SHORT COMMUNICATION

Neotropical Bats from Costa Rica harbour Diverse Coronaviruses

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Impacts

- For a comprehensive analysis of CoV diversity, we sampled 41 species of neotropical bats. CoV sequences were detected in bats belonging to frugivorous (*Artibeus jamaicensis, Carollia perspicillata* and *Carollia castanea*) and nectivorous (*Glossophaga soricina*) species. *Glossophaga soricina* CoV clustered in a different subgroup apart from CoV sequences found in the same bat species.
- The non-clustering of these newly described bat CoV sequences near sequences from known pathogenic human CoVs and the low prevalence found suggests that the risk of spillover of known human pathogenic CoVs carried by bats is low.
- The finding of diverse CoV sequences in one specific bat species emphasizes that the whole diversity of CoV is still unknown and that surveillance should be continued.

Keywords:
Bats; Coronavirus; Costa Rica; phylogenetic analysis

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Summary

Bats are hosts of diverse coronaviruses (CoVs) known to potentially cross the host–species barrier. For analysing coronavirus diversity in a bat species-rich country, a total of 421 anal swabs/faecal samples from Costa Rican bats were screened for CoV RNA-dependent RNA polymerase (RdRp) gene sequences by a pancoronavirus PCR. Six families, 24 genera and 41 species of bats were analysed. The detection rate for CoV was 1%. Individuals ($n = 4$) from four different species of frugivorous (*Artibeus jamaicensis*, *Carollia perspicillata* and *Carollia castanea*) and nectivorous (*Glossophaga soricina*) bats were positive for coronavirus-derived nucleic acids. Analysis of 440 nt. RdRp sequences allocated all Costa Rican bat CoVs to the α-CoV group. Several CoVs sequences clustered near previously described CoVs from the same species of bat, but were phylogenetically distant from the human CoV sequences identified to date, suggesting no recent spillover events. The *Glossophaga soricina* CoV sequence is sufficiently dissimilar (26% homology to the closest known bat CoVs) to represent a unique coronavirus not clustering near other CoVs found in the same bat species so far, implying an even higher CoV diversity than previously suspected.

Approximately 71.8% of emerging infectious diseases have originated from wildlife (Jones et al., 2008). Bats have been involved in outbreaks of emerging diseases around the world and are known reservoirs of coronaviruses (CoVs) capable of crossing the host–species barrier (Calisher et al., 2006). One example is the 2002–2003 severe acute

respiratory syndrome (SARS) CoV outbreak. After the subsequent finding that bats constitute this virus' natural reservoir (Lau et al., 2005), several research groups have detected α- and β-CoVs in a variety of bat species throughout the world (Drexler et al., 2014). Also the newly emerged Middle East respiratory syndrome (MERS) CoV depicts limited sequence similarities with CoVs found in bats (Annan et al., 2013). Around 33% of overall bat species and 75% of the known 113 bat genera are found in the neotropics (Osborne et al., 2011). Costa Rica exhibits an extraordinary bat diversity with more than 109 recorded species so far, representing 10% of the total bat diversity worldwide (Timm and LaVal, 2000). In this study, the diversity of bat coronaviruses in neotropical bats was assessed by analysing putative phylogenetic relationships with previously detected RdRp sequences around the world.

