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ADENOVIRUS IN RURAL CÔTE D`IVOIRE: HIGH DIVERSITY AND EVIDENCE FOR CROSS-SPECIES TRANSMISSION

Pauly, Maude[1,2], Akoua-Koffi, Chantal[3], Buchwald, Nanina[2], Schubert, Grit[1], Weiβ, Sabrina[1], Couacy-Hymann, Emmanuel[4], Anoh, Augustin Etile[3,4], Mossoun, Arsène[3,4], Calvignac-Spencer, Sébastien[1], Leendertz, Siv Aina[1], Leendertz, Fabian H [1], Ehlers, Bernhard[2]

1. Project group "*Epidemiology of highly pathogenic microorganisms*", Robert Koch Institute, 13353, Berlin, Germany
2. Division 12 "Measles, Mumps, Rubella and Viruses affecting immunocompromised patients", Robert Koch Institute, 13353, Berlin, Germany
3. Centre de Recherche pour le Développement, Université Alassane Ouattara of Bouake, Bouake 01 BP V18, Côte d'Ivoire
4. Laboratoire National d`Aide au Développement Agricole/ Laboratoire Central de Pathologie Animale, Bingerville 206, Côte d'Ivoire

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Corresponding Author:

Bernhard Ehlers, Robert Koch-Institut, Division 12

Seestr. 10, D-13353 Berlin, Germany

Phone 030 18754 2347, fax 0039 30 18754 2598

ehlersb@rki.de

Fabian H Leendertz, Robert Koch-Institut, Epidemiology of highly pathogenic microorganisms

Nordufer 20, D-13353 Berlin, Germany

25 Phone 0039 30 18754 2592, fax 0039 30 18754 2181

26 leendertz@rki.de

27

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ADENOVIRUS IN RURAL CÔTE D`IVOIRE: HIGH DIVERSITY AND CROSS-SPECIES DETECTION

Abstract

The Taï region in Western Côte d`Ivoire is characterized by extensive overlap of human and animal habitats. This could influence patterns of adenovirus transmission between humans and domestic animals. Fecal samples from humans and various domestic animals were tested for the presence of adenoviruses by PCR. Phylogenetic and species delineation analyses were performed to further characterize the adenoviruses circulating in the region and to identify potential cross-species transmission events. Among domestic animals, adenovirus shedding was frequent (21.6 % of domestic mammals and 41.5% of chickens) and the detected strains were highly diverse, several of them representing novel types. Although no evidence for zoonotic transmission of animal adenovirus was obtained, the present study provides concordant evidence in favor of common cross-species transmission of adenoviruses between different animal species and first indications for adenovirus transmission from humans to animals. These findings underline the thus far underestimated importance of reverse zoonotic transmission of viruses and of the role of domestic animals as pathogen reservoirs, “bridge species” or intermediate hosts.

INTRODUCTION

Between 1996 and 2009, more than 25% of the emerging infectious diseases (EID) in humans were caused by viruses. The majority originated from animal hosts (Jones et al. 2008) and emerged in tropical Africa (Chan et al. 2010). A major driver for disease emergence was likely the modification and intensification of agriculture, since it resulted in novel wildlife-livestock-human interactions (Pearce-Duvet 2006; Jones et al. 2013). It has been shown that livestock can play key roles as intermediate host for the transmission of wildlife pathogens to humans (Daszak et al. 2000; Wood et al. 2012). In fact, the majority of the pathogens of domestic animals are multiple host pathogens and many of them have zoonotic potential (Cleaveland et al. 2001). Thus far, research mainly focused on zoonotic transmission of pathogens from animals to humans, even though “anthropozoonoses” or “reverse zoonoses” are not infrequent and can have dramatic consequences for animal health (Messenger et al. 2014).

Recently, several cross-species transmission and recombination events have been reported for different adenoviruses (AdV) (Walsh et al. 2010; Chen et al. 2011; Chiu et al. 2013; Robinson et al. 2013; Yu et al. 2013). Close contact between the infected animal and human care takers was consistently reported as a major risk factor for the host switch. Although most AdV infections are asymptomatic and self-limiting in human and animal hosts, AdV-induced diseases occur. In humans, these include gastroenteritis, keratoconjunctivitis and pneumonitis (Harrach et al. 2008). Bacterial co-infection, young age and immunosuppression enhance the risk to develop severe symptoms (Kojaoghlanian et al. 2003; Echavarria 2008). AdV have been detected worldwide (Horwitz and Wold 2007), but little is known about prevalence, epidemiology, and phylogeny of AdV in humans and animals living in remote regions, such as the Taï region in Western Côte d`Ivoire. The population in the Taï region largely consists of breeders, cultivators and hunters, hence contact to livestock and wildlife is frequent and intense. It is thus the perfect environment to investigate whether overlapping human and animal habitats results in AdV transmission between humans and domestic animals.

As AdV prevalence and diversity have already been described for humans and wild non-human primates of the region (Wevers et al. 2011; Pauly et al. 2014), the present study focused mainly on AdV in domestic animals.

MATERIALS

In 2012, fecal swabs from 189 humans (Pauly et al. 2014) and rectum/cloacal swabs from 306 domestic animals were collected in the Taï region in Western Côte d`Ivoire, situated next to the protected rain forest of the Taï National Park. A basic clinical examination was performed by a trained medical professional and a veterinarian, respectively. When necessary, free treatment was provided. Among the 304 animals screened for AdV were 14 cows, 58 dogs, 60 goat, 7 monkeys, 24 pigs, 50 sheep and 91 chickens. Moreover 17 rats were caught in the villages, mainly inside the human habitations, and tissue samples were obtained during full necropsies, carried out under extensive safety precautions.

The people living at the park boundary are predominantly subsistence hunters, pastoralists and cultivators. Many rear livestock (mainly ruminants, chickens and pigs) for personal consumption, but also as potential cash reserve or as store of wealth and insurance. Thus animal health directly influences human health, since the loss of an animal entails not only loss of protein provision, but also of the cash reserve required in emergency situations (e.g. need for medical treatment). Most of the animals roam freely through the villages, feed on human waste and leftovers and often share water supply with the local population. There is frequently no clear separation between cooking, cleaning, washing and slaughtering area. During the days, cows and on occasion also small ruminant herds are moved by the farmers in search of fresh pasture and water. In several villages, animals are confined overnight in simple pens or enclosures built from local materials. Especially young piglets are regularly kept in small sheds during fattening. Some keep multiple animal species in the same

restricted area for commercial purposes. Dogs in these rural communities serve primarily as hunting animals or as protection for the properties. Most of these animals most likely have never received any vaccinations or primary health care.

