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A novel astrovirus associated with encephalitis and ganglionitis in domestic sheep

Running head: Encephalitis in Domestic Sheep caused by Astrovirus

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Summary

In June 2013, a four-year-old Welsh Mountain ewe and in March 2014 a ten-day-old lamb of the same breed and the same flock presented progressive neurological signs including depressed sensorium, tremor, and unusual behaviour. Neuropathological examination of the brain and spinal cord detected non-suppurative polioencephalomyelitis and dorsal root ganglionitis, characteristic of a neurotropic viral agent in both sheep. Metagenomic analysis of different tissue samples from both animals identified a novel *Ovine Astrovirus* (OvAstV). Presence of viral genome in the central nervous system was confirmed by RT-qPCR. Although the cases presented nine months apart, the identified OvAstV shared nearly identical sequences, differing in only three nucleotide positions across the complete genome. Phylogenetic analysis revealed a close relation of OvAstV to neurotropic bovine astroviruses and an enteric OvAstV. In conclusion, these are the first reported cases of astrovirus infection in domestic sheep that were associated with encephalitis and ganglionitis.

Introduction

Members of the family *Astroviridae* are frequently associated with cases of diarrhoea and gastroenteritis in young individuals and have been described for a multitude of mammals (genus *Mamastrovirus*) and birds (genus *Avastrovirus*). The name astrovirus derives from the star-like appearance of their icosahedral virions, demonstrated by electron microscopy (Madeley and Cosgrove 1975). Typical virions are about 28–30 nm diameter in size, non-enveloped, and contain a single genome molecule, consisting of single-stranded RNA with positive polarity (Monroe 2012), and a size of about 6.4–7.7 kb. The RNA molecule is flanked by untranslated regions (UTR) and contains a poly-A tail at the 3’ terminus, but no 5’ cap. Three open reading frames (ORF) are arranged along the astrovirus genome (ORF1a, ORF1b, and ORF2), coding for at least three polyproteins. Alternatively, ORF1a and ORF1b could form a single ORF, called ORF1ab, utilizing ribosomal frameshifting (Marczinke et al. 1994). During infection, the polyproteins are proteolytically processed into intermediates and several final products, including the RNA-dependent RNA polymerase, a protease, at least three capsid proteins (Méndez et al. 2003) and a genome-linked protein VPg (Fuentes et al. 2012). The virus is transmitted orofaecally and no vector or natural reservoir has been described. As mentioned above, human astroviruses are a major cause of acute gastroenteritis in infants, young children, immunocompromised patients, and the elderly (Glass et al. 1996).

Shortly after the description of the first human astrovirus, a viral agent of similar size and shape was detected in faeces during an outbreak of diarrhoea in lambs (Snodgrass and Gray 1977). The advent of modern molecular diagnostics led to the discovery of novel astroviruses in several domestic animals including cattle, pigs, and poultry, as well as in wild aquatic and terrestrial animals (De Benedictis et al. 2011). Despite being mainly associated with enteric diseases, there are reported observations of extra-intestinal localization of astroviruses, causing hepatitis in ducks (Gough et al. 1984) and nephritis in chicken (Imada et al. 1979) (initially attributed to picornavirus). The first recognized case of fatal encephalitis caused by a human astrovirus affected a 15-year-old boy suffering from X-linked agammaglobulinemia (Quan et al. 2010) and since then, astroviruses have been increasingly recognized as human neurotropic pathogens, mainly in immunocompromised patients (Brown et al. 2015; Frémond et al. 2015; Naccache et al. 2015; Lum et al. 2016). Astrovirus encephalitides have also been reported in cattle (Li et al. 2013; Bouzalas et al. 2014; Bouzalas et al. 2016; Schlottau et al. 2016; Seuberlich et al. 2016) and minks (Blomström et al. 2010). In a representative study, reviewing 1570 cases of human encephalitis, aetiological agents were identified in only 16% (Glaser et al. 2006). This is a remarkably low rate, since the diagnostic efforts in human medicine are relatively high in comparison to veterinary health. Therefore, the likelihood of neurological disorders in domestic animals being unexplained could be even higher. In these cases, high-throughput sequencing in combination with metagenomic analysis provides a powerful and unbiased survey of nucleic acid sequences present in a sample.