For an intensive screening of bats potentially infected with CoV, permission for bat capturing and sampling was obtained from the Institutional Committee of Care and Use of Animals of the University of Costa Rica (CICUA-124-12) according to international animal welfare standards and by University of Costa Rica project B2-285. Bats were sampled between 2012 and early 2014 at 11 distinct locations in Costa Rica (Fig. 1a) subjected to different levels of human intervention, that is minimal (site A Palo Verde 10°21′14.2″N 85°19′45.7″W, Site G Santa Rosa 10°50′08.5″N 85°37′27.2″W), agricultural/rural with patches of forest (site B Virgen del Socorro 10°17′31.9″N 84°07′21.3″W, site C Tirimbina Biological Station 10°25′01.4″N 84°07′25.4″W°, Site E Nandayure 10°03′25.6″N 85°15′55.2″W, Site F Sarapiqui 10°24′11.0″ N 84°08′01.6″W, Site H Bolson 10°21′05.6″N 85°25′05.8″ W, Site J Parrita 9° 32′ 26.0″ N 84° 24′ 02.5″ W) and urban (site D San José 9°56′19.3″N 84°02′42.8″W, Site I Puntarenas 9° 58′ 39.4″ N 84° 50′ 16.7″ W, Site K Nicoya 10° 8′35.64″N, 85°26′47.33″W). Bats were captured using three mist nets at a height of 2 metres (Platt et al., 2000) set at each location in open areas near drinking or feeding sites, to maximize capture success (Newman et al., 2011). Once captured, bats were collected and transported to the field laboratory. Bats were examined for signs of disease and taxonomically classified (Timm and LaVal, 2000). Anal swabs or faecal pellets for a total of 421 bats were collected and dissolved in 400 μl of RNA stabilizing reagent (RNAlater®, Life Technologies, Carlsbad, CA, USA) and