An individual study number was assigned to every participant in order to protect privacy. Written informed consent was obtained from every study participant before sampling and the collection was performed according to the declaration of Helsinki. The sampling missions were approved in November 2010 by the ethic commission “Comité national d’éthique et de la recherche (CNER)” from the “Ministère de la santé et de l’hygiène publique - République de Côte d`Ivoire” (permit number 101-10/MSHP/CENR/P). Sampling of domestic animals was done according to the Directive 86/609/EEC on the Protection of Animals Used for Experimental and Other Scientific Purposes. The permit for sampling of domestic animals was issued by LANADA/LCPA, Laboratoire national d`appui au développement agricole/Laboratoire Nationale de la Pathologie Animale, Bingerville, CI.

The DNA extraction from human fecal samples was performed at LANADA/LCPA using the roboklon stool kit (roboklon, Berlin, DE), according to the manufacturer`s instructions. All samples were transferred to a -80°C freezer in Côte d’Ivoire and transported to Germany on dry- ice. DNA extraction from the rectum/cloacal swabs of animals was performed at the Robert-Koch Institute (RKI) in Germany using the Qiagen Blood and Tissue Kit (Qiagen, Hilden, DE), according to the manufacturer`s instructions.

METHODS

PCR:

Two approaches for the investigation of zoonotic transmission, i.e., the detection of animal-derived AdV in human samples were applied. First, the consensus Animal-HEX-PCR (see below) together with

129 blocking primers against *Human mastadenovirus D* (HAdV-D) (Vestheim and Jarman 2008), the
130 common human AdV in the region (Pauly et al. 2014), was applied (Blocking-HAdVD PCR) to
131 selectively mask human HAdV-D DNA during the amplification process (*Table 1*). Degenerate blocking
132 primers were designed based on an alignment of the hexon genes from a selection of animal AdV
133 and the HAdV-D sequences, which were detected in humans from the investigated area (Pauly et al.
134 2014). The specificity of these blocking primers was tested on animal samples, which had already
135 been tested positive for animal AdV with the Animal-HEX-PCR and on HAdV-D positive human fecal
136 samples. After optimization, we opted out for a 5-fold excess of the blocking primers compared to
137 the hexon-primer (ratio 5:1) and blocking primers were added at each step of the semi-nested PCR
138 (as opposed to using them in only one of the steps of the semi-nested PCR). Cycling was performed
139 as follows: activation of the polymerase at 95°C for 12 min and 45 cycles of denaturation (95°C, 30 s),
140 annealing (56°C, 30 s), and elongation (72°C, 2min), final elongation at 72°C for 10 min. Second, all
141 human samples were tested with primers specifically targeting fowl AdV (FAdV) and ruminant AdV
142 (Short HEX-FAdV PCR and Ruminant-HEX PCR, respectively) *(Table 1)*.
143 To estimate the diversity of AdV shed by domestic animals, different primer pairs were applied.
144 All mammalian samples were initially tested with a generic semi-nested PCR that targets the hexon
145 gene of all mastadenoviruses *(*Animal-HEX PCR) and all chicken with a generic nested PCR that
146 targets the hexon gene of all fowl AdV (Short HEX-FAdV PCR). Both PCR systems were established
147 using supernatant fluid of FAdV-A Celo or FAdV-1 and bovine AdV-3-infected cells (kindly provided by
148 Mohamed H. Hafez, Freie Universität Berlin, and Balázs Harrach, Hungarian Academy of Science).
149 Longer AdV genome fragments from the positive samples were obtained with long-distance (LD) PCR
150 (LD-HEX-MastAdV PCR and Long HEX-FAdV PCR, respectively) (Meulemans et al. 2001; Lehmkuhl and
151 Hobbs 2008) *(Table 1)*.
152 PCRs were performed as previously described *(Table 1)* (Meulemans et al. 2001; Lehmkuhl and
153 Hobbs 2008; Wevers et al. 2011; Pauly et al. 2014). All AdV PCR products were purified using the
154 purification kit, MSB® Spin PCRapace (Stratec Molecular, Birkenfeld, DE), or purified using the gel
155 extraction kit, Invisorb® Spin DNA Extraction Kit (Stratec Molecular, Birkenfeld, DE), according to the

manufacturer`s instructions, and sequenced using the Big Dye Terminator v3.1 system (Life Technologies, Grand Island, NY) on an ABI PRISM 3730xl capillary sequencer (Life Technologies, Grand Island, NY).

Phylogenetic analysis

All sequences obtained were cleaned and assembled in Geneious v6.1.6. After BLAST confirmation of their identity, these sequences were added to a dataset consisting of homologous AdV sequences available in Genbank. They were aligned with the ClustalW multiple alignment method (Thompson et al. 1994). As it has been shown that the removal of poorly aligned regions from an alignment increases the quality of subsequent analyses, conserved blocks from the alignment were selected, using Gblocks as implemented in SeaView v4 or online (Castresana 2000; Talavera and Castresana 2007; Gouy et al. 2010). With default settings in the Recombination Detection Program v.4.16 (RDP4), potential recombination events were analyzed (Martin et al. 2005; Martin et al. 2010). No strong signal for recombination could be revealed for the datasets (data no shown).

