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Phylogeny and prevalence of kobuviruses in dogs and cats in the UK

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ABSTRACT

The kobuviruses represent an emerging genus in the *Picornaviridae*. Here we have used next generation sequencing and conventional approaches to identify the first canine kobuvirus (CaKoV) from outside the USA. Phylogenetic analysis suggests that a single lineage genotype of CaKoV now exists in Europe and the USA with 94% nucleotide similarity in the coding region. CaKoV was only identified in a single case from a case–control study of canine diarrhoea, suggesting this virus was not a frequent cause of disease in this population. Attempts to grow CaKoV in cell culture failed. Sequence analysis suggested CaKoV was distinct from human Aichi virus (AiV), and unlikely to pose a significant zoonotic risk. Serosurveys by ELISA, immunofluorescence and neutralisation tests, using AiV as antigen, suggested kobuvirus infection is prevalent in dogs. In addition, IgG antibody to AiV was also detected in cat sera, indicating for the first time that cats may also be susceptible to kobuvirus infection.

1. Introduction

Kobuvirus is a relatively recently described genus of the *Picornaviridae* family. Human Aichi virus (AiV), its first member, was isolated from faecal samples of human patients with oyster-associated nonbacterial gastroenteritis in Japan in 1989 (Yamashita et al., 1991). Since then, AiV has been detected in many countries around the world, especially in outbreaks associated with consumption of oysters and other shell-fish, and in sporadic disease in children (Ambert-Balay et al., 2008; Kaikkonen et al., 2010; Oh et al., 2006; Pham et al., 2007; Reuter et al., 2009; Sdiri-Loulizi et al., 2008; Yang et al., 2009). Other members of the *Kobuvirus* genus have now been described causing enteric infections in cattle (Barry et al., 2011; Khamrin et al., 2008; Park et al., 2011), pigs (Barry et al., 2011; Khamrin et al., 2009; Reuter et al., 2008), wild boar (Reuter et al., 2012), bats (Li et al., 2010), sheep (Reuter et al., 2010) and rodents (Phan et al., 2011). Their role as a primary pathogen remains uncertain (Kaikkonen et al., 2010; Lorrot et al., 2011), and mixed infections are common (Ambert-Balay et al., 2008). Although detection of AiV itself is rare, seroprevalence of antibody to AiV is high in humans, approaching 100% in adults, suggesting infection is common (Ribes et al., 2010; Yamashita et al., 1993).

Human AiV has a linear, positive sense ssRNA genome of ~8270 nucleotides, and exists as non-enveloped, 22–30 nm icosahedral particles (Yamashita et al., 2003). The RNA molecule contains one large open reading frame encoding a single polyprotein which like other members of the *Picornaviridae* family is cleaved into structural and non-structural proteins. Initially two genotypes of AiV

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were identified based on differences of nucleotide sequence in the 3C–3D junction (Yamashita et al., 2000). More recently a third genotype type has been identified (Ambert-Balay et al., 2008). Some unique molecular features have been described compared to other picornaviruses. Yamashita et al. (1998) showed that the VP0 capsid protein is expressed in the mature particle in Aichi virus and does not undergo cleavage (Yamashita et al., 1998) in contrast to other picornaviruses (Hellen and Wimmer, 1992). Also the 2A and L protein of Aichi virus have no protease or autocatalytic motifs as documented for other picornaviruses and their function remains unknown (Yamashita et al., 1998).

In dogs, the first kobuviruses were described in 2011. In a study of acute gastroenteritis in canine shelters in the United States, 5 of 18 dogs from two of three outbreaks tested positive for kobuviruses. Sequence analysis and distances from AiV genotypes A and B led the authors to propose these viruses be called canine kobuvirus (CaKoV) (Kapoor et al., 2011). In a separate study published around the same time, a similar virus was detected in the stools of three dogs with diarrhoea (Li et al., 2011). Based on sequence analysis, the canine virus was independently named CaKoV and tentatively classified as a novel species, closely related to AiV, in the genus *Kobuvirus*. Follow up PCRs of 200 sick and healthy dogs found CaKoV in 14 healthy and six diarrhoeic dogs. Both of these studies failed to identify a clear role for CaKoV in disease, suggesting that any pathogenicity was likely to be low, at least in the mostly adult canine population they tested.

