Original article

Unique genetic features of canine adenovirus type 1 (CAdV-1) infecting red foxes (*Vulpes vulpes*) in Northern Norway and arctic foxes (*Vulpes lagopus*) in Svalbard

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Acknowledgements

We thank all the trappers and hunters that provided the fox samples. Funding for sampling the fox tissues were provided from the Norwegian Polar Institute and financed by the Norwegian Environment Agency to Climate –ecological Observatory for Arctic Tundra (COAT). We also acknowledge Dr. Javier Sánchez Romano for help with the tissue samples and DNA extraction.

Abstract

Canine adenovirus type 1 (CAdV-1) is the aetiological agent of infectious canine hepatitis (ICH) in domestic dogs (*Canis familiaris*). In spite of the widespread use of vaccination, CAdV-1 continues to circulate in the dog population. Although a high number of serological screenings have indicated that CAdV-1 is widespread in fox species, little is known about the potential role of foxes as reservoirs of CAdV-1. Furthermore, very little data exist on the molecular features of this virus in foxes. To add to existing knowledge on CAdV-1 circulating in wild carnivores, tissue samples from CAdV-seropositive red foxes (*Vulpes vulpes*, n = 10) from the northern mainland of Norway and arctic foxes (*Vulpes lagopus*, n = 10) from the Svalbard archipelago, Norway, were investigated using a molecular approach to detect CAdV-1 DNA and important structural and non-structural genes of the detected viruses were sequenced and analysed. Amplicons characteristic for CAdV-1 were amplified from 14 out of 20 foxes (7 red foxes and 7 arctic foxes) and spleen and lymph node tissues resulted optimal targets for the viral DNA detection. The nucleotide sequences showed unique features that distinguished the viruses detected in this study from the CAdV-1 to date identified in wild carnivores and dogs. Greater attention should be given to genetically different CAdV-1 circulating in wild carnivores that may be transferred to dogs, potentially causing disease and reducing the effectiveness of available vaccines.

KEYWORDS

Canine adenovirus; fox; genetic characterization; mastadenovirus; Norway; wildlife.

Introduction

Canine adenovirus type 1 (CAdV-1) is the aetiological agent of infectious canine hepatitis (ICH) in domestic dogs (*Canis familiaris*). During the last decades, the widespread use of a modified live CAdV type 2 (CAdV-2) vaccine has greatly reduced the incidence of ICH in dogs (Decaro et al., 2008). Nevertheless, the high prevalence of CAdV infection in domestic dogs (Gür and Acar, 2009, Belsare and Gompper, 2013, Balboni et al., 2014) along with reported clinical cases of CAdV-1 (Pratelli et al., 2001, Caudell et al., 2005, Decaro et al., 2007, Headley et al., 2013, Balboni et al., 2017) support the hypothesis that CAdV-1 continues to be a circulating pathogen in dogs.

Canine AdV-1 is also widespread in wildlife, primarily as a subclinical infection, but can cause epizootics in wild carnivores belonging to the *Canidae*, *Mustelidae* and *Ursidae* families (Woods, 2001). Evidence of exposure to CAdV has been reported for different fox species in several geographic areas (McCue and O'Farrell, 1988, Truyen et al.,1998, Robinson et al., 2005, Clifford et al., 2006, Gerhold et al., 2007, Akerstedt et al., 2010, Thompson et al., 2010, Balboni et al., 2013, Walker et al., 2016; Hechinger et al., 2017) but very little data exist on the molecular features of CAdV-1 in foxes.

The possible transmission of the virus to the domestic canine population, as well as a possible transmission from dogs to wildlife populations, require detailed knowledge of the CAdV-1 strains circulating in wild carnivores. The aim of this study was to investigate the genetic features of CAdV-1 circulating in two species of foxes in Norway, the red fox (*Vulpes vulpes*) from the northern mainland and the arctic fox (*Vulpes lagopus*) from the Svalbard archipelago.

