ORIGINAL ARTICLE

Analysis of adenovirus DNA detected in rodent species from the Democratic Republic of the Congo indicates potentially novel adenovirus types

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Abstract

Different species of adenoviruses (AdVs) infect humans and animals and are known for their role as pathogens, especially in humans, with animals, primarily rodents, often serving as model systems. However, although we know over 100 types of human AdVs, we know comparatively little about the diversity of animal AdVs. Due to the fact that rodents are the most diverse family of mammals and a standard model system for human disease, we set out to sample African rodents native to the Democratic Republic of the Congo and test them for AdV DNA using a semi-nested consensus PCR. A total of 775 animals were tested, and viral DNA was detected in four of them. The AdV DNA found belongs to three different AdVs, all being closely related to murine adenovirus 2 (MAdV-2). Considering the genetic differences of the amplicon were 9%, 11% and 19% from MAdV-2 and at least 10% from each other, they seem to belong to up to three different novel types within the *Murine mastadenovirus B* species. This evidence of genetic diversity highlights the opportunities to isolate and study additional AdVs that infect rodents as models for AdV biology and pathology.

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Introduction

Adenoviruses (AdVs) are double-stranded DNA viruses with a linear, non-segmented genome. The genome has an approximate size of 36 kb and is packaged into a non-enveloped icosahedral capsid of about 90 nm. There are five genera in the *Adenoviridae* family, namely *Mastadenovirus*, *Aviadenovirus*, *Atadenovirus*, *Siadenovirus* and *Ichtadenovirus*. Mammals are primarily infected by members of the *Mastadenovirus* genus, but also by some members of the *Atadenovirus* genus. The known AdVs are largely host-specific, and numerous distinct members of the *Adenoviridae* family have been identified in many vertebrate species including such diverse hosts as humans, turkeys, corn snakes, northern leopard frogs and white sturgeon [1,2].

Although they are pathogens of both humans and animals, most infections with AdVs are believed to take an asymptomatic course. Infections with human AdV (HAdV) can cause a variety of symptoms, such as respiratory diseases, conjunctivitis and gastroenteritis, especially in infantile and immunocompetent individuals. Newborns and immunocompromised individuals can suffer from severe pneumonia, hepatitis, or encephalitis as a result of AdV infections [2].

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The diversity of AdVs is very high, with over 100 different HAdV types described so far [2,3]. Despite the high AdV diversity, much of what we know about their biology is based on work with rodent-based model systems, and murine AdV-1 (MAdV-1) is probably one of the best-understood rodent specific AdVs today [2,4–6]. Much like in humans, AdV infections of immunocompetent mice rarely result in clinical disease, whereas immunocompromised or newborn mice can suffer from similar symptoms to their human counterparts [4,5]. Serological studies suggest that infections among wild rodents may be common, but only a handful of different rodent AdVs were known until recently [7–11]. Increasing evidence, such as the amplification of novel sequences from rodents in China and Cameroon, suggests that there are many more yet to be discovered [12–14]. Finding and studying such viruses could reveal important information about AdV prevalence, epidemiology and evolution, and may provide opportunities to establish new relevant model systems for AdV biology and pathology regarding the human disease. Due to its high biodiversity, central Africa is certainly a promising place to search for such viruses, and so far, no rodent AdVs had been reported from the Democratic Republic of the Congo (DRC). Our goal for this study was therefore to screen a diverse set of rodents found in DRC for evidence of AdVs and evaluate their relation to known types.

Materials and methods

Samples were collected between December 2010 and June 2013 from rodents in several locations in DRC (Fig. 1). These animals were sampled as part of a larger research programme that aimed to investigate the sharing of viruses between animals and humans, and rodent infections were of critical importance to the research goal. People who reported hunting or butchering wild animals in rural areas volunteered to collect blood samples from the wild animals they interacted with. They were trained in personal safety measures and in the correct sampling techniques to ensure sample quality and minimize the risks of contamination. Samples were collected as dried blood spot (DBS) samples on Whatman paper during butchering and allowed to air dry before they were stored in individually labelled envelopes. All DBS samples were individually stored in clip-lock plastic bags with silica gel at ambient temperature until further processing. Hunters were not compensated to avoid an increase in hunting above normal rates. Animal capture and specimen collection were approved by the UC Davis Institutional Animal Care and Use Committee (IACUC) and the Ministry of Agriculture, Fisheries and Livestock in DRC.

