Species-Specific Transmission of Novel Picornaviruses in Lemurs

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Abstract

The roles of host genetics versus exposure and contact frequency in driving cross-species transmission remains debated. Here, we used a multi-taxon lemur collection at the Saint Louis Zoo in the USA as a model to gain insight into viral transmission in a high inter-species contact setting. Lemurs are a diverse and understudied group of primates that are highly endangered. Endemic to the island of Madagascar, the speciation of lemurs occurred in geographic isolation apart from continental African primates. Although evidence of endogenized viruses in lemur genomes exist, no exogenous viruses of lemurs have been described to date. Here we identified two novel picornaviruses in fecal specimens of ring-tailed lemurs (Lemur catta) and black-and-white ruffed lemurs (Varecia variegata). We found that the viruses were transmitted in a species-specific manner (lesavirus 1 was detected only in ring-tailed lemurs while lesavirus 2 was detected only in black-and-white ruffed lemurs). Longitudinal sampling over a one year interval demonstrated ongoing infection in the collection. This was supported by evidence of viral clearance in some animals and new infections in previously uninfected animals, including a set of newly-born triplets that acquired the infection. While both viruses were found to be co-circulating in a mixed species exhibit of ring-tailed lemurs, black-and-white ruffed lemurs and black lemurs, there was no evidence of cross-species transmission. This suggests that despite high intensity contact, host species barriers can prevent cross-species transmissions of these viruses.
Importance (105 words)

Up to seventy-five percent of emerging infectious diseases in humans today are the result of zoonotic transmission. However, a challenge in understanding transmission dynamics has been the limited models of cross-species transmission. Zoos provide a unique opportunity to explore parameters defining viral transmission. We demonstrated that ongoing virus transmission in a mixed lemur species exhibit was species-specific. This suggests that despite high contact intensity, host species barriers contribute to protection from cross-species transmission of these viruses. While the combination of species might differ, most zoological parks worldwide commonly feature mixed species exhibits. Collectively, this study demonstrates a widely applicable approach towards understanding infectious disease transmission.
Introduction

The origin of many emerging infectious diseases can be traced to transmissions between humans and non-human animals. For example, the SARS outbreak resulted from the transmission of SARS coronavirus from civets to humans, and the ongoing HIV-AIDS pandemic originated from cross-species transmissions of SIV from chimpanzees and related primates (1, 2). Host genetic factors, such as cellular receptors and immunity genes, can act as species barriers to viral transmission (3-5). For RNA viruses, it has been proposed that host barriers that share closer genetic similarities between species correspond to the flattened fitness valley that viruses can traverse in their adaptation to new hosts (4, 6). Consequently, species-specific barriers can be overcome by virus evolution through adaptive mutations and neofunctionalization (7-10). Alternatively, it has been argued that high contact rate is the key driver of virus emergence (11-13). However, a major challenge to studying the dynamics of cross-species transmission has been the lack of models in relevant settings. Hence, most studies have relied on prospective inference and reconstruction.

Zoological parks feature collections that house different animal species within an enclosure (i.e. mixed species exhibits). Mixed species exhibits benefit both the animals and public visitors by providing a more enriched environment and increasing the educational experience (14, 15). Mixed species exhibits also provide a practical solution to the limited space available at most zoos. However, this creates an environment where inter-species interactions may occur through physical contact (16).
Lemurs, endemic to Madagascar, are prosimians that diverged from other primates on the African mainland approximately 62 million years ago (17). Lemurs are highly diverse in part because, unlike African and Asian prosimians that are strictly nocturnal, they evolved in the absence of anthropoid primates (monkeys and apes), branching out to occupy the diurnal and nocturnal niches of the island’s different ecosystems. There is only limited data regarding viruses that infect lemurs. Serological studies suggest that lemurs have been exposed to pathogens similar to West Nile virus and lentiviruses (18). Moreover, endogenous gammaherpesvirus, lentivirus and spumavirus sequences have been identified in lemur genomes (19-23). However, there has been no direct evidence to date of extant exogenous viruses in lemurs.