Materials and methods

Fox samples

Ten red foxes (2007 - 2009) from the low-Arctic region of Finnmark County (Norway; 70-20°N, 29-38°E; numbered from 602-01 to 602-10) and 10 arctic foxes (1997 - 2002) from the high-Arctic Svalbard archipelago (Norway; 74-81°N and 10-30°E; numbered from 603-05 to 603-14) tested positive for CAdV antibodies using an immunofluorescence test (Tryland et al., 2018) were selected (high antibody titre) for molecular analyses. A *post mortem* examination had been carried out on all foxes at the end of each hunting season, and biological samples stored at -20 °C were available for this study (Tryland et al., 2018). Histopathological examinations were not performed due to freezing and thawing of the carcasses and autolysis of tissues. Several biological matrices were tested for each fox (Table 1). From the 10 red foxes, spleen and liver samples were available, except for one animal (number 602-10) from which only liver was available. From the 10 arctic foxes, spleen, liver, kidney and mesenteric lymph node samples were available, except for individual number 603-09 and 603-11, from which we did not have access to the spleen, and the liver and lymph node, respectively.

PCR for canine adenovirus detection and amplification of hexon and fiber viral genes

Viral DNA extraction from tissues was carried out by using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Canine adenovirus screening and amplification of hexon and fiber viral genes were carried out according to Hu et al. (2001) and Balboni et al. (2017), respectively. Primers used were reported in Online Resource 1. The PCR assay used for the CAdV screening amplified the 3'-end fragment of the E3 gene and flanking regions (a subsequent non coding fragment and the U-exon gene) and was able to distinguish between CAdV-1 and CAdV-2 (Hu et al., 2001).

Sequencing and sequence analysis

PCR amplicons obtained were sequenced, assembled and translated into amino acid sequences according to Balboni et al. (2017). For foxes that showed CAdV specific PCR products in more than one organ, the amplicon that showed the highest amount of PCR product and no non-specific products, was sequenced. Assembled nucleotide sequences of partial E3 gene and flanking regions, and of hexon and fiber genes, were aligned with reference sequences (GenBank) of canine, skunk and bat adenoviruses (Table 2) using the ClustalW method implemented in BioEdit 7.2.5. The variability of the different nucleotide residues of hexon and fiber genes was evidenced using entropy (H(x)) plot function implemented in BioEdit; only the CAdV-1 reference strains, from which both hexon and fiber genes sequences were available, were used for the analysis (Table 2). The total number of polymorphic sites, the total number of

synonymous and non-synonymous differences, and the number of haplotypes were calculated on hexon and fiber genes sequences using DnaSP package version 5.10.01 (Librado and Rozas 2009). In order to evaluate the potential biological effects of the detected amino acid mutations, the immunogenicity of hexon and fiber proteins was predicted by using Protean DNASTAR Lasergene 11 package software. Antigenicity (Welling et al., 1985, Jameson and Wolf, 1988), B-cell epitopes (DNASTAR), MHC II epitopes (Sette et al.,1989), and T-cell epitopes (Margalit et al., 1987) analysis were performed on deduced hexon and fiber proteins. The analyses were performed on representative red and arctic fox viruses and CAdV-1 reference strains (Table 2). Phylogenetic relationships among the partial E3 gene and flanking regions sequences and multiple gene sequences (concatenated hexon and fiber genes sequences) were evaluated using MEGA version 7.0.26.

Results

CAdV detection and sequence analysis of partial E3 gene and flanking regions

No gross pathological changes had been observed in any of the 20 foxes. A CAdV-1-specific PCR fragment was detected in seven red foxes (7/10, numbers: 602-01, 02, 03, 04, 05, 06, and 07) and in seven arctic foxes (7/10, numbers: 603-05, 06, 07, 10, 11, 12, and 13) (Table 1). A DNA fragment of approximately 500 bp, corresponding to CAdV-1, was present in all the PCR positive foxes, whereas none of the samples generated a CAdV-2 characteristic fragment of 1030 bp (Hu et al., 2001). Nine out of the 14 PCR positive foxes showed CAdV-1 specific amplicons in all sampled organs. The remaining five foxes showed specific PCR products in at least one of the tested tissue samples (Table 1). Canine AdV-1 was detected in the spleen of all PCR positive foxes, with the exception of arctic fox 603-11 from which the spleen sample was not available. CAdV-1 was also detected in mesenteric lymph node of all the PCR positive arctic foxes (with the exception of arctic fox 603-11 from which this organ was not available). The mesenteric lymph node was not available from the red foxes (Table 1).

