JOURNAL OF GENERAL VIROLOGY

SHORT COMMUNICATION

Barr *et al.*, *Journal of General Virology* 2022;103:001785

DOI 10.1099/jgv.0.001785

MICROBIOLOGY SOCIETY

OPEN ACCESS

Detection of filovirus-reactive antibodies in Australian bat species

Jennifer Barr[1,*,†], Victoria Boyd[1†], Shawn Todd[1], Ina Smith[2], Diana Prada[3,4], Mark O'Dea[3], Bethany Jackson[4], Lesley Pearce[5], Timothy E. Adams[5], Eric Vanderduys[6], David Westcott[7], Adam McKeown[7], Michelle L. Baker[1] and Glenn A. Marsh[1]

Abstract

Bats have been implicated as the reservoir hosts of filoviruses in Africa, with serological evidence of filoviruses in various bat species identified in other countries. Here, serum samples from 190 bats, comprising 12 different species, collected in Australia were evaluated for filovirus antibodies. An in-house indirect microsphere assay to detect antibodies that cross-react with Ebola virus (*Zaire ebolavirus*; EBOV) nucleoprotein (NP) followed by an immunofluorescence assay (IFA) were used to confirm immunoreactivity to EBOV and Reston virus (*Reston ebolavirus*; RESTV). We found 27 of 102 Yinpterochiroptera and 19 of 88 Yangochiroptera samples were positive to EBOV NP in the microsphere assay. Further testing of these NP positive samples by IFA revealed nine bat sera that showed binding to ebolavirus-infected cells. This is the first report of filovirus-reactive antibodies detected in Australian bat species and suggests that novel filoviruses may be circulating in Australian bats.

Of the zoonotic viral diseases for which bats are known or suspected to be reservoir hosts, the ecology and epidemiology of filoviruses are comparatively understudied, particularly in the Australian context. Filoviruses are single-stranded negative-sense RNA filamentous viruses within the family *Filoviridae,* which includes four genera found in mammals: *Ebolavirus*, *Marburgvirus, Cuevavirus* and *Dianlovirus*. The Ebolavirus genus comprises six viral species: *Zaire ebolavirus*, *Sudan ebolavirus*, *Bundibugyo ebolavirus*, *Tai Forest ebolavirus*, *Bombali ebolavirus* and *Reston ebolavirus* and are represented by Ebola virus (EBOV), Sudan virus (SUDV), Bundibugyo virus (BDBV), Tai Forest virus (TAFV), Bombali virus (BOMV) and Reston virus (RESTV), respectively [1]. Most of the *Ebolavirus* species cause severe disease and death in humans, with sporadic outbreaks in humans in Africa since their discovery in 1976 and a major outbreak in 2014–2016 resulting in the deaths of over 11000 people in West Africa [2]. RESTV, which causes a relatively mild disease or no disease in humans [3], is considered less pathogenic and BOMV, of which there have been no detections in humans [4], has unknown pathogenicity. EBOV, SUDV, BDBV, TAFV and BOMV are all found in Africa whereas RESTV is geographically distinct from the more pathogenic viruses, having been discovered in non-human primates from the Philippines [5]. Owing to their high mortality rate (up to 90%), ebolaviruses are classified as WHO Risk group 4 pathogens requiring high biocontainment, including RESTV [6]. Filoviruses remain one of the viral families of interest during pathogen surveillance of bat populations.

