, (g) Middle East respiratory syndrome coronavirus and (h) Australian bat lyssavirus using a multiplex microsphere-based immunoassay. Samples were considered cross-reactive if they exceeded both the median florescence intensity (MFI) threshold of 1000 (lnMFI value of 6.9; solid red line) and the threshold (dotted red line) established by the shifted-Gompertz upper percentile

**TABLE 2** Multiplex microsphere-based immunoassay antigen-bound median fluorescence intensity (MFI) readings for Christmas Island flying-foxes (*Pteropus natalis*) with bound antibodies to henipaviruses (Cedar, Hendra and Nipah virus), Menangle virus and Middle East respiratory syndrome coronavirus (MERS-CoV)

| | Christmas Island flying-fox ID number | | | | | | |
|---|---|---|---|---|---|---|---|
| | 15 | 19 | 21 | 22 | 174 | 201 | 245 |
| **Paramyxoviruses** | | | | | | | |
| **Henipaviruses** | | | | | | | |
| Cedar virus | **3415** | 364 | 223 | 166 | 103 | 124 | 143 |
| Hendra virus | **4988** | **1066** | 471 | 341 | 105 | 395 | 193 |
| Nipah virus | 253 | 144 | 157 | 111 | 91 | 763 | 109 |
| **Pararubulaviruses** | | | | | | | |
| Menangle virus | **3648** | 589 | 410 | 232 | **1188** | **1229** | 129 |
| Tioman virus | 3029 | 1084 | **13,716** | **9997** | 290 | 2881 | 433 |
| **Betacoronaviruses** | | | | | | | |
| MERS-CoV | **1011** | 249 | 256 | 168 | 151 | 216 | **7650** |
| SARS-CoV-1 | 880 | 397 | **5891** | **1674** | 268 | 354 | **1274** |
| **Lyssaviruses** | | | | | | | |
| ABLV | 173 | 335 | 513 | 243 | 114 | 242 | 115 |

*Note*: MFI readings in bold are considered positive or cross-reactive.
ABLV, Australian bat lyssavirus; SARS-CoV-1, severe acute respiratory syndrome coronavirus.

Table S1), 0.5% (95% CI: 0.03–2.9%; $n = 1$ adult) of CIFFs had binding antibodies (bAb) to CedV, 1.1% (95% CI: 0.3–3.8%; $n = 2$ adults) to HeV, 1.6% (95% CI: 0.5–4.5%; $n = 3$, $n = 1$ juvenile and $n = 2$ adults) to MenV, 10.5% (95% CI: 6.9–15.7%; $n = 20$; $n = 2$ juveniles, $n = 5$ sub-adults and $n = 13$ adults) to TioV, 4.7% (95% CI: 2.5–8.8%, $n = 9$; $n = 4$ juveniles and $n = 5$ adults) to SARS-CoV-1, and 1.1% (95% CI: 0.3–3.8%; $n = 2$ adults) to MERS-CoV (Supporting Table S2 and Supporting Figure S4). Of the few CIFFs that had bAb to CedV, HeV and MenV, all but one of these also had increased MFI values (>1000 MFI) for TioV (Table 2). Additionally, of the two CIFFs that had bAb to MERS-CoV, one also had bAb to SARS-CoV-1 and the other had a bAb MFI value that was approaching the threshold for SARS-CoV-1 (Table 2). No bAb's were reactive with NiV or ABLV in any of the CIFF serum or plasma samples tested.

### 3.2 | Multiplex X-TAG and PCR analysis

Similar to the MMIA data, all X-TAG data fit a one-component shifted-Gompertz model (Figure 3), which was supported by bootstrapping (Supporting Figure S3). Using an MFI threshold of 1000 (lnMFI value of 6.9) and the thresholds determined from the shifted-Gompertz models (Supporting Table S1), no paramyxovirus or coronavirus viral nucleic acids were detected in the CIFF samples. However, two CIFF urine swab samples had X-TAG MFI values that were approaching thresholds for multiple viruses. Of these samples, one had increased MFI values for TioV (MFI of 985) and MenV (MFI of 994) and moderate MFI values for HeV (MFI of 829). The second sample had increased MFI values for MenV (MFI of 900) and TioV (MFI of 888), and moderate MFI values for the other henipaviruses tested (MFI range from 800 to 856). No paramyxovirus viral nucleic acid was detected in urine and urine swabs, oral swabs and faeces samples screened by qRT-PCR and no coronavirus viral nucleic acid was detected in faeces screened by conventional RT-PCR.

### 3.3 | Virus isolation

Virus isolation was attempted on thirteen urine samples. Of these, four samples were discarded due to bacterial contamination. No CPEs were observed from the other nine urine samples.

