

## Molecular evidence of kobuviruses in free-ranging red foxes (*Vulpes vulpes*)

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**Abstract** Red foxes (*Vulpes vulpes*) are susceptible to viral diseases of domestic carnivores. In this study, by screening rectal swabs collected from 34 red foxes in Italy, we identified kobuvirus RNA in five samples. Based on analysis of partial RdRp and full-length VP1 genes, all of the strains shared the highest identity with canine kobuviruses (CaKVs) recently detected in the US, the UK and Italy. These findings provide the first evidence of the circulation of these novel viruses in foxes.

Picornaviruses are small, non-enveloped viruses of approximately 27–30 nm in diameter with single-stranded, positive-polarity RNA genomes of 8.2–8.4 kb. According to the International Committee on Taxonomy of Viruses (<http://ictvonline.org/virusTaxonomy.asp?version=2012>), the family *Picornaviridae* is currently divided into 17 genera: *Aphthovirus*, *Aquamavirus*, *Avihepatovirus*, *Cardiovirus*, *Cosavirus*, *Dicipivirus*, *Enterovirus*, *Erbovirus*, *Hepatovirus*,

*Kobuvirus*, *Megrivirus*, *Parechovirus*, *Salivirus*, *Sapelovirus*, *Senecavirus*, *Teschovirus* and *Tremovirus*.

The genus *Kobuvirus* consists of three species [1], *Aichivirus A* (formerly *Aichi virus*), *Aichivirus B* (formerly *Bovine kobuvirus*) and *Aichivirus C* (porcine kobuvirus).

Human Aichi virus (AiV) was first recognized in 1989 as the cause of oyster-associated nonbacterial gastroenteritis in humans in Aichi Prefecture, Japan [27]. Several investigations worldwide have revealed that AiVs are involved in 0.9–4.1 % of sporadic cases of pediatric gastroenteritis [2, 10, 11, 21].

Novel kobuviruses that are genetically and antigenically closely related to human AiVs have been found recently in domestic carnivores [5, 6, 12, 14], raising public health concerns of potential cross-species transmission. Carnivore kobuviruses were first discovered in diarrhoeic and asymptomatic dogs [12, 14]. Based on sequence analysis of the complete genome, the novel virus designated canine kobuvirus (CaKV) was found to be genetically closest to the recently identified mouse kobuvirus M-5/USA/2010 (84.0 % amino acid [aa] sequence identity) [17] and to human AiVs (80.0 % aa sequence identity). Accordingly, CaKV may be considered a distinct genotype (CaKV type 1) from both murine kobuvirus (MuKV type 1) and human AiV (AiV type 1), within the species *Aichivirus A* (<http://ictvonline.org/proposals/2012.014aV.A.v1.Kobuvirus-Sp,Ren.pdf>).

Subsequently, kobuviruses that are genetically related to CaKVs were identified in the faecal samples of cats with diarrhoea [6]. Nucleotide sequence identity between CaKVs and feline kobuviruses in the RdRp region ranged from 82.2 % to 89.3 %, while identity to MuKV and to human AiV was 82.4–85.5 % and 81.9–82.4 %, respectively.

Also, a novel kobuvirus more close related to bovine and ovine kobuviruses within the species *Aichivirus B* has been identified recently in a rectal swab from a ferret [23],

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demonstrating that non-domestic carnivores are susceptible to kobuvirus infections.

Here we report the detection of kobu-like viruses in faecal samples obtained from Italian red foxes (*Vulpes vulpes*) that are genetically very similar to CaKVs.

Between September 2009 and May 2013, individual rectal swabs were collected from 34 red foxes in northern Italy (Valle d'Aosta and Piemonte regions) submitted to the National Reference Center for Wild Animal Diseases (CeRMAS). The samples were obtained from animals of both genders (14 females and 20 males) found dead or shot during the regular hunting season. Twelve foxes were under 1 year of age, and 22 were adults. Fresh faecal samples were placed into sterile containers and stored at  $-80^{\circ}\text{C}$  until tested.

RNA was extracted from 200  $\mu\text{l}$  of 10 % (wt/vol) faecal suspension using TRIzol LS (Invitrogen, Ltd, Paisley, UK) procedure. The final RNA pellet was resuspended in 50  $\mu\text{l}$  of RNase-free water and used directly in RT-PCR assays or stored at  $-80^{\circ}\text{C}$ . Viral DNA was extracted from the supernatants of faecal homogenates by boiling for 10 min and chilling on ice as described previously [7].