Bats are known to harbour various zoonotic viruses of public health significance such as Severe Acute Respiratory Syndrome coronavirus (SARS-CoV), Middle Eastern Respiratory Syndrome coronavirus (MERS-CoV) and the henipaviruses (Hendra and Nipah viruses) and are suspected to be the natural reservoir hosts for filoviruses [7, 8]. Although no live ebolaviruses have been isolated from bat samples, serological and PCR data from fruit bat species in Africa support their role as reservoir hosts

Received 18 May 2022; Accepted 18 July 2022; Published 16 August 2022

Author affiliations: [1]CSIRO, Health and Biosecurity, Australian Centre for Disease Preparedness, Geelong, Australia; [2]CSIRO, Health and Biosecurity, Canberra, Australia; [3]Harry Butler Institute, Murdoch University, Western Australia; [4]College of Veterinary Medicine, Murdoch University, Western Australia; [5]CSIRO, Manufacturing, Parkville, Australia; [6]CSIRO, Land and Water, Dutton Park, Australia; [7]CSIRO, Land and Water, Cairns, Australia.
***Correspondence:** Jennifer Barr, Jennifer.barr@csiro.au

Keywords: Australia; bats; filovirus; serology.

Abbreviations: ACDP, Australian centre for disease preparedness; AEC, animal ethics committee; BDBV, Bundibugyo ebolavirus; BOMV, Bombali ebolavirus; BSA, bovine serum albumin; CSIRO, Commonwealth Scientific and Industrial Research Organisation; EBOV, Zaire ebolavirus; ELISA, enzyme linked immunosorbent assay; GP, glycoprotein; IFA, immunofluorescence assay; LLOV, Lloviu virus; MARV, Marburg virus; MERS, Middle Eastern respiratory syndrome; MFI, median fluorescent intensity; MOI, multiplicity of infection; NA, not applicable; NBF, neutral buffered formalin; NP, nucleoprotein; NT, northern territory; PBS, phosphate buffered saline; PCR, polymerase chain reaction; QLD, Queensland; RESTV, Reston ebolavirus; RNA, ribonucleic acid; SARS, severe acute respiratory syndrome; SUDV, Sudan ebolavirus; TAFV, Tai Forest ebolavirus; Vic, Victoria; WA, Western Australia; WHO, World Health Organisation.

†These authors contributed equally to this work

Table 1. List of species (common name), the location and year collected, and number tested. N/A=not applicable

Yinpterochiroptera	Location 1	Location 2	No. tested
Pteropus alecto (Black flying fox)	Brisbane, QLD (2008, 2009)	Katherine, NT (2016)	40+7=47
Pteropus scapulatus (Little red flying fox)	Charters Towers, QLD (2017)	Katherine, NT (2016)	15+8=23
Pteropus poliocephalus (Grey headed flying fox)	Geelong, VIC (2012, 2013)	N/A	22
Rhinolophus megaphyllus (Eastern horseshoe bat)	Kenilworth, QLD (2005, 2007)	N/A	10
			Total=102
Yangochiroptera	**Loctaion 1**	**Location 2**	**No. tested**
Chalinolobus morio (Chocolate wattled bat)	WA Site 1 (2017)	N/A	12
Falsistrellus mackenziei (Western false pipistrelle)	WA Site 1 (2017)	N/A	5
Nyctophilus geoffroyi (Lesser long-eared bat)	WA Site 1 (2017)	WA Site 2 (2017)	1+4=5
Nyctophilus gouldi (Gould's long-eared bat)	WA Site 1 (2017)	N/A	7
Nyctophilus major (Western long-eared bat)	WA Site 1 (2017)	N/A	1
Vespadelus regulus (Southern forest bat)	WA Site 1 (2017)	N/A	23
Austronomus australis (White-striped free-tailed bat)	WA Site 2 (2017)	N/A	11
Chalinolobus gouldii (Gould's wattled bat)	WA Site 2(2017)	N/A	24
			Total=88

[9–12]. In addition, the closely related filovirus, Marburg virus (MARV), was isolated from samples from the Egyptian fruit bat, *Rousettus aegpytiacus* in 2007/2008 [13, 14] and another filovirus, Lloviu virus (LLOV), was isolated from *Miniopterus schreibersii* in Hungary earlier this year [15]. In recent years, serological evidence of filovirus infection in bats has been detected in various countries including China, the Philippines, Bangladesh, Singapore, Spain, India and Trinidad [16–23].

