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DNA indicative of human bocaviruses detected in non-human primates in the Democratic Republic of the Congo

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Abstract

Bocaparvoviruses are members of the family *Parvovirinae* and human bocaviruses have been found to be associated with respiratory and gastrointestinal disease. There are four known human bocaviruses, as well as several distinct ones in great apes. The goal of the presented study was to detect other non-human primate (NHP) bocaviruses in NHP species in the Democratic Republic of the Congo using conventional broad-range PCR. We found bocavirus DNA in blood and tissues samples in 6 out of 620 NHPs, and all isolates showed very high identity (>97 %) with human bocaviruses 2 or 3. These findings suggest cross-species transmission of bocaviruses between humans and NHPs.

Bocaviruses are small, single-stranded DNA viruses that belong in the subfamily *Parvovirinae* of the *Parvoviridae*. They form the genus *Bocaparvovirus*, with two species. One species is *Primate bocaparvovirus 1*, which includes the members human bocavirus 1 (HBoV1) and HBoV3, and the other species is *Primate bocaparvovirus 2*, with the members HBoV2 and HBoV4 [1, 2]. Human bocaviruses were first described in Sweden in 2005 in children with respiratory symptoms of unknown origin, and have since been found to infect people all over the world [2–4]. While HBoV2-4 are potentially involved in the development of gastrointestinal symptoms, HBoV1 is considered to be a respiratory pathogen [2, 4, 5]. Many aspects of HBoV pathobiology remain unclear, partly due to the lack of an adequate animal model [2, 6].

The DNA of related bocaviruses has also been found in the faeces of gorillas and chimpanzees and, based on the existing sequence data, it was suggested that human and non-human primate (NHP) bocaviruses might have diverged from common ancestors rather recently – between 60 and 300 years ago – and have since coevolved with their host species [7–10]. In a previous study researchers failed to detect bocavirus DNA in other species of NHPs, but antibodies were detected in a drill (*Mandrillus leucophaeus*), a mona monkey (*Cercopithecus mona*) and a Preuss's monkey (*Allochrocebus preussi*), indicating distinct bocaviruses in these and other Old World monkeys [9].

The goal of the present study was to screen African NHPs in the Democratic Republic of the Congo (DRC) for the presence of bocaviruses and assess their relationship to known isolates based on their DNA sequences. This study was part of the global USAID-funded Emerging Pandemic Threats (EPT) PREDICT project that aims to increase global capacity for the detection and discovery of viruses at the human–animal interface.

Specimens from various species of NHPs were collected opportunistically between December 2010 and December 2013 in DRC (Fig. 1). Samples from 620 animals were tested, with 92.9 % of them being blood or buffy coat, 5.6 % liver or spleen and 1.5 % colon or lung. Bush-meat hunters who

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Keywords: Bocavirus; emerging disease; zoonosis; primate; Africa; Congo.

Abbreviations: BC, buffy coat; DRC, Democratic Republic of the Congo; EPT, Emerging Pandemic Threats; HBoV, human bocavirus; IACUC, The Institutional Animal Care and Use Committee; ICCN, Institut National de Conservation de la Nature; INRB, Institut National de Recherche Biomedicale; NHP, non human primate; NP1, nuclear protein gene 1; NS1, non-structural protein gene 1; PL, plasma; SPV, simian parvovirus; USAID, United States Agency for International Development; VP1, viral protein gene 1; VP2, viral protein gene 2.

The sequences reported in this manuscript are deposited in GenBank under the accession numbers MF959513-MF959518.

Two supplementary tables are available with the online version of this article.

