Novel enteric viruses in fatal enteritis of grey squirrels

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Abstract

Astro- and kobu- viruses infect both humans and animals. Here, we report disease history, detection and genomic characterisation of novel astro- and kobuviruses from fatal diarrhoea of two juvenile grey squirrels. The virus particles had enterovirus-like morphology and a diameter of 28-32nm. Next generation sequencing confirmed astro- and kobuviruses and sequence analysis revealed typical astrovirus and picornavirus genome organisations. The astrovirus ORF2 sequence clustered with a clade of unassigned astroviruses, with marmot and rodent mamastroviruses as closest relatives. For the kobuvirus, divergences greater than 49.4% for P1 and 43.5% in the non-structural proteins indicated a novel species. However, phylogenetic analysis of the 3D polymerase showed that it clustered with that of the newly classified ludopivirus A1, suggesting a previous recombination event in the evolution of the kobuvirus. Our data provide further insights into diversity of astro- and kobuviruses and broadens the spectrum of viruses infecting grey squirrels.

Keywords

Kobuvirus, mamastrovirus, grey squirrel, enteritis, phylogeny, genomic organisation

Author Notes

One supplementary figure is available with the online version of this article.

Abbreviations

VP, Virus protein; ORF, Open reading frame; UTR, untranslated region; ICTV, International Committee on Taxonomy of Viruses; AiV, aichivirus; NGS, next generation sequencing; nt, nucleotide; aa, amino acid; VPg, viral protein genome-linked; RdRp, RNA-dependent RNA polymerase; sgRNA, single guide RNA; AstV, astrovirus; MAstV, mamastrovirus;

Full text

Astroviruses are classified into the genera *Mamastrovirus* and *Avastrovirus* infecting mammalian and avian species respectively (ICTV: https://talk.ictvonline.org/taxonomy/) [1, 2]. Astroviruses were first identified in 1975 in children with diarrhoea [3] and are most commonly associated with gastroenteritis in the elderly, immunocompromised and young children [2]. They have also been associated with respiratory illness and encephalitis in immunocompromised subjects [4]. The first reports in animals were from lambs and calves suffering from diarrhoea [5, 6], followed by further reports in a range of domestic and wild mammals in both terrestrial and aquatic environments [7]. Astrovirus encephalitides have been reported in cattle [8-12], mink [13], sheep [14] and pigs [15] and hepatitis in ducks [16] and nephritis in chickens [17].

Astroviruses possess a non-enveloped, icosahedral morphology with a positive-sense, single-stranded RNA genome [18]. The genome includes 5′ and 3′ untranslated regions (UTRs) and three open reading frames (ORFs). ORF1a and ORF1b encode non-structural proteins including a virally encoded serine protease, a genome-linked viral protein (VPg), and an RNA-dependent RNA polymerase (RdRp). ORF1b is translated through a frameshift mechanism. ORF2 encodes the capsid protein [1, 18].

The *Kobuvirus* genus in the *Picornaviridae* family includes pathogens infecting humans and animals. Aichivirus A1 (AiV-A1), the exemplar virus of species *Aichivirus A*, was first isolated from acute gastroenteritis patients in Japan consuming raw oysters [19, 20] and followed by other outbreaks linked to oyster or seafood consumption [21, 22]. Kobuviruses have also been identified in a variety of domestic and wild animal species. Bovine viruses of the species *Aichivirus B* were first identified in Japan, AiV-B1 [23] and since, they have been identified in mustelid (AiV-B2) [24] and ovine species (AiV-B3) from Hungary [25]. AiVs of the species C are widely distributed in the pig population (AiV-C1) in both diarrhoeic and healthy pigs [26]. Further, AiV-C1 has been found in wild boars in Hungary suggesting a potential role as a reservoir [27], whereas a second type, AiV-C2, was reported from Korean black goats (*Capra hircus coreanae*) with diarrhoea [28]. There are also recent reports of other aichiviruses (*Aichivirus D-F* and unassigned aichiviruses) from cattle, European roller, rabbits and bats [29-32]. The clinical significance of many AiVs, however, remains undetermined.

Kobuviruses are icosahedral non-enveloped positive-sense single-stranded RNA viruses with a genome of up to 8.5 kb. Genomic organisation is typical of the *Picornaviridae* members with a large ORF encoding a single polyprotein precursor and 5′ and 3′ UTRs. The polyprotein generates structural (VP0, VP3, and VP1) and nonstructural (2A–2C and 3A–3D) proteins (33).

This study reports novel astro- and kobuviruses detected from two grey squirrels (*Sciurus carolinensis*) with fatal diarrhoea and enteric pathology. The two orphaned juvenile male animals, assumed to be three to four months old, developed diarrhoea and died in 2010 within a month of their arrival at a UK wildlife hospital. On post mortem examination the animals were emaciated and dehydrated with no abdominal fat. Both stomachs were distended with clotted milk with smooth cream mucosal surfaces. The small intestines of each were also filled with creamy fluid content with yellow/green tinges distally and pale pink mucosal surfaces. Liquid yellow/green content was also present from caecum to rectum. Both sets of lungs were congested with mid dark red and wet cut surfaces. Both spleens showed a wrinkled capsule with friable dark red cut surfaces and lymph nodes were typically small, pink or cream with wet pink/purple mediastinal thymus. Femoral bone marrow from each showed dark red content and each kidney capsule stripped readily from smooth friable cortical surfaces. Brains were autolysed. No remarkable lesions were seen in any other tissues examined. No histopathology was carried out on the intestinal tissues from either animal.