For every alignment the best-fit model of nucleotide substitution given the data was selected statistically using jModelTest v2 (Darriba et al. 2012). Phylogenetic analyses were performed on two separate datasets respectively gathering sequences from AdV likely to belong to the genera *Aviadenovirus* and *Mastadenovirus (Table 2)*. For every analysis, Bayesian, as well as maximum-likelihood phylogenetic approaches were used. Maximum likelihood phylogenies were estimated using the PhyML online web interface (Guindon and Gascuel 2003; Gouy et al. 2010). Tree search was performed using the SPR&NNI algorithm. Each analysis was started with 5 random trees. Both topology and branch length were optimized in order to maximize the likelihood. The reliability of internal branches was assessed using nonparametric bootstrap with 1000 pseudo-replicates. Bayesian phylogenies were estimated using BEAST v1.8.0. (Drummond et al. 2012; Bouckaert et al. 2014). A relaxed lognormal molecular clock was chosen to model rate heterogeneity among lineages. The prior assumption of a constant population size throughout the time spanned by the genealogy was specified. Two to three MCMC were run and convergence as well as appropriate sampling sizes

were assessed using Tracer v1.5 (combined effective sample sizes of >200). Separate run outputs were combined using Logcombiner v1.8.0. A maximum clade credibility (MCC) tree was generated from this combination using Treeannotator v1.8.0. As both inference methods resulted in similar trees, only the Bayesian trees are depicted in this article and only posterior probability values of >0.95 are shown.

Species delineation analysis

The Generalized Mixed Yule Coalescent (GMYC, (Pons et al. 2006)) method was applied on an alignment comprising sequences of at least one FAdV isolate of every recognized FAdV serotype (n = 41) and the study sequences (n=22). In a first step, a Bayesian MCMC analysis was performed as described above with the evolutionary model HKY+G previously selected with jModelTest v2 (Darriba et al. 2012). Delineation analysis was conducted in R (R-Core-Team 2014) with the package "splits" (Fujisawa and Barraclough 2013) using the single threshold approach.

Genbank Accession Numbers:

FAdVD_CDAO107: KP274018; FAdVD_CKEI522: KP274019;
FAdVD_CKEI519: KP274020; FAdVD_CZAI444: KP274021; FAdVD_CDAO120: KP274022;
FAdVD_CDAO164: KP274023; FAdVE_CGOU266: KP274024; FAdVE_CGOU267: KP274025;
FAdVE_CGOU234: KP274026; FAdVE_CPON002: KP274027; FAdVE_CDAO197: KP274028;
FAdVB_CGOU223: KP274029; FAdVE_CPAU281: KP274030; FAdVE_CZAI462: KP274031;
FAdVE_CPON012: KP274032; FAdVE_CZAI445: KP274033; FAdVB_CGOU224: KP274034;
FAdVB_CPON047: KP274035; FAdVB_CPAU286: KP274036; FAdVB_CPON040: KP274037;
FAdVE_CDAO182: KP274038; FAdVC_CDAO110: KP274039; DGAH350_HAdVD: KP274040;
DGOU241_HAdVF: KP274041; DGOU263_HAdVD: KP274042; DKEI526_HAdVD : KP274043
DPON033_HAdVC: KP274044; GGAH386_caAdV2: KP274045; GTAI086_HAdVD: KP274046;
GZAI440_caAdV2: KP274047; MTAI277_SAdV: KP274048; PGAH389_HAdVD: KP274049;

PGAH389_PAdV: KP274050; PGOU244_PAdV3: KP274051; PKEI502_PAdV3: KP274052; SPAU316_ovAdV4: KP274053

RESULTS

Human fecal samples were tested for the presence of animal AdV. Two different PCR approaches were used to ensure detection of animal AdV even from samples with low AdV copy numbers, and to simultaneously avoid the amplification of the related and highly prevalent human AdV (HAdV-D). From the 189 human fecal samples, animal AdV sequences (fowl AdV and animal derived mastadenoviruses) could not be reproducibly identified.

In the mammalian samples, an average AdV prevalence of 21.7 % (50/230; 95% CI 16.6-27.6%) was obtained with the Animal-HEX PCR. More specifically, AdV were detected in 28 % (16/58) of dogs, 18 % (9/50) of sheep, 17 % (10/60) of goat, 7 % (1/14) of cows, 38 % (9/24) of pigs, 24% (4/17) of rats and 14 % (1/7) of monkeys. Among the AdV types detected in mammals were typical caprine (n=4, 8%), porcine (n=6, 12%), simian (n=2, 4%), murine (n=4, 8%) and ovine (n=14, 28%), but also different human types (n=17, 34%). For some AdV-positive samples, AdV species identification failed as the chromatograms were of bad quality. Several spill-over events in domestic animals were identified by BLAST analysis: ovine AdV sequences were amplified from goats, caprine AdV sequences from sheep, ovine AdV sequences from dogs and a porcine AdV sequence from a cow. Moreover, human AdV were detected in rectum swabs of pigs, dogs, goats and sheep. No canine AdV could be identified among the different detected AdV types in dogs.

In spite of numerous trials, the amplification of the nearly complete hexon gene with the LD-HEX-MastAdV PCR was only successful for a small proportion of the previously detected AdV (28.8 %). In total, we obtained 15 AdV sequences: 1 from a sheep, 3 from goats, 4 from pigs, 1 from a monkey, 1 from a rat and 5 from dogs. The sequences were assigned to the following AdV species: ovine AdV-5

(n=1), caprine AdV-2 (n=2), porcine AdV-3 (n=3), murine AdV-2 (n=1), HAdV-2 (HAdV-C) (n=1), HAdV-36, -49, -67, -25, -32 (HAdV-D) (each n=1) and HAdV-41 (HAdV-F) (n=1). For the simian AdV detected, species or type assignment was not feasible as the pairwise observed genetic distance of the amino acid sequence to every known simian AdV was at least 12.5%. Most of the human AdV sequences detected from the animal rectal swabs were highly similar to already published HAdV types, but – surprisingly – not to those previously characterized in local human populations (Pauly et al. 2014).

The phylogenetic tree confirmed and reinforced the findings from the BLAST analysis *(Figure 1)*: except for MTAI277 (detected in a *Cercopithecus mona)*, the study sequences clustered within the different recognized human and animal AdV species clades.

