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Short Communication

Coronaviruses Detected in Bats in Close Contact with Humans in Rwanda

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Abstract: Bats living in close contact with people in Rwanda were tested for evidence of infection with viruses of zoonotic potential. Mucosal swabs from 503 bats representing 17 species were sampled from 2010 to 2014 and screened by consensus PCR for 11 viral families. Samples were negative for all viral families except coronaviruses, which were detected in 27 bats belonging to eight species. Known coronaviruses detected included the betacorona viruses: Kenya bat coronaviruses, Eidolon bat coronavirus, and Bat coronavirus HKU9, as well as an alphacoronavirus, Chaerephon Bat coronavirus. Novel coronaviruses included two betacorona viruses clustering with SARS-CoV, a 2d coronavirus, and an alphacoronavirus.

Keywords: Rwanda, Bats, Coronaviruses, Human–wildlife interfaces

Introduction

Bats are natural reservoirs for a number of pathogens of public health concern (Plowright et al. 2015; Shi 2013). For example, in Southeast Asia, *Pteropus* fruit bats are the natural reservoirs of the zoonotic paramyxoviruses Hendra and Nipah (Chua et al. 2000). In 2002–2003, an epidemic of severe acute respiratory syndrome (SARS) caused by a novel coronavirus (SARS-CoV) emerged in China (Drosten et al. 2003, Ksiazek et al. 2003), and bats were determined to be natural reservoirs and the possible source of the virus (Ge et al. 2013; Lau et al. 2010). In 2012, a pathogenic paramyxovirus, Sosuga virus, which caused severe illness in a patient following contact with bats in Uganda, was subsequently detected in Egyptian fruit bats (*Rousettus aegyptiacus*) (Amman et al. 2015). In Africa, Egyptian fruit bats (*R. aegyptiacus*) are reservoirs of Marburg virus (Towner et al. 2008), and antibodies against Zaire ebola virus have been detected in the same species (Pourrut et al. 2009),

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while antibodies against *Bombali ebolavirus* have been detected in Little free-tailed bat (*Chaerephon pumilus*), Angolan free-tailed bat (*Mops condylurus*) (Goldstein et al. 2018).

To assess the risk presented by human-bat contact, the USAID Emerging Pandemic Threats PREDICT project has been conducting viral surveillance in wildlife in more than 35 countries to detect viruses of zoonotic potential, including in Rwanda (https://ohi.vetmed.ucdavis.edu/programs-projects/predict-project).

Rwanda is continental Africa's most densely populated country (Butler 2004), and bats are frequently observed in/around urban centers and adjacent to wildlife protected areas. Ecotourism centered on mountain gorillas in Volcanoes National Park also drives tourism to the nearby bat roosting "Musanze Caves" (Spenceley et al. 2010; Joachim 2013).

Between 2010 and 2014, biological sampling of bats was conducted at urban and rural sites in Rwanda characterized by an intense human–wildlife interface, including in and around Volcanoes National Park and the Musanze Caves. Anthropogenic activities around sampling sites were classified according to human livelihoods and activities, including ecotourism, crop farming, and national parks.

A total of 503 bats belonging to 17 species were captured at 25 sites (Fig. 1) following established PREDICT protocols for bat capture and sampling (PREDICT 2017) (Table 1). Following capture, bats were photographed, measured, and identified to species level as close as possible (Kingdon et al. 2013; Patterson and Webala 2012). Date, site name, season, apparent species, sex, age class (determined by the degree of epiphyseal-diaphyseal fusion) (Anthony 1988), reproductive status, and mass data were recorded. For handling and restraining larger fruit bats, light anesthesia was induced using inhalational isoflurane and oxygen (Fluriso^{TM}, Teva UK, Limited, Castleford, UK). Smaller fruit bats and insectivorous bats were physically restrained during sampling. All bats were released at the capture site within 3 h of capture.

Sampling for viral family screening included collection oral and rectal mucosal or fecal swabs. Swabs were placed into viral transport media (BD Universal Viral Transport^{TM} medium, Becton, Dickinson and Co., Sparks, Maryland) and NucliSENS Lysis Buffer (bioMerieux SA^{TM}, Marcy l'Etoile, France) and stored in liquid nitrogen for transport and transfer to a − 80 °C freezer.