Direct light microscopy of a pooled faecal sample visualised small numbers of coccidial oocysts, but macroscopic changes (e.g. necrotising enteritis) that would typically be expected with clinical coccidiosis were absent. Transmission electron microscopy [34] on pooled intestinal contents detected many round particles of approximately 28-32nm diameter (Fig. 1). Their size, shape and observable morphology indicated they were enterovirus-like particles. The sample was then prepared for a pan viral microarray analysis [35] which indicated the presence of only an aichivirus-like virus. To confirm the microarray finding and obtain the virus genome sequence, sample RNA was prepared for Next Generation Sequencing (NGS) [36]. The virus sequences were assembled either by mapping 4,156,838 NGS sequence reads to those of GenBank virus reference sequences or through *de novo* assembly using the SeqMan NGen software version 11 (DNASTAR, USA). A total of 879,695 of the *de novo* assembled sequence reads matched the coding sequence of an astrovirus (coverage of 2437). The *de novo* contigs were assembled using the SeqMan Pro software (DNASTAR, USA). Kobuvirus specific sequence reads, 5768 sequences (average length 138 nt) were assembled through mapping assembly (coverage of 108). No other virus sequences were identified in the samples.

The assembled astrovirus genome sequence comprises the complete coding region of 6495 nt. The genome exhibits a typical mamastrovirus gene organisation consisting of ORF1a encoding the nonstructural protein 1A (protein p19, transmembrane protein 1A, 3CL protease, VPg, protein p20), ORF1b (RdRp) and ORF2 (capsid protein precursor), an AAAAAAC frameshift signal and a putative single guide RNA (sgRNA) promotor (AUUUGGAGGGGAGGACCAAAGAAGUGUGAUG) (Fig. 2a). Conserved protein cleavage sites of the nonstructural protein 1a were predicted using UniProtKB (www.uniprot.org/uniprot/Q67726). Phylogenetic analysis of the ORF2 sequence revealed that the grey squirrel mamastrovirus clustered with a clade of yet unassigned astroviruses. Closest relatives are four marmot astroviruses and other rodent astroviruses, with viruses in the sister clade from diverse hosts including bats, porcupines, various even-toed ungulates and a mamastrovirus detected in faeces from the European roller (Fig. 3a).

The genome of grey squirrel kobuvirus has a length of at least 8182 nt. The open reading frame of 7392 nt encodes a polyprotein of 2464 amino acids (aa) and is flanked by a 5'-UTR of at least 641 nt and a 3'-UTR of at least 149 nt. Grey squirrel kobuvirus displays a typical picornavirus genome layout comprising the structural proteins 1AB (VP0), 1C (VP3), 1D (VP1) and eight non-structural proteins (L, 2A-2C, 3A-3D) (Fig. 2b). The proteinase cleavage sites have been predicted on the basis of conserved cleavage sites of known kobuviruses and other picornaviruses of supergroup 2 (dicipiviruses, galliviruses, hemipiviruses, livupiviruses, ludopiviruses, megriviruses, myrropiviruses, osciviruses, passeriviruses, pemapiviruses, poeciviruses, rafiviruses, rosaviruses, sakobuviruses, saliviruses, siciniviruses, symapiviruses, tropiviruses). Conserved motifs of picornaviruses are (i) the H-box/NC motif of the 2A protein (aa positions 1121-1124 and 1179/1180), (ii) the nucleotide binding motif GxxGxGKS of the 2C protein at aa position 1513, (iii) the Tyr-3 residue of the 3B (VPg) peptide at aa 1784, (iv) the active site motif GxCG of the 3C proteinase at aa 1948, and (v) the KDE, PSG, YGDD and FLKR motifs at aa 2156, aa 2286, aa 2324, and aa 2373, respectively. The conserved proteins P1 (precursor of capsid proteins 1AB, 1C, 1D), $2C^{hel}$, $3C^{pro}$ and $3D^{pol}$ were compared to the corresponding proteins of all acknowledged 34 species of the picornavirus supergroup 2 plus enterovirus A71 of supergroup 3 as outgroup. The data reveal divergences greater than 49.4% for P1 and greater than 43.5% for the non-structural proteins indicating a novel picornavirus species. The coalescent trees propose a relationship of the virus with other members of the genus *Kobuvirus* (Fig. 3b, c). This assignment is supported by the P1 phylogenetic tree, and also the 2C and 3C trees are compatible with this classification (Supplementary data). The 3D polymerase sequence, however, clustered with 3D^{pol} of the newly classified ludopivirus A1 (goose picornavirus 1) [37].

