Novel paramyxoviruses in Australian flying-fox populations support host–virus co-evolution

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Understanding the diversity of henipaviruses and related viruses is important in determining the viral ecology within flying-fox populations and assessing the potential threat posed by these agents. This study sought to identify the abundance and diversity of previously unknown paramyxoviruses (UPVs) in Australian flying-fox species (Pteropus alecto, Pteropus scapulatus, Pteropus poliocephalus and Pteropus conspicillatus) and in the Christmas Island species Pteropus melanotus natalis. Using a degenerative reverse transcription-PCR specific for the L gene of known species of the genus Henipavirus and two closely related paramyxovirus genera Respirovirus and Morbillivirus, we identified an abundance and diversity of previously UPVs, with a representative 31 UPVs clustering in eight distinct groups (100 UPVs/495 samples). No new henipaviruses were identified. The findings were consistent with a hypothesis of co-evolution of paramyxoviruses and their flying-fox hosts. Quantification of the degree of co-speciation between host and virus (beyond the scope of this study) would strengthen this hypothesis.

Hendra virus (HeV) and Nipah virus (NiV) (genus Henipavirus, family Paramyxoviridae) cause fatal encephalitic and respiratory diseases in animals and humans (Field et al., 2010; Playford et al., 2010). A third member of the genus Henipavirus, Cedar virus (CeV) (Marsh et al., 2012), and an undescribed henipavirus in Africa (Baker et al., 2013a; Drexler et al., 2009) have recently been identified. The natural reservoir hosts of these viruses in Australia and Southern Asia are bats of the genus Pteropus (order Chiroptera), commonly known as flying foxes (Yob et al., 2001; Young et al., 1996).

It is recognized that bats have played an important role in the evolution of paramyxoviruses, both as historical and current reservoir hosts (Drexler et al., 2012). Multiple studies of the family Pteropodidae have reported broad diversity of unknown paramyxoviruses (UPVs) (Anthony et al., 2013; Baker et al., 2013a; Barr et al., 2015; Drexler et al., 2009; Sasaki et al., 2012). Phylogenetic analyses suggest that some of these UPVs are either members of the genus Henipavirus (Drexler et al., 2009) or belong to novel genera closely related to henipaviruses (Baker et al., 2012, 2013a; Sasaki et al., 2012). This study investigated the diversity of UPVs, with an emphasis on henipaviruses, in flying-fox populations endemic to Australia and Christmas Island to inform emerging disease risk prediction and to gain insight into the evolutionary biology of bat paramyxoviruses.

Pooled urine samples were collected from underneath 10 roosting sites of Pteropus alecto, Pteropus poliocephalus, Pteropus conspicillatus and Pteropus scapulatus during 16 sampling events between May 2012 and July 2013 (Table 1). Samples were collected as described by Field et al. (2011). Individual urine samples were collected from 28 Pteropus melanotus natalis (Christmas Island flying fox) between July and August 2010, as described by Hall et al. (2014). The samples were aliquoted into lysis buffer (Life...
Technologies) before being transported at 4 °C and then stored at −80 °C. RNA extractions were performed using a MagMAX Viral RNA Isolation kit (Life Technologies).

The RNA samples were screened for the presence of respirovirus/morbillivirus/henipavirus (RMH) using a previously described semi-nested PCR (Tong et al., 2008). Phylogenetic analysis of the nucleotide sequences were carried out with maximum-likelihood analysis using the GTR (general time reversible) model with Ψ distribution and bootstrapping (n=1000). Representative UPVs were submitted to GenBank (accession numbers KF871289–KF871319).

A total of 495 samples from Australia (n=467) and Christmas Island (n=28) were tested using the RMH PCR. Of these, 100 samples (Australia n=97, Christmas Island n=3) yielded UPVs from 15 of the 17 sampling events (Table 1). Phylogenetic analysis (Fig. 1) and comparative analysis of the p distances of the UPVs found no molecular evidence of henipaviruses in P. melanotus natalis or of any new henipaviruses in Australian flying foxes. CeV RNA was detected in a P. poliocephalus roost site in Sydney (n=2), and in a mixed roost site of P. alecto and P. poliocephalus in Boonah (n=1) (Fig. 2). This is the first published finding of CeV since it was characterized (Marsh et al., 2012) and the lowest latitude (33.900° S) at which any henipavirus has been identified.