We extracted RNA from oral and rectal swab samples using the Qiamp Viral Mini kit^{TM} (Qiagen Inc., Valencia, CA) and reverse transcribed into cDNA using SuperScript III^{TM} (Invitrogen Corp, Carlsbad, CA). Primers targeting the housekeeping gene β-actin were used to ensure the presence of amplifiable nucleic acid in RNA extracts (Goldstein et al. 2004). Samples were screened by consensus PCR targeting conserved gene fragments using established assays know to detect corona (Quan et al. 2010; Watanabe et al. 2010), alpha (Sánchez-Seco et al. 2001), arena (Lozano et al. 1997), bunya (Briese et al. 2007), filo (Zhai et al. 2007), flavi (Moureau et al. 2007), hanta (Raboni et al. 2005), influenza (Anthony et al. 2012), paramyxo (Tong et al. 2008), lenti (Courgnaud et al. 2001), and rhabdo (Wray et al. 2016) viruses. Bands of the expected size were excised from 1% agarose and purified using the Qiaquick kit (Qiagen Inc.). Purified PCR products were cloned (pCR4-TOPO vector; Invitrogen Corp.) and sequenced (ABI 3730 Capillary Electrophoresis Genetic Analyzer; Applied Biosystems, Inc., Foster City, CA). Sequences were analyzed and edited using Geneious (Version 6.0.3) and compared with known sequences in the Genbank database. Species identification of PCR-positive individuals was confirmed by DNA bar coding of the cytochrome b (*Cytb*) and cytochrome oxidase subunit 1 (*CO1*) mitochondrial genes (Townzen et al. 2008). Fisher's exact test was used to examine the association of viral positivity with age and season using STATA 13.0 software. The level of significance was set at $P \leq 0.05$ (Raymond and Rousset 1995).

No alpha, arena, bunya, filo, flavi, hanta, influenza, paramyxo, lenti, or rhabdo viruses were detected in oral or rectal swabs. Coronaviruses (CoV) were detected in 27 (5.4%) of the 503 bats sampled. Twenty-two of the 27 coronavirus positive bats belonged to three species: Straw-colored fruit bat (*Eidolon helvum*; 13 positives of 111 sampled, 11.7%), Geoffroy's horseshoe bat (*Rhinolophus clivosus*; 5 of 24, 24.2%), and Egyptian fruit bat (*Rousettus aegyptiacus;* 4 of 36, 11.1%). Coronavirus positive bats were sampled at 11 different sites, including in the Musanze Caves (Table 1). Subadult bats were more likely to be positive for CoV than adults (6/36 vs. 21/429; $P = 0.04$). No coronaviruses were detected in juvenile bats ($n = 11$). There were no observed differences between seasons (Dry/Rainy; 3/90 vs. 24/386; $P = 0.445$).

Coronavirus sequences were classified as belonging to different viral species according to established cutoffs and methods (Anthony et al. 2017b). We detected four known coronaviruses (Table 2) and four new coronaviruses (Table 3; Fig. 1). One of the new betacoronaviruses, Coronavirus PREDICT_CoV-43, was detected in *Hipposideros*

