Co-circulation of diverse paramyxoviruses in an urban African fruit bat population

K. S. Baker,1,2 S. Todd,3 G. Marsh,3 A. Fernandez-Loras,1 R. Suu-Ire,4 J. L. N. Wood,2 L. F. Wang,3 P. R. Murcia5 and A. A. Cunningham1

1Institute of Zoology, Zoological Society of London, Regent's Park, London NW1 4RY, UK
2Cambridge Infectious Diseases Consortium, University of Cambridge, Department of Veterinary Medicine, Madingley Road, Cambridge CB3 0ES, UK
3CSIRO Australian Animal Health Laboratories, Portarlington Road, East Geelong, VIC 3219, Australia
4Wildlife Division of the Forestry Commission, Accra, Ghana
5University of Glasgow Centre for Virus Research, Institute of Infection, Immunity and Inflammation, College of Medical, Veterinary and Life Sciences, Garscube Estate, Bearsden Road, Glasgow G61 1QH, UK

Bats constitute a reservoir of zoonotic infections and some bat paramyxoviruses are capable of cross-species transmission, often with fatal consequences. Determining the level of viral diversity in reservoir populations is fundamental to understanding and predicting viral emergence. This is particularly relevant for RNA viruses where the adaptive mutations required for cross-species transmission can be present in the reservoir host. We report the use of non-invasively collected, pooled, neat urine samples as a robust sample type for investigating paramyxoviruses in bat populations. Using consensus PCR assays we have detected a high incidence and genetic diversity of novel paramyxoviruses in an urban fruit bat population over a short period of time. This may suggest a similarly unique relationship between bats and the members of the family Paramyxoviridae as proposed for some other viral families. Additionally, the high rate of bat–human contact at the study site calls for the zoonotic potential of the detected viruses to be investigated further.

Bats are an increasingly recognized source of emerging zoonoses and are known to harbour a wide range of viruses, including highly virulent zoonoses such as henipa-, filo- and lyssa- viruses (Calisher et al., 2006). Consequently, research into bat viromes is intensifying and results obtained from those investigations show that viruses in bat populations exhibit significant genetic diversity. Marked diversity of lyssaviruses (Delmas et al., 2008), coronaviruses (Tang et al., 2006; Woo et al., 2006), astroviruses (Chu et al., 2008) and, more recently, adenoviruses (Li et al., 2010) and circoviruses (Ge et al., 2011) has been reported. In some cases, this diversity has led to speculation that chiropterans have ancient relationships with these viral families, and consequently act as reservoirs for emergence (Badrane & Tordo, 2001; Gouilh et al., 2011). Bats are known to harbour multiple paramyxoviruses, including rubulaviruses and henipaviruses that have spilled over into humans and/or domestic animals (Calisher et al., 2006; Chant et al., 1998; Lau et al., 2010).

African straw-coloured fruit bats (Eidolon helvum) sampled in Ghana have been shown to have neutralizing antibodies against henipaviruses (Hayman et al., 2008) as well as paramyxoviral RNA in their faeces (Drexler et al., 2009). Paramyxoviruses are known to be excreted in the urine of experimentally infected and wild fruit bats (Middleton et al., 2007; Chu et al., 2002; Smith et al., 2011). Here, we report the paramyxovirus diversity in neat urine samples collected from underneath a large population of E. helvum in Accra, Ghana, which lives in close proximity to humans and domestic animals.

Pooled urine samples (n=72) from E. helvum were collected over 12 sampling intervals between September and November 2010 (Table 1). Samples were collected from underneath a tree holding ~1500 E. helvum (co-roosting with other species has not been observed at this site) that comprise part of a much larger colony of up to 1 000 000 bats in Accra (Hayman et al., 2008). Plastic sheeting was laid out underneath the tree approximately 1 h before dawn (adapted from Chua, 2003). On return from foraging, bats urinated and defecated on the sheets.
and 1 ml pools of neat urine were collected by pipette and divided equally between two vials for PCR analysis and virus isolation. Care was taken to avoid contact with faeces but it is possible that small amounts of faecal contamination were present in the samples. Samples were stored and transported at ~4 °C before freezing and stored at ~80 °C until further processing. RNA was extracted from 500 μl neat urine from each pool using the MagMAX viral RNA isolation kit and a MagMAX Express-96 automated extraction unit (Life Technologies – Applied Biosystems), using a 1:2 ratio of sample to lysis buffer.