We have avoided using taxonomic terminology as it denotes a level of comparative analysis not applicable with partial sequences from unidentified viruses. To aid the discussion of phylogenetic analysis, informal definitions have been applied. When a monophyletic clade contained UPVs with p distances less than the difference between NiV variants from Malaysia and Bangladesh (4.7 %), a single representative sequence was selected and termed 'representative UPV'. A clade of UPVs is considered a UPV group when the p distance between members of the cluster is less than the sequence identity of the two most distant species of the genus Henipavirus (CeV and NiV-Malaysia: 33.0 %) and they are contained on a monophyletic clade that is well supported by bootstrapping (≥75 %) at the common ancestral node.

Table 1. Sampling information and results from respirovirus/morbillivirus/henipavirus (RMH) nested PCR

<table>
<thead>
<tr>
<th>Species</th>
<th>Location</th>
<th>Date collected</th>
<th>RMH nested PCR No. tested</th>
<th>No. positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. poliocephalus</td>
<td>Sydney (Centennial Park), NSW</td>
<td>June 2012</td>
<td>30</td>
<td>4 (13.3)</td>
</tr>
<tr>
<td>P. poliocephalus</td>
<td>Sydney (Centennial Park), NSW</td>
<td>July 2012</td>
<td>30</td>
<td>7 (23.3)</td>
</tr>
<tr>
<td>P. poliocephalus</td>
<td>Sydney (Centennial Park), NSW</td>
<td>July 2012</td>
<td>30</td>
<td>4 (13.3)</td>
</tr>
<tr>
<td>P. alecto</td>
<td>Tannum Sands, QLD</td>
<td>June 2012</td>
<td>36</td>
<td>2 (5.5)</td>
</tr>
<tr>
<td>P. alecto</td>
<td>Tannum Sands, QLD</td>
<td>October 2012</td>
<td>28</td>
<td>7 (25)</td>
</tr>
<tr>
<td>P. alecto</td>
<td>Pine Creek, NT</td>
<td>May 2012</td>
<td>30</td>
<td>2 (6.6)</td>
</tr>
<tr>
<td>P. scapulatus</td>
<td>Duaringa, QLD</td>
<td>June 2012</td>
<td>50</td>
<td>28 (56)</td>
</tr>
<tr>
<td>P. scapulatus</td>
<td>Duaringa, QLD</td>
<td>October 2012</td>
<td>29</td>
<td>14 (48.3)</td>
</tr>
<tr>
<td>P. conspicillatus</td>
<td>Cairns, QLD</td>
<td>May 2012</td>
<td>29</td>
<td>1 (3.4)</td>
</tr>
<tr>
<td>P. conspicillatus</td>
<td>Cairns, QLD</td>
<td>June 2012</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td>P. conspicillatus</td>
<td>Yungaburra, QLD</td>
<td>May 2012</td>
<td>29</td>
<td>0</td>
</tr>
<tr>
<td>P. melanotus natalis*</td>
<td>Christmas Island</td>
<td>August 2010</td>
<td>28</td>
<td>3 (10.7)</td>
</tr>
<tr>
<td>P. poliocephalus and P. alecto</td>
<td>Boonah, QLD</td>
<td>June 2013</td>
<td>17</td>
<td>9 (52.9)</td>
</tr>
<tr>
<td>P. poliocephalus and P. alecto</td>
<td>Boonah, QLD</td>
<td>July 2013</td>
<td>16</td>
<td>4 (25.0)</td>
</tr>
<tr>
<td>P. poliocephalus and P. alecto</td>
<td>Redcliffe, QLD</td>
<td>July 2013</td>
<td>30</td>
<td>8 (26.6)</td>
</tr>
<tr>
<td>P. poliocephalus and P. alecto</td>
<td>Nambucca Heads, NSW</td>
<td>June 2012</td>
<td>23</td>
<td>6 (26.1)</td>
</tr>
<tr>
<td>P. poliocephalus and P. alecto</td>
<td>Byron Bay, NSW</td>
<td>June 2012</td>
<td>30</td>
<td>1 (3.3)</td>
</tr>
</tbody>
</table>

Total 495 100 (20.2)

*Collections that were of individual urine samples.