Nucleotide sequencing of partial E3 gene and flanking regions was performed for all the 14 CAdV-1 PCR positive individuals (Table 1; GenBank ID: MF344652-MF344665). Canine AdV-1 nucleotide sequences obtained from five arctic foxes (603-05, 10, 11, 12, and 13) were 462 bp in length, comprising the last 285 bp of the E3 gene (corresponding to the last 94 amino acid codons of E3 protein), a non-coding region of 8 nucleotides and the entire U-exon gene (168 nucleotides corresponding to 55 amino acid residues). Canine AdV-1 nucleotide sequences obtained from all the red foxes (602-01, 02, 03, 04, 05, 06, and 07) and two of the arctic foxes (603-06 and 07) were 484 bp in length, showing 22 additional nucleotides in the initial tract of the non-coding region between the E3 gene and U-exon gene (nucleotide sequence: AAA TAA ACA CTA TGG AGT TTA A).

Nucleotide alignment showed complete identity between the five CAdV-1 sequences without the additional 22 nucleotides. These sequences showed a complete identity (100%) with several CAdV-1 reference strains identified in dogs and foxes from 1966 to 2015 (Y07760, M60937, KU755713, KU755714, KU755715, KU755716, KU755718, KT853096, KT853097, KC577558, JX416838, JX416839, KF676980, KF676977, KP670422, KP670423, KP670424). Among the identified CAdV-1 sequences with the long non-coding region, an identity of 100% was showed between 602-01, 602-03, 602-05, 603-06 and 603-07, and between 602-02, 602-04 and 602-06. An identity of 94.0-95.4% was observed between the sequences with the 22 additional nucleotides and CAdV-1 reference strains. In Table 3 the nucleotide alignment of the non-coding region between E3 gene and U-exon gene of the detected CAdV-1 with representative CAdV-1, CAdV-2, SkAdV-1, BtAdV-2 and BtAdV-3 reference sequences (Table 2) is summarized. With the exception of the CAdV-1 with 22 additional nucleotides identified in this study, all CAdV-1 up to now sequenced showed the same non-coding region of 8 nucleotides in length, including a putative polyadenylation (polyA)

motif (AATAAA) (Morrison et al., 1997). Canine AdV-2, SkAdV-1, and BtAdV-2 had a non-coding region longer than 8 nucleotides. Bat AdV-3 does not have a non-coding region because U-exon gene overlaps on E3 gene. Another putative polyA motif was present in the elongated 5'-end of the non-coding region of CAdV-1 detected in this study, as well as in SkAdV-2 and BtAdV-2 (BtAdV-2 showed only this putative polyA motif). The phylogenetic tree showed that all the sequences obtained in this study belonged to the CAdV-1 group, clustering together with all CAdV-1 reference strains. Clusters based on non-coding region length, year of sampling, geographical origin and host species (dog or fox) were not evidenced (Online Resource 2).

Amplification and sequence analysis of hexon and fiber genes

PCR products specific for the hexon gene (2718 bp in length, corresponding to 905 amino acid residues) and the fiber gene (1632 bp in length, corresponding to 543 amino acid residues) were generated from six out of the 14 PCR positive foxes: one red fox (602-07; GenBank ID: MF344666 and MF344672) and five arctic foxes (603-06, 07, 10, 12 and 13; GenBank ID: MF344667-MF344671 and MF344673-MF344677) (Table 1). For the hexon gene, all the nucleotide sequences obtained from arctic foxes showed a complete identity between them and an identity of 99.9% with 602-07. An incomplete identity, but still > 99.2%, both at nucleotide and at amino acid level, was also showed between the obtained sequences and reference strains. Some nucleotide mutations, synonymous and non-synonymous, distinguished the CAdV-1 strains. In particular, in the deduced amino acid sequences, codon 234 was asparagine (N) in all reference strains and lysine (K) in all viruses sequenced in this study, and codon 388 was aspartate (D) in all sequences obtained in this study, serine (S) in all Italian reference strains, and asparagine (N) in the other reference strains. For the fiber gene, comparison of the nucleotide sequences allowed a separation of the obtained sequences in three groups showing an intra-group identity of 100% and an identity of 99.8-99.9% between the groups. These three groups were composed by: A) 603-06 and 603-07; B) 603-10, 603-12 and 603-13; and C) 602-07. A nucleotide identity of 99.2-99.6% and an amino acid identity of 98.8-99.4% were also found between obtained sequences and reference strains. Some nucleotide mutations, synonymous and non-synonymous, distinguished the CAdV-1 sequences identified in this study to the reference strains. In particular, amino acid changes 304G→E and 318I→R distinguished the sequences obtained to all the reference strains. Arctic fox viruses 603-10, 603-12 and 603-13 also showed amino acid asparagine (N) in position 283 rather than tyrosine (Y).