Additionally, animal tissue samples were obtained during necropsies in the same villages and bushmeat markets using sterile instruments. They were collected either in RNA-later (Qiagen, Hilden, Germany) and kept at room temperature initially or directly frozen on-site without any medium. Samplers wore N95 masks, examination gloves and protective eyewear during carcass sampling to minimize risks of disease transmission and sample contamination. The samples were stored at −20°C and later transferred to −80°C or directly stored at −80°C until further processing, which happened in the Metabiota laboratory at the Institut National de Recherche Biomédicale (INRB) in Kinshasa.

Species identification was performed in the field by trained field ecologists, as well as retrospectively based mainly on morphology, body size, tail size, tooth organization and pelage colour. Various sources, including Kingdon and Monadjem et al., were used as references for species determination [15,16]. The species of animals that tested positive for AdV DNA were verified by Cytochrome B barcoding [17].

DNA was extracted from the specimens using the Qiagen AllPrep DNA/RNA (tissue samples) or Qiagen QIAamp DNA Mini kit (DBS samples) according to the manufacturer's instructions and was stored at −20°C until analysis. The workflow within the laboratory was optimized to minimize the risks of contamination between different stages of the analysis process. A semi-nested consensus PCR, with the primer combinations ADHEX1F (CAA CAC CTA YGA STA CAT GAA) and ADHEX1R (KAT GGG GTA RAG CAT GTT) for the first round as well as ADHEX2F (CCC ITT YAA CCA CCA CCG) and ADHEX1R for the second round was used to detect AdV DNA [18]. The PCR amplifies a product of approximately 334 nucleotides flanked by the primer binding sites within the conserved hexon gene. PCR conditions were as described before starting with an initial denaturation for 2 min at 94°C followed by 45 cycles of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C and ending with a final elongation step of 5 min at 72°C [18].

PCR products were separated by size on a 1.5% agarose gel, and products of the amplicon's expected size were excised. Any amplified DNA was extracted using either the Qiagen QIAquick Gel Extraction Kit or the MP Biomedicals GeneClean kit and was sent for commercial Sanger sequencing at GATC Biotech (Germany). Sequencing results were evaluated and processed using Geneious (version 11.1.3) and compared with sequences present in the GenBank database (BLAST N and BLAST P, NCBI). Novel viral sequences were deposited in GenBank with submission numbers MN318952–MN318955.

For a maximum likelihood phylogenetic analysis, multiple sequence alignments were made in Geneious (ClustalW), and positions supported by <50% of the sequences were removed.

