Infection, Genetics and Evolution 44 (2016) 162–168

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Research paper

# Full-genome based molecular characterization of encephalitis-associated bovine astroviruses

Ilias G. Bouzalas [a], Daniel Wüthrich [b], Senija Selimovic-Hamza [a,c], Cord Drögemüller [d], Rémy Bruggmann [b], Torsten Seuberlich [a,*]

[a] NeuroCenter, Division of Neurological Sciences, Vetsuisse Faculty, University of Bern,Bremgartenstrasse 109a, CH-3012 Bern, Switzerland
[b] Interfaculty Bioinformatics Unit, University of Bern and Swiss Institute of Bioinformatics, Baltzerstrasse 6, CH-3012 Bern, Switzerland
[c] Graduate School for Cellular and Biomedical Sciences, University of Bern, Freiestrasse 1, CH-3012 Bern, Switzerland
[d] Institute of Genetics, Vetsuisse Faculty, University of Bern, Bremgartenstrasse 109a, CH-3012 Bern, Switzerland

ARTICLE INFO

*Article history:*
Received 21 May 2016
Received in revised form 29 June 2016
Accepted 30 June 2016
Available online 1 July 2016

*Keywords:*
Encephalitis
Cattle
Astrovirus
Epidemiology
Neurology

ABSTRACT

Novel types of astrovirus have been identified recently in association with neurological disease in cattle. Among those viruses is bovine astrovirus CH13 (BoAstV CH13) that has been identified in Switzerland in a cow with encephalitis. Molecular testing by a combination of reverse transcription (RT-) PCR and in situ hybridization (ISH) indicated that astrovirus infection accounts for around one quarter of viral encephalitis cases of unknown etiology in cattle. Yet, it remained to be explored whether these animals were infected by BoAstV CH13 or other astrovirus species. In the present study we sequenced the entire astrovirus genome in brain tissues of eight RT-PCR and/or ISH positive cattle. Phylogenetic comparison of the genomic RNA and the encoded non-structural and structural proteins revealed that all these astrovirus strains were very similar to BoAstV CH13 as well as to a bovine encephalitis strain reported from the USA (BoAstV NeuroS1), and clearly distinct from other previously reported astroviruses. Conserved 5′ and 3′ untranslated regions (UTRs) were predicted to display distinct secondary RNA structures, which likely play a role in viral RNA replication and/or protein translation. Based on these data we propose that BoAstV CH13/NeuroS1 represents a new genotype species within the genus *Mammastrovirus*. The high degree of similarity between the strains and their relative distance to other genotype species suggest that during evolution some astroviruses acquired factors that specifically contribute to neuroinvasion.



## 1. Introduction

Astroviruses are small, non-enveloped viruses with a non-segmented, positive sense, single-stranded RNA genome of approximately 6.2–7.7 nucleotides. The viral genomes are poly-adenylated, comprising of short 5′ and 3′ untranslated regions (UTRs) and show three partially overlapping open-reading frames (ORF1a, ORF1b, and ORF2) (De Benedictis et al., 2011). ORF1a and 1b are located towards the 5′ end of the RNA and encode for polyproteins that are translated into the precursor non-structural proteins (nsp) 1a and 1ab via a ribosomal frameshift mechanism. Posttranslational cleavage of these polyproteins yields the RNA-dependent RNA polymerase (RdRP), a viral protease (v-Pro) and a number of proteins of unknown function (Kiang and Matsui, 2002). The polyprotein encoded by ORF2 is translated from a subgenomic RNA, and further processed by cellular caspases and trypsin to mature proteins which form the viral capsid (Mendez et al., 2002).

The family *Astroviridae* consists of two genera: Avastrovirus (AAstV), infecting avian species and *Mammastrovirus* (MAstV), infecting mammalian species. MAstVs have been identified in >14 mammalian species, including humans, wildlife, companion animals as well as livestock. Conventionally, astrovirus strains are named according to the host species of origin (Bosch et al., 2014). However in 2010, the International Committee on Taxonomy of Viruses (ICTV) redefined astrovirus classification into 19 distinct MAstV species, MAstV 1–19. Recently, the additional species MAstV 20–33 have been proposed (Guix et al., 2013). The classical type of human astrovirus (MAstV 1, also referred to as HAstV 1–8) is a leading cause of gastroenteritis in children worldwide (Wilhelmi et al., 2003). In other mammalian species the causal relation of astrovirus infection and disease is not apparent, and most strains were identified in feces samples of healthy individuals. Advances in viral identification techniques such as generic PCR protocols and