Of the 100 UPVs, a total of 31 representative UPVs were identified. These representative UPVs formed eight distinct groups (Fig. 1). None had sufficient sequence similarity to be defined as members of a known genera of the family Paramyxoviridae (Fig. 1). This level of UPV group diversity is consistent with the variety reported in Asia and Africa (Anthony et al., 2013; Baker et al., 2013a; Drexler et al., 2009; Sasaki et al., 2012), and supports the existence of a diverse range of unidentified viruses that are not associated with known diseases (Anthony et al., 2013). It should be noted that diversity may be higher in the flying-fox populations studied as the primers used would not have identified all paramyxoviruses. We propose that these UPV groups represent new genera within the family. Isolation and characterization of representative strains and detailed comparative analyses are needed to confirm this.

Comparative analysis identified the same UPV groups in flying foxes from both Australia and Christmas Island (Groups 1 and 5), despite the populations being geographi-
It is widely accepted in phylogeography studies that Pteropodidae originated in Asia (Chan et al., 2011; O’Brien et al., 2009) and migrated to Africa through overland routes when forest corridors existed between Africa and Asia ~15–25 million years ago (MYA) (Juste et al., 1999), and colonized Indian Ocean islands using oceanic routes that occurred between 0.37 and 2.58 MYA (Almeida et al., 2014; Bastian et al., 2002). The replacement of forest corridors by deserts and tectonic movements have since isolated pteropid populations to their respective regions, and presumably also their viral assemblages. Thus, we postulate that bat paramyxoviruses originated with ancestral bats in Asia and accompanied their hosts’ dispersal to Africa. This hypothesis is in direct contrast to the proposed origins of henipaviruses in Africa and subsequent global dispersal (Drexler et al., 2012). To further elucidate the origins of bat paramyxoviruses, and whether these relationships represent co-speciation and host–virus specificity, additional research into co-phylogeny and virus diversity is needed.

There is a tendency to view related species of bats (i.e. Pteropodidae, Pteropus genus) as a single host population (Breed et al., 2011, 2013; Plowright et al., 2008), with viruses moving easily between species, rather than considering species or closely related groups of species as alternative hosts that require a host-switch event for viral replication and excretion to occur. In this study there is evidence of UPV specificity to a single species, despite overlapping geographical ranges for the four Australian species (Hall & Richards, 2000). None of the eight UPVs found in P. scapulatus were identified in any other of the three Australian species (Fig. 1). In contrast, there were examples of UPVs detected in multiple roosts with different resident Pteropus species: AU-15 (Group 7) was identified from a P. poliocephalus roost and P. alecto roost, and AU-12 (Group 2) was identified in a P. conspicillatus roost and in a mixed roost of P. alecto and P. poliocephalus. During the evolutionary history of P. scapulatus this species is believed to have undergone an extended period of allopatric speciation (Bastian et al., 2002), and colonized Australia much earlier than the ancestor of P. alecto and P. conspicillatus, and the ancestor of P. poliocephalus (Almeida et al., 2014). Taken together, this is suggestive of ancient association of UPVs with flying foxes that have evolved through periods of geographical isolation.