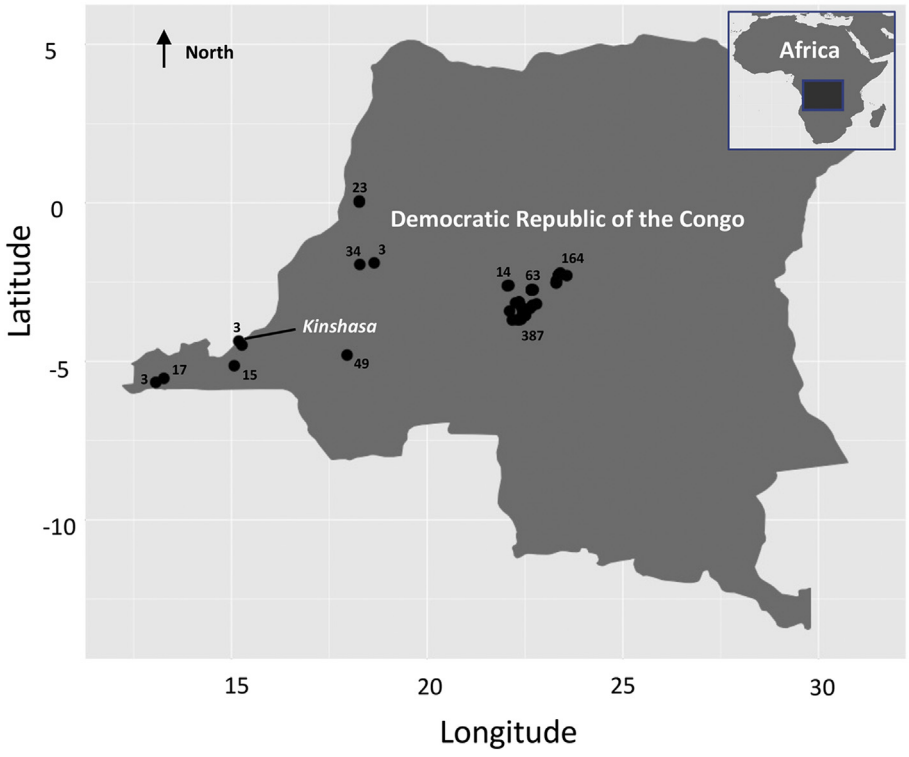

FIG. 1. Map showing the sampling sites; locations (black dots) and the numbers of animals that were sampled in the Democratic Republic of the Congo for the study.

The four novel sequences, 24 other *Mastadenovirus* sequences, and as an outgroup, two *Atadenovirus* sequences were included. Bayesian phylogeny of the polymerase gene fragment was inferred using MrBayes (version 3.2) with a GTR + I + G substitution model [19,20]. We ran four chains of 10 000 000 generations, and trees were sampled after every 1000 steps during the process to monitor phylogenetic convergence. The final average standard deviation of split frequencies was below 0.0038. The first 10% of the trees were discarded and the remaining ones were combined using TreeAnnotator (version 2.5.1; http://beast.bio.ed.ac.uk) and displayed with FIGTREE (1.4.4; http://tree.bio.ed.ac.uk/) [21].

Results

We collected samples from 775 rodents, of which 382 were *Muridae*, 173 *Sciuridae*, 60 *Hystricidae*, 20 *Thryonomyidae*, 5 *Eutheria* and 135 of undetermined family. The sampling sites were predominantly in rural areas in Kasai Oriental, Bandundu, Bas-Congo and Equateur provinces of the DRC (Fig. 1). In most cases, only a single specimen was collected per individual; overall, 618 DBS samples, 180 liver/spleen pooled samples, 11 liver samples, three spleen samples and eight colon samples were collected and tested (Table 1; see also Supplementary material, Table S1).

Adenovirus DNA was detected in four animals (0.5% positive, n = 775), all of them hunted in the Kasai Oriental province, with two of them being giant pouched rats (*Cricetomys* sp.), one a striped mouse (*Hybomys* sp.) and the other one a Muton's soft-furred mouse (*Praomys mutoni*). All four AdV DNA-positive specimens were DBS samples; no other material was available for these four animals. Two of the sequences were identical and upon comparison with the NCBI database, all were most similar to MAdV-2, with 91%, 87% and 81% identities in BLASTN as well as 96%, 90% and 86% identities in BLASTP. On the amino acid level, the newly detected sequences differed by 4.1% (DRC-3582 from MAdV-2), 9.9% (DRC-1479 from MAdV-2) and 11.3% (DRC-2973 and DRC-1502 from DRC-3582) from the next closest AdV sequences. Upon phylogenetic analysis, the four novel sequences also clustered closely with MAdV-2 (Fig. 2).