Australia has around 80 bat species belonging to both suborders of Chiroptera; Yinpterochiroptera and Yangochiroptera [24]. Australian Yinpterochiroptera include 21 species from four families; Pteropodidae, Rhinolophidae, Hipposideridae and Megadermatidae and Australian Yangochiroptera consist of more than 60 species from four families; Emballonuridae, Molossidae, Miniopteridae and Vespertilliondae [25]. To date, no studies have investigated the potential presence of filovirus antibodies in Australian bats using robust serological methods, despite evidence of filoviruses in neighbouring regions. In this study, we tested 190 serum samples, representing 12 different Australian bat species, from both Chiropteran suborders (Yinpterochiroptera and Yangochiroptera) for the presence of filovirus-reactive antibodies.

Although it is unlikely that Australian bats harbour any known ebolaviruses, there could be novel filoviruses circulating, as has been demonstrated in other countries. Based on this hypothesis, we chose the nucleoprotein (NP) of the type species, EBOV, for initial antibody screening rather than the envelope glycoprotein (GP) [26]. The NP sequence is more conserved across filoviruses than the GP and therefore more likely to detect cross-reactive filovirus antibodies, whereas the GP is more likely to detect species-specific antibodies [27]. An indirect microsphere assay was developed in-house for screening bat serum samples for filovirus antibodies using Luminex technology. EBOV NP was produced in HEK293T cells and purified using a caesium chloride (CsCl) gradient [28]. The purified NP was then coupled to microspheres using the same procedure as described previously [29]. The microsphere assay was used to initially screen bat sera for filovirus-reactive antibodies, then positive sera were selected for further testing of antibodies to EBOV and RESTV in immunofluorescence assays (IFA). The IFA was chosen as a confirmatory assay rather than a virus neutralization assay, which is very specific for the filovirus species tested. The serum samples were tested against EBOV and RESTV in the IFA to represent both the type species (EBOV) and the species found closest geographically to Australia (RESTV).

Table 2. Summary of microsphere assay and immunofluorescence assay (IFA) results

A sample was considered positive in the microsphere assay if the MFI was >1000. For the 11 positive *Pteropus alecto* samples, eight were from QLD and three from NT. For the seven positive *Pteropus scapulatus* samples, four were from QLD and three from NT The two IFA reactive *Pteropus alecto* samples reacted with both EBOV infected cells and RESTV infected cells. All other IFA reactive samples reacted with RESTV infected cells only. (See Fig. 1.)

Yinpterochiroptera	**NP positive/total**	**IFA reactive**
Pteropus alecto	11/47	2 (QLD, 2008)
Pteropus scapulatus	7/23	2 (QLD, 2017) and 2 (NT, 2016)
Pteropus poliocephalus	8/22	1 (VIC, 2013)
Rhinolophus megaphyllus	1/10	0
	TOTAL=27/102	TOTAL=7
Yangochiroptera	**NP positive/total**	**IFA reactive**
Chalinolobus morio	3/12	2 (WA, 2017)
Falsistrellus mackenziei	5/5	0
Nyctophilus geoffroyi	0/5	0
Nyctophilus gouldi	2/7	0
Nyctophilus major	0/1	0
Vespadelus regulus	3/23	0
Austronomus australis	1/11	0
Chalinolobus gouldii	5/24	0
	Total=19/88	Total=2

Bat sera tested in this study were sourced from several locations around Australia (Table 1).