Material and Methods

Case Reports

In June 2013, an at least four-year-old Welsh Mountain ewe, brought off a mountain in North Wales (United Kingdom) for shearing was noticed standing apart from the other animals in the flock and trembling. The disability became worse with stimulation and she collapsed in lateral recumbency. She was depressed but aware and did not appear to be blind. However, the centre of the left cornea was cloudy and there was a swelling of periocular skin. The animal was somewhat thin, although small internal fat stores were present, and wool over the back and flanks was loose. The ewe was euthanised two days after clinical signs were noticed.

In March 2014, a ten-day-old Welsh Mountain lamb in the same flock showed unusual behavior, including circling and nibbling at its front legs or at the ground, as well as trembling. When stressed, it showed whole body tremor. Treatment with oxytetracycline over four days resulted in no improvement and the lamb was euthanised ten days after the onset of clinical signs. It was noted, during restraint of each animal for blood sampling and intravenous injection, that turning of the head to one side was resisted.

High-throughput sequencing

RNA was extracted from an organ pool containing brain, spinal cord, and spleen material, for each animal individually, using the Covaris cryoPREP (Covaris, Brighton, United Kingdom) in combination with Trizol LS reagent (Life Technologies, Darmstadt, Germany) and RNeasy columns (Qiagen, Hilden, Germany). A cDNA synthesis system kit (Roche, Mannheim, Germany) together with random hexamer primers (Roche, Mannheim, Germany) was used to generate double stranded cDNA. Libraries were prepared with the GeneRead DNA Library L Core Kit (Qiagen, Hilden, Germany) and sequenced with the IonTorrent PGM according to the manufacturer's instructions.

Metagenomics and sequence assembly

Raw reads of both libraries were subsequently analyzed with the metagenomic pipeline RIEMS (Scheuch et al. 2015). RIEMS classified several reads of both samples as related to the family *Astroviridae*. A de novo assembly of these sequence reads, followed by iterative mapping and assembly using the 454 Sequencing Systems Software Suite (version 3.0; Roche), resulted in the complete genome sequences of two novel astroviruses, provisionally classified as OvAstV. Strains were designated according to sampling place, year, host, and laboratory number as UK/2013/ewe/lib01454 and UK/2014/lamb/lib01455. Genomic termini were confirmed by 5’- and 3’-RACE, as described elsewhere (Schlottau et al. 2016).

Accession numbers

The complete genome sequences for OvAstV UK/2013/ewe/lib01454 and UK/2014/lamb/lib01455 are public available under the accessions LT706531and LT706530, respectively.

RT-qPCR

A *Bovine astrovirus* (BoAstV) BH89/14 specific RT-qPCR system (Schlottau et al. 2016) was used to quantify the viral load in a panel of organ tissues of both animals. To this end, an artificial positive control was designed and ordered as a synthetic oligomer (Biomers, Ulm, Germany), encompassing a T7 promotor, the PCR amplicon with a NotI region and a binding site for a LacZ probe. The artificial positive control was in-vitro transcribed with the RiboMAX Large Scale RNA Production System (Promega, Mannheim, Germany). A DNase I digestion was performed on column during purification of the in-vitro transcript with the RNeasy Mini Kit (Qiagen, Hilden, Germany). The resulting RNA concentration was measured and the number of RNA molecules calculated. A $\log_{10}$ dilution series (1×10^{8} to 1×10^{-1} genome equivalent copies per µl) was prepared and used to absolutely quantify the viral loads in the different organs.

Phylogenetic Analysis

Whole genome sequences of 45 representative astrovirus reference strains, together with the two newly sequenced OvAstV were selected for phylogenetic analysis. The references were either listed in the NCBI Reference Sequence Database (RefSeq) or linked to cases of encephalitis with substantial metadata. The BoAstV CH13/NeuroS1 cluster (Bouzalas et al. 2016) was represented by the prototypic strains BoAstV CH13 and NeuroS1. All sequences were aligned using MAFFT [version 7.017; Katoh et al. (2002)] and refined with MUSCLE (Edgar 2004). A maximum-likelihood tree for the resulting alignment was calculated in IQ-TREE [version 1.3.13; Nguyen et al. (2015)] utilizing the optimal substitution model (GTR+G4+I) and 100,000 ultra-fast bootstrap (Minh et al. 2013) replicates. The analysis was repeated with the ORF2 gene region of the respective astroviruses and an additional partially sequenced OvAstV (OAstV-2/Hungary/2009; 2,474 nt; JN592482), that was found in fecal samples from healthy domestic sheep (Reuter et al. 2012).