One Health has been defined as an initiative that aims to merge animal and human health sciences to benefit both (24). Emerging infectious diseases of animals and humans, along with the continued anthropogenic environmental stressors that challenge wildlife and human health have been the catalyst for the growing One Health approach in the veterinary, medical, and environmental fields (25). Within this framework, mixed species exhibits provide a unique opportunity to examine viral transmission in a high inter-species contact setting. In this study, we demonstrate the species-specific transmission of two novel picornaviruses in lemurs housed in single and mixed species exhibits at the Saint Louis Zoo.
Materials and Methods

Specimens. The study was approved by the Saint Louis Zoo’s Institutional Animal Care and Use Committee. 35 fecal specimens were collected during September - October 2012 from ring-tailed lemurs (Lemur catta), black lemurs (Eulemur macaco macaco), a blue-eyed black lemur (Eulemur macaco flavifrons), mongoose lemurs (Eulemur mongoz), black-and-white ruffed lemurs (Varecia variegata) and Coquerel’s sifakas (Propithecus coquereli). Details of the individual species in the collection are listed in Supplementary Table S1. A fecal specimen from 1 Coquerel’s sifaka was not available at the time of collection. A second set of 33 fecal specimens was collected in September 2013. One ring-tailed lemur and 3 black-and-white ruffed lemurs died since the 2012 collection. Samples from 1 black-and-white ruffed lemur (transferred to another zoo) and 1 black lemur were not available at the time of the 2013 collection.

Sequencing. A subset of specimens from the 2012 collection was subjected to unbiased next generation sequencing. Fecal specimens were diluted in 6:1 in PBS and filtered through a 0.45 µm membrane to minimize recovery of intact bacteria. Total nucleic acid was extracted from the filtrate. The sequencing library for the specimen from lemur Mis101308 was prepared using ScriptSeq (Epicentre, Madison, WI, USA). Total nucleic acid extracted from the specimen from lemur Nai108015 was subjected to random-priming cDNA synthesis and amplification, and the sequencing library was generated using a standard TruSeq (Illumina, San Diego, CA, USA) protocol. Libraries were sequenced on an Illumina MiSeq instrument. High quality reads with no detectable similarity to the reference human genome or NCBI nt database by BLASTn were
analyzed by BLASTx alignment against the NCBI non-redundant (nr) protein database as previously described (26), in order to identify divergent viral sequences. Contigs were assembled from viral sequences using Newbler (27).

**Amplification of complete genome.** PCR primers were designed from contigs assembled from Illumina sequences. The complete genome of the lesavirus 1 was amplified by RT-PCR in five overlapping fragments using SuperScript III Reverse Transcriptase kit (Invitrogen, Grand Island, NY, USA), cloned using TOPO cloning kit (Invitrogen, Grand Island, NY, USA) and Sanger sequenced as previously described (28). The following primers were used: LV1-1F (5’–TCACATTAGCCATGGCTTGC–3’) with LV1-1r (5’–CATCACCTGGGCTGAATTG–3’); LV1-2F (5’–CAAGTACAAGTGAAGCGCAACG–3’) with LV1-2r (5’–GGAGTGGTCCAGTCTCGAC–3’); LV1-3F (5’–TGGAGCTACTTTCCCTGGCTCAGAC–3’) with LV1-4F (5’–ACAGGTCTGTTGATAGCCATCC–3’); LV1-4r (5’–AACCTTGGAGCAGGCAAATGG–3’); LV1-5F (5’–CTGCCAGGCTTCTGTGATC–3’) with LV1-5r (5’–TGGAAATGCTGGTCTGGTCAAAGTGG–3’). 5’ RACE was performed with LV1-5RACE1r (5’–CCATGAAGGGGCTGCTAACCC–3’); 3’ RACE was performed with LV1-3RACE1F (5’–ATGACGAGGAGTACACGCTG–3’).