Extracted RNA was tested for the presence of paramyxovirus polymerase gene RNA using two heminested RT-PCRs (Tong et al., 2008). *Paramyxovirinae* PCR (PMV-PCR) was used to amplify a 531 bp (excluding primers PAR-F2 and PAR-R) fragment of polymerase genes of viruses belonging to the subfamily *Paramyxovirinae*, that spanned positions 13 898–14 428 of the hendra virus genome (GenBank accession no. NC_001906). A PCR targeting an upstream portion of the polymerase gene (~439 bp, excluding primers RES-MOR-HEN-F2 and RES-MOR-HEN-R, corresponding to positions 12 617–13 055 of the hendra virus genome) of respiro-, morbilli- and henipa-viruses (RMH-PCR) was also used.

PCR products were cloned into pGEM-T Easy (Promega) and one or more clones from each sample were sequenced (Big Dye Terminator v3.1; Life technologies – Applied Biosystems). Obtained sequences were aligned with polymerase genes from the subfamily *Paramyxovirinae* using MUSCLE (Edgar, 2004) and CLUSTAL_X (Thompson et al., 1994). Phylogenetic trees were constructed using MrBayes (Ronquist & Huelsenbeck, 2003) under the GTR + I + G model (as determined by ModelTest; Posada & Crandall, 1998).

The urine samples were frequently found to contain paramyxovirus sequences with PCR-positive samples being collected on 8 of the 12 sampling dates (over a 2 month period) and 31 of 72 samples (43 %) being positive. Fifteen of the samples were positive by both PMV-PCR and RMH-PCR, with each PCR detecting a further 10 and 7 (respectively) positive samples uniquely (Table 1, Figs 1 and 2). Phylogenetic analysis of sequences derived from cloned PCR products from the PMV-PCR (43 clones) and RMH-PCR (39 clones) not only revealed the relationships of the novel sequences with each other and known sequences from the subfamily *Paramyxovirinae*, but also allowed for comparison of the assays’ performance on field samples (Figs 1 and 2). GenBank accession numbers for novel paramyxovirus sequences are shown in Figs 1 and 2.

The sequences obtained through PCR indicated the presence of multiple novel paramyxoviruses circulating in the *E. helvum* population in Accra, with sequences having pairwise nucleotide identities of 57–89 % with known paramyxoviruses. These sequences expanded the *Paramyxovirinae* phylogeny, with the PMV-PCR detecting sequences in five clusters throughout the phylogeny (Fig. 1). Some clusters (2, 3 and 5 in Fig. 1) represented phylogenetically diverse subgroups that shared a common ancestor supported by high posterior probability values, and two of these clusters (1 and 2, Fig. 1) did not group confidently with any existing genus in the subfamily *Paramyxovirinae*. Another group of sequences detected by PMV-PCR were phylogenetically related to other unclassified bat-derived paramyxoviruses (menangle, tioman and tuhoko viruses) within the clade containing known rubulaviruses (5, Fig. 1).

Notably, some sequences were related to known human pathogens, including sequences derived from sample U53 using the PMV-PCR that grouped with mumps virus with a high posterior probability value (4, Fig. 1) and highly structured groups of sequences related to, but distinct from, henipaviruses (3, Fig. 1 and C and D, Fig. 2).

