SHORT COMMUNICATIONS

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Multicentric Molecular and Pathologic Study On Canine Adenovirus Type 1 in Red Foxes (Vulpes vulpes) in Three European Countries

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ABSTRACT: Canine adenovirus type 1 (CAdV-1) is the agent of infectious canine hepatitis, a severe frequently fatal disease affecting primarily dogs (Canis lupus familiaris). The virus has been detected in many wild carnivore species. Our aim was to evaluate the prevalence and genetic and histopathologic features of CAdV-1 in wild red foxes (Vulpes vulpes). Kidney and liver samples were obtained from 86 subjects, coming from the UK (n=21), Italy (n=36), and Germany (n=29). We used PCR, targeting the viral E3 gene and flanked regions, to detect the presence of the virus; viral E3, fiber, and E4 genes were sequenced and their sequences were compared with published sequences. Kidneys and liver from foxes in Italy and Great Britain (n=57) were prepared for histologic and immunohistochemical examination for CAdV-1. Viral DNA was detected in 22% (19 of 86) kidney samples, with E3 and E4 genes showing reported and unreported single nucleotide changes. No pathologic changes or viral immunopositive signals were detected in the examined tissues. Our study suggests that red foxes could be considered potential shedders of CAdV-1, as they showed a relatively high prevalence without related pathologic changes in the organs examined.

Key words: Canine adenovirus, immunohistochemistry, molecular epidemiology, red fox, sequencing. Vulpes vulpes.

Canine adenovirus type 1 (CAdV-1) is a double-stranded DNA virus and an agent of infectious canine hepatitis, a frequently fatal disease of carnivores including domestic dogs (Canis lupus familiaris). This infection also occurs in several free-ranging and captive wild carnivores such as brown bear (Ursus arctos), striped skunk (Mephitis mephitis), and Eurasian otter (Lutra lutra), causing subclinical disease and sporadically epizootic episodes (Woods 2001; Knowles et al. 2018).

CAdV-1 circulates in European red fox (Vulpes vulpes) populations (Åkerstedt et al. 2010), with serologic prevalences ranging from 3.5% in Germany (Truyen et al. 1998) to 64.4% in the UK (Walker et al. 2016a). The early detection of possible new genetic variants of CAdV-1 in wildlife by molecular studies could represent an important surveillance tool to evaluate the potential threat for other wild or domestic species, especially in those countries with a high level of urbanization.

We characterized the prevalence and genetic features of CAdV-1 in red foxes in one Mediterranean country (Italy), one western European country (UK), and one central European country (Germany), adding molecular epidemiologic data about this pathogen. Eighty-six red foxes (Italy, n=36; UK, n=21; and Germany, n=29) were examined. All foxes from Italy and from the UK were shot during the regular hunting season (October to February 2018) in the province of Pisa, central Italy (43°43’N, 10°24’E), and in Cheshire County (53°10’N, 2°35’W), respectively. Foxes from Germany were obtained within the framework of the official rabies monitoring program of the German federal states of Berlin (52°31’N, 13°E) and Brandenburg (52°21’N, 12°E). Only nonautolyzed carcasses were included. Age was determined by means of dry eye lens weight (Verin et al. 2010), and foxes were divided into two categories: juveniles, <1 yr old and adults, >1 yr old. Samples of liver and kidney were collected from foxes from Italy and the UK during postmortem examinations for both histopathologic and molecular analyses. Samples from Germany were only used for...
molecular analyses (no histopathologic samples were available).

Once fixed in formalin, the tissues were routinely processed for histopathology on tissues stained with H&E. We used a commercial goat polyclonal antibody cross-reacting with numerous adenoviruses (0151-9004, AbD Serotech, Kidlington, Oxford, UK) for immunohistochemistry. Liver sections from a serology-positive and PCR-confirmed naturally CAdV-1–infected dog were used as positive controls. The samples for molecular analysis were stored at −80°C and later used for DNA extraction.