The complete genome of the lesavirus 2 was amplified by RT-PCR in four overlapping fragments. The following primers were used: LV2-1F (5’–GGAATTCCAGGGAGCCGAC–3’) with LV2-1r (5’–CATTTCCGTCAGTCTACGG–3’); LV2-2F (5’–CAGGTGAATCGGCGCCGAAATG–3’) with LV2-2r (5’–GCTGCCAGCATGGGCTGCT–3’); LV2-3F (5’–TGACTTCAGAGCCAGCTTCTACG–3’) with LV2-3r (5’–GACATCCGCTGGGATTCTGAAG–3’); LV2-4F (5’–
CAGCTCTTAGCTGCAGAGACCCA–3’) with LV2-4r (5’–ACTGGCCCACTGTGTACAGCCAG–3’). 5’
RACE was performed with LV2-5RACE1r (5’–ACCAAGCCATACCTCTTGAC–3’); 3’ RACE was
performed with LV2-3RACE1F (5’–CACCTGCCCCAGAGGATGGAGAC–3’).

The VP1 sequence for LV1 was amplified from nucleic acid extracted from fecal specimens collected in 2012 and 2013 from a ring-tailed lemur Mis101308 using primer set LV1-VP1F (5’–CAGGTGCTACAACACCCACTGATG–3’) with LV1-VP1r (5’–
TGAACCACCAAGCAGAAACACTGC–3’). LV2 VP1 was amplified from nucleic acid extracted from fecal specimens collected in 2012 and 2013 from a black-and-white ruffed lemur Mah951211 using primer set LV2-2F and LV2-2r.

**PCR Amplification of cytochrome B.** Partial mitochondrial cytochrome B gene was PCR amplified from total nucleic acid extracted from fecal specimens using the AccuPrime Taq DNA Polymerase kit (Invitrogen). The following primer set was used: LemurCytB400F (5’–CCATGAGGACAAATATCTTCTGAG–3’) with LemurCytB1032r (5’–CCATGAGGACAAATATCTTCTGAG–3’). PCR products were cloned and sequenced.

**Diversity analyses and phylogenetic methods.** Amino acid sequences of the full-length polyprotein from lesavirus 1, lesavirus 2, hunnivirus A1 (NC_018668), hunnivirus A2 (HM153767) and porcine teschovirus 1 (NC_003985) were aligned by MUSCLE (29). Diversity plots were generated with Simplot (30), employing sliding windows of 250 amino acids in length and a step size of 10 amino acids, with Kimura (2-parameter) correction.

Phylogenetic trees were constructed from alignments of the concatenated 2C3CD and P1 (VP4231) regions from the following picornaviruses: enterovirus A (NC_001612), simian
sapelovirus (NC_004451), food-and-mouth disease virus (NC_004004), cosavirus A
(NC_012800), equine rhinitis B virus (NC_003983), encephalomyocarditis virus (NC_001479),
seneca valley virus (NC_011349), porcine teschovirus 1 (NC_003985), hunnivirus A1
(NC_018668), hunnivirus A2 (HM153767), aichi virus (NC_001918), salivirus A (GQ253930),
cadicivirus A (JN819202), melegrivirius A (HM751199), human parechovirus (FM178558), duck
hepatitis A virus (NC_008250), hepatitis A virus (NC_001489), aquamavirus A (EU142040), avian
encephalomyelitis virus (NC_003990), mosavirus A (JF973687), mischivirus A (JQ814851),
gallivirus A (JQ691613), passerivirus A (GU182406), oscivirus A (GU182408), rosavirus A
(JF973686), avisivirus A (KC465954), pasivirus A (KM259923). Phylogenies were constructed
with PhyML v3.0 (31) by the maximum likelihood (ML) method using the LG substitution model.
A discrete γ distribution of 4 rate categories was used to model among-site heterogeneity.
Analyses were performed at least twice, and support for ML trees was assessed by 1,000
nonparametric bootstraps. The best-fit model of protein evolution was determined by ProtTest
v 2.4 (32). Bayesian Markov Chain Monte Carlo (MCMC) inference (WAG +I +G +F) was
performed with BEAST v1.7.5 (33). 10,000,000 MCMC states were run with a 25% burn-in
period, under a lognormal relaxed clock and Yule prior. Convergence and mixing were assessed
with Tracer v1.5 and AWTY (34, 35). The two methods yielded trees with similar topologies.