Here, we describe canine kobuvirus in the UK, and provide serological evidence for widespread kobuvirus infection in cat and dog populations. The relationship of these carnivore viruses with AiV is discussed.

2. Methods

2.1. Samples

The faecal samples in this study were from an existing case–control study to determine the role of canine enteric coronavirus (CECoV) and other potential pathogens in enteric disease (Stavisky et al., 2011). As well as using PCR to detect known pathogens, electron microscopy was also conducted on all cases ($N = 86$) and on 60 controls. In two cases and one control, myxovirus-like particles were described. These samples tested negative using conventional PCR for canine distemper and parainfluenza (data not presented). One of these samples (UK003) was from a two month-old Staffordshire-bull terrier-cross that presented collapsed with diarrhoea and vomiting. This puppy was also shedding type II CECoV and *Campylobacter upsaliensis*, as well as *Toxacara canis* and *Toxacara leonina*. Sample UK003 was used for next generation sequencing.

AiV strain A846/88 (genotype A), was isolated by Yamashita et al. (1991), and kindly provided by Pierre Pothier (University of Dijon, France). This strain was propagated in Vero cells, recovered from cell lysates, and clarified by centrifugation, and the supernatant was divided into aliquots, which were stored at −80 °C. The stock virus was titrated by immunofluorescence on Vero cells.

Anonymized canine ($N = 198$) and feline ($N = 97$) serum samples were obtained as part of routine diagnostic work being carried out on animals attending the University of Liverpool Small Animal Teaching Hospital. For confidentiality reasons, clinical data could not be obtained with the samples.

2.2. Sample preparation, sequencing and informatics

Faecal samples were purified and nucleic acid extracted essentially according to published protocols with minor modification (Finkbeiner et al., 2008; Wang et al., 2003). Briefly, chips of frozen archived faeces (~30–150 mg) were resuspended in 6 volumes of PBS, clarified by centrifugation ($9700 \times g$, 10 min), and the supernatants passed through 0.45 μm filters. RNA was extracted from 100 μl of the filtrate (Qiagen RNA extraction kit) without the DNase step, reverse transcribed (Superscript) using primer A (5′-GTTTCCCAGTCACGATCNNNNNNNNN-3′), and amplified using primer B (5′-GTTTCCCAGTCACGATC-3′). Smears of DNA were gel purified and submitted for library preparation according to standard protocols.

Whole-genome shotgun pyrosequencing was performed by generating a standard DNA fragment library (Roche Applied Sciences, Indianapolis, IN) and sequenced with a GS-FLX using Titanium chemistry (454 Life Sciences, Roche Applied Sciences). The 454 reads were assembled with Newbler (v2.3 Roche Applied Sciences), and results compared to a viral protein database downloaded from NCBI Jan 2009 using BlastX.

The full genome of UK003 was determined by sequencing overlapping amplicons generated by multiple PCRs using primers based on the draft next generation genome, and 5′/3′ RACE. Maximum likelihood trees with 1000 bootstrap replicates were calculated using PHYML 3.0 (Guindon and Gascuel, 2003) using the most appropriate model of evolution, which was identified using the model selection approach implemented in Topali v2.5 (Milne et al., 2009).

2.3. Viral prevalence by PCR detection

Based on the results of next generation sequencing, a diagnostic PCR assay was designed to screen the case–control study (80 cases and 147 controls) for the presence of a putative Aichi/kobu viruses. RNA was extracted from 140 μl of virus transport medium containing the faecal sample using the QIAamp viral RNA extraction kit (Qiagen), and first strand cDNA synthesis was carried out using the RNA SuperscriptIII kit (Invitrogen), following the manufacturer's instructions. Reactions contained 1 μl oligodT or random hexamers, 3 μl RNA, 2 μl 5 mM dNTP mix, 8 μl H_2O, 4 μl first-strand buffer, 1 μl 0.1 M DTT and 1 μl SuperscriptIII. Reactions were incubated at 25 °C for 5 min, 50 °C for 60 min and 70 °C for 15 min. The resulting cDNA was used as the template for the PCR assay.

Five separate oligonucleotide primers were designed (three forward and two reverse), based on the known sequences of three kobuviruses: AiV (AB010145), porcine kobuvirus (EU787450) and our putative CaKoV. The primers sequences were chosen to match our CaKoV