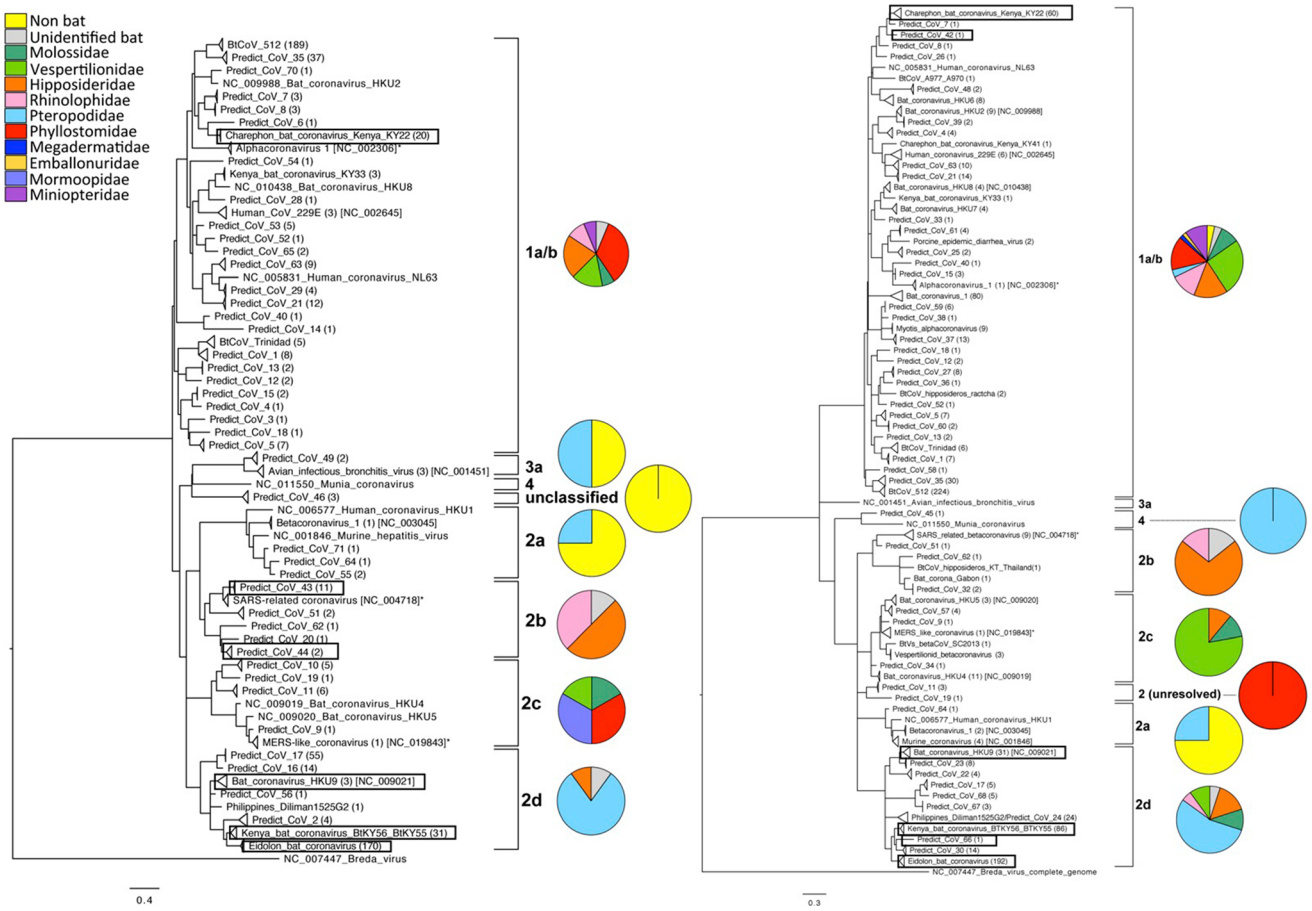

Figure 1. Coronaviruses phylogenetic analysis performed in Anthony et al. (2017b). Sequences are collapsed into clades, representing operating taxonomic units (sequences sharing equal or more than 90%). The sequences included in this manuscript are indicated in the boxes.

ruber and *R. clivosus* bats co-roosting in bat tourism caves (Site 10; Fig. 1; Table 3). Comparison of the conserved polymerase gene fragment sequences to other known coronaviruses indicated that Coronavirus PREDICT_CoV-43 clustered near the SARS-like coronaviruses but suggests it may be a distinct virus based on the conserved fragment sequence, as it showed only 84% nucleotide similarity to SARS-CoV (Genbank accession no. NC_009694). The second new betacoronavirus, PREDICT_CoV-44, was detected in two *Hipposideros caffer* bats trapped in Nyungwe National Park (Site No. 11) and in a *R. clivosus* bat in tourism caves at Site No. 10 (Fig. 2). Although the conserved sequence fragment also clustered with other betacoronaviruses, it was quite divergent, showing only 79% nucleotide similarity to others in the group.