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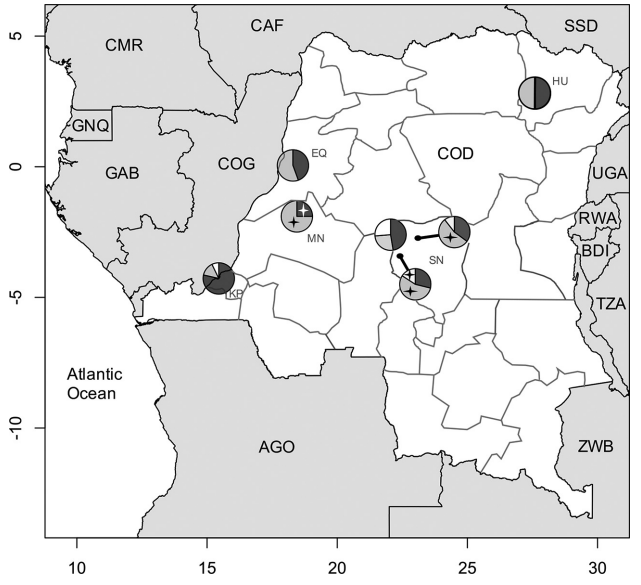

Fig. 1. Map of the sampling sites for non-human primates in the Democratic Republic of the Congo. Pie charts indicate the proportion of species collected at each location. Dark grey represents 'other', light grey represents 'red-tailed monkeys (*Cercopithecus ascanius*)' and white represents 'Wolf's monkeys (*Cercopithecus wolfi*)'. *Cercopithecus ascanius* and *Cercopithecus wolfi* are included individually because they are the two highest sampled species and contain positive individuals. The category 'other' included *Allenopithecus nigroviridis, Cercocebus* spp., *Cercocebus chrysogaster, Cercopithecus erythrogaster, Cercopithecus hamlyni, Cercopithecus denti, Cercopithecus neglectus, Cercopithecus nictitans, Colobus angolensis, Erythrocebus patas, Galagoides demidovii, Lophocebus aterrimus, Lophocebus albigena, Pan paniscus, Pan troglodytes, Papio cynocephalus, Perodicticus potto* and *Piliocolobus tholloni.* Pie segments with a star represent the sampling of red-tailed monkeys, Wolf's monkeys, or Putty-nosed monkeys (white, within the 'other' category) that tested positive for bocavirus DNA. See the Results section and Table S2 for the sampling size for each location. Countries indicated by ISO 3166-1 alpha-3 codes. Provinces: EQ, Equateur; HU, Haut Uele; KP, Kinshasa Province; MN, Mai-Ndombe; SN, Sankuru.

were recruited to participate in the sample collection were trained by PREDICT staff in personal safety measures and in the correct sampling techniques to ensure sample quality and minimize the risks of contamination. Whole-blood samples were collected by the hunters from freshly killed wild animals on filter paper (Whatman) and placed in pre-labelled zip-lock plastic bags with envelope and desiccant sponge. Tissue samples (colon, liver, spleen and lung) from freshly killed wild animals were collected by PREDICT staff in cryotubes with 500 µl of RNAlater (Ambion/Qiagen). Samples in RNAlater were stored at room temperature in the field and transferred to a −80 °C freezer upon arrival at the laboratory. Fresh blood samples were collected from monkeys and great apes housed in a bonobo sanctuary, the Kinshasa Zoo and several touristic sites in 7 ml Vacutainer tubes containing EDTA (VWR International). Within 24 h, samples were transferred to the laboratory for separation of plasma (PL) and buffy coat (BC) using centrifugation (10 min at 3000 ***g***); the resulting aliquots were stored at –80 °C until analysis. Overall, 94 % of the sampled animals were living in the wild, while 6 % were captive.