Results and Discussion

The significant necropsy finding in both sheep was moderate to severe non-suppurative polioencephalomyelitis particularly involving the cerebellar cortex and spinal cord in the ewe, and more extensively in the lamb, together with non suppurative dorsal root ganglionitis. The histological features in both animals were characteristic of neurotropic viral infections. Louping ill, a tick-borne encephalitis caused by *Louping ill virus*, a member of the family *Flaviviridae* occurs commonly in sheep mainly in upland areas of the British Isles. However, the neuropathological results, in particular presence of dorsal root ganglionitis, and immunohistochemical analyses (data not shown) of brain material argued against *Louping ill virus* infection as the cause of the encephalitides.

Using a metagenomic workflow, we identified several sequences in pooled organ samples of both animals, related to sequences of the family *Astroviridae*. From the available reads, complete genome sequences were assembled de-novo for UK/2013/ewe/lib01454 and UK/2014/lamb/lib01455, and genomic termini were confirmed by 5'- and 3'-RACE. The sequences shared a nucleotide identity of 99.96%, differing in only three positions from a total of 6,454 nt, causing two amino acid changes in ORF1a (A631T) and ORF2 (N554D). The genomic arrangement of predicted ORF1a, ORF1b, and ORF2 and the presence of a ribosomal frameshifting site for expression of ORF1ab represented the typical gene composition common to all astroviruses.

A specific RT-qPCR system (Schlottau et al. 2016) was used to quantify the viral load in a panel of organ tissues from each animal (Tab. 1). The highest OvAstV genome loads were detected in regions of the central nervous system (CNS) for both animals, including the obex and spinal cord. Additionally, the cerebellum of the ten-day-old lamb showed comparable high viral loads and peak values were present in the cerebrum. Other organs, such as spleen, ileum, and pooled intestine showed remarkably low viral loads in comparison with organs of the CNS. Furthermore, low to moderate OvAstV genome loads were detected in lymphoid tissue of the tonsil of both animals. Lymph nodes of the lamb were negative. The presence of high levels of viral RNA in regions of the CNS provides strong evidence for OvAstV being the causative agent of neurological disorders in both animals.

In order to determine the phylogenetic relationship of the newly sequenced astroviruses to other members of the family *Astroviridae*, we compared a total of 47 full length astrovirus genome sequences. The resulting phylogenetic tree (Fig. 1) separated sequences of the genera

Avastrovirus and *Mamastrovirus* into two distinct clusters. The *Mamastrovirus* cluster was further divided into genogroup I, containing classical strains, and genogroup II, encompassing the human-mink-ovine (HMO) strain complex (Kapoor et al. 2009) as well as the BoAstV CH13/NeuroS1 strains (Bouzalas et al. 2016). Both novel OvAstV strains were grouped into the *Mamastrovirus* genogroup II, forming a monophyletic group with sequences of BoAstV BH89/14 (Schlottau et al. 2016), CH13 (Bouzalas et al. 2014), CH15 (Seuberlich et al. 2016), and NeuroS1 (Li et al. 2013), that have previously been associated with fatal encephalitides, and the only other full-length OvAstV genome (NC_002469). The phylogenetic analysis of the ORF2 gene region resulted in the same genogroup assignment as based on full-length sequences and the partial sequenced OvAstV OAstV-2/Hungary/2009 was placed in genogroup I (Fig. S1 in the Supporting Material). Ovine astroviruses are therefore present in both genogroup I and II.

A retrospective classification of analyzed *Mamastrovirus* strains revealed three major types of associated pathogenesis: diarrhoea or gastroenteritis, asymptomatic cases, and encephalitis. Remarkably, all of the described strains that have been associated with encephalitis in humans and mink, as well as in cattle and sheep, including both novel OvAstV strains, are located in genogroup II, indicating a similar genetic ancestry or common genetic features. Whether the capacity for neuronal invasion and unusual tissue tropism of some astroviruses could be reflected by phylogenetic analysis alone, is however questionable.