Discussion

We screened specimens from different rodent species in DRC and detected AdV DNA in four individuals. Compared with other studies, the proportion of positive animals (0.5%, n = 775) is very low, but direct comparison is difficult, as several methodological factors are very different. Although most of the samples we collected and tested were DBS,

TABLE 1. Sample types

	One sample tested	Two samples tested	Three samples tested
Total no. of animals	735	35	5
DBS	580		
Liver/Spleen[a]	147		
Liver	7		
Spleen	1		
DBS + Liver/Spleen[a]		28	
DBS + Liver		2	
DBS + Colon		5	
DBS + Liver/Spleen[a] + Colon			3
Liver + Spleen + Liver/Spleen[a]			2

DBS, dried blood spot.
[a]Pooled sample.

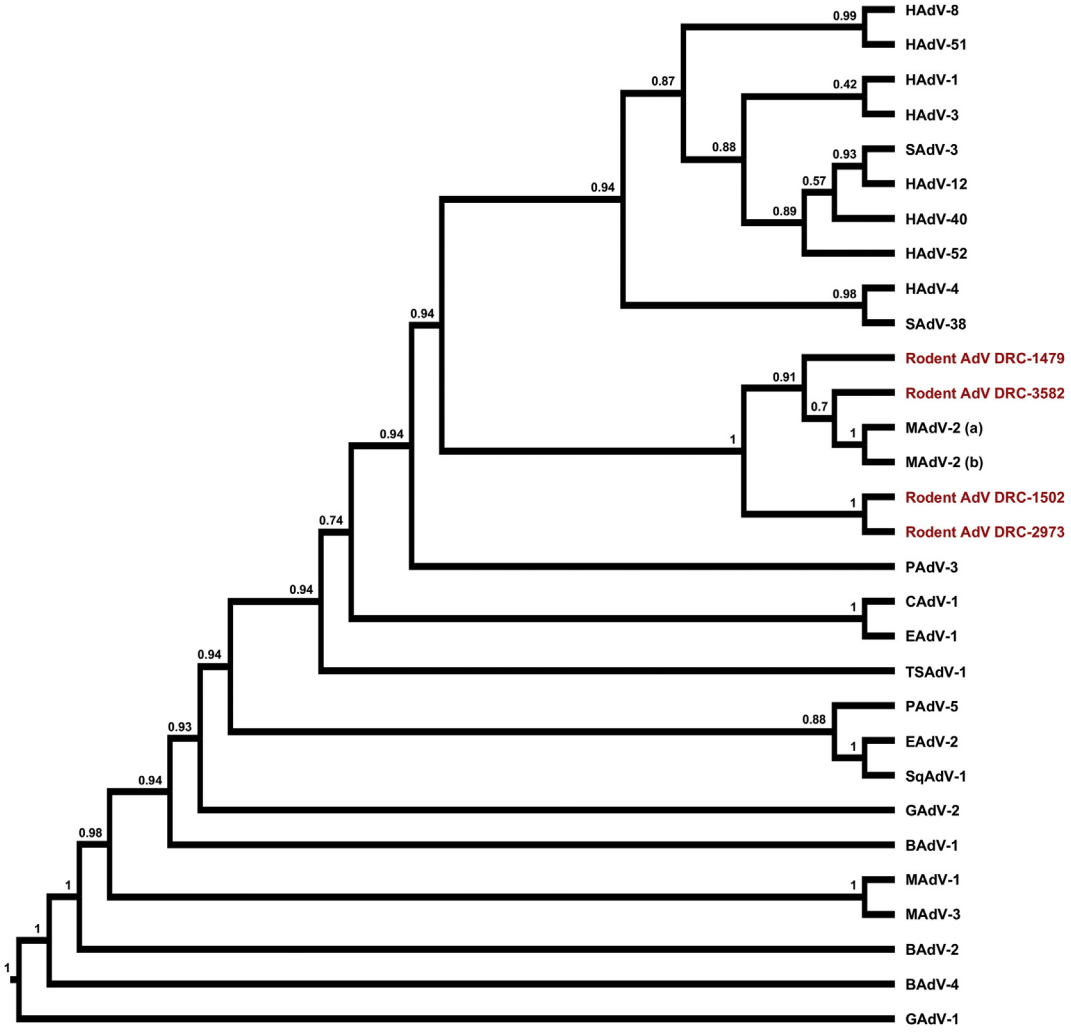

FIG. 2. Phylogenetic tree of adenoviruses. Phylogenetic tree of adenoviruses based on the PCR target region within the hexon gene. The tree is presented as a proportional cladogram. Numbers at knots are indicating bootstrap support. Sequences included are human adenoviruses HAdV-1 (AF534906), HAdV-3 (DQ086466), HAdV-4 (AY487947), HAdV-8 (AB448767), HAdV-12 (NC_001460), HAdV-40 (NC_001454), HAdV-51 (JN226765) and HAdV-52 (DQ923122), simian adenoviruses SAdV-3 (AY598782) and SAdV-38 (FJ025922), murine adenoviruses MAdV-1 (M81889), MAdV-2 (a: MF416385 and b: NC_014899) and MAdV-3 (NC_012584), bovine adenoviruses BAdV-1 (BD269513) and BAdV-2 (AF252854), equine adenovirus EAdV-1 (L79955) and EAdV-2 (KT160425), porcine adenoviruses PAdV-3 (AF083132) and PAdV-5 (AF289262), canine adenovirus CAdV-1 (AC_000003), goat adenovirus GAdV-2 (DQ630760), tree shrew adenovirus TSAdV-1 (AF258784), squirrel adenovirus SqAdV-1 (KY427939) from the *Mastadenovirus* genus, and bovine adenovirus 4 (AF036092) and goat adenovirus 1 (AF207660) from the *Atadenovirus* genus. Novel sequences are depicted in red.

previous studies primarily included faecal or tissue samples, and the time for which virus or viral DNA is present and preserved in these may vary greatly [12,14]. However, considering the relative stability of the non-enveloped AdVs with their DNA genome, the choice of PCRs might be the even more important factor. The mentioned studies previously conducted in Cameroon and China used a broad-range PCR targeting the DNA polymerase gene, whereas we used a PCR targeting the hexon gene [12,14]. Both PCRs probably have their limitations and it seems reasonable to assume that the actual AdV prevalence, in either case, will be higher than what was detected.