The 2d betacoronavirus, PREDICT_CoV-66, was detected in one *Rousettus angolensis* bat in Nyungwe National Park (Site 11; Fig. 2) and showed 84% nucleotide similarity to the closest recognized coronavirus, Kenya bat Coronavirus BtKY84 (Genbank accession no. GU65428) found previously in *E. helvum*. The only alphacoronavirus PREDICT_CoV-42 was detected in a *R. clivosus* bat in tourism caves (Site No. 10). This virus sequence showed only 85% nucleotide similarity to the closest recognized coronavirus, Kenya bat Coronavirus BtKY69 (Genbank accession no. GU65413), found previously in horseshoe bats (*Rhinolophus* species).

Phylogenetic analyses of complete genome sequences of coronaviruses from bats, humans, and other vertebrates suggest that bats may be the reservoir hosts from which all coronavirus lineages originated (Vijaykrishna et al. 2007; Anthony et al. 2017a), and several studies document the diversity of bat coronaviruses globally (Dominguez et al. 2007; Annan et al. 2013; Anthony et al. 2017b).

In this study, sequences representing two novel coronaviruses that clustered with the SARS-like coronaviruses

Table 1. Bat Species Sampled at 25 Sites in Rwanda for 11 Viral Families, with Numbers and Percentages of Bats Testing Positive for CoV RNA.

Site no.*	Species	Risk interface	Season	Total tested (oral and rectal swabs)	No. of positive	% Positive
2	*Epomophorus labiatus*	Homestead	Rainy	17	0	0
25	*Mops condylurus*	Homestead	Rainy	78	0	0
18	M. condylurus	Homestead	Dry	20	0	0
12	*Hipposideros caffer*	Ecotourism site	Rainy	15	1	7
	Rhinolophus clivosus	Ecotourism site	Rainy	19	4	21
	Rousettus aegyptiacus	Ecotourism site	Rainy	9	0	0
	Otomops martiensseni	Ecotourism site	Rainy	1	0	0
7	*R. clivosus*	Ecotourism site	Rainy	5	1	20
	R. aegyptiacus	Ecotourism site	Rainy	13	0	0
	Hipposideros ruber	Ecotourism site	Rainy	2	0	0
	Nycteris hispida	Ecotourism site	Rainy	1	0	0
3	*Eidolon helvum*	Ecotourism site	Rainy	53	4	7,5
16	*E. helvum*	Homestead	Dry	15	0	0
8	*E. helvum*	Homestead	Rainy	9	2	22
5	*E. helvum*	Crop farming	Rainy	9	6	67
6	*R. Aegyptiacus*	Ecotourism site	Rainy	9	2	22
11	*Neoromicia tenuipinnis*	National park	Rainy	5	0	0
	Myonicteris angolensis	National park	Rainy	6	1	17
	H. caffer	National park	Rainy	1	0	0
23	*Stenonycteris lanosus*	National park	Rainy	1	0	0
	M. angolensis	National park	Rainy	23	0	0
10	*Epomophorus labiatus*	Crop farming	Rainy	6	0	0
	S. lanosus	Crop farming	Rainy	5	0	0
	R. Aegyptiacus	Crop farming	Dry	5	2	40
4	*E. helvum*	Ecotourism site	Rainy	5	1	20
22	*N. hispida*	Fishing area	Rainy	1	0	0
	E. Labiatus	Fishing area	Rainy	3	0	0
	Lavia frons	Fishing area	Rainy	1	0	0
21	*N. hispida*	Ecotourism site	Rainy	1	0	0
	E. Labiatus	Ecotourism site	Rainy	2	0	0
24	*M. condylurus*	Homestead	Rainy	20	0	0
20	*Chaerephon pumilus*	National park	Rainy	8	0	0
	M. condylurus	National park	Rainy	12	0	0
9	*E. Labiatus*	Homestead	Rainy	5	0	0
13	*E. Labiatus*	Ecotourism site	Rainy	35	0	0
	N. tenuipinnis	Ecotourism site	Rainy	1	0	0
	Neoromicia cf. zuluensis\	Ecotourism site	Rainy	2	0	0
17	*E. helvum*	Homestead	Rainy	20	0	0
19	*Scotophilus viridis*	Homestead	Rainy	4	0	0
	C. pumilus	Homestead	Rainy	3	1	33
	E. Labiatus	Homestead	Rainy	18	0	0
15	*E. Labiatus*	Homestead	Rainy	2	0	0
	S. viridis	Homestead	Rainy	1	0	0

Table 1. continued

Site no.*	Species	Risk interface	Season	Total tested (oral and rectal swabs)	No. of positive	% Positive
1	*M. Angolensis*	Homestead	Rainy	16	0	0
	E. Labiatus	Homestead	Rainy	9	1	11
	S. viridis	Homestead	Rainy	1	0	0
14	*N. hispida*	Homestead	Dry	6	0	0
Total				503	27	54

*Bats sampling sites can be visualized in Figure 2 by site number.