Entry plot analysis showed that nucleotide variation was equally distributed throughout the hexon gene, whereas greater nucleotide variability was present in the 3' portion of the fiber gene, in particular between residues 847 and 984 (Online Resource 3 and 4). DnaSP analysis (Table 4) showed the following. A) The fiber gene sequences had a total number of polymorphic sites higher than the hexon gene sequences (20 and 12, respectively) despite the fact that the fiber gene is

shorter of about 1000 nucleotides. B) The obtained sequences had less synonymous and non-synonymous mutations than reference strains. C) The number of synonymous and non-synonymous mutations increased significantly for both genes combining obtained sequences with reference sequences, and D) two more haplotypes were identified analyzing fiber gene sequences as compared to the hexon gene sequences.

Immunogenicity prediction carried out on amino acid positions of the deduced hexon and fiber proteins that were able to distinguish between viruses identified in this study and reference strains showed the following results. A) No change in the antigenicity was indicated for the amino acid mutations in residue 234 of hexon protein and in residues 283 and 304 of fiber protein; only residue 234 of hexon protein was part of a potential antigenic epitope (231-237) both in reference strains and in viruses detected in red and arctic foxes. B) The residue 388 of hexon protein showed no antigenic importance in the reference strains, while it was part of a potential antigenic epitope (387-396) in viruses detected in red and arctic foxes. Finally, C) residue 318 of fiber protein was part of a potential MHC II and T-cell epitope (318-323) in reference strains and was predicted as part of an antigenic epitope (315-319) in viruses detected in this study.

In the rooted phylogenetic tree constructed from the concatenated nucleotide sequences of hexon and fiber genes, the viruses identified were grouped in the CAdV-1 cluster in a clade separated from other viruses. Furthermore, the Norwegian sequences were subdivided in three groups: 1) ID 603-06 and 603-07; 2) ID 603-10, 603-12 and 603-13; and 3) ID 602-07 (Fig. 1).

185 **Discussion**

186 In this study, we detected CAdV-1 DNA in 14 out of 20 seropositive foxes (7 red foxes from Finnmark county on the
187 mainland of Norway and 7 arctic foxes from the Svalbard archipelago, Norway) and gene sequences from the viruses
188 were amplified and analysed. The absence of gross pathological lesions in the foxes that tested positive may suggest
189 that CAdV-1 infection was asymptomatic, but the inability to do histology due to the freezing and thawing of the tissue
190 samples did not allow us to address potential microscopic alterations.

191 Since IFA is unable to distinguish between CAdV-1 and CAdV-2, foxes that had CAdV specific antibodies could have
192 been infected by both virus types (Millán et al., 2016), but only CAdV-1 DNA was amplified by PCR. Inferring
193 population prevalence from these data is inappropriate due to the small sample size and the sampling design,
194 nevertheless, from the data obtained it seems to be a preferential circulation of CAdV-1 rather than to CAdV-2. The
195 absence of CAdV-2 DNA in the analysed foxes may also be due to the type of tissues examined, since CAdV-2 is
196 mainly detected in the upper respiratory tract or occasionally in feces and urine samples, which were not available for
197 our study (Greene, 2012, Balboni et al., 2013, Headley et al., 2013, Balboni et al., 2014). Further analyses are needed to
198 evaluate the prevalence of the two viral types in the two fox species.