Kobuvirus RNA was detected by RT-PCR using a broadly reactive primer pair, UNIV-kobu-F/UNIV-kobu-R, which amplifies a 217-bp fragment of the 3D gene of all kobuviruses, following reaction conditions described previously [20].

The samples were also tested by RT-PCR for canine coronaviruses (CCoVs) [18] and canine noroviruses (NoVs) [16, 25], and by PCR for canine parvovirus (CPV-2) [4].

Novel primers (VP1<sub>rf</sub>-F, 5'-GCGGGCGAATCCCTTCAAC-3, and VP1<sub>rf</sub>-R, GCGACCTTTCGGAGCGCC-3') to amplify the complete VP1 gene were designed by visual inspection of an alignment containing the kobu-like sequences obtained in this study and the corresponding conserved regions of the CaKVs and AiVs available on the NCBI website.

All of the amplicons were purified using a QIAquick Gel Extraction Kit (QIAGEN GmbH, Hilden, Germany) and sequenced directly using BigDye Terminator cycle chemistry and a 3730 DNA analyzer (Applied Biosystems, Foster City, CA). RT-PCR products for which no sequences were obtained by direct sequencing were cloned into the pCR2.1 vector (Invitrogen, Ltd, Paisley, UK). Plasmid DNA was purified using a Miniprep Kit (QIAGEN GmbH, Hilden, Germany); three clones per product were sequenced. Basic Local Alignment Search Tool (BLAST; <http://www.ncbi.nlm.nih.gov>) and FASTA (<http://www.ebi.ac.uk/Tools/sss/fasta/>) with default values were used to find homologous hits. Sequence editing and multiple alignments were performed with the BioEdit software package, version 2.1 [9]. A phylogenetic tree (neighbor joining and p-distance model) with bootstrap analysis

(1,000 replicates) was constructed using the MEGA software package, version 3.0 [13].

Out of 34 faecal specimens, five (14.7 %) were found to be positive for kobuvirus RNA, and two (5.8 %) for CCoV RNA. Canine NoVs and CPV-2 were not detected. Kobuvirus was the single identified enteric virus in four specimens, while a mixed infection with both pathogens (CCoV + kobuvirus,  $n = 1$ ) was found in one sample. All of the samples were obtained from adult animals. None of the foxes that were positive for CCoV or kobuvirus RNA showed pathological lesions indicative of enteritis.

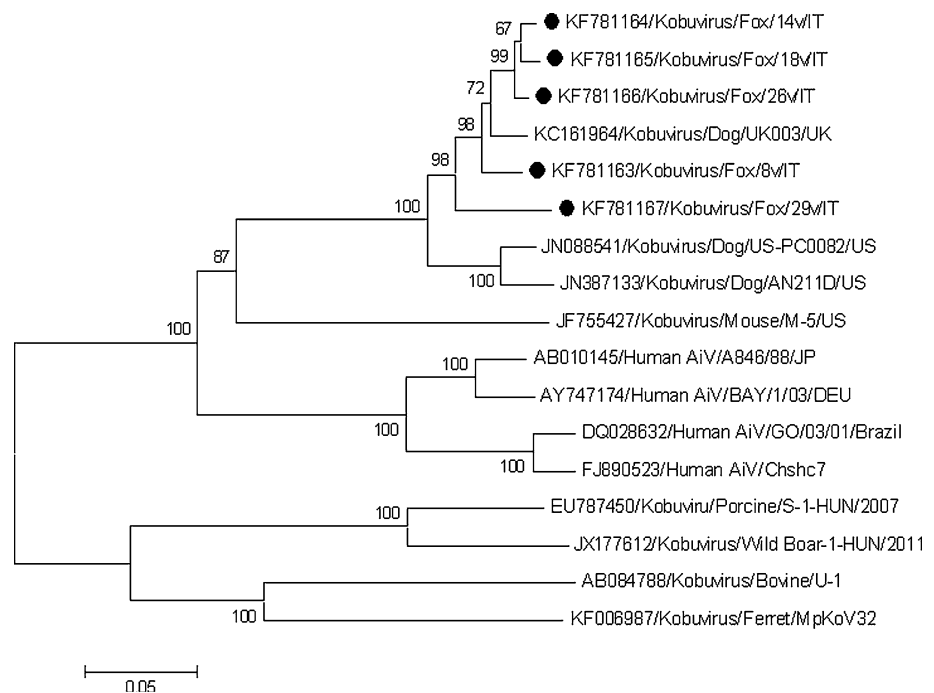
By FASTA and BLAST analysis of the partial 3D region amplified with the primers UNIV-kobu-F/UNIV-kobu-R, the five fox strains (GenBank accession no. KF781168-KF781172) shared 95.4-96.8 % nt sequence identity with each other and displayed the closest relatedness (92.7-96.2 % nt) with the CaKVs previously found in the US, the UK and Italy [5, 8, 12, 14]. Identity to feline kobuviruses [6] ranged from 81.1 % to 87.7 %, while nt sequence identity to human AiVs was 81.6-85.0 %.