The occurrence of recombination in picornaviruses' genomes is variable, even between viruses within a genus [38]. Generation of recombinants is reportedly favoured in genomic regions with restricted genetic variability, e.g. polymerase gene, to guarantee enhanced likelihood of biologically compatible genome combinations [38, 39]. The unexpected clustering of the 3D polymerase of the grey squirrel kobuvirus with 3D^{pol} of the goose picornavirus 1 may indicate a previous recombination event in the evolution of the grey squirrel kobuvirus as documented for kobuviruses of other mammalian species [40, 41]. For instance, a kobuvirus detected in one specimen from a six-month old pig in Hokkaido, Japan was, for the partial 3D^{pol} sequence, phylogenetically most closely related to bovine kobuvirus [41]. A natural inter-species recombinant bovine/porcine enterovirus is also reported in sheep [42], suggesting that picornaviruses may infect different animals sharing close habitats. Squirrels, more than any other mammals, are likely to share a territory with avian species around water bodies, which could potentially explain this recombination event. On the other hand, clustering with the ludopivirus does not necessarily indicate a recombination event with an avian picornavirus. The branch length of the 3D^{pol} trees indicate similar distances to other picornavirus genera, hence, it is also likely that another, yet unknown picornavirus may have served as donor for the 3D^{pol} gene.

Detection of the two viruses in the grey squirrels may indicate a viral cause for the fatal diarrhoea, with astroviruses being the main suspect based on 150fold higher NGS reads, indicating efficient viral replication in the gut. However, further studies such as *in-situ* hybridisation, immunohistochemistry or virus isolation and challenge are required to verify the viruses' pathogenicity and their role in the disease. The orphaned squirrels died within a month of their arrival at the hospital therefore, lacked maternal IgA from their mothers' milk which otherwise would have potentially protected them against such enteric viruses. They were also at an age that they would likely had been weaned naturally in the wild. It could be postulated that the animals were infected in the hospital from previous admissions or in-contact animals and lack of maternal IgA and their naïve/immature immune system have contributed to failure of animals in controlling the infection.

Grey squirrels also host other viral agents, but they are invariably detected as an asymptomatic infection. The most widely known asymptomatic viral infection associated with this species is squirrelpox virus, with only a single known case of pathogenic disease [43]. The virus, however, when transmitted to its sympatric native red species (*Sciurus vulgaris*) has proven to be invariably fatal. Adenovirus is another enteric virus increasingly being detected in the grey squirrel across Britain [44-47], while paramyxovirus presence was also determined in animals from around the UK [48]. Further afield, Hoff and Bigler (1980) [49] reported the presence of human derived asymptomatic echovirus infection in grey squirrels from an area of Florida in the USA. Greenwood and Sanchez (2002) [50] further determined, in a small study at a single site in Wales, exposure of grey squirrels to a wide range of pathogenic agents including enteroviruses, some zoonotic in nature, but with no outward effect. These novel kobu- and astro- viruses increase the spectrum of viruses detected in grey squirrels but their impact on either of the UK's squirrel species is yet to be investigated.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

Samples used in this study were obtained from animal carcasses, so no ethical approval was required.

Fig. 1. Electron micrograph of enterovirus-like particles from a pooled faecal sample visualised by transmission electron microscopy. (Mag. bar = 100nm).

Fig. 2. Genomic organisation of the grey squirrel mamastrovirus and kobuvirus. The astrovirus (a) exhibits typical mamastrovirus genome organisation consisting of ORF1a encoding the nonstructural protein 1A (protein p19, transmembrane protein 1A, 3CL protease, VPg, protein p20), ORF1b (RdRp) and ORF2 (capsid protein precursor). The kobuvirus (b) displays a typical picornavirus genome layout comprising of the structural proteins 1AB (VP0), 1C (VP3), 1D (VP1) followed by eight non-structural proteins (L, 2A-2C, 3A-3D). Proposed proteinase

cleavage sites are indicated. Abbreviations: L, leader protein; 2C^{hel}, 2C helicase; VPg, virus peptide, genome-associated; 3C^{pro}, 3C proteinase; 3D^{pol}, 3D polymerase.

Fig. 3. Phylogenetic analysis of novel grey squirrel astro- and kobuviruses. Phylogenetic analyses were conducted with MrBayes using the GTR+G+I model of nucleotide substitution. Convergence was reached after 2 million generations. The scale bar indicates nt substitutions per site. Numbers at nodes indicate posterior probabilities. AstV, Astrovirus; MAstV, Mamastrovirus. (a) ORF2 phylogenetic analysis of grey squirrel astrovirus, other viruses in the *Mamastrovirus* genus and unassigned mamastroviruses. Four major clustering of viruses are seen in the phylogenetic tree with further divisions within each cluster. Avastrovirus 2 was used as an outgroup. (b, c) Phylogenetic analysis of grey squirrel kobuvirus, other virus species in the *Kobuvirus* genus and representative viruses of other genera in the *Picornaviridae* family, of the P1 and 3D polymerase (3D^{pol}) gene regions.

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