In the first screenings for AdV of fowl with the very sensitive nested Short HEX-FAdV PCR, 42.9% of the cloacal swabs from chickens were FAdV positive (39/91; 95% CI 33-53%). FAdV positivity was confirmed for 71.8% (28/39) of the tested samples with the Long HEX-FAdV PCR. 79% (22/28) presented a good chromatogram quality and phylogenetic and species delineation analyses were based on these sequences. Fowl aviadenovirus B (FAdV-B) (n=4), C (FAdV-C) (n=1), D (FAdV-D) (n=10) and E (FAdV-E) (n=13) were identified with the Long HEX-FAdV PCR. No FAdV-A member (FAdV-1) was detected. In 36% (10/28) of the cases, the AdV detected with the short and Long HEX-FAdV PCR systems differed.

With the FAdV sequences obtained with the Long HEX-FAdV PCR a phylogenetic tree was constructed (*Figure 2*). Five well supported monophyletic clusters, representing the 5 fowl AdV species, were identified. The FAdV-5 strain TR22 was only distantly related to these species. While most of the study sequences formed sister groups with recognized FAdV types, others were located on separate branches and were thus only distantly related to recognized types. The clustering of most of the study sequences with the recognized FAdV was supported by high pp values (pp>0.95) and thus species assignment based on the tree topology was feasible. Consistent results were obtained when species and type assignment was made according to the demarcation criteria proposed by Marek et al, 2010 (Marek et al. 2010).

In order to make statistically supported statements on the FAdV species circulating in the Taï region and to elucidate the diversification process of FAdV, species delineation analysis was performed applying the Generalized Mixed Yule Coalescent (GMYC) method (with a single threshold; (Fujisawa and Barraclough 2013)). The best model of species delineation had a significantly higher likelihood than the null model which assumes all sequences were sampled from a single species (p-value=0.04). According to this model, the number of clusters (species) was estimated to be 12 (confidence interval: 3-16). The only recognized species, which was monophyletic and for which the species delineation analysis confirmed the classification into a single species was FAdV-A (AIC weight = 0.9). The other previously recognized FAdV species were divided into several novel evolutionary entities (possibly species). Furthermore, some of the study sequences (e.g. those from the samples CDAO110, CPON012, CDAO120) clustered in one of the newly defined species with recognized FAdV types, whilst others stand on a separate branch, identified as sole types of separate new species by the GMYC method (e.g. CDAO182, CGOU223, CZAI402) *(Figure 3)*. The Japanese strain TR22 (presently FAdV-5) was recognized as a member of a separate species (AIC weight <0.1). Partly consistent with the species subdivision proposed by Marek et al, 2010 (Marek et al. 2010), the species identified here were named by adding a number to the presently used species letter (e.g. FAdV-B1 to B3) (*Figure 3*).

DISCUSSION

Despite frequent exposure of the local population in Côte d`Ivoire to blood, organs and feces of AdV-infected domestic animals (this study) and NHP (Wevers et al. 2011), there was no evidence for zoonotic transmission of AdV. This finding alone reinforces the notion that AdV are predominately host-specific; and possibly that their main mode of evolution is through host-driven vicariance (Benkö and Harrach 2003; Davison et al. 2003). Nevertheless, as we detected HAdV in different

animal species, interspecies transmission of AdV certainly occurs, but appears to often result in evolutionary dead-ends. Several factors might have contributed to the occasional transmission of HAdV to animals and the absence of reverse transmission. AdV are so stable in the environment that they are often used to trace fecal environmental contamination and evaluate water quality (Sibley et al. 2011). Hence animals are probably continuously exposed to objects contaminated with human feces including infectious AdV (and likely other viruses). In comparison, the amount of infectious virus ingested by humans might be reduced, as animal-derived food is typically stewed for hours and intense contact with animal feces might be limited to butchers. In addition, the human access to hygienically improved drinking water in the region could be considered as possible explanation. Several covered and well-maintained wells were recently constructed in different villages. This certainly benefitted the local population by reducing the risk for water contamination with animal waste or rainwater and might have contributed to decrease indirect transmission.

Another explanation for the obvious lack of non-human mammalian AdV in human feces could come from the applied detection method. The blocking primers in our study were designed to be HAdV-D specific. However, it cannot be excluded that they blocked amplification of other AdV species, leading to false-negative results. However, as the negative result was confirmed with species-specific PCR systems, unintentional blocking of ruminant and fowl AdV is unlikely. False negative results due to inhibitors in animal rectal swabs samples should also be taken into account. For the human samples however, the extraction kit was specifically chosen to effectively remove inhibitors, the influence on the results should be insignificant. Furthermore, the limited sample number might have resulted in an underestimation of the circulating AdV diversity and AdV prevalence obtained from a larger dataset would possibly more correctly mirror the actual situation in the study region. Because of incongruence in detection method, study population and study design, direct comparison of our cross-sectional study to studies from other regions of the world (*Supplementary Table 1*) was not feasible.

With the help of the LD PCR further characterization of the identified AdV was achievable. Reasons for the low success rate of LD amplification compared to the rates obtained with generic

313 PCR (32%; 15 out of 46) were probably poor sample quality and/or insufficient system sensitivity.
314 Observed pairwise distance and phylogenetic analyses revealed that the detected animal AdV might
315 represent novel types of recognized AdV species (*Figure 1)*. One criterion for species designation
316 requests 5-15% of amino acid sequence difference (Harrach et al. 2011). Hence there is strong
317 evidence that the sole detected SAdV might not only be a new type related to HAdV-F, but the first
318 isolate of a novel species (pairwise observed genetic distances to every known simian and human
319 AdV was >10%). Closely related to this new SAdV/HAdV-F clade was SAdV-18, which was detected
320 previously from fecal samples of asymptomatic rhesus macaques (*Macaca mulatta*). Of note, SAdV-
321 18 also shares molecular characteristics (e.g. fiber sequence) with the HAdV-F types. These findings
322 were interpreted as indication for probable cross-species transmission of SAdV-18 between humans
323 and monkeys (Roy et al. 2012). The SAdV sequence in the current study was detected from a mona
324 monkey (*Cercopithecus mona)*, belonging to the habituated mona group living in Taï village. Close
325 contact between caring humans and captive NHP in research centers or sanctuaries has already
326 resulted in cross-species transmission of AdV (Chen et al. 2011; Wevers et al. 2011).
327 The local husbandry conditions and the inexistent separation between human and animal habitats
328 increase the risk for fecal contamination of the environment and of open water bodies. One
329 consequence might be the shedding of both animal and human AdV by small ruminants, pigs and
330 dogs *(Figure 3)*. Cross-species transmissions of AdV between different ruminant species have been
331 reported before (Belák and Pálfi 1974; Barbezange et al. 2000; Lehmkuhl and Hobbs 2008; Intisar et
332 al. 2010). The present study provides concordant evidence in favor of common cross-species
333 transmission of AdV between ruminants and the more distantly related dogs. Whether animals
334 shedding HAdV can be considered to be an intermediate reservoir of HAdV or mixing vessels for the
335 appearance of recombinants between HAdV and animal AdV or whether the shedding results from
336 passive passage only due to the ingestion of contaminated material, is not clear. All these scenarios
337 can be of importance for human health. Knowing that most of the animals have no restricted
338 territory, animals excreting AdV might contribute to the virus spread within, but also between
339 villages. Thus, one might consider them not as AdV host, but as AdV vector or carrier. By shedding