We extracted DNA for 25 mg of tissues using the DNAeasy mini kit (Qiagen, Manchester, UK), following the manufacturer’s instructions, and the samples were submitted to CAdV PCR. The assay amplifies the canine adenovirus E3 gene and flanked regions, producing fragments of different lengths that allowed for differentiation between the two adenovirus types: 508 bp for CAdV-1 and 1,030 bp for canine adenovirus type 2 (CAdV-2), respectively (Hu et al. 2001). We used DNA extracted from a paraffin-embedded liver of a dog with confirmed canine infectious hepatitis as a positive control for CAdV-1. We used DNA extracted from the Nobivac vaccine (MSD Animal Health Srl, Milton Keynes, UK) and the Eurican Epta vaccine (Merial SpA, Lyon, France) as CAdV-2 positive controls.

We submitted each positive sample for additional PCRs, targeting the E3, fiber, and E4 genes (Walker et al. 2016b), and the amplicons were directly sequenced (BMR Genomics, Padova, Italy). Nucleotide sequences were assembled using BioEdit 7.2.5 (Hall 1999) and aligned with the reference sequence of canine adenoviruses from GenBank (AC_000003.1). Phylogenetic relationships were evaluated using MEGA 6.0.6 (Tamura et al. 2013).

Histopathologic examination of livers and kidneys did not reveal relevant pathologic changes (i.e., presence of intranuclear viral inclusion bodies, hepatic necrosis, and vasculitis) related to adenoviral infection. Similarly, immunohistochemistry did not show positive immunostaining in any of the samples examined.

Amplicons of 508 bp corresponding to CAdV-1 were detected in 22% (19 of 86) of kidney samples, whereas none of the hepatic samples tested positive in the PCR.

Kidneys were positive in 38% (8 of 21) of foxes from the UK, 28% (10 of 36) of foxes from Italy, and 3% (1/29) of foxes from Germany. Positive foxes from the UK were all classified as adults, and only one of eight foxes was a female. Positive samples from Italy were from five male and five female foxes and equally distributed in age classes (five adults and five juveniles). The only positive fox from Germany was an adult male (Table 1). All sequences shared a high identity rate from 99% to 100%, irrespective of the geographic origin. The phylogenetic analysis showed a strict relation between all the CAdV-1, in particular between the Italian and English clusters (Fig. 1). By comparing the sequences that we determined to those identified in a previous study (Walker et al. 2016b), two single nucleotide (nt) changes were confirmed: nt 29912 from A to G and nt 30070 from G to A in E4. These changes were observed in all positive samples from the UK and in the positive fox from Germany. Unreported single nucleotide changes were identified in E3 gene sequences (nt 25513 from G to A) and E4 gene sequences (nt 29763 from G to A; 29987 from G to A) obtained from all the Italian positive samples (Table 2). Interest-

<table>
<thead>
<tr>
<th>Country</th>
<th>Adult Male</th>
<th>Adult Female</th>
<th>Juvenile Male</th>
<th>Juvenile Female</th>
<th>Total Male</th>
<th>Total Female</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Italy</td>
<td>17 (3/18)</td>
<td>25 (2/8)</td>
<td>50 (2/4)</td>
<td>50 (3/6)</td>
<td>28 (10/36)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UK</td>
<td>47 (7/15)</td>
<td>20 (1/5)</td>
<td>0 (0/1)</td>
<td>——</td>
<td>38 (8/21)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Germany</td>
<td>9 (1/11)</td>
<td>0 (0/5)</td>
<td>0 (0/8)</td>
<td>0 (0/5)</td>
<td>3 (1/29)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>23 (14/62)</td>
<td>21 (5/24)</td>
<td></td>
<td></td>
<td>22 (19/86)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* — not sampled.
ingly, the mutation in nucleotide position 25513 of the E3 gene resulted in an amino acid substitution from glycine to glutamate, whereas the mutation in nucleotide position 29987 of the E4 gene resulted in a change from phenylalanine to serine. We did not observe the nucleotide mutations recently reported in the hexon and fiber genes of *Canis familiaris*.

**Figure 1.** Molecular phylogenetic analysis by maximum likelihood method for E3 and E4 sequences. The evolutionary history was inferred by using the maximum likelihood method based on the Kimura two-parameter model. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 18 nucleotide sequences (E3 amplicons) and 20 nucleotide sequences (E4 amplicons). The sequences obtained in this study are highlighted in bold.