199 Spleen and lymph node were always positive for CAdV-1 DNA in all positive foxes when these organs were available.
200 On the contrary, liver and kidney, that are recognized as the primary site of viral persistence (Greene, 2012), were not
201 always positive. These data raise questions about the role spleen and lymph nodes can play in viral persistence and to
202 the possible use of these organs as target in diagnostic tests.

203 Analysis of the generated nucleotide sequences showed that CAdV-1 detected in all the red foxes and in two arctic
204 foxes (603-06 and 07) had 22 additional nucleotides in the initial tract of the non-coding region between the E3 gene
205 and U-exon gene that has never been reported for this virus. These 22 nucleotides can represent a relatively recent
206 insert, or an ancestral hallmark of CAdV-1. The presence of the long non-coding region in all the red fox viruses and in
207 only two arctic fox viruses might also suggest that CAdV-1 with this mutation originates from the Norwegian mainland
208 and that it later arrived in Svalbard. Red foxes from the Norwegian mainland do not get in direct contact with arctic
209 foxes in Svalbard, but the red fox population may have contact with other European wildlife populations (Norén et al.,
210 2015). One explanation for the arrival of the virus to Svalbard can be through migration of arctic foxes from other
211 Arctic regions, as suggested for other pathogens arrived in Svalbard from Russia (Henttonen et al., 2001, Mørk et al.,
212 2011). It is known that arctic foxes in Svalbard get in contact with other high-Arctic wildlife populations by using the
213 sea ice as platform for migration (Geffen et al., 2007). Another explanation for the arrival of this particular virus is
214 through contact with dogs brought from the Norwegian mainland to Svalbard. Further studies would be needed to
215 explore this issue and it would be interesting to investigate the genetic features of CAdV-1 circulating in the domestic

dogs in Arctic regions. Since these 22 nucleotides are in a non-coding region of the viral genome, it is reasonable to suggest that they do not have biological effects, but the presence of a putative polyA motif could have a role in transcription of messenger RNA. Furthermore, the presence of a long non-coding region and of a putative polyA motif in some of the detected CAdV-1 and in reference SkAdV-1 and BtAdV-2, can give evidence of a high phylogenetic correlation between these viruses, as previously suggested by Kozak et al. (2015). Further studies investigating the presence of a putative common ancestor of these viruses and the biological effects of a longer non-coding region are warranted.

Investigations have also focused on two genes, hexon and fiber genes, that code for structural proteins. Proteins encoded by these two genes play an important role in the pathogenicity and infectivity by eliciting the immune response. Accordingly, the two genes are theoretically more variable since they have been subjected to a high selective pressure, which also makes them suitable for the characterization of genetically different viruses. The two genes were completely sequenced for six of the 14 detected viruses, in one red fox (602-07) and in five arctic foxes (603-06, 07, 10, 12 and 13). A high grade of identity was demonstrated between hexon and fiber gene sequences, respectively, of the obtained CAdV-1 PCR amplicons. More differences emerged by comparing CAdV-1 detected in this study with reference strains. Four amino acid mutations, two in the hexon protein (234K and 388D) and two in the fiber protein (304E and 318R), distinguished the sequences in this study from all CAdV-1 strains sequenced so far both in wild carnivores and dogs. All amino acid mutations evidenced in the fiber gene are encoded by the more variable genomic tract comprised between nucleotides 847 and 984 and it could be the consequence of a greater selective pressure exerted on this portion of the protein. Position 388 of the hexon protein is quite variable for CAdV-1 and seems able to differentiate viruses belonging to some different geographical regions as previously supposed by Balboni et al. (2017). Furthermore, the change of amino acid residue in position 388 of the deduced hexon protein is the only one who determines an important change in predicted immunogenicity. Therefore, even if a greater number of nucleotide mutations are shown by analyzing fiber gene sequences (20) rather than analyzing hexon gene sequences (12) and that the analysis of fiber gene distinguish more genetic types compared to the analysis of hexon gene, the study of both these structural genes together was important for the characterization of CAdV-1. This is further demonstrated by phylogenetic analysis performed with the two concatenated genes, which together generated a good resolution, clustering the sequences obtained in this study in a separate clade inside the CAdV-1 cluster.