HAdV, animals would contribute to the maintenance of infection in humans and to the spread of HAdV in the region. A similar scenario has been described for other zoonotic pathogens (Duffy and Moriarty 2003; Rimmelzwaan et al. 2006). Moreover, it is possible that HAdV evolve in the unintentional animal host and spill back to humans. Particularly dogs and pigs are suspected to play a role as amplifier hosts or mixing vessels, as they seem to be susceptible to viruses from different species. Pigs have been identified as amplifiers for different human infections and have been implicated in severe disease outbreaks (e.g. Nipah virus) (Chua et al. 2000). It has been assumed that pathogens accumulate in carnivore and scavenger species (e.g. dogs), as they feed on various prey species and hence are exposed to many pathogens circulating in the prey population (Cleaveland et al. 2006; Halliday et al. 2012). This accumulation effect could also explain the high diversity of AdV species detected in dogs and pigs. Moreover, the susceptibility to pathogens from diverse host might favor coinfection with different virus types and subsequent genetic recombination. Mixed infections with different AdV types have been repeatedly observed in the present study and might be a common feature of AdV. Surprisingly, none of the “animal” HAdV-D types was identical to the types found in the local population. However, profound conclusions with regard to transmission ways cannot be drawn from this observation, as only a small proportion of the HAdV-D circulating in animals and humans in the study region were compared and hence shedding of identical HAdV-D types might have been overlooked. The application of quantitative PCR on diverse sample materials from animals, shedding human and animal AdV, would be of interest to draw conclusions as to infection progression, severity and to virus distribution. One might reconsider the standard approach that the natural host of an AdV is necessarily the host in which the AdV was first detected. Moreover, the results of species delineation analysis challenge the presently recognized species concept within the *Aviadenovirus* genus, which is mainly based on serological and biological properties *(Figure 2)*. It may prove beneficial if precise criteria for typing of animal AdV based on genomics would be proposed to permit type identification when the recognized reference methods are not applicable, particularly as currently many studies focus on phylogenomics (Ojkic et al. 2008; Mase et al. 2009;

366 Steer et al. 2009; Marek et al. 2010; Lim et al. 2011; Kajan et al. 2013). The Human AdV Working
367 Group supports already such an approach for HAdV (Brister et al. 2009).
368
369

370 **CONCLUSION**

371

372 Our results provide evidence that habitat overlap among humans, livestock, and wildlife can
373 influence pathogen transmission ways and facilitate especially the cross-species transmission of
374 environmentally stable pathogens, such as AdV. Even if the pathogenicity of AdV is limited, these
375 widespread viruses might represent a valuable tool to assess the risk for cross-species transmission
376 of more pathogenic viruses in regions with high opportunity for animal-to-human exposure.
377 Furthermore, this study underlines the thus far underestimated importance of studies investigating
378 reverse zoonotic transmission of viruses and the role of domestic animals as “bridge species” or
379 intermediate hosts.

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Table 1. Description of the applied PCR systems

PCR name	Primer name	Primer sequence (5`-3`)	Primer type	Target AdV	Target gene	Annealing temperature (C°)	Product length (bp)	Designed by
Animal-HEX PCR	6500s	CGCAGTGGKCNTWCATGCACAT	genus-specific degenerate consensus	MastAdV	Hexon gene	56	292	
	6500as	GTGCCGGTGTANGGYTTRAA						
	6501s	ACCCACGAYGTSACNACNGA				56	160	
Blocking-HAdVD PCR	6542s	CGCAGTGGGCGTACATGCACATCGCCG GGCAGGACGCCTCG--C3spacer	Blocking primer	Blocking primer for HAdV-D	Hexon gene	56		
	6543s	ACCCACGATGTGACCACGGACCGGTCC CAGCGT--C3spacer						
LD-HEX-MastAdV PCR	6676s	ATGGCKACSCCKTCGATG	genus-specific degenerate consensus	MastAdV	Hexon gene	45	2690	(Lehmkuhl and Hobbs 2008)
	6676as	GGTRGCGTTSCCGGCBGA						
	6677s	GATGMTGCCGCARTGGTC				45	2673	
	6677as	CGTTSCCGGCBGAGAA						
Short HEX-FAdV PCR	6569s	CCTTCTTTTAAACCnTACkGnGGmAC	species-specific degenerate	FAdV	Hexon gene	45	390	
	6569as	CGAGGCGCTAWAkTCYTCNAC						
	6570s	GGAGGAACSGCNTAYAAYCC				45	306	
	6570as	AAGGAGTGGGGTTAADNGAYTGNGA						
Long HEX-FAdV PCR	6708s	CAARTTCAGRCAGACGGT	species-specific degenerate	FAdV	Hexon gene	54	862	(Meulemans et al. 2001)
	6708as	TAGTGATGMCGSGACATCAT						
	6709s	SKCSACYTAYTTCGACAT				54	547	
	6709as	TTRTCWCKRAADCCGATGTA						
Ruminant-HEX PCR	6856s	TGATCCTTACTTTACTTATTCDGGCACT	ruminant-specific degenerate	ovine and caprine AdV	Hexon gene	61	394	
	6856as	GTAAGGGTGGCCTTCYCTDGGYA						
	6857s	AGTGTCTATTCAGTTTGAYTCTTCDGTW CA				61	286	
	6857as	GTTACCAAAAGCDGTAAAKCCDGA						