Nevertheless, the monophyletic group of BoAstV and OvAstV in genogroup II could indicate cross species transmission of astroviruses between cattle and sheep. Most strains of this group have been reported from cases with neurological disease and are only fairly related to enteric or asymptomatic BoAstV and OvAstV, of genogroup I. It could be speculated that astroviruses that cause encephalitis in cattle could originate from small ruminants in close contact. Such mixed animal species rearing has been reported by Schlottau et al. (2016), where 21 goats were held together with a single cow that developed fatal encephalitis. Screening of the goats for astroviruses, however, was negative four months after the encephalitis case. Whether the goats had already cleared the virus or an unknown reservoir host was involved remained unclear.

The described cases of OvAstV affected a lamb as well as a mature ewe from the same flock and two nearly identical astroviruses have been obtained in different years. Repeated outbreaks of a single neurotropic OvAstV might indicate a yet unknown reservoir host, present at the pasture or in the lambing shed. The virus could also be maintained in the flock,

causing mainly undiagnosed enteric signs or asymptomatic infection in healthy animals, whereas only some animals develop encephalitis. Neither theory could be proved by the available data and more screening of the herd and animals in close contact needs to be done. Furthermore, for unresolved cases of ovine neurologic disease from which tissue samples have been preserved, a retrospective diagnostic investigation should be carried out using the established RT-qPCR system published by Schlottau et al. (2016) to further elucidate the role of astroviruses as a cause of encephalitis in sheep. Since *Louping Ill virus* is generally known to be the major cause of neurological disorders in sheep on the British Islands (Jeffries et al. 2014), special caution in diagnostics is needed in order to identify further cases that are related to this potential emerging OvAstV. If neurotropic OvAstV is transmitted by a yet unknown host or is related to cross species transmission between small ruminants and cattle, there may be significant implications for livestock farming and breeding.

Conclusion

Using metagenomic analyses, we identified two nearly identical astroviruses in the brains of two temporally separated cases of fatal encephalitis in domestic sheep of the same breed and the same flock. Using RT-qPCR we estimated the viral load for different organs, reaching peak values in the central nervous system. This is the first report of neurotropic OvAstV, providing complete genome sequences along with substantial clinical metadata. Besides arthropod-borne viruses, such as *Louping ill virus*, OvAstV needs to be considered in differential diagnosis of encephalitis and ganglionitis in domestic sheep. Whether the close phylogenetic relationship of BoAstV and OvAstV is addressable to interspecies transmission between small ruminants and cattle remains an open question for further studies.

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Figure Legends

Fig. 1 Phylogenetic relationship of representative Astrovirus strains, based on full-length genomes. This Maximum-likelihood tree is based on a full-length genome alignment of 45 reference strains together with the two newly sequenced OvAstV (highlighted in red). Three genetic clades are highlighted: Avastroviruses (pink), Mamastrovirus genogroup I (blue) and Mamastrovirus genogroup II (purple). GenBank accession numbers are in parentheses. Colored symbols indicate certain pathology: encephalitis (red circle), diarrhoea or gastroenteritis (blue square) and faecal samples from asymptomatic cases (black triangle). AvAstV, avian astrovirus; BoAstV, bovine astrovirus; CaAstV, canine astrovirus; FeAstV, feline astrovirus; HuAstV, human astrovirus; DcAstV, dromedary camel astrovirus; MiAstV, mink astrovirus; MuAstV, murine astrovirus; OvAstV, ovine astrovirus; PoAstV, porcine astrovirus; RaAstV, rabbit astrovirus; WBAstV, wild boar astrovirus.

Supporting Information

Fig. S1 Phylogenetic relationship of representative Astroviruses based on the ORF2 gene region.