The sequences obtained using the RMH-PCR were, as expected, restricted to the portion of the *Paramyxovirinae* phylogeny pertaining to these genera (Fig. 2). However, consistent with the results obtained using the PMV-PCR, the sequences detected using the RMH-PCR similarly expanded this portion of the phylogeny, with sequences being distributed in four main clusters (A–D, Fig. 2). These clusters were related to, but distinct from, henipaviruses and respiroviruses but were otherwise unable to be confidently placed in existing genera of the *Paramyxovirinae* phylogeny. Clusters B and C (Fig. 2) were related to paramyxovirus sequences previously obtained from *E. helvum* faecal material in Ghana (Drexler et al., 2009). However, some sequences in cluster B were still phylogenetically distinct from these (Fig. 2), sharing as little as 74 % nucleotide identity, perhaps suggesting that the sequences obtained thus far still represent only a fraction of the paramyxovirus diversity in *E. helvum*.

These results illustrate the high levels of paramyxovirus diversity in the sampled population, with the sequences detected by PMV-PCR having nucleotide identities as low as 42 % among them, and those detected through RMH-PCR as low as 57 %. Even subsequent to phylogenetic grouping, the nucleotide identities of sequences within the same clades were as low as ~65 % (e.g. 3, Fig. 1 and B, Fig. 2). Notably, individual sample pools were also genetically diverse. PCR products (where >1 clone was sequenced) often contained phylogenetically distinct sequences. For example, sequences U9B and U9D generated by PMV-PCR belonged to phylogenetic groups 5 and 2, respectively (see Fig. 1, and other sequences marked with crosses in Figs 1 and 2). This phenomenon was more commonly observed in samples using PMV-PCR (7/15) than those using RMH-PCR (2/17).

Here, we have detected a high incidence and diversity of paramyxoviruses in *E. helvum* in Accra, Ghana, using previously published consensus PCR assays together with sequencing of cloned PCR products. Both the PMV-PCR and RMH-PCR assays were highly effective at detecting novel paramyxovirus sequences and consequently will likely continue to be used in other field studies. However, they were not equivocal in their results and these
Table 1. Collection dates, PCR results and clone notations of pooled urine samples used in this study

| Date       | Sample ID | PMV-PCR | Clone(s)* | RMH-PCR | Clone(s)* |
|------------|-----------|---------|-----------|---------|-----------|
| 24/09/2010 | U1        |         |           |         |           |
|            | U2        |         |           |         |           |
|            | U3        |         |           |         |           |
|            | U4        |         |           |         |           |
|            | U5        | +       | B, D      | +       | A, B, C   |
|            | U6        | +       | A, B      | +       | A, B      |
| 30/09/2010 | U7        |         |           |         |           |
|            | U8        |         |           |         |           |
|            | U9        | +       | B, D      |         | A         |
|            | U10       |         |           |         |           |
|            | U11       |         |           |         |           |
| 04/10/2010 | U12       |         |           |         |           |
|            | U13       |         |           |         |           |
|            | U14       |         |           |         |           |
|            | U15       |         |           |         |           |
|            | U16       |         |           |         |           |
| 08/10/2010 | U17       |         |           |         |           |
|            | U18       |         |           |         |           |
|            | U19       |         |           |         |           |
|            | U20       |         |           |         |           |
| 12/10/2010 | U21       |         |           |         |           |
|            | U22       | +       | C         |         |           |
|            | U23       |         |           |         |           |
|            | U24       |         |           |         |           |
|            | U25       |         |           |         |           |
|            | U26       |         |           |         |           |
| 14/10/2010 | U27       |         |           |         |           |
|            | U28       |         |           |         |           |
|            | U29       |         |           |         |           |
|            | U30       |         |           |         |           |
|            | U31       |         |           |         |           |
| 19/10/2010 | U32       | +       | C         |         |           |
|            | U33       |         |           |         |           |
| 21/10/2010 | U34       |         |           |         |           |
|            | U35       |         |           |         |           |
|            | U36       |         |           |         |           |
|            | U37       |         |           |         |           |
|            | U38       |         |           |         |           |
| 02/11/2010 | U39       |         |           |         |           |
|            | U40       |         |           |         |           |
|            | U41       |         |           |         |           |
| 10/11/2010 | U42       | +       | A, B      | +       | A, B      |
|            | U43       | +       | B         |         |           |
|            | U44       | +       | A, B      |         |           |
|            | U45       | +       | B, G, H   | +       | A, B      |
|            | U46       | +       | B, G, H   |         |           |
|            | U47       | +       | C, E, F, G, H |   |           |
|            | U48       |         |           |         |           |
| 15/11/2010 | U49       | +       | B         | +       | B         |
|            | U50       | +       | A, B      | +       | B, C      |
|            | U51       |         |           |         |           |
|            | U52       | +       | B, C      |         |           |
|            | U53       | +       | A, C      | +       | A, B      |
|            | U54       | +       | C, D      | +       | A, B      |
|            | U55       | +       | C         |         |           |
analyses provide important insights into their differential capabilities that are likely applicable across other species and sites. The assays differed in their specificities, with the PMV-PCR detecting sequences throughout the *Paramyxovirinae* phylogeny (with the notable exception of sequences closely related to avulaviruses) and the RMH-PCR being restricted to respiro-, morbilli- and henipaviruses. This is likely caused by the greater redundancy of the PMV-PCR primers and is further evidenced by the results from 10 samples in which sequence was detected using PMV-PCR but not using RMH-PCR. The sequences derived from these 10 samples showed phylogenetic clustering (Fig. 1), with the majority within the genus *Rubulavirus*. The broader specificity of the PMV-PCR is also what likely allowed this assay to detect intrasample diversity more frequently than was observed with the RMH-PCR. It should be noted, however, that sequences derived from the same sample could be derived from different viruses and/or bats due to the nature of the sampling method.