Table 2. Description of the phylogenetic analyses

Name of tree	PCR applied	Number of taxa	Lenght of alignment	Evolution model selected	Amount of Boostrap replicates	Figure N°
Phylogenetic tree of Mastadenovirus	LD-HEX-MastAdV PCR	39	1856	GTR+I+G	1000	Figure 1
Phylogenetic tree of the FAdV	Long HEX-FAdV PCR	57	469	HKY85+G	1000	Figure 2 and 3

Figure 1. Maximum clade credibility tree of mastadenoviruses

Bayesian analysis of a 1856 bp long alignment of nearly complete hexon gene sequences, comprising at least one reference strain of every *Mastadenovirus* species. The alignment comprised adenovirus sequences identified in this study and recognized reference strains from Genbank. The reference strains are represented by host name, type and Genbank accession number. The study sequences are in red and the animal host can be retrieved from the first letter (P=pig, G=goat, S=sheep, M=monkey, D=dog). Adenovirus is abbreviated to AdV. Posterior probabilities are plotted and considered a measure of branch robustness (well supported branches exhibit pp>0.95). This tree was built under a clock model and therefore is rooted. The pictograms represent the different hosts from which the AdV were detected. The two AdV marked by a red star were detected in the same sample.

Figure 2. Maximum clade credibility of fowl adenoviruses

Bayesian analysis of a 471 bp long alignment of partial hexon gene sequences, comprising at least one reference strain of every fowl adenovirus species and sequences identified in this study. The reference strains are represented by host name, type and Genbank accession number. The study sequences are in red and were all detected from chicken cloacal swabs. Adenovirus is abbreviated to AdV. Posterior probabilities are plotted and considered a measure of branch robustness (well supported branches exhibit pp>0.95). This tree was built under a clock model and therefore is rooted. The colored boxes represent the different recognized fowl adenovirus species.

Figure 3. Species delineation analysis of fowl adenoviruses

Bayesian analysis of a 471 bp long alignment of partial hexon gene sequences, comprising at least one reference strain of every fowl adenovirus species and sequences identified in this study. The reference strains are represented by host name, type and Genbank accession number. The study

sequences were all detected from chicken cloacal swabs (code: first letter “C”). Adenovirus is abbreviated to AdV. The delineation analysis was run applying the Generalized Mixed Yule Coalescent (GMYC) method. GMYC species are highlighted in red. Akaike information criterion weights are shown for all GMYC species. The colored boxes represent the different recognized adenovirus species. On the right side, the GMYC species are further highlighted by thick vertical lines

AdV detected in:

sheep

dog

monkey

goat

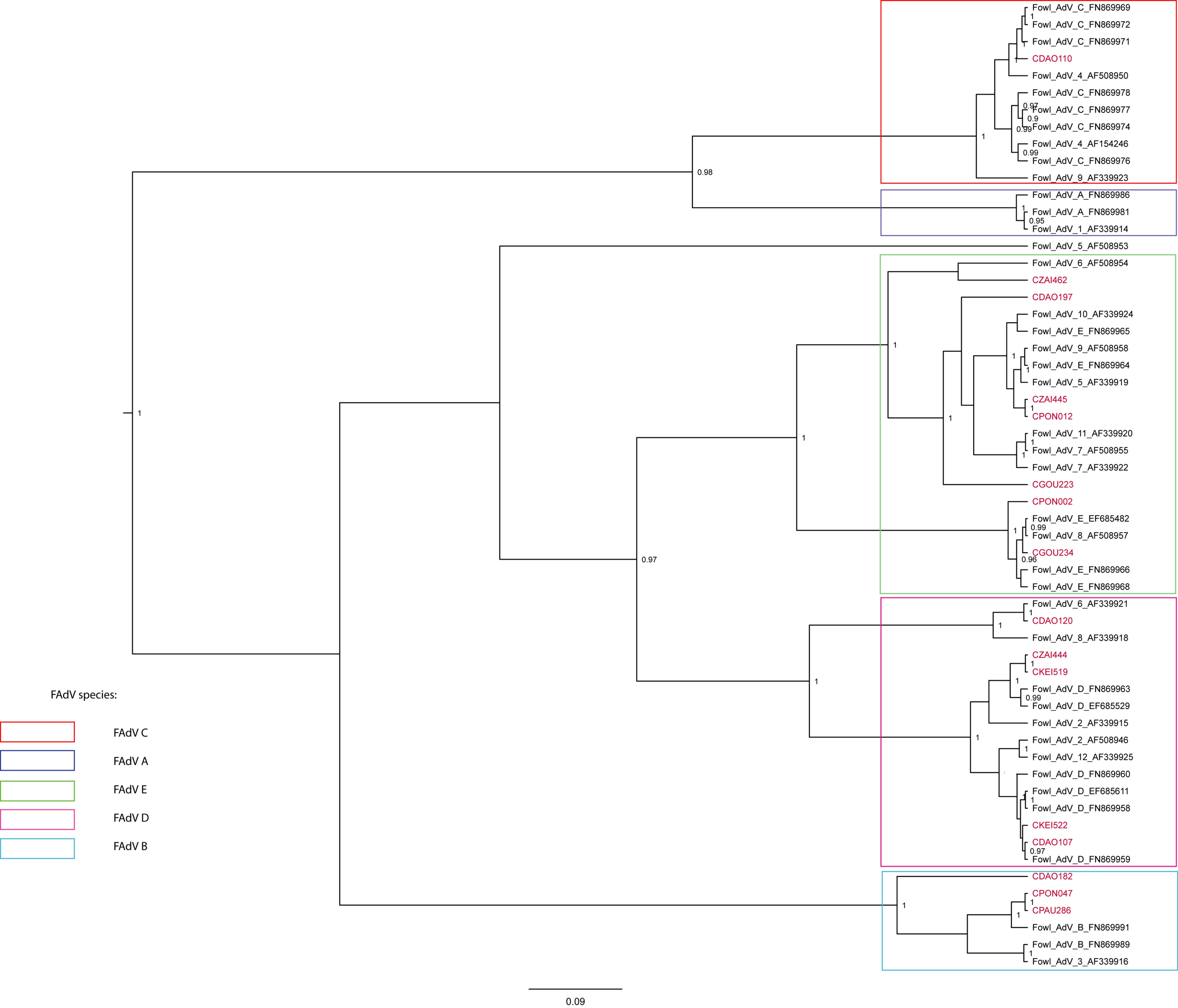
pig

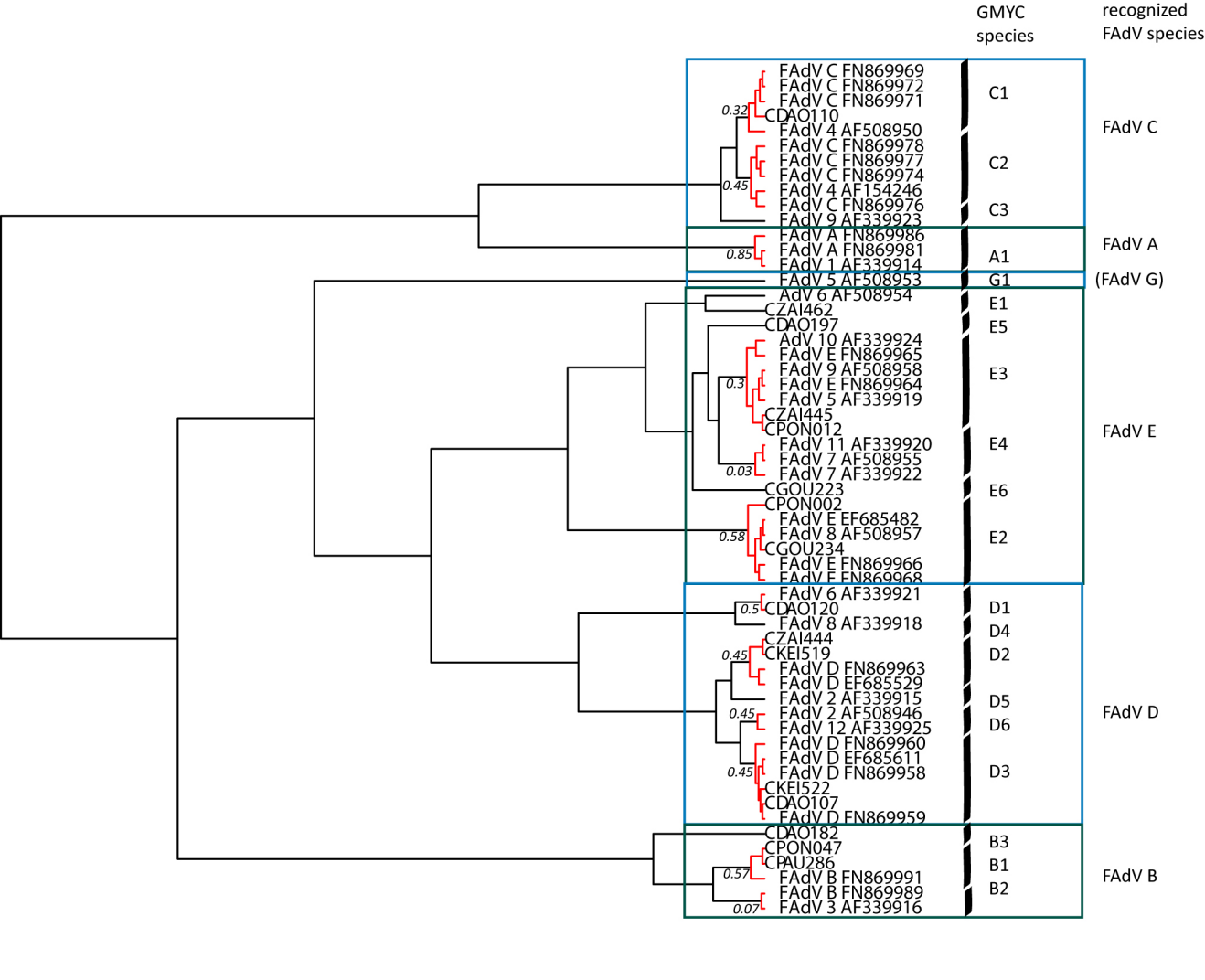
rat

AdV detected in identical sample:

Human_AdV_C_AC_000007
DPON033
Human_AdV_B_DQ105654
Chimpanzee_AdV_Y25_JN254802
DGOU263
PGAH389_a
DKEI526
Human_AdV_D_KF268325
DGAH350
DGOU241
Human_AdV_F_AB610521
MTAI277
Simian_AdV_18_FJ025931
Simian_AdV_A1258_JN880451
Human_AdV_52_DQ923122
Human_AdV_A_AC_000005
Simian_AdV_3_NC_006144
Porcine_AdV_5_AF289262
Ovine_AdV_2_DQ630755
Bovine_AdV_2_AF252854
Ovine_AdV_3_DQ630756
Ovine_AdV_5_DQ630758
SPAU316
Ovine_AdV_4_DQ630757
Bovine_AdV_A_AC_000191
GGAH386
Ovine_AdV_1_DQ630754
Caprine_AdV_2_DQ630760
Tree_shrew_AdV_1_AC_000190
PGAH389_b
PGOU244
Porcine_AdV_3_AC_000189
PKEI502
Bovine_AdV_B_AC_000002
Canine_AdV_1_AC_000003
Bat_AdV_TJM_GU226970
Equine_AdV_1_JN418926
Murine_AdV_A_AC_000012
Murine_AdV_2_HM049560
RDAO132