For the phylogenetic analysis of cytochrome B genes and lesavirus sequences obtained
through the screening assay, nucleotide sequences were aligned by Muscle (29) and primer
sequences were trimmed from the alignment. A phylogeny was constructed by the neighbor-
joining method using the Jukes-Cantor model of nucleotide substitution and maximum
likelihood method. Both methods yielded similar phylogenies.
Diagnostic RT-PCR amplification. Standard precautions to avoid end product contamination were taken for all PCR assays, including the use of PCR hoods and maintaining separate areas for PCR set up and analysis. Seven no-template negative controls were interspersed between the actual samples. OneStep RT-PCR (Qiagen, Valencia, CA, USA) was used to amplify 5 μl of extracted samples using the following PCR program: 50°C for 35 min, 95°C for 15 min, 40 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 21 sec, followed by 72°C for 10 min. The following consensus-degenerate primer pair was used to screen samples for the presence of lesavirus 1 and lesavirus 2: LVScreenF (5′–TTGTMACCTTYYCTCAARGATGAGAC–3′) in combination with the LVScreenr (5′–GTGTAYTCCCTCRTCCAGATRTG–3′) that together generated a 388 nt amplicon from the 3Dpol region, one of the most highly conserved region of the genomes. Products were visualized following electrophoresis on 1.25% agarose gels. Amplicons were cloned and sequence verified.

Accession numbers. The sequences of the complete genome of lesavirus 1, lesavirus 2, VP1 sequences, amplicons obtained through screening and cytochrome B amplicon sequences have been entered into the GenBank database under accession numbers: KM396707–KM396752.
Results

Two novel picornaviruses in lemurs

35 fecal specimens from 6 taxa of lemurs at the Saint Louis Zoo were collected for this study in 2012. The lemurs were housed in 9 single species exhibits (ring-tailed lemurs, black-and-white ruffed lemurs, Coquerel’s sifakas, mongoose lemurs, a blue-eyed black lemur and black lemurs) and a mixed species exhibit (4 ring-tailed lemurs with 4 black-and-white ruffed lemurs and 2 black lemurs in one exhibit) (Figure 1A). As there have been no known exogenous viruses of lemurs described to date, we first sought to identify viruses associated with lemurs by performing unbiased deep sequencing on total nucleic acid extracted from fecal specimens from a subset of lemurs.

From 3,349,958 total sequencing reads in a ring-tailed lemur (Mis101308), we identified 20 reads that had limited sequence identity to known picornaviruses. De novo assembly of the picornavirus-like sequence reads yielded five contiguous sequences (contigs) that shared between 37 – 65% amino acid identity to hunnaviruses, picornaviruses previously identified from cattle and sheep (36). Picornaviruses are single stranded RNA viruses. The genome of typical picornaviruses encodes a single open reading frame, flanked by untranslated regions at the 5’ and 3’ ends. Using a combination of RT-PCR and RACE methods, the complete genome of 7,687 nucleotides (nt) was obtained and verified to more than 3x coverage by Sanger sequencing (Figure 1B). This virus was named lesavirus 1 (LV1, lemur stool-associated picornavirus 1).
Analyses of 579,108 reads from a black-and-white ruffed lemur (Nai108015) identified 341 reads that assembled into 3 contigs with limited sequence identity to picornaviruses (Figure 1C). Sequence comparison demonstrated that the 3 contigs only shared 64.1% nucleotide identity with LV1, suggesting that the viral sequences in each specimen were distinct. Therefore, we sequenced the complete genome (7,593 nt) of the virus and named it lesavirus 2 (LV2, lemur stool-associated picornavirus 2). Sliding window analysis demonstrated that LV1 and LV2 were indeed distinct viruses, and that the limited similarity to the next most closely related hunnivirus and porcine teschovirus 1 was observed throughout the genome (Figure 1D).