## 4 | DISCUSSION

Using multiplex serological and molecular assays, this study sought to determine whether the CIFF population is maintaining known, or serologically cross-reactive, viruses found in continental flying-fox species. We then hypothesize on the implications of these findings in small, geographically isolated bat populations. Due to the cross-reactive nature of the N protein used for the TioV, SARS-CoV-1 and MERS-CoV MMIA assay, the bAb detected for these viruses may represent cross-reactivity against known (e.g. Peel et al., 2019; Smith et al., 2016) or as yet undescribed viruses within the same genus. For this reason, we subsequently refer to TioV bAb findings as pararubulavirus antibodies and SARS-CoV-1 and MERS-CoV bAb findings as betacoronavirus antibodies. Our serological findings suggest the CIFF population is maintaining a pararubulavirus and a betacoronavirus, but not CedV, HeV, MenV, NiV or ABLV.

Of the CIFFs sampled in the present study, 10.5% had bAb to a pararubulavirus. Two previous studies have reported bAb to TioV, or

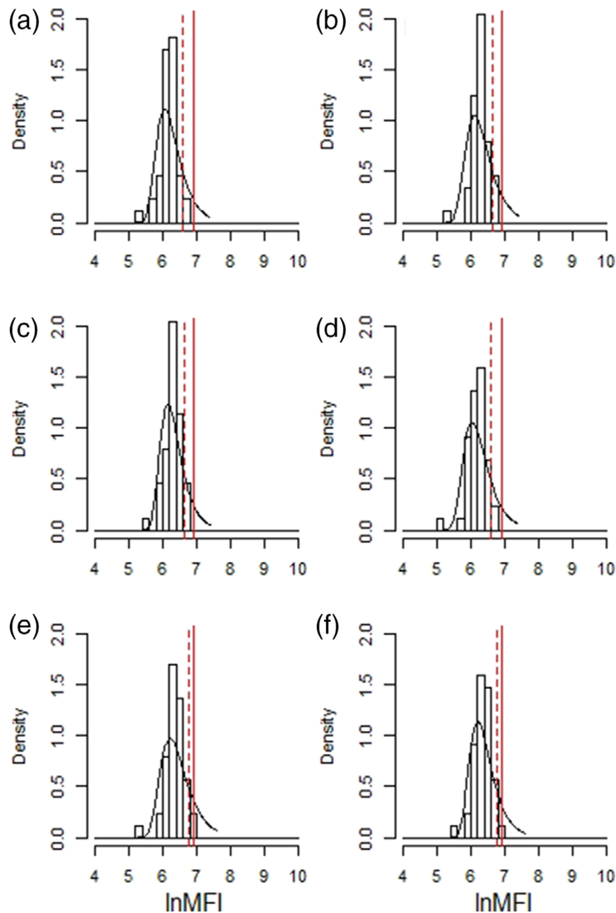


**FIGURE 3** Histogram of data overlaid with density of the fitted one-component shifted-Gompertz model for detection of viral nucleic acid to (a) Cedar virus, (b) Hendra virus, (c) henipaviruses, (d) Nipah virus, (e) Menangle virus and (f) Tioman virus using the multiplex X-TAG assay. Samples were considered cross-reactive if they exceeded both the median florescence intensity (MFI) threshold of 1000 (lnMFI value of 6.9; solid red line) and the threshold (dotted red line) established by the shifted-Gompertz model upper percentile. No samples were above the established thresholds

a cross-reactive pararubulavirus, in Papua New Guinea and Australian flying-foxes (Boardman et al., 2020b; Breed et al., 2010). A recent survey in Australian grey-headed flying-foxes demonstrated serological evidence of bAb against a pararubulavirus, using the same protein and antigen-based MMIA used in our study (Boardman et al., 2020b). Although a different statistical approach was used in that study, the pararubulavirus serology upper threshold estimated for CIFFs in the present study (TioV lnMFI 8.04) was higher than the upper threshold estimated for grey-headed flying-foxes (TioV lnMFI 7.38) by Boardman et al. (2020b). Furthermore, 5% of CIFFs had pararubulavirus bAb MFI values that were higher than the maximum MFI value reported for grey-headed flying-foxes (maximum TioV bAb MFI of 4971; Boardman et al., 2020b). This further increases the confidence in our findings that CIFFs are maintaining a pararubulavirus. Our study thus adds to the existing knowledge of the host range of pararubulaviruses and provides further evidence to support the hypothesis that these viruses are endemic to flying-foxes across Australasia (Tsang et al., 2021).