The full-length sequence of the VP1 gene was determined for each of the five kobuvirus strains (GenBank accession no. KF781163-KF781167). Sequence analysis indicated that the fox kobuviruses were highly related to each other (91.7-98.5 % nt and 97.8-100 % aa identities) and to the CaKV strain UK/003 (accession no. KC161964) (92.6-96.4 % nt and 98-99 % aa identities) recently identified in a healthy dog in the UK [5]. Neighbor-joining phylogenetic analysis based on the VP1 sequences was carried out with a selection of strains representative of the genus *Kobuvirus*, including murine kobuvirus [17], human AiVs, wild-boar kobuvirus [22], the ferret kobuvirus strain MpKoV32 [23], and the prototype strains porcine kobuvirus S-1 [19] and bovine kobuvirus U1 [26]. Based on inspection of the tree (Fig. 1), all of the fox kobuvirus sequences formed a tight cluster with the CaKVs recently identified in the UK and in the US [5, 12, 14], sharing a common root with murine kobuvirus and human AiVs.

Historical evidence shows that foxes are susceptible to viral diseases of domestic carnivores, including infections with canine distemper virus [15, 24], CPV-2 [24], canine adenoviruses [3, 24], CCoV [28], and canine herpesvirus [24].

In the present study, we detected kobuviruses in free-ranging red foxes with a prevalence rate of 14.7 % (5/34). Investigations based on the molecular analysis of the full-length VP1 gene revealed that the five fox strains were most closely related to the CaKVs (97.8-100 % aa) recently identified in dogs [5, 12, 14]. Therefore, the fox kobuviruses detected in this study may be considered members of genotype CaKV-1 within the species *Aichivirus A*. In addition, this is the first study demonstrating the presence of CCoVs in free-ranging red foxes in Italy.

**Fig. 1** Neighbor-joining phylogenetic tree based on the VP1 gene (834 bp) of the kobuvirus strains detected in red foxes. The tree was generated using the neighbor-joining method and p-distance correction, supplying a statistical support with bootstrapping of 1000 replicates. The scale bar indicates nucleotide substitutions per site. The markers indicate the sequences detected in this study



Although CaKVs were found in both asymptomatic and diarrhoeic dogs, the pathogenic potential of these novel kobuviruses in carnivores remains to be elucidated. In this preliminary study, all of the CaKV-like viruses detected were identified in adult foxes found dead or shot during the regular hunting season without apparent pathological enteric lesions. Accordingly, it is unclear if these viruses may have a role in the aetiology of diarrhoea in foxes. Future large-scale virologic investigations by screening faecal samples collected from both diarrhoeic and healthy animals are needed to assess the distribution and pathogenic potential of CaKV-like viruses in foxes.

In conclusion, this study provides direct evidence of the circulation of CaKV-like viruses in red foxes, reinforcing the previous observations that kobuvirus infections are probably as frequent in wild animals as they are in domestic animals [22, 23]. Additional studies are warranted in order to determine the significance of these findings and to evaluate the epidemiological and clinical importance of these novel viruses in wild carnivores. Furthermore, as fox kobuviruses are newly recognized viruses and information on their diversity is still limited, a definitive characterization should rely on sequence analysis of the complete genome.

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**Conflict of interest** All authors declare that there are no financial or other relationships that might lead to a conflict of interest. All authors

have seen and approved the manuscript and have contributed significantly to the work.

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