* Corresponding author.
*E-mail addresses:* ilias.bouzalas@vetsuisse.unibe.ch (I.G. Bouzalas), daniel.wuethrich@bioinformatics.unibe.ch (D. Wüthrich), senija.selimovic@vetsuisse.unibe.ch (S. Selimovic-Hamza), cord.droegemueller@vetsuisse.unibe.ch (C. Drögemüller), remy.bruggmann@bioinfromatics.unibe.ch (R. Bruggmann), torsten.seuberlich@vetsuisse.unibe.ch (T. Seuberlich).

http://dx.doi.org/10.1016/j.meegid.2016.06.052

**Table 1**
Details of astrovirus-positive encephalitis cases and results of next generation sequencing and read mapping to BoAstV CH13.

| Case ID | Year of diagnosis | In-situ hybridization | RT-PCR | NGS results [total reads] | Mapping to BoAstV CH13 | |
|---|---|---|---|---|---|---|
| | | | | | Mean read-depth | Fraction covered by reads [%] |
| 23871 | 1995 | + | + | 14,354,647 | 33,403 | 99.9 |
| 23985 | 1995 | + | + | 7,435,880 | 36 | 51.4 |
| 26730 | 1998 | + | nd | 25,407,275 | 35 | 99.9 |
| 26875 | 1998 | + | + | 14,928,092 | 37,519 | 99.9 |
| 36716 | 2004 | + | nd | 14,367,372 | 2849 | 99.8 |
| 42799 | 2007 | + | + | 22,359,907 | 1916 | 99.9 |
| 43660 | 2009 | + | nd | 17,445,920 | 101 | 98.9 |
| 43661 | 2009 | nd | + | 6,742,907 | 16 | 48.7 |

nd, not done.

unbiased next-generation sequencing (NGS), have led to a vast expansion of the number of known astrovirus strains. Some of them, such as HAstV MLB (MAstV 6), HAstV HMO-A (MAstV 8) and HAstV VA1 (MAstV 9) are novel genotype species and form previously unknown phylogenetic clades (Finkbeiner et al., 2008; Finkbeiner et al., 2009).

Most recently, a number of novel strains were found in brain tissues or cerebral spinal fluid of human patients with neurological disease and encephalitis. This suggested a role for astrovirus infection in diseases beyond those of the enteric system (Blomstrom et al., 2010; Brown et al., 2015; Cordey et al., 2016; Fremond et al., 2015; Naccache et al., 2015; Quan et al., 2010; Sato et al., 2016; Wunderli et al., 2011). In the context of neurologically diseased animals, the mink astrovirus strain (MiAstV-SMS) was identified in the brains of farmed minks affected by shaking mink syndrome (Blomstrom et al., 2010). Moreover, two lineages of porcine astrovirus were detected in piglets with congenital tremor, but the presence of the virus was not clearly associated with the disease (Blomstrom et al., 2014).

In cattle four astrovirus strains were identified by NGS in brain tissue samples by independent studies originating from the USA and Europe: BoAstV NeuroS1 (Li et al., 2013), BoAstV CH13 (Bouzalas et al., 2014), BoAstV CH15 (Seuberlich et al., 2016) and BoAstV BH89/14 (Schlottau et al., 2016), and full genome sequences of these viruses were reported. By reverse transcription-PCR (RT-PCR) and in situ hybridization (ISH), we found evidence for BoAstV CH13 infection in around one quarter of cattle with non-suppurative encephalitis of unresolved etiology in Switzerland (Bouzalas et al., 2014). However, knowledge of the molecular epidemiology of these strains remained limited. The aim of the present study was to obtain full astrovirus genome sequences for these cases by NGS and to analyze them at the molecular level. Our results show that these strains are remarkably conserved genetically and are closely related to BoAstV CH13 and BoAstV NeuroS1.