Molecular analysis and isolation attempts did not detect pararubulaviruses or other paramyxoviruses; however, sample sizes of the preferred sample type for paramyxovirus detection were small (urine $n = 19$ and urine swabs $n = 25$; includes 13 duplicate samples in VTM). Additionally, the X-TAG multiplex molecular bead assay used here is highly specific to targeted viruses (Boyd et al., 2015) known to exist in mainland Australia and neighbouring countries; therefore, it is possible that a novel virus, present only in this isolated population, remained undetected. Two urine swab samples had X-TAG MFI values approaching the threshold (MFI of 1000) for MenV and TioV simultaneously suggesting that non-specific binding to a related pararubulavirus may be occurring. This further supports our MMIA results that a novel pararubulavirus is likely circulating in the CIFF population. Further molecular studies, ideally targeting urine samples, are required to determine the pararubulavirus circulating in the population. The three partially characterized paramyxoviruses previously reported in the CIFF (Vidgen et al., 2015) were genetically distinct from known paramyxovirus genera and were suggested to constitute a new group of paramyxoviruses that likely evolved due to geographical isolation. This suggests that they are unlikely to be associated with the pararubulavirus serological results seen here. Instead, it seems most plausible that an endemic pararubulavirus and other novel bat paramyxoviruses are circulating in the CIFF.

Bats host a diverse array of alphacoronaviruses and betacoronaviruses (Drexler et al., 2014), with the latter being a primary focus since they include viruses with implications for human and animal health. Betacoronavirus antibodies were detected in 5.3% ($n = 10$) of CIFFs sampled in this study. This included 8 CIFFs with bAb to SARS-CoV-1, 1 CIFF with bAb to MERS-CoV, and 1 CIFF with bAb to MERS and a bAb value that was approaching the threshold for SARS-CoV-1. A recent survey demonstrated bAb to a betacoronavirus in 42.5% of Australian grey-headed flying-foxes tested using the same proteins and MMIA used in the present study (Boardman et al., 2020a). Although a different statistical approach was used, the betacoronavirus serology thresholds estimated for CIFFs (SARS-CoV-1 lnMFI 6.65 and 6.91) were higher than those previously estimated for grey-headed flying-foxes (SARS-CoV-1 lnMFI 6.21; Boardman et al., 2020b). Coronavirus nucleic acid was not detected in any CIFF faecal samples analysed in this study. Additional studies with larger sample sizes, ideally targeting faecal samples or rectal swabs, will be required to elucidate what coronaviruses are circulating in the CIFF population.

Taken together, the data in the present study suggest that henipaviruses, MenV and ABLV are not maintained in the CIFF population, are circulating at a very low prevalence (<2% based on our sample size) or exhibit a seasonality in transmission that did not overlap with our sampling periods. Of the few CIFFs that had bAb to other paramyxoviruses, all but one also had increased MFI values for TioV, suggesting that the bAb in these individuals are most likely cross-reacting with a pararubulavirus. Additionally, serological evidence of ABLV was not found in CIFFs. Using different assays and non-Bayesian approaches, previous studies reported a low seroprevalence of 0–3% in wild

Australian flying-foxes and microbats (Boardman et al., 2020b; Field, 2018; Prada et al., 2019a). However, in the Philippines, up to 20% of small flying-foxes had neutralizing antibodies against lyssaviruses (Arguin et al., 2002). These findings suggest that ABLV is either absent in the CIFF population, is circulating at a very low prevalence, seropositivity is short lived, or there is non-detection due to resultant mortalities from infection.

Studies on small, isolated island bat populations may provide insights into viral persistence mechanisms within individual bats and bat populations more broadly. However, the very features of small, isolated bat populations that make them excellent model systems for understanding viral maintenance also make them challenging to study. The resultant paucity of studies means that viral diversity of isolated bat populations is not well documented. In this study, bAb to a pararubulavirus and betacoronavirus was identified in sub-adult and juvenile (>7 months) CIFFs, respectively, suggesting that these viruses have been circulating within the lifespan of these individuals (within the last 7–24 months). Experimental studies of HeV in *Pteropus* spp. pups found that maternally derived antibodies wane significantly in the first month of age and the duration of immunity lasts about 7 months (Epstein et al., 2013). We would expect that the sub-adult CIFFs with bAb to the pararubulavirus are beyond the age at which maternal antibodies would be expected to wane. To the authors' knowledge, comparable longitudinal age-specific seroprevalence and waning maternal antibody studies have not been conducted for coronaviruses in bats. In the present study, two juvenile CIFFs had the highest betacoronavirus bAb MFI values (SARS-CoV-1 MFI values of 7968 and 5891) and were captured approximately 7 months after peak birthing season (Todd et al., 2018) – a time when pups would be expected to have low levels of maternal coronavirus antibodies, whereas high antibody levels would be more likely indicative of a recent infection.