Serum samples from eight species (*Chalinolobus morio, Falsistrellus mackenziei, Nyctophilus geoffroyi, Nyctophilus gouldi, Nyctophilus major, Vespadelus regulus, Austronomus australis* and *Chalinolobus gouldii*) collected in 2017 were from two sites in Western Australia's (WA) Wheatbelt region and were part of a joint project between CSIRO and Murdoch University. Due to the size of the bats and blood volume collected, these sera were diluted 1:10 in PBS prior to shipping to the CSIRO Australian Centre for Disease Preparedness (ACDP). Seven *Pteropus alecto* sera and eight *Pteropus scapulatus* sera from Katharine, Northern Territory (NT) were collected in 2016 and an additional 15 *Pteropus scapulatus* sera were collected from Charters Towers, Queensland (QLD) in 2017 during surveillance activities. The remaining bat sera analysed were historical and used advantageously for this study. Sera from *Rhinolophus megaphyllus* was obtained from animals captured near Kenilworth in South East QLD in 2005 and 2007. Forty *Pteropus alecto* sera from Brisbane, QLD, in 2008 and 2009 and 22 *Pteropus poliocephalus* sera were collected from Geelong, Victoria (Vic) in 2012 and 2013 (Table 1). The number of samples used in this study overall were limited, due to the difficulty in catching and collecting serum from bats (particularly the smaller species).

All 190 bat sera were tested against EBOV NP in our in-house microsphere-based assay and the median fluorescent intensity (MFI) was obtained for each sample. A serum sample resulting in an MFI less than 250 has been considered negative previously and a positive cut-off value of at least three times the mean MFI of negative sera has been used for interpreting bat serology studies published from our laboratory and elsewhere using the same Bio-Plex platform [30, 31]. For this study, we considered a sample with an MFI greater than 1000 to be reactive.

Based on this MFI threshold, 27 of 102 Yinpterochiroptera sera were classified as positive for antibodies to EBOV NP (Table 2). In comparison, 19 of 88 Yangochiroptera sera were classified as positive for antibodies to EBOV NP (Table 2). There was a range of positive MFIs from 1023 to 4533 for the Yinpterochiroptera samples and 1060 to 6576 for the Yangochiroptera samples (data not shown). All five of the *Falsistrellus mackenziei* sera were positive. No reactivity was observed for the five *Nyctophilus geoffroyi* or the single *Nyctophilus major* serum tested. Sera was observed to react with the EBOV NP in the microsphere assay across species, collection locations and sample dates, but no significant trends were observed.

The 46 bat sera considered positive in the microsphere assay were then tested by IFA against uninfected cells, EBOV-infected cells and RESTV-infected cells. Confluent Vero cell monolayers were infected at a m.o.i. of 1 with EBOV and RESTV and incubated for 4 days at 37 °C. Cell-culture media was removed, and 10% neutral-buffered formalin (NBF) added prior to staining. After blocking in 1% BSA in PBS, primary antibodies were added. The positive control antibody (Anti-RESTV NP Rabbit sera, produced