We then examined the genomes for molecular features characteristic of picornaviruses. The NPGP cleavage motif in 2A was conserved in LV1 (N\text{970}PGP) and LV2 (N\text{949}PGP). The putative 2C proteins had both the GXXGXGKS NTP binding motif (LV1: G\text{1250}RPGQGKS and LV2: G\text{1231}KPGQGKS) and DDLXQ helicase activity motif (LV1: D\text{1299}DLGQ and LV2: D\text{1280}DLGQ). Additionally, the GXCG cysteine active site in 3C was also conserved (LV1: G\text{1725}FCG and LV2: G\text{1698}YCG). Finally, the putative 3D maintains the YGDD active site (LV1: Y\text{2101}GDD and LV2: Y\text{2074}GDD), KDELR (LV1: K\text{1936}DETR and LV2: K\text{1909}DETR), FLKR (LV1: F\text{2149}LKR and LV2: F\text{2122}LKR) and GGLPSG motifs (LV1: G\text{2063}GLPSG and LV2: G\text{2036}GLPSG). Thus, LV1 and LV2 encode conserved molecular hallmarks of picornaviruses.

Lesavirus 1 and lesavirus 2 define a novel genus in the family Picorniridae

The Picorniridae family consists of 26 genera (37). We examined the evolutionary relationship of LV1 and LV2 in the family Picorniridae. Phylogenetic trees were constructed
with Bayesian and maximum likelihood methods using a concatenated amino acid alignment of 2C and 3CD genes that included representative members from 26 picornavirus genera. Identical topologies were obtained when reconstructed with Bayesian and maximum likelihood methods. The phylogenetic analyses strongly supported that LV1 and LV2 formed a monophyletic clade, and that they be placed sister to hunniviruses (Figure 2A). These findings were also well supported by phylogenetic reconstruction using the P1 (VP4321) region (Figure 2B). This indicated that LV1 and LV2 have a distinct evolutionary history from other picornaviruses.

ICTV guidelines for picornavirus species demarcation is <70% amino acid identities in the P1 and 2C3CD regions, and within-genus criteria as >40% in P1, >40% in P2 and >50% in P3 regions (37, 38). The pairwise amino acid identity of LV1 compared to LV2 in the P1, 2C3CD, P2 and P3 region was 54.8, 75.2, 68.9 and 72.8%, respectively (Table 1). While the 2C3CD region supports LV1 and LV2 being the same species, the P1 region had <70% identity, suggesting that they are a different species. Nonetheless, this indicated that LV1 and LV2 should be placed within the same genus. We next performed pairwise comparisons to hunniviruses and porcine teschovirus 1 which were most similar to LV1 and LV2. Comparison between LV1 and LV2 to hunniviruses in the P1, P2, P3 regions ranged from 39.0 – 42, 35.0 – 36.2, and 46.2 – 46.6% respectively. Sequence comparison against porcine teschovirus 1 ranged from 30.3 – 31.3, 36.5 – 37.9, and 40.5 – 40.9% in the P1, P2 and P3 regions respectively. Taken together, this indicated that LV1 and LV2 define a novel picornavirus genus.
Lemur picornaviruses are highly prevalent and species-specific. We examined the epidemiology of the two novel picornaviruses in the lemur collection using longitudinally collected fecal specimens. We designed and validated a consensus-degenerate RT-PCR assay to amplify a 388 nt product from the 3Dpol region of LV1 and LV2 (Figure 3A). Thirty-five fecal specimens representing 6 lemur taxa (ring-tailed lemurs, black lemurs, a blue-eyed black lemur, mongoose lemurs, black-and-white ruffed lemurs and Coquerel’s sifakas) collected from September and October 2012 were screened by the RT-PCR assay. Additionally, 33 fecal specimens collected approximately a year later (September 2013) were evaluated. In the period between the two samplings, 4 lemurs had died (3 black-and-white ruffed lemurs and 1 ring-tailed lemur), a triplet of black-and-white ruffed lemurs was born, and a black-and-white ruffed lemur was transferred to a different zoo. To verify the species origin of the specimens, we sequenced the mitochondrial cytochrome B gene from nucleic acid extracted from the fecal specimens. Mitochondrial gene sequences from lemur species clustered into well supported clades that matched the generally accepted phylogeny of lemurs (Figure 3B).