All laboratory analyses were performed in the Metabiota Laboratory on the premises of the Institut National de Recherche Biomedicale (INRB) in Kinshasa. The personnel were trained in personal and environmental safety as well as in good laboratory practice to ensure safe and accurate workflow and results. The workflow within the laboratory was optimized to minimize the risks of contamination between different stages of the analysis process. Samples were subjected to DNA extraction using the Qiagen Allprep DNA/RNA kit (tissue samples) or the Qiagen QIAamp DNA Mini kit (dried blood spot samples). Samples were tested for the presence of bocaparvovirus DNA using a semi-nested broad range PCR assay targeting the non-structural gene 1 (NS1) of human and other primate bocaviruses as previously described [8]. PCR was repeated on any positive results, starting from the original DNA to rule out contaminations downstream of the extraction. To follow up on PCR-positive samples, a set of three additional nested PCR assays targeting known human bocaviruses was designed to target NP1, VP1/VP2 and a different fragment of the NS1 gene (Table S1, available in the online version of this article). Samples that were positive for viral DNA were additionally tested with a cytochrome b PCR assay to confirm the host species [11]. PCR products were run on a 1.5 % agarose gel and the corresponding size fragments excised. DNA was extracted using either the Qiagen QIAquick Gel Extraction kit or the Promega Wizard SV Gel and PCR Clean-Up System, and were sent for commercial Sanger sequencing at GATC Biotech (Germany). The sequencing results were assessed and processed using Geneious 7.1.

Phylogenetic trees based on the PCR target regions in NS1, NP1 and VP1/VP2 were constructed with amplified sequences and previously published bocavirus isolates (Fig. 2). First, multiple sequence alignments were made (Geneious version 10.1.2, MUSCLE alignment), and regions supported by less than 50 % of the sequences were removed. The Bayesian phylogeny of the NS1, NP1 and VP1/VP2 partial sequences was inferred using MrBayes (version 3.2) [12]. Trees were made using the Markov chain Monte Carlo model with the SYM substitution matrix (Pol) or the GTR substitution matrix (Ter), variable gamma rates, invariant sites, two runs and four chains of 10 000 000 generations. Bat bocavirus isolate YNJH (NC_029300) was defined as the outgroup to root each tree. Trees were sampled after every 1000 steps during the process to monitor phylogenetic convergence. The average standard deviation of split frequencies was below 0.0054 (NS1 short), 0.0080 (NS1 long), 0.0027 (NP1), or 0.0030 (VP1/VP2) (MrBayes recommended final average <0.01). The first 10 % of the trees were discarded and the remaining ones were combined (TreeAnnotator version 1.8.2; http://beast.bio.ed.ac.uk) and displayed with FIGTREE (1.4.2; http://tree.bio.ed.ac.uk/).