**Table 2.** Nucleotide changes among canine adenovirus type 1 (CAdV-1) complete genome reference sequence AC_000003.1 and those obtained in this study divided by country.*

<table>
<thead>
<tr>
<th>Sequence source</th>
<th>E3 ORFA</th>
<th>Hexon</th>
<th>E4 ORFA</th>
<th>E4 ORFA</th>
<th>E4 ORFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC_000003.1 residue position</td>
<td>25513</td>
<td>29763</td>
<td>29912</td>
<td>29957</td>
<td>30070</td>
</tr>
<tr>
<td>AC_000003.1 nucleotide</td>
<td>G</td>
<td>G</td>
<td>A</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>Walker et al (2016b)</td>
<td>Nd</td>
<td>Nd</td>
<td>A&gt;G</td>
<td>Nd</td>
<td>G&gt;A</td>
</tr>
<tr>
<td>Italian foxes</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>G</td>
</tr>
<tr>
<td>English foxes</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>A</td>
</tr>
<tr>
<td>German foxes</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>A</td>
</tr>
</tbody>
</table>

*Nd = no nucleotide change detected compared with CAdV-1 sequence AC_000003.1. A>G or G>A: detection of nucleotide changes in a set of sequences recently reported in red foxes (Walker et al 2016b). A = adenine; G = guanine.
CAdV-1 in two Italian dogs (Balboni et al. 2017).

Our study, along with others, supported the presence of CAdV-1 in red foxes in several European countries. The evidence of the virus in kidneys of healthy subjects may support the hypothesis that red foxes represent a source of infection for other species. The absence of pathologic lesions referable to CAdV-1 infection in the liver and kidney, as well as the positive results obtained by PCR in kidneys, reinforces the role of the red fox as a possible shedder for this virus; nevertheless, a limit of our study could have been the restriction of sampling to two organs, without including other target organs such as brain (despite the minor epidemiologic role this organ can play).

We observed PCR positivity only in kidney samples, suggesting a potential for excretion in urine, as reported in dogs (Greene 2012). In fact, red fox, based on the results of our study and recent literature (Balboni et al. 2013; Walker et al. 2016b; Hechinger et al. 2017), not only harbors low CAdV-1 loads but also seems to have developed tolerance, as CAdV-1-typical lesions (at least in the tested organs) or outbreaks are rarely reported (Thompson et al. 2010; Walker et al. 2016a). In the UK, the red fox is the only free-ranging canid and is deemed to be the primary wildlife shedder of CAdV-1, whereas in other countries, additional canid species may share this epidemiologic role. This possibility should be investigated further, and more species should be included in future surveys.

Genetic variations were identified previously (Walker et al. 2016b) as a result of a genetic drift in relation to distinct and separate geographic areas, suggesting a variable rate of divergence in different CAdV-1 genes. This indicates that the sequences from CAdV-1 field strains circulating in wild animals differ among them and differ from sequences reported in domestic dogs in Europe. We found three previously unreported new mutations in the Italian strains. The new mutations suggest a geographic pressure in Italy for CAdV-1 antigenic drift, as in the UK (Walker et al. 2016b). Nevertheless, in silico studies, performed to predict possible protein and RNA folding changes introduced by the amino acid substitution in the E3 gene, did not show any potential differences with the reference strains and should be interpreted as synonymous mutations. The absence of relevant pathologic differences compared with the animals infected with different strains should also be considered.

Future studies are needed on the stability of the mutations that we reported herein. Also, it would be important to understand whether the genetic variants reported in this study are present in red foxes as well as in domestic dogs of other regions. Because of the number of nonvaccinated stray dogs in Italy, it is pivotal to consider these animals as playing an active role in the circulation of CAdV-1. Stray dogs may have more chances to interact with wild animals than owned dogs. These studies would be essential to quantify a possible risk of infection for wild and domestic animals.

**LITERATURE CITED**


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3. Author: Are edits to sentence “The absence of relevant . . . also be considered” OK? Please confirm or amend as appropriate for intended meaning. Copy editor

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