## 2. Materials and methods

### 2.1. Tissue samples

Frozen brain tissues of eight neurologically diseased cattle (1.5 to 7 years of age) diagnosed in Switzerland between 1995 and 2009 were selected from the archive of the Division of Neurological Sciences, Vetsuisse Faculty, University of Bern. These animals had been diagnosed with non-suppurative encephalitis by histopathological examination of the brain. The spectrum of clinical signs included abnormal gait (or recumbency), behavioral changes and hyper-reactivity or depression. All of them had been notified as clinical suspicious for Bovine spongiform encephalopathy, and were tested negative for BSE and some (23871, 23985, 26730 and 26875) for additional pathogens, including Rabies virus, Borna virus, Tick Borne Encephalitis virus and Chlamydia (Theil et al., 1998). Four animals were tested positive for the presence of AstV RNA by RT-PCR targeting a conserved region of ORF 1b and/or in situ RNA hybridization using in vitro transcribed digoxigenin-labeled RNA probes in the brain, and have been reported in a previous study (Bouzalas et al., 2014). Using the same methods, four additional cases were diagnosed as AstV positive in an extended retrospective study, which is still ongoing in our laboratory (unpublished data). However, both molecular tests detect short genomic regions which were not sufficient for molecular characterization of the strain. Therefore, all eight astrovirus positive cases were submitted for full genome determination. Details of these cases are shown in Table 1.

### 2.2. Sample pretreatment and viral RNA extraction

Approximately 100 μg of frozen brain tissue per case were collected at the level of the caudal brainstem and homogenized in 1 ml sterile PBS using a Fastprep homogenization device and TeSeE grinding tubes (Bio-Rad). Homogenates were transferred to LoBind 1.5 ml tubes (Eppendorf) and centrifuged for 10 min at 10,000 g and 4 °C. The supernatant was filtered through a 0.22 μm syringe filter and 249 μl of the filtrate was mixed with 30 μl of 10× DNase buffer (Roche), 150 U DNAse I (Roche), as well as 4 μg RNAse (Promega) to a final volume of 300 μl and incubated for 120 min at 37 °C. RNA was then extracted with Trizol reagent (Thermo Fisher Scientific) following the manufacturers protocol. The quality and quantity of RNA was assessed by a Bioanalyzer 2100 (Agilent Technologies).

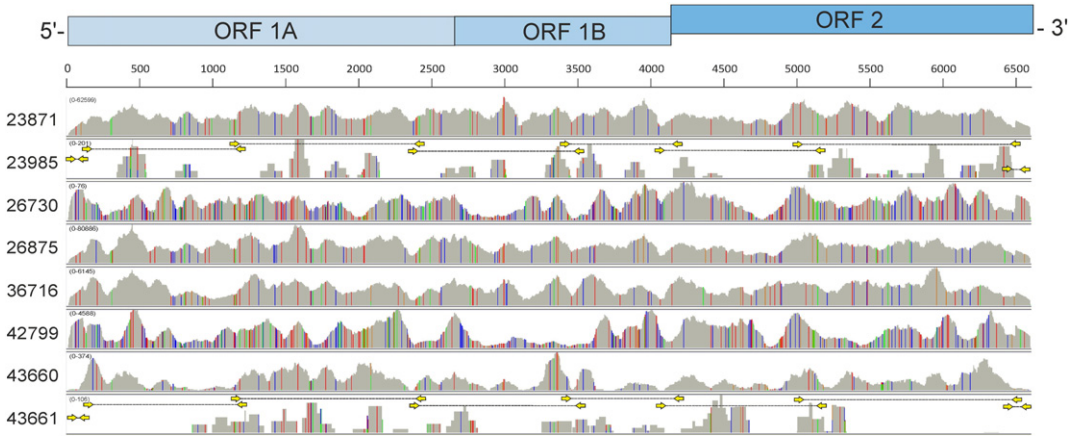



**Fig. 1.** Next Generation Sequencing coverage plots of bovine astrovirus-positive animals. For each animal the read depth (y-axis) is presented over the entire lengths of the BoAstV CH13 reference sequence (x-axis). The corresponding genome organization is shown on top. Grey colors indicate sequences identical to the BoAstV CH13 reference and colour bars indicate the type and proportion of nucleotide polymorphisms (C, blue; T, red; G, brown; A, green). Numbers in brackets show the range of read depth for each sample. Arrows indicate positions of oligonucleotides for RT-PCR and Sanger Sequencing.