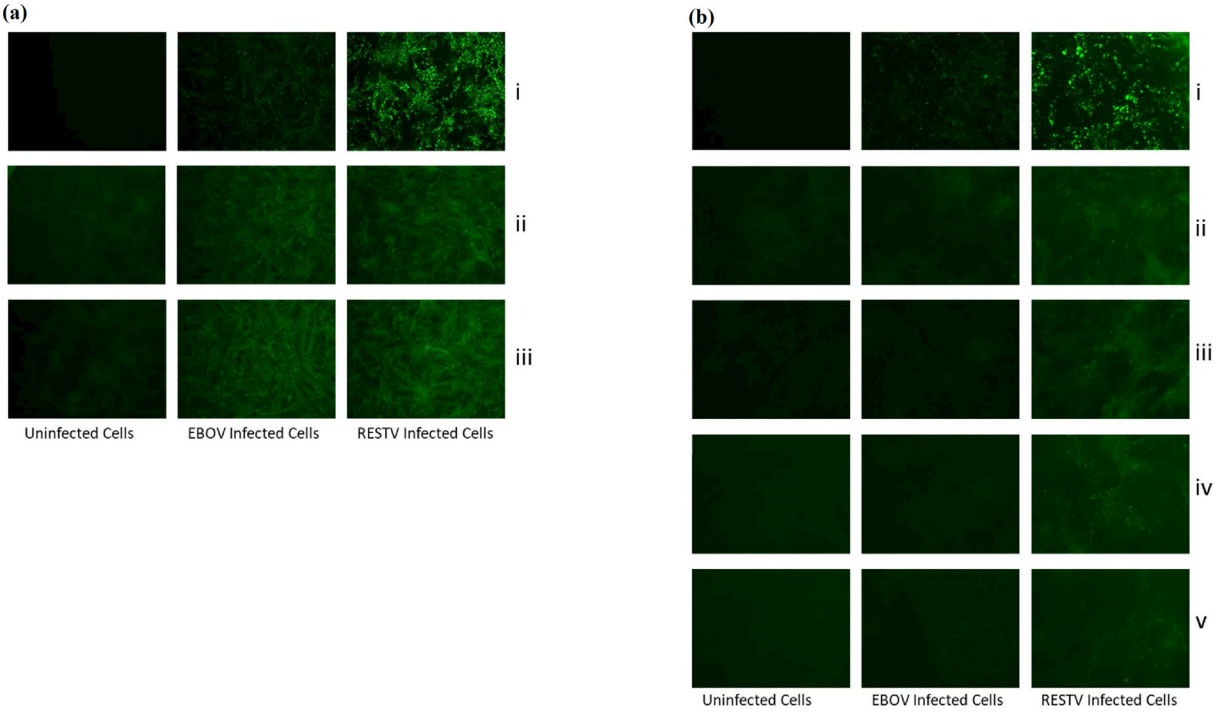

Fig. 1. (a) Immunofluorescence assay. Immunofluorescence assay demonstrating diffuse staining observed in two *Pteropus alecto* serum samples reacting with EBOV and RESTV infected cells compared to uninfected cells. (i) Anti-RESTV NP Rabbit positive control sera, (ii) *Pteropus alecto* (no. 45) and (iii) *Pteropus alecto* (no. 51) (10x magnification). (b) Immunofluorescence assay demonstrating particulate staining observed in two *Pteropus scapulatus* and two *Chalinolobus morio* serum samples reacting with RESTV-infected cells compared to uninfected cells and EBOV-infected cells. (i) Anti-RESTV NP Rabbit positive control sera, (ii) *Pteropus scapulatus* (No. 70), (iii) *Pteropus scapulatus* (no. 83), (iv) *Chalinolobus morio* (no. 2) and (v) *Chalinolobus morio* (no. 11) (tested at dilution of 1:150) (20x magnification).

in-house) was diluted 1:2000 in blocking buffer. The bat sera were diluted 1:50 for samples that had sufficient volume and 1:100, 1:150 or 1:200 for samples where the volume of sera was limiting. A mixture of Protein A-488 and Protein G-488 (Invitrogen) was used for detection of bound primary antibodies and visualization was achieved using a fluorescent microscope (EVOS FL, Life Technologies). The infected cells were observed for virus-specific fluorescence compared to the uninfected cell control for each sample. Patterns of staining were recorded as being either diffuse or particulate. Virus-specific staining was seen in a total of nine serum samples, included four different bat species and was observed against both viruses (Table 2).

Of the 27 microsphere assay-positive Yinpterochiroptera sera, seven reacted with the RESTV-infected cells, with two of those seven also reacting with the EBOV-infected cells in the IFA. The two samples that reacted to both viruses showed a diffuse pattern of staining compared to their respective uninfected cell controls (Fig. 1a). Interestingly, these two samples were also the only two *Pteropus alecto* that were reactive. The samples reactive to RESTV only, showed a particulate pattern of staining consistent with the pattern observed with the anti-RESTV NP rabbit positive control sera, although much weaker in intensity (Fig. 1b). The five reactive Yinpterochiroptera samples showing particulate staining included one *Pteropus poliocephalus* serum (Geelong VIC 2013) and four *Pteropus scapulatus* sera (two from Katherine NT 2016 and two from Charters Towers QLD 2017).