LV1 was detected in 5 out of 7 (71.4%) ring-tailed lemur fecal specimens collected in 2012, and in 5 out of 6 (83.3%) ring-tailed lemur specimens in the 2013 collection (Figure 3C). LV1 was not detected in black lemurs, a blue-eyed black lemur, mongoose lemurs, black-and-white ruffed lemurs or Coquerel’s sifakas. We detected LV2 in 6 out of 12 (50%) black-and-white ruffed lemurs in the 2012 collection, and 7 out of 11 (63.6%) black-and-white ruffed lemurs in 2013 (Figure 3C). We did not detect LV2 in ring-tailed lemurs, black lemurs, a blue-
eyed black lemur, mongoose lemurs or Coquerel’s sifakas. A phylogenetic tree constructed with all LV1 and LV2 sequences overlaid with each host species origin confirmed that all 10 specimens that were positive for LV1 were ring-tailed lemurs, and all 13 LV2-positive specimens were black-and-white ruffed lemurs (Figure 3D). Picornaviruses evolve rapidly due to the error-prone RNA-dependent RNA polymerase. Therefore, we compared the VP1 sequences of LV1 from fecal specimens collected in 2012 and 2013 from the same ring-tailed lemur (Mis101308). Similar analysis was done for the VP1 of LV2 from a black and white ruffed lemur (Mah951211) that was positive at both time points. The estimated mean rate of LV1 and LV2 VP1 evolution was approximately $9.22 \times 10^{-3}$ and $8.26 \times 10^{-3}$ nucleotide substitutions per site per year respectively, within the range of previous estimates for enteroviruses (39, 40).

We next examined the virus prevalence in the context of their single-species or mixed-species housing. Examples of both new infection and viral clearance were observed. In one single-species exhibit, a previously LV2-positive black-and-white ruffed tested negative in 2013 (Exhibit 3, Supplementary Table S1). A set of black-and-white ruffed lemur triplets born after the 2012 sampling and kept in a single-species exhibit were all positive for LV2 at the 2013 testing. In the mixed species exhibit that housed 4 ring-tailed lemurs, 4 black-and-white ruffed lemurs, and 2 black lemurs together for approximately 5 months, both LV1 and LV2 were detected (Figure 3E). Initially, in 2012, two ring-tailed lemurs were positive for LV1 and one black-and-white ruffed lemur was positive for LV2. In 2013, both LV1 positive ring-tailed lemurs remained positive while an additional ring-tailed lemur became infected with LV1. Both black lemurs remained negative for LV1 and LV2. Approximately 2 months after the first sampling,
the four black-and-white ruffed lemurs were transferred to a separate exhibit, after which one lemur was found to have acquired LV2 in 2013 and the initially LV2-positive individual died. These observations demonstrated that even in a high contact mixed species exhibit, the viruses were transmitted in a species-specific manner.

A ring-tailed lemur (Geo101895) that was positive for LV1 in 2012 subsequently died prior to the 2013 sampling. Two out of the three black-and-white ruffed lemurs (Man105690 and Bon101605) that died prior to the 2013 sampling were positive for LV2 in 2012. An additional positive black-and-white ruffed lemur (And113831) died after the 2013 evaluation. However, the cause of death was different among the 5 lemurs – malignant neoplasia (Geo101895), progressive neurological disease (Man105690, Bon101605 and Jir105691) and suppurative meningoencephalitis (And113831) (Supplementary Table S1).