Supplementary Information for

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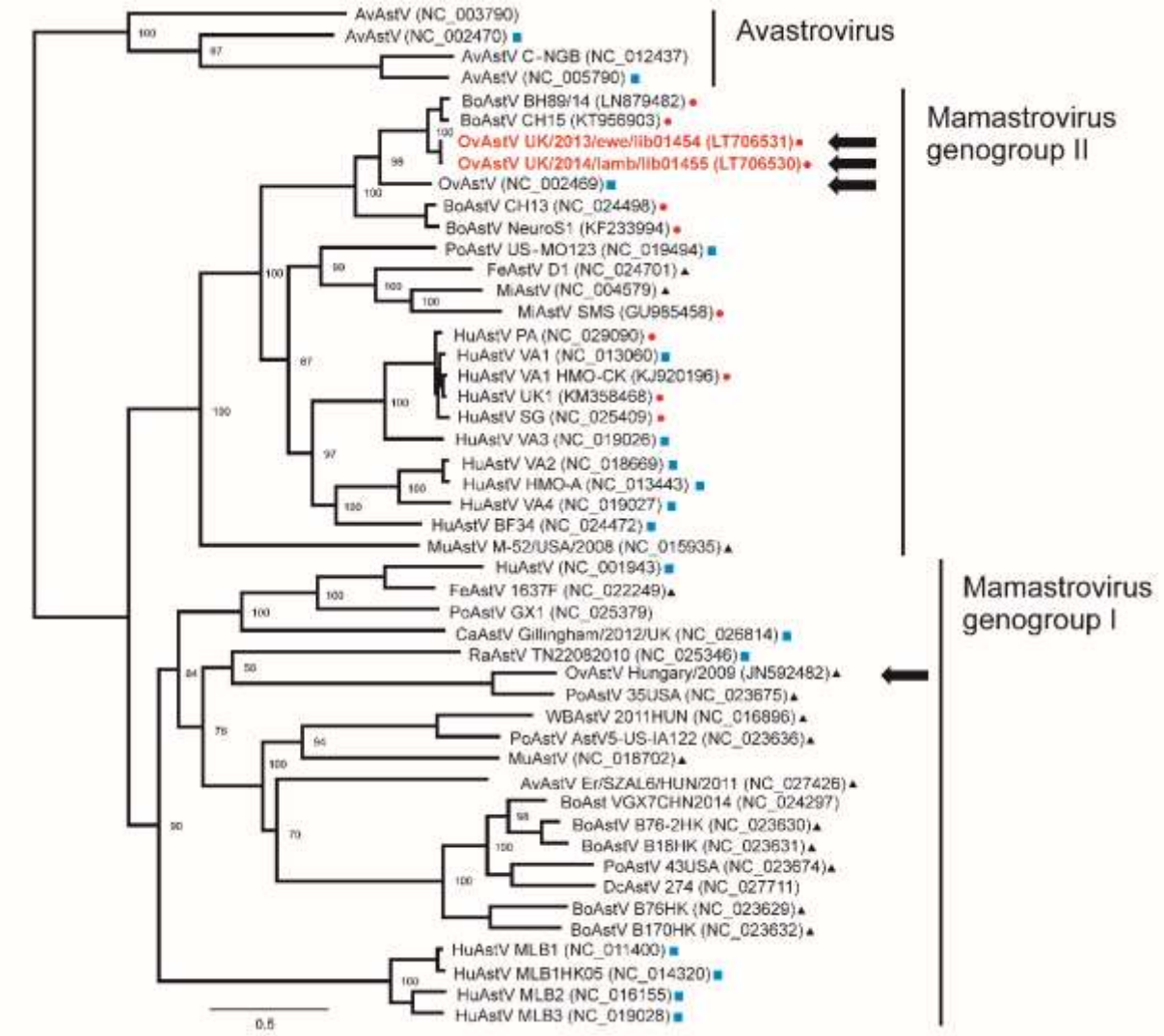

Fig. S1 Phylogenetic relationship of representative Astroviruses based on the ORF2 gene region.

This Maximum-likelihood tree is based on an alignment of the ORF2 gene region of 46 reference strains together with the two newly sequenced OvAstV (highlighted in red). Three genetic clades are indicated: Avastroviruses, Mamastrovirus genogroup I and Mamastrovirus genogroup II. GenBank accession numbers are in parentheses. Colored symbols indicate certain pathology: encephalitis (red circle), diarrhoea or

gastroenteritis (blue square) and faecal samples from asymptomatic cases (black triangle). Ovine astroviruses are (indicated with black arrows) are present in both genogroups. AvAstV, avian astrovirus; BoAstV, bovine astrovirus; CaAstV, canine astrovirus; FeAstV, feline astrovirus; HuAstV, human astrovirus; DcAstV, dromedary camel astrovirus; MiAstV, mink astrovirus; MuAstV, murine astrovirus; OvAstV, ovine astrovirus; PoAstV, porcine astrovirus; RaAstV, rabbit astrovirus; WBAstV, wild boar astrovirus.