0.1


Fowl_AdV_C_FN869969
Fowl_AdV_C_FN869972
Fowl_AdV_C_FN869971
CDAO110
Fowl_AdV_4_AF508950
Fowl_AdV_C_FN869978
Fowl_AdV_C_FN869977
Fowl_AdV_C_FN869974
Fowl_AdV_4_AF154246
Fowl_AdV_C_FN869976
Fowl_AdV_9_AF339923
Fowl_AdV_A_FN869986
Fowl_AdV_A_FN869981
Fowl_AdV_1_AF339914
Fowl_AdV_5_AF508953
Fowl_AdV_6_AF508954
CZAI462
CDAO197
Fowl_AdV_10_AF339924
Fowl_AdV_E_FN869965
Fowl_AdV_9_AF508958
Fowl_AdV_E_FN869964
Fowl_AdV_5_AF339919
CZAI445
CPON012
Fowl_AdV_11_AF339920
Fowl_AdV_7_AF508955
Fowl_AdV_7_AF339922
CGOU223
CPON002
Fowl_AdV_E_EF685482
Fowl_AdV_8_AF508957
CGOU234
Fowl_AdV_E_FN869966
Fowl_AdV_E_FN869968
Fowl_AdV_6_AF339921
CDAO120
Fowl_AdV_8_AF339918
CZAI444
CKEI519
Fowl_AdV_D_FN869963
Fowl_AdV_D_EF685529
Fowl_AdV_2_AF339915
Fowl_AdV_2_AF508946
Fowl_AdV_12_AF339925
Fowl_AdV_D_FN869960
Fowl_AdV_D_EF685611
Fowl_AdV_D_FN869958
CKEI522
CDAO107
Fowl_AdV_D_FN869959
CDAO182
CPON047
CPAU286
Fowl_AdV_B_FN869991
Fowl_AdV_B_FN869989
Fowl_AdV_3_AF339916
0.98
0.97
0.09
FAdV species:
FAdV C
FAdV A
FAdV E
FAdV D
FAdV B


GMYC species
recognized FAdV species
FAdV C FN869969
FAdV C FN869972
FAdV C FN869971
CDAO110
FAdV 4 AF508950
FAdV C FN869978
FAdV C FN869977
FAdV C FN869974
FAdV 4 AF154246
FAdV C FN869976
FAdV 9 AF339923
FAdV A FN869986
FAdV A FN869981
FAdV 1 AF339914
FAdV 5 AF508953
AdV 6 AF508954
CZAI462
CDAO197
AdV 10 AF339924
FAdV E FN869965
FAdV 9 AF508958
FAdV E FN869964
FAdV 5 AF339919
CZAI445
CPON012
FAdV 11 AF339920
FAdV 7 AF508955
FAdV 7 AF339922
CGOU223
CPON002
FAdV E EF685482
FAdV 8 AF508957
CGOU234
FAdV E FN869966
FAdV E FN869968
FAdV 6 AF339921
CDAO120
FAdV 8 AF339918
CZAI444
CKEI519
FAdV D FN869963
FAdV D EF685529
FAdV 2 AF339915
FAdV 2 AF508946
FAdV 12 AF339925
FAdV D FN869960
FAdV D EF685611
FAdV D FN869958
CKEI522
CDAO107
FAdV D FN869959
CDAO182
CPON047
CPAU286
FAdV B FN869991
FAdV B FN869989
FAdV 3 AF339916
0.32
0.45
0.85
0.3
0.03
0.58
0.5
0.45
0.45
0.45
0.57
0.07
C1
C2
C3
A1
G1
E1
E5
E3
E4
E6
E2
D1
D4
D2
D5
D6
D3
B3
B1
B2
FAdV C
FAdV A
(FAdV G)
FAdV E
FAdV D
FAdV B

SUPPLEMENTARY FILE

Supplementary Table 1. Prevalence of AdV in animals around the world

Animal species	Sample origin	AdV species	Detection method	Prevalence	Reference
arctic foxes	Norway	canine	serology	38%	(Akerstedt et al. 2010)
bats	Kenya	bat	PCR	2%	(Conrardy et al. 2014)
	China	bat	PCR	8%	(Li et al. 2010)
	Hungary	bat	PCR	5%	(Janoska et al. 2011)
bonobos	different countries	different	PCR	46%	(Roy et al. 2009)
camel	Sudan	bovine	serology	90%	(Intisar et al. 2010)
cats	Czech Republiy	feline	serology	35%	(Lakatos et al. 1999)
cattle	USA	bovine	PCR	13%	(Sibley et al. 2011)
	USA	bovine	serology	82%	(Lehmkuhl and Cutlip 1999)
	Nigeria	different	serology	4%	(Obi and Taylor 1984)
	Zambia	bovine	serology	87%	(Ghirotti et al. 1991)
	Zaire (DRC)	bovine	serology	44%	(Jetteur et al. 1988)
	Spain	bovine	PCR	75%	(Maluquer de Motes et al. 2004)
	Irland	bovine	serology	55%	(Adair et al. 1996)
chimpanzee	different countries	different	PCR	63%	(Roy et al. 2009)
dogs	South Africa	canine	serology	50%	(Wright et al. 2013)
	Turkey	canine	serology	28-100%	(Gür and Acar 2009)
	Galapagos	canine	serology	67%	(Levy et al. 2008)
fox	Italia	canine	PCR	9%	(Balboni et al. 2013)
goat	Nigeria	different	serology	18%	(Obi and Taylor 1984)
	USA	caprine	serology	60%	(Lehmkuhl and Cutlip 1999)
gorillas	different countries	different	PCR	40%	(Roy et al. 2009)
horses	Nigeria	different	serology	5%	(Obi and Taylor 1984)
mink	France	canine	serology	2-10%	(Philippa et al. 2008)
monkey	China	simian	PCR	46%	(Banyai et al. 2010)
macaque	China	simian and human	PCR	46%	(Wang et al. 2007)
non-human primates	Africa	simian and human	PCR	59%	(Wevers et al. 2011)
panda	China	canine	serology	9%	(Qin et al. 2010)
pigs	Spain	porcine	PCR	70%	(Maluquer de Motes et al. 2004)
	Quebec	porcine	serology	15%	(Dea and El Azhary 1984)
red fox	Norway	canine	serology	57%	(Akerstedt et al. 2010)
sheep	Nigeria	different	serology	18%	(Obi and Taylor 1984)
	Australia		serology	71%	(Peet et al. 1990)
	USA	caprine	serology	80%	(Lehmkuhl and Cutlip 1999)
	Irland	bovine	serology	70-90%	(Adair et al. 1984)
wolves	Norway	canine	serology	68%	(Akerstedt et al. 2010)
	Yellowstone NP, USA	canine	serology	94%	(Almberg et al. 2009)

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