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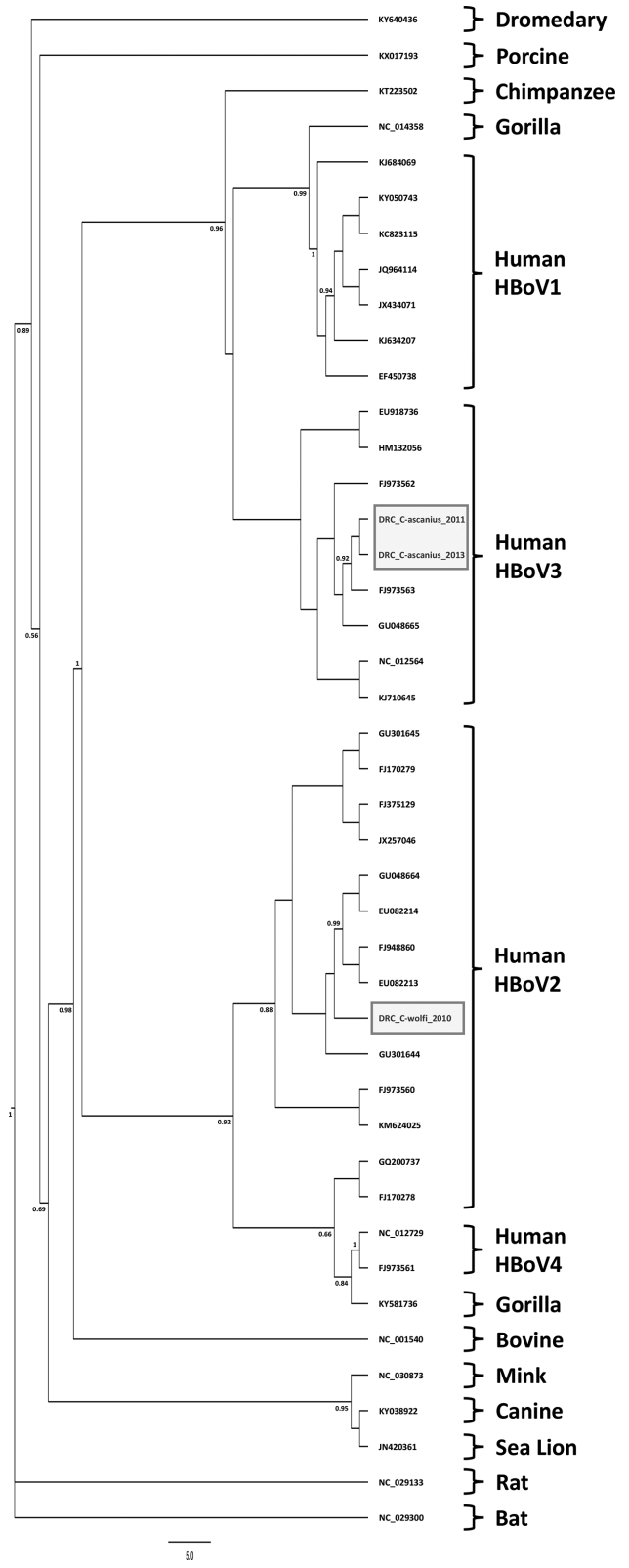

Fig. 2. Bocavirus phylogenetic tree. Phylogenetic tree of 43 bocavirus isolates, including the sequences of isolates DRC_C-ascanius_2011 from the Sankuru province, DRC_C-ascanius_2013 from the Mai-Ndombe province and DRC_C-wolfi_2010 from the Sankuru province, as well as 40 previously published human and animal isolates. The tree is based on 180 nucleotides of the NS1 fragment amplified by the Kapoor PCR assay [8] and shown as a proportional cladogram, with the numbers at branches indicating posterior probabilities; only probabilities >0.5 are shown. The presumed host species on the right are based on previous reports. The partial NS1 sequences of isolates DRC_C-ascanius_2011, DRC_C-ascanius_2013 and DRC_C-wolfi_2010 have been deposited in GenBank under accession numbers MF959513–MF959515. The other included virus sequences are *Dromedary*, KY640436 – dromedary camel bocavirus 1; *Porcine*, KX017193 – porcine bocavirus strain CH/HNZM; *Chimpanzee*, KT223502 – primate bocavirus 1 isolate CPZh2; *Gorilla*, NC_014358 – bocavirus gorilla/GBoV1/2009; *Human Bocavirus 1*, KJ684069 – human bocavirus isolate 2012GZ1169; KY050743 – human bocavirus strain 03EI-02–10103/VietNam/2014; KC823115 – human bocavirus strain Irish; JQ964114 – human bocavirus isolate Rus-Nsc10-N1117; JX434071 – human bocavirus isolate CQ802; KJ634207 – human bocavirus 1 isolate 307AR09; EF450738 – human bocavirus isolate HK22; *Human Bocavirus 3*, EU918736 – human bocavirus 3 strain W471; HM132056 – human bocavirus 3 isolate 46-BJ07; FJ973562 – human bocavirus 3 strain HBoV3B-TU-A-210–07; FJ973563 – human bocavirus 3 strain HBoV3A-NI-374; GU048665 – human bocavirus 3 strain CU2139UK; NC_012564 – human bocavirus 3; KJ710645 – human bocavirus isolate Rus-Nsc11 N2512; *Human Bocavirus 2*, GU301645 – human bocavirus 2 isolate LZ55602; FJ170279 – human bocavirus 2 isolate PK-2255; FJ375129 – human bocavirus isolate SH3; JX257046 – human bocavirus 2 strain BJQ435; GU048664 – human bocavirus 2 strain CU1557UK; EU082214 – human bocavirus 2 strain W208; FJ948860 – human bocavirus 2 strain W298; EU082213 – human bocavirus 2 strain W153; GU301644 – human bocavirus 2 isolate LZ53819; FJ973560 – human bocavirus 2b NI strain HBoV2b-NI-213; KM624025 – human bocavirus strain LZFB080; GQ200737 – human bocavirus 2 isolate KU1; FJ170278 – human bocavirus 2c PK isolate PK-5510; *Human Bocavirus 4*, NC_012729 – human bocavirus 4 NI strain HBoV4-NI-385; FJ973561 – human bocavirus 4 NI strain HBoV4-NI-385; *Gorilla*, KY581736 – gorilla bocaparvovirus 2 isolate GBOV2/GAB1; *Bovine*, NC_001540 – bovine parvovirus; *Mink*, NC_030873 – mink bocavirus clone 1; *Canine*, KY038922 – canine bocavirus isolate GZHD15; *Sea lion*, JN420361 – California sea lion bocavirus 1 isolate 1153; *Rat*, NC_029133 – rat bocavirus strain HK15; *Bat*, NC_029300 – bat bocavirus isolate YNJH.