Table 2. Known Coronaviruses Detected in Bats by Species and Specimen Type.

Bat species	c-PCR positive	Sample tested	Year of collection/ season	Site no	Risk interface	Virus name	Genbank No.
Epomophorus labiatus	1	Rectal swab	2013/Rainy	1	Home stead	Strain of Kenya bat coronavirus/BtKY56/BtKY55	KX285830
Chaerephon pumilus	1	Rectal swab	2013/Rainy	6	Ecotourism site	Strain of Chaerephon bat/coronavirus/Kenya/KY22/2006	KX285828
Eidolon helvum	4	Rectal swab	2012/Rainy	3	Ecotourism site	Strain of Eidolon bat coronavirus/Kenya/KY24/2006	KX285106
Eidolon helvum	1	Rectal swab	2012/Rainy	4	Ecotourism site	Strain of Eidolon bat coronavirus/Kenya/KY24/2006	KX285107
Eidolon helvum	6	Rectal swab	2013/Dry	5	Crop farming	Strain of Eidolon bat coronavirus/Kenya/KY24/2006	KX285108
Rousettus aegyptiacus	2	Rectal swab	2013/Rainy	6	Ecotourism site	Strain of Bat coronavirus HKU9	KX286259
Rousettus aegyptiacus	2	Rectal swab	2013/Rainy	1	Home stead	Strain of Kenya bat coronavirus/BtKY56/BtKY55	KX285819
Eidolon helvum	2	Rectal swab	2012/Rainy	3	Ecotourism site	Strain of Eidolon bat coronavirus/Kenya/KY24/2006	KX285822
	19						

were detected in bat tourism caves and other sites where people and bats come into close contact in Rwanda. One virus (PREDICT CoV-43) was detected in both Sundevall's roundleaf bat (*H. ruber*) and Geoffrey's horseshoe bat (*R. clivosus*) that were co-roosting in the Musanze Caves (Site No. 10). The high sequence similarity of the viral fragment detected in both bat species suggests that this virus may have the ability to be maintained in more than one host or that cross-species transmission may occur. Studies have found that viral sharing and cross-species transmission may be important factors that contribute to emergence of novel coronaviruses and recombination of bat coronaviruses (Lau et al. 2010; Johnson et al. 2015).

While the known coronaviruses detected in this study have been identified in other geographical areas and in different bat species (Tao et al. 2012; Drexler et al. 2010), we report their first detection in Rwanda. The bat coronavirus HKU9 was previously detected in *Rousettus leschenaulti* bats in China (Tang et al. 2006), and now a strain of this virus has been detected in bats in Rwanda. Similarly,

Table 3. Novel Coronaviruses Detected in Bats by Species and Specimen Type.

Bat species	c-PCR positive	Sample tested	Year of collection/ season	Site no.	Risk interface	Virus name	Genbank no.
Rhinolophus clivosus	1	Oral swab	2011/Rainy	12	Ecotourism sites	PREDICT_CoV-43	KX285821
Rousettus angolensis	1	Rectal swab	2013/Rainy	11	National park	PREDICT_CoV-66	KX285426
Hipposideros caffer	1	Rectal swab	2011/Rainy	11	National park	PREDICT_CoV-44	KX285826
Rhinolophus clivosus	1	Rectal swab	2013/Rainy	11	National park	PREDICT_CoV-44	KX286327
Hipposideros ruber	1	Rectal swab	2013/Rainy	10	Crop farming	PREDICT_CoV-43	KX286324
Rhinolophus clivosus	2	Rectal swab	2011/Rainy	12	Ecotourism site	PREDICT_CoV-43	KX286325
Rhinolophus clivosus	1	Rectal swab	2011/Rainy	12	Ecotourism site	PREDICT_CoV-42	KX285111
	8						