Regarding assay sensitivity, although the PMV-PCR and RMH-PCR appear superficially to have similar sensitivities (24 vs 21 of 72 positives, respectively), the RMH-PCR detected seven samples as positive that were negative on PMV-PCR. However, the phylogenetic clustering of these samples' sequences was not as marked as in the previous case (Fig. 2). Thus, it is likely that the uniquely detected samples in the case of the RMH-PCR are the result of increased assay sensitivity as reported in the original *in vitro* studies (Tong et al., 2008). Due to differences in phylogenetic tree topology produced by different polymerase regions, and the differing sensitivity and specificity of the assays, it is difficult to comment on the relationships of sample sequences generated by different PCRs. However, sample U6 notably generated unique and poorly placed clusters in phylogenetic analysis of both regions.

Irrespective of assay differences, novel paramyxovirus sequences were detected at a high incidence in pooled urine samples collected from underneath a single roost-tree of *E. helvum* in Accra, Ghana. This demonstrates that neat urine pools are a robust sample type for investigation into paramyxoviruses in bat populations. Indeed, novel paramyxoviruses have since been isolated and characterized from this population (K. S. Baker and others, unpublished data). Not only is collecting urine from underneath roosts highly efficacious for qualitative virus population studies, but the non-invasive nature of the collection method would facilitate the study of infection dynamics free from sampling-induced stress. However, one disadvantage is that the viral sequences detected here cannot be absolutely confirmed as having been derived from *E. helvum*. Although co-roosting with other species has not previously been observed, and sheeting was supervised throughout the sampling period, detection of these sequences in samples directly collected from individual bats would be required to confirm their origin.