Arguably, other host species may be contributing to viral maintenance on Christmas Island. Non-native insectivorous bat species have occasionally been spotted on the island, likely originating from ships porting from Malaysia and Indonesia (Woinarski, 2018). However, these bats have not been observed in subsequent surveys, suggesting that if these bats are still present on the island it is highly unlikely that they would be contributing to the persistance of these viruses in the CIFF population. While various scenarios are plausible, the most parsimonious interpretation of our data is that that an endemic pararubulavirus and betacoronavirus are circulating in the CIFF population. Thus, our study adds to previous viral detections in this species and provides support that this small, isolated population of insular flying-foxes is likely maintaining coronaviruses and various paramyxoviruses.

Limited studies have hypothesized that henipaviruses (Peel et al., 2012; Plowright et al., 2016; Tsang et al., 2021; Wang et al., 2013) and coronaviruses (Dominguez et al., 2007; Jeong et al., 2017) can be maintained in bat populations through recrudescence of latent infection with increased shedding occurring during reproduction or during times of stress. Rapidly waning immunity may also contribute to viral persistence in bat populations as has been previously proposed for henipaviruses (Glennon et al., 2019; Plowright et al., 2016). Indeed, increasing and waning serological patterns and seroconversion in seronegative captive flying-foxes has been reported, suggesting recrudescence, virus shedding and reinfection could support maintenance of paramyxoviruses in flying-foxes (Sohayati et al., 2011). Overall, the present study provides further support that these viruses can be maintained in isolated populations through recrudescence of latent infections or rapidly waning immunity resulting in reinfection susceptibility of individuals within the population. The reason for the absence of other paramyxoviruses and ABLV in the CIFF is unknown, but could reflect the inability to maintain the viruses in a small population, the non-detection of seropositive animals, non-detection due to highly specific antigens used or that only select virus species have the ability to establish latent infection in CIFFs. Alternatively, the low diversity of viruses may be a result of what was brought to the island by the founding population, similar to the founder effects described for viral diversity of insular house mouse populations (Moro et al., 2003).

From conservation and biosecurity perspectives, the lower prevalence and apparent absence of some paramyxoviruses and ABLV may make the CIFF immunologically naïve and susceptible to an introduced disease. Although rare or underreported, mass mortality events suggestive of introduced viral pathogens have been reported for insular flying-foxes (O'Shea et al., 2016). A novel pathogen could be introduced through a reverse zoonotic event from humans through the introduction of a non-native bat species, or another vector. Thus, to reduce exposure of CIFF, and flying-foxes more broadly, to human pathogens, researchers should adopt biosecurity protocols such as those outlined by the IUCN Bat Specialist Group (IUCN, 2020). Considering non-native insectivorous bat species have occasionally been spotted on the island (Woinarski, 2018), increased biosecurity protocols for ships docking on Christmas Island are also warranted to decrease the opportunity for introduced pathogens from non-native species. As a conservation breeding program is being considered as a possible management tool for the CIFF population, the presence of a pararubulavirus and betacoronavirus in the population may have implications for public health. Further studies are required to characterize these viruses before any risk can be assessed. Regardless, people handling CIFFs should wear appropriate personal protective equipment to prevent virus spillover and reverse spillover events.

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## CONFLICT OF INTEREST

There are no conflicts of interest.

## AUTHOR CONTRIBUTIONS

Laura A. Pulscher: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, validation, project administration, writing – original draft. Alison J. Peel: data curation, formal analysis, supervision, writing – review & editing. Karrie Rose: conceptualization, funding acquisition, investigation, resources, project administration, supervision, writing – original draft, writing – review & editing. Justin A. Welbergen: funding acquisition, project administration, resources, writing – review & editing. Michelle L. Baker: investigation, validation, resources, writing – review & editing. Vicky Boyd: investigation, methodology, validation, resources, writing – review & editing. Samantha Low-Choy: data curation, formal analysis, writing – review & editing. Daniel Edson: conceptualization, funding acquisition, writing – review & editing. Christopher Todd: investigation, writing – review & editing. Annabel Dorrestein: data curation, investigation, writing – review & editing. Jane Hall: investigation, writing – review & editing. Shawn Todd: investigation, writing – review & editing. Christopher C. Broder: investigation, methodology, validation, writing – review & editing. Lianying Yan: methodology, validation, writing – review. Kai Xu: Investigation, validation, writing – review & editing. Grantley R. Peck: methodology, validation, writing – review & editing. David N. Phalen: conceptualization, funding acquisition, resources, project administration, supervision, writing – original draft, writing – review & editing.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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