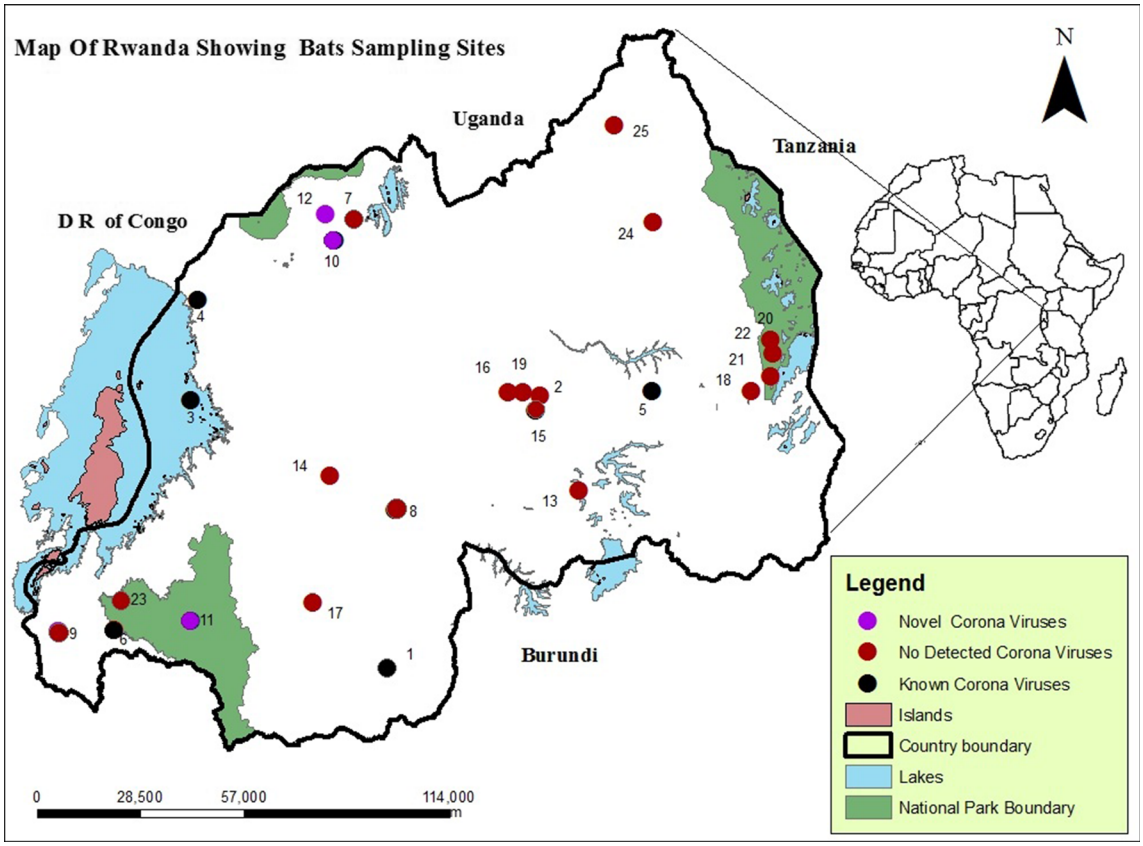

Figure 2. Map of Rwanda showing the bats sampling sites for corona viral surveillance during the study.

Kenya bat coronavirus/BtKY56/BtKY55 in *R. aegyptiacus*, Chaerephon bat coronavirus/Kenya/KY22/2006 in *Chaerephon pumilus*, and Eidolon bat coronavirus/Kenya/KY24/2006 in *Eidolon helvum* were first detected in Kenya in 2006 (Tao et al. 2012). We report the presence of these viruses in these same bat species in Rwanda, indicating a wider geographic distribution of these viruses in Eastern Africa, likely due to the widespread distribution of their bat hosts (Drexler et al. 2010; Gloza-Rausch et al. 2008).

In conclusion, bats in Rwanda carry novel and known coronaviruses, a family of viruses from which novel viruses have caused human pandemics. However, bats play important ecological roles and their elimination as a control measure is not recommended or warranted. We rec-

ommend additional surveillance and longitudinal studies to further understand the ecology of bat coronaviruses and the extent of human–bat interactions to identify strategies for public health protection and bat conservation.

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