Conclusions

In conclusion, the CAdV-1 sequences generated in this study, allowed us to distinguish the viruses of Svalbard and mainland Norway from other reference CAdV-1 strains currently identified in dogs and wild carnivores. Some of the identified mutations can play a role both in the viral pathogenicity and in evoking the host's immune response. Thus, the transmission of these viruses to dogs could have important consequences for their health and to the vaccination coverage. In light of the genetic peculiarities found in the identified viruses, an extension of the study to other geographical regions and involving other carnivore host species would be important to get more information about the CAdV-1 strains circulating in wildlife populations. Currently, very little is known about the pathogenic role of adenovirus in wild carnivores and even less on the possible transmission of the virus between wild animals and dogs. Greater attention should be given to viral pathogens that may emerge or re-emerge both in domestic dogs, and in wildlife populations.

Data availability

The nucleotide sequences obtained have been lodged within the GenBank sequence database under accession numbers: MF344652-MF344677.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-profit sectors.

Conflict of interest

The authors declare that they have no conflict of interest.

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357 **TABLES**

358

359 **Table 1** Red foxes (*Vulpes vulpes*) from the low-Arctic region of Finnmark County (northern mainland of Norway) and
360 arctic foxes (*Vulpes lagopus*) from the high-Arctic archipelago of Svalbard (Norway) tested for canine adenovirus 1
361 (CAdV-1) by a diagnostic PCR

	Fox	Date of sampling	Sex	Age (years)	Weight (grams)	Sampled organs	Diagnostic PCR
Red foxes (*Vulpes vulpes*)	602-01	2007	F	0	5607	Spleen	**CAdV-1**
						Liver	N
	602-02	2008	M	1	5251	Spleen	**CAdV-1**
						Liver	N
	602-03	2008	M	2	5937	Spleen	**CAdV-1**
						Liver	CAdV-1
	602-04	2008	M	1	5727	Spleen	**CAdV-1**
						Liver	N
	602-05	2008	M	3	4544	Spleen	**CAdV-1**
						Liver	CAdV-1
	602-06	2008	M	1	4733	Spleen	**CAdV-1**
						Liver	N
	602-07	2008	M	1	6926	Spleen	**CAdV-1** (**H**; **F**)
						Liver	CAdV-1
	602-08	2009	F	2	4608	Spleen	N
						Liver	N
	602-09	2009	M	7	7018	Spleen	N
						Liver	N
	602-10	2009	M	2	6031	Liver	N
Arctic foxes (*Vulpes lagopus*)	603-05	2001-2002	M	1	3900	Spleen	CAdV-1
						Liver	N
						Kidney	N
						Lymphnode	**CAdV-1**
	603-06	1997-1998	M	3	NA	Spleen	CAdV-1
						Liver	**CAdV-1** (**H**; **F**)
						Kidney	CAdV-1
						Lymphnode	CAdV-1
	603-07	1997-1998	F	3	NA	Spleen	CAdV-1
						Liver	CAdV-1
						Kidney	**CAdV-1** (**H**; **F**)
						Lymphnode	CAdV-1
	603-08	1998-1999	M	6	NA	Spleen	N
						Liver	N
						Kidney	N
						Lymphnode	N
	603-09	1997-1998	M	5	NA	Liver	N
						Kidney	N
						Lymphnode	N
	603-10	1999-2000	F	1	NA	Spleen	**CAdV-1** (**H**; **F**)
						Liver	CAdV-1
						Kidney	CAdV-1
						Lymphnode	CAdV-1
	603-11	2001-2002	M	2	NA	Liver	CAdV-1
						Kidney	**CAdV-1**
	603-12	2001-2002	F	2	2850	Spleen	**CAdV-1** (**H**; **F**)

						Liver	CAdV-1
						Kidney	CAdV-1
						Lymphnode	CAdV-1
	603-13	1999-2000	M	1	NA	Spleen	CAdV-1
						Liver	CAdV-1
						Kidney	CAdV-1
						Lymphnode	**CAdV-1** (**H**; **F**)
	603-14	1997-1998	M	1	NA	Spleen	N
						Liver	N
						Kidney	N
						Lymphnode	N