Only two of the 19 microsphere assay-positive Yangochiroptera sera showed virus-specific staining and were reactive to RESTV-infected cells only. Both samples were from *Chalinolobus morio* bats collected from WA Site 1 in 2017. One of the two samples had sufficient volume to test at a dilution of 1:50 (no. 2), whilst the other was tested at 1:150 (no. 11) (Fig. 1b).

Sera from the three *Pteropus* species tested in this study showed reactivity with the EBOV NP in the microsphere assay and virus-specific antibodies were further demonstrated in the IFA. In comparison, sera from six of the eight Yangochiroptera species tested in this study showed reactivity with the EBOV NP in the microsphere assay; however, only one species (*Chalinolobus morio*, collected from WA Site 1 in 2017) showed additional reactivity in the IFA. A positive result in the microsphere assay did not always correlate to reactivity being observed in IFA. In total, 46 bat samples out of 190 were classified as positive for EBOV NP antibodies in the microsphere assay but only nine samples displayed specific staining in the IFA. Furthermore, there was no correlation between the intensity of the MFI and an IFA reaction. For example, the sample giving the highest MFI (4533

for a *Pteropus scapulatus*) was reactive in the IFA, as was the sample with one of the lowest positive MFIs (1077 for a *Pteropus scapulatus*).

Owing to the small sample sizes by species and region generally, combined with variation in the randomisation of sampling effort and thus inherent bias in the study population, it is challenging to report numbers as anything other than crude proportions. Thus, we cannot infer the impact of species, or region, on the reported prevalence of infection. Broadly, the proportion of EBOV NP-positive individuals in the microsphere assay was similar across Yinpterochiroptera samples and Yangochiroptera samples. However, we note the difference in the proportion of positive microbats from within the same region, with *Nyctophilus geoffroyi* and *Nyctophilus major* all testing negative (n=5 and n=1, respectively), whereas all five *Falsistrellus mackenziei*, tested positive. Such differences may form the basis for future hypotheses and surveillance efforts, albeit sample sizes as noted limit our capacity to draw robust conclusions at the species level.

Two different patterns of staining were observed in the IFA; diffuse and particulate staining. The diffuse staining was only observed in two *Pteropus alecto* samples and it is arguable whether this staining was virus-specific. However, there was an observable difference between ebolavirus-infected cells and uninfected cells in these two samples that was not seen in any other samples. The significance of this finding is unknown. Conversely, the particulate staining observed in all other IFA-positive samples was consistent with virus-associated staining patterns, and specific for RESTV only. It appears the antibodies detected were cross-reacting with the NP of RESTV, as the pattern of staining was similar to the positive control rabbit sera, which was directed against RESTV NP. We found that samples from *Pteropus scapulatus* were more likely to be reactive in the IFA than the other two *Pteropus* species and only *Chalinolobus morio* was IFA-reactive from the Yangochiroptera samples. Again, given the very low sample numbers, no strong conclusions can be made about prevalence of filovirus antibodies in species, collection locations or sample dates. Interestingly, the reactive samples showing particulate staining were only seen with RESTV, which is geographically the closest known filovirus to Australia.