Samples from 620 NHPs were collected and tested for bocavirus DNA; the samples originated from five provinces in DRC: Equateur (n=9), Haut Uele (n=10), Kinshasa Province (n=70), Mai-Ndombe (n=20) and Sankuru (n=511) (Fig. 1). The majority of samples collected came from rural and semi-rural areas, from hunted and butchered NHPs (all provinces except Kinshasa Province). Most sampling sites were in lowland forests and tropical and subtropical savannas, with some being located near a river or lake. The main occupation of the local communities in these areas is hunting for personal consumption, local sale and bush-meat trade. Some habitats surrounding the samplings sites are experiencing moderate to severe changes, mainly due to ongoing deforestation and land use change for new human settlements, agriculture, or livestock production. All sampled touristic sites, including the main bush-meat markets, were located in semi-urban and urban areas of Kinshasa. In these more urban sites, wild animals are housed in

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sanctuaries, kept as pets in human dwellings and/or presented in the bush-meat markets alive or freshly killed for sale (Table S2).

The samples came from 20 different NHP species, not including 4 individuals for which the exact species could not be determined. Half of the sampled animals were *Cercopithecus ascanius* (red-tailed monkey, *n*=310), while other common species included *Cercopithecus wolfi* (Wolf's monkey, *n*=77) and *Lophocebus aterrimus* (black crested mangabey, *n*=64) (for more details about sampled species see Table 1).

During NS1 broad-range PCR screening, 99 % of the samples produced a negative result, while putative bocavirus DNA was amplified from six of them (1 %). Half of these were from dried blood spot samples, and the other half were from liver samples. Bocavirus DNA was found in four red-tailed monkeys, one De Brazza's monkey and one Wolf's monkey; all animals had been hunted as bush meat (Table S2). Amplified PCR products were sequenced and upon comparison with the GenBank database (BLASTN) were confirmed as bocaviruses. Four of the sequences are identical (isolates DRC_C-ascanius_2010, DRC_C-ascanius_2011, DRC_C-ascanius_2013b and DRC_C-neglectus_2013) and match the sequence of a human bocavirus 3 isolate (FJ973563) 100 % (nt). A fifth positive sample (DRC_C-ascanius_2013) differs by two nucleotides (nt) and shares 99 % (nt) identity with the same human bocavirus 3 isolate and differs by one amino acid (aa) on the protein level from human bocavirus 3 isolate HM485564. The sixth sequence (DRC_C-wolfi_2010) resembles human bocavirus 2, sharing 100 % (nt) of the sequence with several HBoV2 isolates, including JX257046. Follow-up PCRs for the alternative NS1 fragment, NP1 and VP1/VP2, only amplified DNA from isolate DRC_C-wolfi_2010, and failed on the other samples. In line with the initial sequencing results for DRC_C-wolfi_2010, the follow-up sequencing products were most similar to human bocavirus 2 sequences: NP1 99 %(nt)/100 %(aa) HQ398864/JX257046, NS1 99 %(nt)/99 %(aa) KM624025/FJ973559 and VP1 97 %(nt)/100 %(aa) FJ375129/FJ170279.