The date of sampling is referring to the year of abatement for red foxes (because these foxes were shot by hunters that recorded the date of kill) and to the winter season of catch, of two consecutive years, for arctic foxes (because these foxes were caught by trappers that did not record the date of kill). M: male; F: female; CAdV-1: specific CAdV-1 product of 508 bp obtained by diagnostic PCR (Hu et al., 2001); N: negative PCR result; In bold: diagnostic PCR product sequenced; H: hexon gene sequenced; F: fiber gene sequenced; NA: not available

Table 2 Reference sequences used for sequence analysis

Virus	Geographical origin	Year [a]	Host	GenBank ID	E	H	F	D	I	P
CAdV-1 field strain B579	IN	2009	dog	GQ340423	■					
CAdV-1 field strain	IN	2006 [b]	dog	EF057101	■					
CAdV-1 field strain Utrecht	NL	1992 [b]	dog	S38238	■					
CAdV-1 field strain 384-FFPEL	IT	1966	dog	KP670422	■					
CAdV-1 field strain 300-RS	IT	2012	dog	KF676980	■					
CAdV-1 field strain 313-Lparaf.	IT	2010	dog	KF676977	■					
CAdV-1 field strain 09-13F	IT	2011	fox	JX416838	■					
CAdV-1 field strain 220515/1	UK	2015	fox	KU755712	■					
CAdV-1 field strain 201114/2	UK	2014	fox	KU755713	■					
CAdV-1 field strain 201114/1	UK	2015	fox	KU755714	■					
CAdV-1 field strain 111114/1	UK	2014	fox	KU755715	■					
CAdV-1 field strain 090315/2	UK	2015	fox	KU755716	■					
CAdV-1 field strain 090315/1	UK	2015	fox	KU755717	■					
CAdV-1 field strain 061014/2	UK	2014	fox	KU755718	■					
CAdV-1 field strain 030415/1	UK	2015	fox	KU755719	■					
CAdV-1 field strain 17157	UK	2015	fox	KU755721	■					
CAdV-1 field strain 17154	UK	2015	fox	KU755722	■					
CAdV-1 field strain 15195	UK	2015	fox	KU755723	■					
CAdV-1 field strain 17066	UK	2015	fox	KU755724	■					
CAdV-1 field strain 15620	UK	2015	fox	KU755725	■					
CAdV-1 field strain 16036	UK	2015	fox	KU755726	■					
CAdV-1 field strain 15346	UK	2015	fox	KU755727	■					
CAdV-1 field strain 15622	UK	2015	fox	KU755728	■					
CAdV-1 field strain 16606	UK	2015	fox	KU755729	■					
CAdV-1 field strain 13-0086	AU	2013	dog	KT853096	■					
CAdV-1 field strain 13-0067	AU	2013	dog	KT853097	■					
CAdV-1 field strain CADRAD	IN	2015	dog	KX181846	■					
CAdV-1 field strain PT/12	PL	2012	dog	KC577558	■					
CAdV-1 field strain ITL2015	IT	2015	wolf	KX545420	■	■	■	■		■
CAdV-1 vaccine strain CLL	/	1996 [b]	/	U55001	■	■	■	■		■
CAdV-1 field strain RI261	UK	1996 [b]	dog	Y07760	■	■	■	■	■	■
CAdV-1 vaccine strain GLAXO	/	1991 [b]	/	M60937	■		■			
CAdV-1 field strain	IN	2007 [b]	dog	EF206692		■				
CAdV-1 field strain CCC-V6	CN	2007 [b]	dog	EF559262		■				
CAdV-1 field strain 113-5L	IT	2011	fox	JX416839	■					
				KP840544			■	■	■	■
				KP840545		■		■	■	■
CAdV-1 field strain 417-L	IT	2013	dog	KP670423	■					
				KP840546			■	■		■
				KP840547		■		■		■
CAdV-1 field strain 574-RS	IT	2013	dog	KP670424	■					
				KP840548			■	■		■
				KP840549		■		■		■
CAdV-2 strain Toronto A26/61	CA	1961	dog	CAU77082	■					■
BtAdV-2 field strain PPV1	DE	2007	bat	JN252129	■					■
BtAdV-3 field strain TJM	CN	2007	bat	GU226970	■					
SkAdV-1 field strain	CA	2005	skunk	KP238322	■					