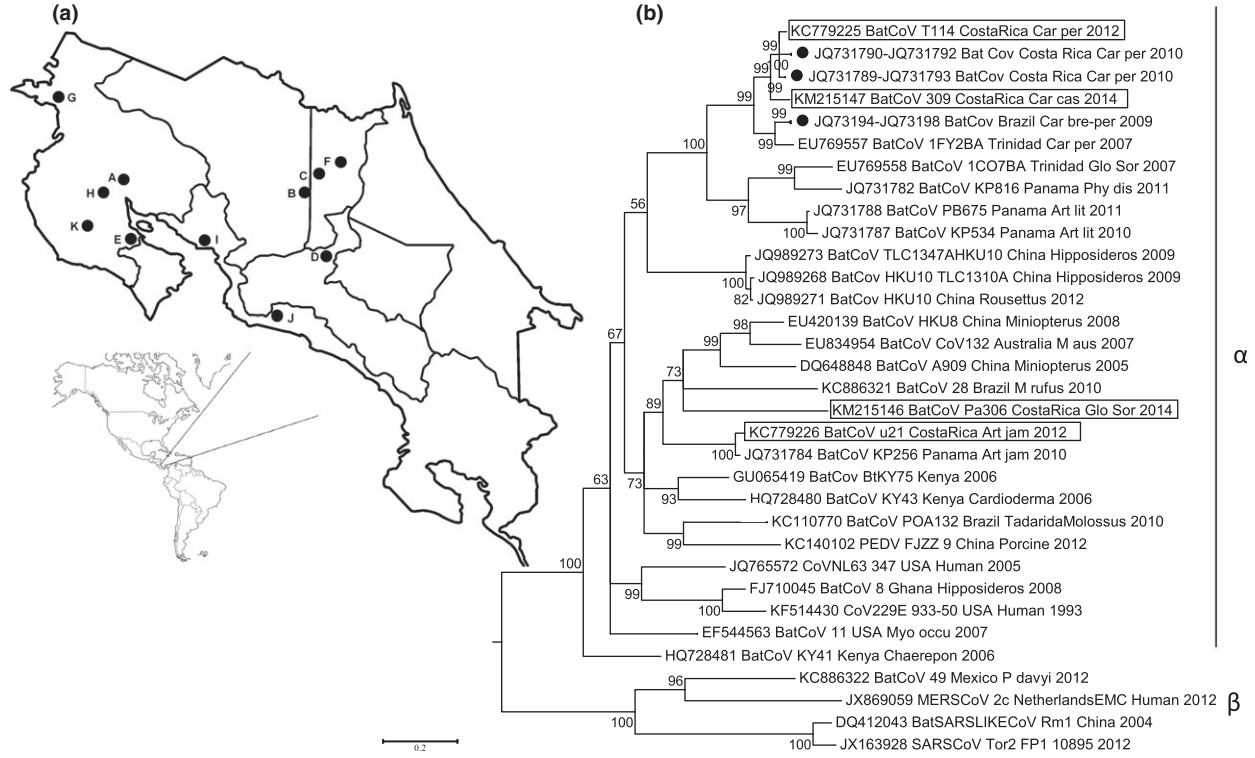

Fig. 1. (a) Map of Costa Rica showing the 11 locations where bat sampling was conducted. Sites were at least 10 km. apart from each other. Five of the seven provinces were sampled. Letters indicate each site. (b) Phylogenetic relationships based on a 440-nt sequence in a conserved region of gene 1b. Bayesian inference phylogenetic tree generated using TN93 + G Model using Mr Bayes 3.2.1 software. The numbers on the branches correspond to posterior probabilities. Black circles correspond to subtrees of CoV found in the same species of bat. Constructed with 4 million generations. Costa Rican sequences found in this work are shown encased. Scale bar at the lower left indicates 0.2 nucleotide substitutions per site.

promptly transported at 4°C to the laboratory. TRIzol method (Invitrogen™ Life Technologies, Carlsbad, CA, USA) was used for RNA extraction, and reverse transcription was conducted using a commercially available kit (Thermo Scientific RevertAid First Strand cDNA Synthesis Kit, Waltham, MA, USA). CoV screening was performed by a previously described pancoronavirus RT-PCR assay (Poon and Peiris, 2008). Sequencing reactions were prepared using Big Dye® Terminator Cycle Sequencing kit version 1.1 (Applied Biosystems, Waltham, MA, USA) and sequenced on an ABI PRISM 3130 Automated Capillary DNA Sequencer (Applied Biosystems). Sequences derived during this study ($n = 4$) were aligned with 40 Bat CoV RdRp sequences from Genbank using Megalign software (DNAStar, Madison, WI, USA) and then trimmed to a common length of 440 bp. Sequences in the data set where chosen based on length, region of the RdRp, geographical location, CoVs of significance for public health and CoV sequences derived from bats found in America. All duplicate sequences were removed. A Bayesian inference (BI) tree using Bayesian Monte Carlo Markov Chain method was constructed using 4 million generations in MrBayes software version 3.2.1. under the general time reversible (GTR) substitution model and a discrete Gamma distributed with invariant sites (G+I) across-site rate (5 categories). The best-fit models for the tree was selected by a model test in MEGA6.0 (Tamura et al., 2013). To attempt coronavirus isolation, CoV RNA-positive faecal samples were inoculated after debris and bacterial clarification in *Desmodus rotundus* embryo primary kidney-, fibroblasts- and intestinal-cell-derived confluent monolayers. Briefly, low passage cells were seeded in 24-well plates with D-MEM 10% NCS and incubated at 37°C in a 5% CO_2 atmosphere until 70% confluence. Cell culture medium was then removed, and cells were inoculated with Log_{10} diluted samples. After a 2-hour virus adsorption period, D-MEM 2% NCS was added. Cells were daily observed during 22 days to detect any changes in cell morphology (CPE). Cells and supernatants were analysed for CoVs presence by RT-PCR at days 7, 14 and 21 post-inoculation.

We sampled 41 neotropical bat species, comprising 24 genera and 6 of the existing 9 neotropical bat families (Supplementary Table S1) and representing a wide variety of trophic guilds. The overall finding for the targeted RdRp sequence was approximately 1% (4 of 421 bats). Four bat species *Artibeus jamaicensis*, *Carollia perspicillata, Carollia castanea* and *Glossophaga soricina* were found to be CoV positive, but none showed any obvious signs of disease, consistent with other reports (Tang et al., 2006; Dominguez et al., 2007). The finding of CoV positivity in these species may be related to the fact that these bats are highly adaptable, resistant to habitat fragmentation and widespread throughout Costa Rica (Medellín et al., 2000; Timm and LaVal, 2000). Genera found previously positive such as *Mollossus sp* (Lima et al., 2013) were negative. This suggests that bat CoV prevalence might be low, although relative sampling for this species in this study was high.

Sequences found (one each from *C. perspicillata*, *A. jamaicensis*, *G. soricina* and *C. castanea*) were submitted to Genbank (Accession Numbers KC779225, KC779226, KM215146 and KM215147, respectively).