Discussion

It is widely accepted that many emerging infectious diseases in humans are the result of zoonotic transmissions. However, the conditions that facilitate or prevent these transmissions are less-well understood. This poses an urgent challenge in predicting disease emergence. Here, we investigated the transmission of two previously-undescribed picornaviruses in lemurs at the Saint Louis Zoo. We chose lemurs because their species diverged on comparable evolutionary timescales to continental African primates, thus providing a parallel model for primate host genetic divergence. For example, black-and-white ruffed lemurs and ring-tailed lemurs diverged from their common ancestor approximately 26 and 21 million years ago, respectively (41). This
evolutionary timescale is comparable to the divergence of the *Catarrhini* parvorder of primates that includes humans, gibbons, great apes and old world monkeys. Using their housing organization, we studied viral transmission in a high inter-species contact setting (mixed species exhibit) and minimal inter-species contact setting (single species exhibit). Mixed species exhibits are common in most zoological parks worldwide and this study illustrates an approach that can be widely applied to other zoo settings to study viral transmission.

The role of host genetic barriers in helping to prevent cross-species transmission and viral adaptation, and whether transmission between species is primarily driven by contact intensity remains debated (4, 6, 13). In this study, we found that both lemur picornaviruses were highly prevalent and species-specific in the lemur collection (Figure 4C and 4D). LV1 was only detected in ring-tailed lemurs and LV2 only detected in black-and-white ruffed lemurs; neither virus was detected in black lemurs, mongoose lemurs or Coquerel’s sifakas. In other studies, enteroviruses and parechoviruses have been found co-circulating between humans and non-human primates (42, 43). For example, rhesus macaques and baboons in a multi-species cage at the Dhaka Zoo harbored human enterovirus 112 (43). In contrast, we found that the two lemur viruses were species-specific despite co-circulating in a high physical exposure and contact environment within a mixed-species exhibit (Figure 3E). A previously-negative ring-tailed lemur and black-and white ruffed lemur in the mixed-species exhibit was positive at the second sampling time, demonstrating that infection could be newly acquired in the exhibit during this time frame. Together, these observations suggest that the lemur species have evolved host barriers to prevent cross-species transmission of these viruses, possibly shaped by selection to survive past pathogenic pressures (44). In addition to understanding the host...
All three lemur species in the mixed species exhibit were fed the same diet, suggesting that the viruses were not simply the result of dietary ingestion. We are unable to exclude the possibility that the viruses originated from other host sources at the Zoo, such as mice and insects that may be commonly encountered despite efforts to control their environment. Nonetheless, regardless of the prior host origin, we have demonstrated that both viruses can be detected in sequential samples in lemurs. Samples collected from 2012 and 2013 showed that the majority of the lemurs positive in 2012 remained positive one year later, with viral clearance observed in only a minority of them. It is possible that the viruses cause persistent infection or alternatively, there may be clearance followed by re-infection. Additionally, black-and-white ruffed lemur triplets born after the initial sampling were found positive for LV2 in 2013 suggesting de novo virus infection occurred. The dam (Lul105694) of the triplets was negative at both time points, suggesting that the infections are the result of horizontal transmission. The host range of these picornaviruses remains to be experimentally determined. This might be difficult to address in vivo as many lemur species, including the critically endangered black and white ruffed lemur and endangered ring-tailed lemur, are threatened with extinction and are the focus of multifaceted conservation efforts (45). However, our studies are noninvasive (fecal collection) and could help assess the potential risk of viral infections on lemur survival.
No exogenous viruses have been described in lemurs prior to this study. The discovery of the lemur picornaviruses raises important questions about infectious causes of morbidity and mortality in lemurs. While we have detected viral nucleic acid in fecal specimens, this finding may not reflect the site of disease or provide clues to the pathogenicity of the virus. For example, poliovirus (a picornavirus) is shed in feces but causes neurologic disease. While four out of the five lemurs that died also tested positive for the viruses at the prior evaluation, the cause of death was different among the individuals. Further studies are necessary to determine the potential pathogenicity of these lemur picornaviruses and to better define the epidemiology of infection in captive and wild lemurs.