In recent years, serological evidence of filovirus infection in bats has been discovered in various other countries. In addition, some of these other studies have collected appropriate samples for detection of filovirus RNA (tissue samples, swabs, faeces) but have had limited success. In China in 2012, several species of bats were found to have antibodies reacting with EBOV and RESTV NP by ELISA and Western Blot [16]. Then in China in 2017, further NP-positive sera were discovered, in addition to filovirus RNA from lung tissue samples from fruit bats (*Eonycteris spelaea* and *Rousettus* sp.) [17]. RESTV antibodies were discovered in bats in the Philippines in 2015 using ELISA and Western Blot [18]. In Bangladesh, 25 potentially filovirus-positive bat serum samples from *Rousettus leschenaultia* were identified by ELISA and then five samples confirmed to be positive by Western Blot against RESTV and EBOV NP. However, no PCR-positives were found despite extensive swab sampling [19]. In Spain in 2019, antibodies to LLOV GP were found in *Miniopterus schreibersii* using immunoblots [21]. Finally, similar to the strategy used in this study, Singapore, India and Trinidad have recently used Luminex assays to screen bat serum samples, however, they used EBOV GP rather than NP. Low numbers of filovirus antibody-positive samples were found in each country, suggesting filoviruses are likely circulating in multiple bat species in many countries [20, 22, 23]. Collecting appropriate samples for filovirus RNA detection compared to collecting blood for serum collection is more challenging, requires additional handling or euthanasia of bats and often results in no detection of filovirus RNA. Our study examined serum samples only for antibody detection, but samples for RNA extraction would be worth collecting in future studies.

In summary, this is the first report of filovirus antibodies detected in Australian bat species and suggests ebolavirus-like viruses may be circulating in bat species in Australia and have been since at least 2005. Our results indicate that it would be worthwhile to conduct a more extensive serosurvey to better determine the prevalence of filovirus antibodies in Australian bats. Given the preliminary results shown here, a future serosurvey could be targeted to include *Pteropus scapulatus* and *Chalinolobus morio* species. It would also be worthwhile collecting appropriate samples for sequencing or virus isolation to identify and further characterize Australian bat filoviruses. The discovery of filovirus-reactive antibodies in Australian bat species has unknown implications for human and animal health. However, if these indicate viral carriage, it is prudent to consider that a spillover event may occur and cause disease in humans and/or animals.

Funding information
This project was funded internally by CSIRO.

Acknowledgements
The authors wish to thank Dr Amy Burroughs for allowing us to use *Pteropus poliocephalus* serum samples collected during her PhD for our study, and Dr Hume Field and team for assistance with catching *Pteropus alecto* bats in Brisbane, QLD in 2008 & 2009 and *Rhinolophus megaphyllus* bats in Kenilworth, QLD in 2005 & 2007, used in this study.

Author contributions
J.B.: writing – original draft preparation, methodology, formal analysis, investigation, visualization. V.B.: writing – review and editing, methodology, formal analysis, investigation, visualization, project administration. S.T.: writing – review and editing, methodology, investigation. I.S.: writing – review and editing, conceptualization. M.O'D.: writing – review and editing, conceptualization, investigation, resources, supervision, funding. B.J.: writing – review and editing, supervision, methodology, investigation. D.P.: methodology, investigation. L.P.: writing – review and editing, methodology, investigation. T.A.:

writing – review and editing, methodology, investigation. E.V.: methodology, investigation. D.W.: methodology, investigation. A.Mc.K.: writing – review and editing, methodology, investigation. M.B.: writing – review and editing, conceptualization, funding. G.M.: writing – review and editing, conceptualization, supervision, funding.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
Bat sera used in this study were sourced from several locations around Australia and approved for use by various Animal Ethics Committee's (AEC). The bat sera collected in the WA Wheatbelt region were part of a joint project between CSIRO and Murdoch University. Information regarding permits/animal capture and sampling details can be found in [32, 32]. Bat sera was collected from Katharine, NT in 2016 and from Charters Towers, QLD in 2017 during surveillance activities. Capture and handling of flying-foxes by CSIRO staff in QLD and the NT were undertaken under CSIRO Wildlife and Large Animal AEC permits 2019-14 and 2016-17, a Queensland Government Department of Environment and Science section 173P special authorisation, and Northern Territory Parks and Wildlife Commission permit 65136. Information regarding *Pteropus poliocephalus* CSIRO AEC permits, bat capture and serum collection details can be found in [33, 33]. Capture and sampling of *Rhinolophus megaphyllus* and *Pteropus alecto* was approved by QLD Parks and Wildlife Service under permit WISP03333105 and CSIRO AEC 1222.

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