The sequences we found are closely related to MAdV-2 and could be MAdV-2 subtypes. The polymerase gene rather than the hexon gene is used for classification purposes, and an amino acid difference of between 5% and 15% would constitute different AdV types [1]. Based on the comparisons of the amino acid sequences of the full polymerase and hexon genes, as well as of the full hexon gene and the PCR target region of 32 AdVs of different species we were able to make a rough prediction with regards to the distance between MAdV-2 and the AdVs detected. The full hexon sequence had identities that were, on average, 11% higher than the full polymerase gene, and the PCR amplified region, on average, has 17% higher identities to other AdVs compared with the full hexon gene (see Supplementary material, Table S2). We therefore conclude two things, first, that the hexon gene is generally more conserved than the polymerase gene, and second, that the amplified region is consistently more conserved than the full hexon sequence. Taking that and the different hosts into account, the sequences detected could represent up to three different novel murine AdV types within or closely related to the *Murine mastadenovirus B* species. Such a conclusion would also be supported by the structure of the phylogenetic tree (Fig. 2). However, only more in-depth studies, including full genome sequencing and virus isolation will be able to bring full clarity.

In conclusion, we amplified partial AdV hexon-gene DNA indicating the presence of three different novel murine AdV types or subtypes in African rodents. These new sequences, in the light of other recent findings unveiling more and more different AdVs, support the idea of a diverse viral family with most of its members still unknown [12,14]. The diversity also highlights a need for more in-depth studies on prevalence and epidemiology, as well as vast opportunities for isolating additional murine AdVs to learn more about AdV biology and pathology in rodent-based model systems.

Conflicts of interest

All authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nmni.2019.100640.

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