### 2.3. Next generation sequencing and bioinformatics

Individually barcoded RNA libraries were constructed from RNA extracts according to the Illumina's TruSeq Stranded Total RNA kit (Illumina). RNA libraries were pooled and sequenced in one lane of an Illumina HiSeq2500 apparatus. Paired-end reads of 2 × 100 bp were collected and reads of each library were mapped to the RefSeq viral genome database (6th May 2015, supplemented with the BoAstV CH15 genome) usingBowtie2 (version 2.2.1–sensitive) (Langmead and Salzberg, 2012) and scaffolds were assembled as described previously (Wuthrich et al., 2016). Coverage plots were generated with the Integrative Genomics Viewer (Robinson et al., 2011).

### 2.4. Sanger sequencing and RACE

Cases in which reads covered only parts of the BoAstV CH13 genome were reanalyzed by conventional Sanger sequencing. To this end, extracted RNA was reverse transcribed to cDNA with the ThermoScript® reverse transcriptase (Invitrogen) using oligo-dT and gene-specific primers, and the cDNA was amplified by PCR with GoTaq® G2 Green Master Mix (Promega) and primer pairs that generate overlapping amplicons covering the entire viral genome. The 5′ and 3′ ends of the viral genome in all 8 cases were determined by rapid amplification of cDNA ends (RACE) following the instructions of the 5′ RACE kit (Life Technologies) (Fig. 1). Amplicons were purified with the QIAquick PCR Purification kit (QIAGEN) and bi-directionally sequenced. Primer sequences are available upon request.

### 2.5. Phylogenetic comparison

Nucleotide and protein sequences of representative members of the *Astroviridae* were aligned to the strains included in our study using the MEGA 5.2 software (Tamura et al., 2011). A neighbor-joining tree based on the full genome nucleotide sequences was constructed using the Jukes-Cantor model with 1000 bootstrap replicates. The same methodology was used to compare protein sequences of the capsid polyprotein using the Jones-Taylor-Thornton model by a maximum-likelihood tree. Pairwise sequence comparison was conducted using the CLC Genomics Workbench 8.5 software (CLCbio).

### 2.6. RNA secondary structure prediction

RNA secondary structures of the 3′ UTR, the 5′ UTR and the putative ribosomal frameshift signal were predicted with m-fold (Zuker, 2003) and RNA sequence alignments and sequence logo graphs were generated with the geneious software package (Version 9.4.0, Biomatters).

## 3. Results

### 3.1. Full genome sequencing of astrovirus strains

Next-generation-sequencing of viral RNA enriched brain tissue extracts of astrovirus positive cattle yielded between 6,742,907 (case 43661) and 25,407,275 (case 36730) reads per sample. In all 8 cases, reads were identified that mapped to the BoAstV CH13 genome (GenBank accession number: NC_024498.1). In six animals >99% and in two animals (23985 and 43661) ~50% of the BoAstV CH13 genome were covered by reads (Table 1, Fig. 1). In the latter two cases the entire viral genome was re-sequenced by Sanger sequencing following RT-PCR and for all strains the 5′ and 3′ ends of the viral genomes were determined by RACE. This resulted in full virus genome sequences of 6529 nt in animals 26730 and 42799 and of 6530 nt in all others. As reported previously, in case 42799 we also found reads that mapped to BoAstV CH15 (GenBank accession number: KT956903), and this animal was likely co-infected by BoAstV CH13 and BoAstV CH15 (Seuberlich et al., 2016). In one animal (26875), we additionally found reads that