According to ICTV guidelines for picornavirus taxonomy (37), the criteria for species demarcation is <70% amino acid identity in the P1 and 2C3CD regions, and genera defined as sharing greater than 40%, 40% and 50% in the P1, P2 and P3 regions respectively. While the 2C3CD comparison of LPV1 and LPV2 falls within the species guidelines indicating that they belong to the same species, the P1 region identity is lower than the 70% cutoff and would be considered separate species. Based on the P1 divergence and the observed species specificity of infection, we propose that the two viruses are distinct species. Regardless, there is consistent agreement in the broader comparison of P1, P2 and P3 regions supporting LV1 and LV2 to be classified within a novel genus in the Picornaviridae family. Thus, we propose the name 'lesavirus' for the novel genus.
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Figure Legends

Figure 1. Identification of 2 novel picornaviruses in lemurs. (A) Species allocation of the lemur exhibits at the time of the 2012 collection are shown. (B) Diagram shows the complete genome of lesavirus 1 (above) and lesavirus 2 (below) confirmed by RACE/RT-PCR. Contigs assembled from the Illumina sequencing reads are indicated in grey. (C) Diversity plots of amino acid sequences are shown comparing the lesavirus 1 polyprotein to lesavirus 2 (red), hunniviruses (light blue and dark blue) and porcine teschovirus 1 (black).

Figure 2. Lesavirus 1 and lesavirus 2 form a monophyletic clade in the Picornaviridae family. Phyllogenetic relationships of representative members of the Picornaviridae family were inferred from the concatenated 2C3CD (A) and P1 (B) amino acid alignment, generated by the Bayesian MCMC method. The P1 phylogeny was consistent with phylogenetic analyses of the
VP1 region (data not shown). Internal branch labels indicate the posterior probability. The maximum likelihood method yielded trees with similar topologies.

**Figure 3.** Species-specific prevalence of lesavirus 1 and lesavirus 2. (A) RT-PCR analysis of lesavirus is shown for water (control), or representative specimens found to be negative (Gay88020), and positive for lesavirus 1 (Giz107097) or lesavirus 2 (Mah951211). Band corresponds to a 388 nt PCR product. (B) Maximum likelihood phylogenetic tree constructed from partial cytochrome B sequences is shown. The alignment was based on sequences from samples found to be positive for either lesavirus 1 (LV1) or lesavirus 2 (LV2), and representative individuals from other species’ sample. Reference sequences from GenBank were included for *L. catta* (LCU53575), *V. variegata* (AB371089) and *P. coquereli* (AF285528). The phylogeny was out-grouped to an aye-aye sequence (DMU53569). Branch labels indicate bootstrap proportion. Individuals in the mixed species exhibit are marked with an asterisk. (C) The prevalence of LV1 and LV2 in specimens collected in 2012 and 2013 are shown. (D) A phylogeny inferred from the nucleotide sequences of all LV1 and LV2 strains screened positive from (C) using the neighbor-joining method is shown. Virus sequences are highlighted with the host species origin as either ring-tailed lemur (open boxes) or black-and-white ruffed lemur (grey boxes) as determined by the cytochrome b genotype (B). Virus sequences from individuals in the mixed species exhibit are marked with an asterisk. (E) Prevalence of lemurs in the mixed species exhibit in 2012 and 2013 is shown. The exhibit consists of 4 ring-tailed lemurs (circles), 4 black-and-white ruffed lemurs (squares) and 2 black lemurs (triangles). Individuals infected by LV1 (grey) and LV2 (black) are shaded. The black-and-white ruffed lemurs were housed separately (Exhibit XI) in
2013. Two previously-LV2-infected black-and-white ruffed lemurs died prior to the 2013 sampling (crossed square).

References


B

Lesavirus 1 (7706 nt)

5' UTR

P1

P2

P3

3' UTR

Contigs

Lesavirus 2 (7603 nt)

5' UTR

P1

P2

P3

3' UTR

Contigs
Table 1. Pairwise amino acid comparison between Lesavirus 1 and Lesavirus 2.

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<th>P2</th>
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<th>3CD</th>
<th>2C3CD</th>
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