Table 1. NHP species sampled and tested for the presence of bocavirus DNA in DRC

Species scientific name*	Species common name in English*	No. tested (no. POS)
Allenopithecus nigroviridis	Allen's swamp monkey	17
Cercocebus chrysogaster	Golden-bellied mangabey	4
Cercocebus spp.	Unidentified	3
Cercopithecus spp.	Unidentified	1
Cercopithecus ascanius	Red-tailed monkey	310 (4)
Cercopithecus erythrogaster	Red-bellied monkey	1
Cercopithecus hamlyni	Owl-faced monkey	1
Cercopithecus denti	Dent's monkey	1
Cercopithecus neglectus	De Brazza's monkey	33 (1)
Cercopithecus nictitans	Greater spot-nosed monkey	4
Cercopithecus wolfi	Wolf's monkey	77 (1)
Colobus angolensis	Angolan colobus	27
Erythrocebus patas	Patas monkey	4
Galagoides demidoff	Demidoff's dwarf galago	4
Lophocebus albigena	Grey-cheeked mangabey	5
Lophocebus aterrimus	Black crested mangabey	64
Pan paniscus	Bonobo	17
Pan troglodytes	Chimpanzee	1
Papio cynocephalus	Yellow baboon	4
Perodicticus potto	Potto	3
Piliocolobus tholloni	Thollon's red colobus	39
Total		620 (6)

*All names used according to the ITIS nomenclature.

Upon phylogenetic analysis of the isolates and other known bocaviruses based on a 180 bp fragment of NS1, isolates DRC_C-ascanius_2010, DRC_C-ascanius_2011, DRC_C-ascanius_2013, DRC_C-ascanius_2013b and DRC_C-neglectus_2013 cluster with human bocavirus 3 sequences (Fig. 2). Isolate DRC_C-wolfi_2010 clusters robustly with human bocavirus 2 sequences in the NP1 fragment (355 bp), the two NS1 fragments (180 and 496 bp) and the VP1 fragment (371 bp) (Fig. 2 and not shown data). The sequences are stored in GenBank with accession numbers MF959513–MF959518.

We screened for potential bocavirus infections in African NHP species from 620 individual animals and detected viral DNA in several of the samples that was highly suggestive of a bocaparvovirus infection. While only six of these NHPs were confirmed to contain bocavirus DNA, the number of infected animals may be higher given that primarily blood samples were tested, and that viraemia only occurs during a short window in the infection cycle of bocaviruses [6]. Many previous studies have screened for and detected human bocaviruses in respiratory tract or stool samples, and found a high prevalence in symptomatic and asymptomatic individuals. It was, however, concluded that humans shed virus for long periods, resulting in this high detection rate in respiratory tract and stool samples [2, 4, 13]. Unlike human parvovirus B19V, human bocaviruses do not seem to persist in the blood for extended periods [2].