The detected sequences are highly novel and demonstrate significant genetic diversity of paramyxoviruses within a single bat population over a short period of time. Furthermore, their close relationships with previously detected paramyxovirus sequences in Ghanaian *E. helvum* (Drexler et al., 2009) and sequences detected in urine

| Date       | Sample ID | PMV-PCR | Clone(s)* | RMH-PCR | Clone(s)* |
|------------|-----------|---------|-----------|---------|-----------|
| 20/11/2010 | U56       | +       | B, C      |         |           |
|            | U57       | +       |           |         | B         |
|            | U58       | +       | C         | +       | A, B      |
|            | U59       | +       |           | +       | A, B      |
|            | U60       |         |           |         |           |
|            | U61       | +       |           |         | A, B      |
|            | U62       |         |           | +       | A, B      |
|            | U63       |         |           | +       | A, B      |
|            | U64       | +       | A         | +       | A, B      |
|            | U65       |         |           |         |           |
|            | U66       | +       | A         | +       | A, B      |
|            | U67       | +       | J, N      |         | A         |
|            | U68       | +       | E, G      |         | A, B      |
|            | U69       | +       | C, D      |         |           |
|            | U70       | +       | C         | +       | A, B      |
|            | U71       |         |           | +       | A, B      |
|            | U72       |         |           |         |           |
| **Total**  | **Samples** | **Positive** | **PMV-PCR clones** | **Positive** | **RMH-PCR clones** |
|            | 72        | 24      | 43        | 21      | 39        |

*Individual cloned sequences from PCR products are noted by individual letters.*
collected from *E. helvum* roosts in Uganda and Tanzania (A.J. Peel and others, unpublished results) supports these findings and increases our understanding of the diversity of paramyxoviruses in Africa. Indeed, the sequences described here support the further expansion of the genus *Rubulavirus*, which appears to have commenced with the detection of other bat-derived paramyxoviruses namely menangle, tioman and tuhoko viruses. Also, our data give rise to numerous phylogenetically distinct, but poorly placed clusters of viral sequences, perhaps indicating that viruses belonging to novel genera may be circulating in the study population. Such diversity and expansion of the paramyxovirus phylogeny within a single population implies that adaptive mutations required for emergence may be present.

Fig. 1. Diversity of paramyxoviruses in pooled *E. helvum* urine samples detected by using *Paramyxovirinae*-targeted PCR. Phylogenetic tree for a 531 bp segment of the polymerase gene of members of the subfamily *Paramyxovirinae*, including sequences generated in this study and publicly available paramyxovirus sequences (Newcastle disease virus, sendai virus, human parainfluenza virus 3, mossman virus, tupai paramyxovirus, J virus, beilong virus, canine distemper virus, measles virus, rinderpest, hendra virus, nipah virus (Bangladesh), nipah virus (Malaysia), human parainfluenza virus 4, mumps virus, simian parainfluenza virus 41, human parainfluenza virus 2, simian virus 5, porcine rubulavirus, mapuera virus, tuhoko virus 1, menangle virus, tioman virus, tuhoko virus 3, tuhoko virus 2). Relevant posterior probability values are shown. Bar, 0.3 expected nucleotide substitutions per site. Individual sample IDs are followed by letters denoting the clone and the GenBank accession number for the sequence. Groups containing previously uncharacterized sequences that display a common phylogenetic origin supported by high posterior probability values (≥0.95) are highlighted by numbered grey boxes. Adjacent to each box number is the range of nucleotide identities among sequences in the clade. Clones derived from samples that contained sequences belonging to more than one of these phylogenetically distinct clades were marked with a cross. Sequences derived from samples that were positive only with this PCR (and negative with RMH-PCR) are marked with a dot.
in the reservoir host, and also may suggest that a co-evolutionary relationship between Chiroptera and Paramyxoviridae exists, as has been proposed for other viral families (Badrane & Tordo, 2001; Gouilh et al., 2011). The presence of intrasample diversity is intriguing and may reflect co-infection of an individual, or alternatively may have resulted from the pooling of co-circulating viruses from different bats. Further studies, such as examination of urine samples from individual bats would be required to differentiate between these possibilities.

Sequences were detected that had close relationships with known human pathogens. E. helvum is widely hunted in Ghana for bush meat (Kamins et al., 2011) and the population under study has a high potential for both direct and indirect contact with human beings and domestic animals. This information, coupled with the knowledge that many bat paramyxoviruses are already known to be zoonotic, makes it imperative that our findings are investigated further.

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