Phylogenetic analysis of the nucleotide sequences clustered all of the CoVs detected to the α-CoV group (Fig. 1b). Sequences found did not cluster near so far known pathogenic human CoVs suggesting that the risk of spillover of known human pathogenic CoVs carried by bats is low. Our *C. perspicillata* CoV and *C. castanea* sequences (KC779225 and KM215147, respectively) clustered with previously published *Carollia* CoVs from Brazil, Costa Rica and Trinidad with 99% posterior probability. The sequence from *C. perspicillata* has 96.8–97.8% nucleotide sequence identity and shows 1 or 2 amino acid differences (98.6–99.3% amino acid sequence identity for the RdRp) with the *Carollia* sp. sequences described by Corman et al. (2013). The sequence of *C. castanea* CoV has a nucleotide sequence identity of 92.5–92.9% with the other *Carollia* sp. CoVs detected in the country by us and by Corman et al. (2013), which represents an amino acid identity of 98.6% with only two amino acid substitutions. This is the first detection of CoV in *C. castanea* so far reported. All *Carollia* sp. CoV sequences found to date cluster together in a genus-specific monophyletic clade. The *A. jamaicensis* CoV sequence (KC779226) was most closely related to *A. jamaicensis* CoV II found in Panama by Corman et al. (2013) (JQ731784), having 97.3% nucleotide sequence identity and clustering together on a branch with 100% posterior probability. The observed nucleotide sequence difference translates into 4 amino acid substitutions resulting in an amino acid identity of 96.8%. Finding the same species of bats carrying similar CoVs even when geographically distant suggests for species specificity of bat CoVs (Corman et al., 2013). Therefore, these findings support the theory that CoVs coevolve with individual bat species or genera.

Interestingly, the *G. soricina* CoV RdRp sequence (KM2 15146) represents a divergent sequence. In fact, the highest homology (76% sequence identity) corresponds to an Australian *Rhinolophus megaphyllus* bat CoV (bat coronavirus R.meg/Australia/CoV100/2007 EU834953.1) and one found by us in a Costa Rican *A. jamaicensis* bat (KC779226). Its sequence identity to CoV sequences from the same species of bat [*G. soricina* in Trinidad EU769558 (Carrington et al., 2008)] is only 70% at the nucleotide level and 79.4% at the amino acid level. Posterior probability of 73% makes the placing of this bat CoV sequence in the phylogeny

uncertain. Also, our *G. soricina* bat CoV sequence did not cluster near previously reported CoVs from the same species of bat in Trinidad (Carrington et al., 2008). Nonetheless, the detection of such a divergent sequence of CoV in *G. soricina* suggests that the diversity of bat CoVs in a given species may be greater than previously known. It is tempting to speculate that nectivorous bats could be more exposed to different CoVs because of their feeding habits (Corman et al., 2013). Additionally, *G. soricina* is known to roost with bats of eight different families (Alvarez et al., 1991), which presents a plausible scenario of virus exchange among different species.

Attempts for virus isolation were unsuccessful. No CPE was observed in any of the primary embryonic cell lines, and none of the cell cultures were positive by the CoV-specific RT-PCR. We hypothesize that at least these embryonic bat cell lines originating from *D. rotundus* are not susceptible to bat CoV infection or that the sample contained non-infectious virus.

Our study emphasizes that even if sampling has been already performed in a given country, continuous surveillance aids in finding novel viruses which may expand the information regarding CoV diversity. The phylogenetic analysis performed in the current study show that the derived bat CoVs are unrelated to coronaviruses from other animals or humans, suggesting that recent zoonotic spillover events have not occurred. For an accurate understanding of the events involving an emergence of a non-described pathogen, for example a bat coronavirus, more profound ecological and taxonomical studies in their natural hosts have to be performed. These studies will support a more accurate surveillance and proper conservation management for these mammals. Moreover, because Latin America has been suggested as an emerging disease hotspot (Jones et al., 2008), more studies on these rich biodiversity countries are encouraged, as it might aid in the forecasting readiness of emerging infectious diseases.

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Authors' Contribution

Conceived and designed experiments: AMS, LTC, BRH, CJ and ECA. Performed the experiments: AMS, NVV and BRH. Analysed the data: AMS, LTC and ECA. Wrote the manuscript: AMS and ECA.

Conflict of Interest

The authors disclose no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Bat species collected, tested and listed according to location and CoV positivity.