Bocavirus DNA has previously been detected in NHPs, specifically in gorillas and chimpanzees. However, these viruses were distinct from human isolates and differed by $\geq$8 % from the next closest human bocavirus in their nucleotide sequence [8–10]. The bocavirus sequences detected here in three *Cercopithecus* species share a very high degree of identity with human bocaviruses 2 or 3, and phylogenetic analysis supports clustering with human isolates, rather than with the gorilla or chimpanzee bocaviruses. This is an unexpected finding, especially given that *Cercopithecus* species as hosts are phylogenetically much further away from humans than are great apes. A contamination from a human source was considered, but dismissed as a likely explanation for the findings for a variety of reasons: measures to minimize the

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risk of contamination were in place in the field and in the laboratory, all PCR results were reconfirmed from the original DNA, viral DNA was detected in different sample types, most samples were collected on different days in different locations and tested at different days, and the sequencing results represent three different sequences.

The very close relationship between human bocaviruses and the bocavirus isolates from red-tailed monkeys, De Brazza's monkeys and Wolf's monkeys is indicative of recent or current zoonotic events. The monkeys harbouring viral DNA were hunted in rural and remote areas that are currently undergoing a process of land use change, which is a setting that has been predicted to foster the emergence of new human pathogens due to increased human–animal contact [14]. Given our findings, we see two potential explanations: a zooanthroponotic or an anthropozoonotic scenario. Should the detected viruses have been introduced into the monkeys from humans, then this might enable novel recombinations and the possible emergence of new bocaviruses with the potential to jump species barriers in the future. To identify such mosaic bocavirus genomes, large parts of or the full genome need to be sequenced, and it is possible that the reason we were not able to obtain additional sequence from the HBoV3-like isolates stems from this phenomenon. If it is the other way around, and human bocaviruses (or at least some of them) indeed have their origin in recent transmission events from NHPs, then this might partly explain the high diversity in human bocaviruses 2 and 3, since species jumps can lead to an accelerated evolution in the process of host adaptation shortly after [15]. The human bocavirus isolates that have been analysed in many recent studies do not show any clear temporal or regional pattern, however, which might be due to the limited data available and high human mobility, and this topic should be thoroughly evaluated in future studies with these new findings in mind.

Events where a virus establishes itself in a new species are rare and parvoviruses are generally rather host-specific. Yet, a similar development was observed in dogs following the pandemic with canine parvovirus 2, an event that also demonstrated the potential of parvoviruses to jump species barriers, at least within distantly related hosts with the same taxonomical order (mink, cat, dog) [15–17]. Based on serological and experimental cell culture data, it has also been proposed that simian parvovirus (SPV), a pathogenic macaque virus and distant relative of *Erythroparvovirus* B19V, has the ability to infect humans [18].

Since we could not obtain full sequences from the isolates, we compared the genome regions of the fragments our PCR assays amplified to test whether these were potentially conserved regions and hence not representative. However, this does not seem to be the case. Comparing the two gorilla bocavirus isolates to human bocaviruses revealed differences of at least 11 % (NP1), 8 % (NS1 short), 12 % (NS1 long) and 13 % (VP1), and while these differences are slightly smaller than over the entire open reading frame, they are overall generally similar.

We conclude that bocaparvoviruses, like protoparvoviruses and potentially erythroparvoviruses, may be able to cross species barriers. Consequently, *Cercopithecus* species and potentially other NHP species could be a source or reservoir for bocaviruses that have the ability to infect humans, regardless of whether these NHPs are the original source of these viruses or if the viruses have been introduced through human contact. Additional studies specifically addressing the situation in wild African NHPs are hence required to test this hypothesis and evaluate the situation. Since the data suggest that red-tailed monkeys, De Brazza's monkeys and Wolf's monkeys can be infected naturally with human or human-like bocaviruses, they might be a good model to study the natural course, biology and pathobiology of bocavirus infections. This could open new doors for research on these viruses, about which we still know relatively little.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
Specimen collection from the wild animals included in this study was approved by IACUC University of California Davis (protocol #16067) and the DRC Institut National de Conservation de la Nature (ICCN). All sample collection was opportunistic, no animal was killed for the purpose of this study.

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