This association is further supported by research into natural HeV infection dynamics in Australian flying foxes. Despite a substantial seroprevalence in the Australian species (Breed et al., 2011; Field, 2005; Plowright et al., 2008), it is suggested that P. alecto and P. conspicillatus are the predominant natural hosts, with other species playing a limited role in the maintenance of the infection or spillover of HeV (Smith et al., 2014). Based on the UPV specificity observed in P. scapulatus in this study, and mounting evidence of species specificity in relation to HeV infection, we suggest that flying foxes have developed a unique infection dynamic with individual paramyxoviruses that has resulted in species-specific or group-specific relationships similar to those reported between coronaviruses and bats (Gouilh et al., 2011). Exposure of a flying-fox species to an unfamiliar paramyxovirus may result in no or limited infection, characterized by a lack of virus replication and excretion, but simply an immunological response to the antigen. If this is so, the use of seroprevalence/serosurveillance as proxy of infection dynamics as a means of risk assessment associated with bat-borne paramyxoviruses should be used with caution (Gilbert et al., 2013).

An alternative explanation of bat and paramyxovirus interactions is localized regional association in an evolutionary timescale. In this study, UPV representatives in Group 2...
and 7 were detected in multiple single-species roosts with different resident *Pteropus* species (Fig. 1). In a localized regional association scenario, the assumption is that the origins of a viral group or host-switch event occurred in a location where multiple species were regularly cohabitating and then evolved in multiple distantly related species. We do not suggest that the proposed evolutionary scenarios are mutually exclusive, but rather that they may co-occur depending on prevailing host dynamics.

NiV could be an example of co-occurring evolutionary scenarios. The host of NiV-Bangladesh, *Pteropus giganteus* (Yadav et al., 2012), is proposed to have diverged ~0.42 MYA (Almeida et al., 2014) from ancestors of the hosts of NiV-Malaysia, *Pteropus lylei* or *Pteropus vampyrus* (Rahman et al., 2010; Wacharapluesadee et al., 2005). Conversely, *P. lylei* and *P. vampyrus* ancestors diverged ~0.5–1.18 MYA, but both remained on the Southeast Asian mainland (Almeida et al., 2014). This suggests NiV-Malaysia evolved in multiple species through localized regional association in an evolutionary timescale. In our study, an Australian UPV from Group 3 was closely related to a UPV identified from *P. vampyrus* (Sasaki et al., 2012) ($p$ distance of 7.4%). This is more variation than is observed between NiV-Malaysia and NiV-Bangladesh (6.4%), but less than observed between NiV and HeV (21.4–21.6%). If the rate of evolution in bat paramyxoviruses is consistent, the divergence between these two UPVs would be equivalent to those observed in NiV-Malaysia.
Bangladesh and NiV-Malaysia (>0.5 MYA). This time frame is consistent with the introduction of *P. alecto* ancestor to Australia from Indonesia/Papua New Guinea.

A number of studies postulate an ancient association between Pteropodidae and their associated viruses, including paramyxoviruses and particularly henipaviruses (Badrane & Tordo, 2001; Drexler et al., 2012; Gouilh et al., 2011). Here, we suggest that ancient associations with their host or a closely related group of hosts may have resulted in host specificity. If this is the case, there are limitations to the ability of these viruses to produce an infection in other species of flying foxes. An earlier quantitative risk assessment (Breed, 2012) identified that the probability of NiV becoming endemic in Australian flying foxes was very low. Our study suggests the improbability of NiV establishing infection in Australian mainland species regardless of any movement of flying foxes between Australia, Indonesia and Papua New Guinea (Breed et al., 2010).

In recent years, there has been an increased effort to understand virus diversity beyond the scope of known pathogens in flying foxes. In this study we found numerous and diverse UPV RNA in Australian and Christmas Island flying foxes, but no novel henipaviruses. The patterns of regional distribution and host specificity observed led us to propose an ancient association with flying foxes and their paramyxoviruses due to species specificity or host-group specificity, owing either to periods of geographical isolation or localized regional association in an evolutionary time-scale. Together, this hypothesis presents a plausible scenario for the co-evolution of paramyxoviruses and their hosts as they originated in Asia. However, further research into bat–virus relationships is needed, as it has implications for the interpretation of seroprevalence studies and biosecurity risk management.

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References