[a] Year of virus identification or sequence submission to the GenBank database

[b] Year of submission to the GenBank database

CAdV-1: canine adenovirus type 1; CAdV-2: canine adenovirus type 2; BtAdV-2: bat adenovirus type 2; BtAdV-3: bat adenovirus type 3; SkAdV: skunk adenovirus; E, H, F, D, I, P: reference sequences used for partial E3 gene and flanking regions alignment and phylogenetic analysis (E), hexon gene alignment (H), fiber gene alignment (F), entropy

(H(x)) plot and DnaSP analysis (D), immunogenicity prediction (I) and phylogenetic analysis of concatenated hexon and fiber genes (P)

Table 3 Multiple sequence alignment of the non-coding region between E3 and U-exon genes

Viral sequence	Sequence alignment	Sequence length and its position in GenBank sequence
CAdV-1_ Y07760	----------AAATAAAC	8 (25713-25720)
CAdV-2_U77082	----------CTTGTC.....	14 (26412-26425)
SkAdV-1_KP238322	----------GAAATAAA.....	16 (26727-26742)
BtAdV-2_JN252129	---TAAGTTAAGAAAATAAATA...A.C..	27 (26627-26653)
BtAdV-3_GU226970	----------ATC.....-	9 (26470-26477)
CAdV-1_602-07_MF344658	AAATAAACACTATGGAGTTTAA.....	30 (286-315)
CAdV-1_603-10_MF344662	----------.....	8 (286-293)

One representative sequence was reported for CAdV-1, CAdV-2, SkAdV-1, BtAdV-2, BtAdV-3, CAdV-1 identified in this study with long non-coding region (602-07) and with "classical" non-coding region (603-10). The BtAdV-3 reference sequence didn't have a non-coding region because U-exon gene overlaps on E3 gene. For this viral sequence, the sequence fragment after the end of the E3 gene that aligns with the non-coding region of the CAdV-1 sequences was reported. In gray: Putative poly(A) motif (AATAAA)

Table 4 Summaries of sequence variability

CAdV-1 Sequences		*η*	*SynDif*	*NSynDif*	*h*
Hexon gene 2715 bp [a]	NS (n = 6)	1	1	0	2
	R (n = 6)	7	4	3	5
	NS+R (n = 12)	12	7	5	7
Fiber gene 1629 bp [a]	NS (n = 6)	2	1	1	3
	R (n = 6)	14	7	7	6
	NS+R (n = 12)	20	10	10	9

[a] The gene length does not consider the stop codon

NS: mainland Norway and Svalbard CAdV-1 sequences; R: reference CAdV-1 sequences; n: sample size; η: total number of polymorphic sites; dSynDif: total number of synonymous differences; NSynDif: total number of non-synonymous differences; h: number of haplotypes

FIGURES

Fig. 1 Rooted phylogenetic tree constructed with the multiple gene approach: concatenated nucleotide sequences of the hexon and fiber genes generated in this study and CAdV-1, CAdV-2 and Bat adenovirus reference sequences available from GenBank (Table 2)

The best-fit model of nucleotide substitution was determined using the Find Best DNA/Protein Model function implemented in MEGA 7.0.26. Hasegawa-Kishino-Yano model with invariant sites resulted optimal for the sequence data. Phylogenetic trees were constructed using Maximum Likelihood method and bootstrap values were determined by 1000 replicates to assess the confidence level of each branch pattern. Bootstrap values greater than 60% are indicated on the respective branches. On the top of the figure, a portion of the obtained tree is enlarged to better visualize the phylogenetic relationships existing between the CAdV-1 nucleotide sequences and the bootstrap values. For some viruses two GenBank accession numbers are reported (the hexon and fiber genes sequences, respectively). In bold: Nucleotide sequences generated in this study