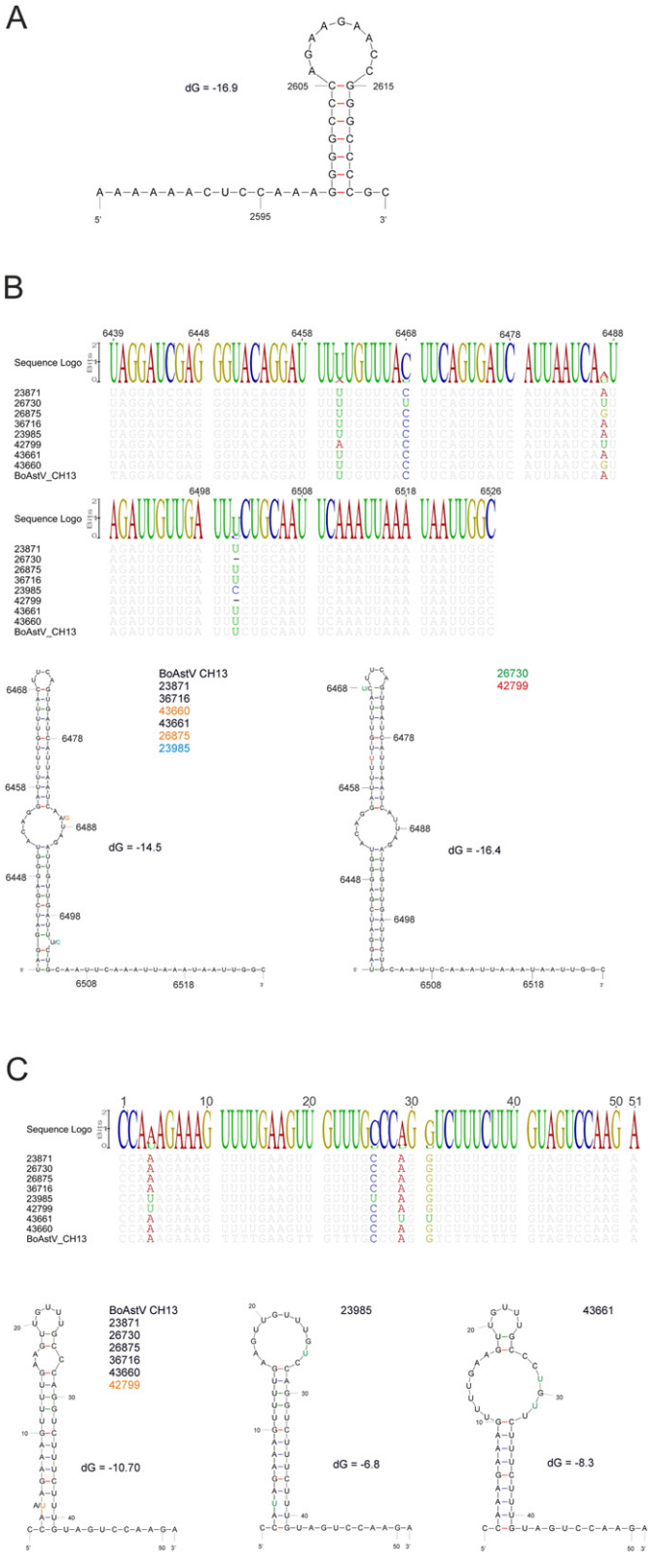



**Fig. 2.** Analysis of primary and secondary RNA structures in bovine astrovirus strains. (A) Predicted secondary structure of the ribosomal frameshift signal at the ORF 1a–1b junction. The sequence was fully conserved among the bovine encephalitis strains and is composed of the 5′-AAAAAAC-3′ ribosomal slippery sequence and a 23 nt stem-loop motif. Sequence alignment and predicted secondary RNA structure of the 3′ UTR (B) and 5′ UTR (C). Polymorphisms of individual strains are indicated by colored identification numbers and corresponding nucleotides. Numbering refers to the nucleotide position in the viral genome.

**Table 2**
Pairwise molecular comparison of encephalitis-associated astrovirus strains of cattle. Nucleotide (bold) and amino acid (grey) sequence identities [%] are shown for ORF 1ab encoding for the nonstructural polyprotein 1ab and ORF 2 encoding for the structural capsid polyprotein. BoAstV CH15 was used as an outgroup.

| Strain | BoAstV CH13 | 23871 | 23985 | 26730 | 26875 | 36716 | 42799 | 43660 | 43661 | BoAstV CH15 |
|---|---|---|---|---|---|---|---|---|---|---|
| Nonstructural polyprotein 1ab / ORF 1ab | | | | | | | | | | |
| BoAstV–CH13 | – | 99.63 | 99.34 | 98.9 | 99.42 | 99.49 | 98.69 | 99.49 | 99.27 | 57.73 |
| 23871 | 98.51 | – | 99.56 | 99.12 | 99.63 | 99.71 | 98.9 | 99.71 | 99.49 | 57.88 |
| 23985 | 98.08 | 98.93 | – | 98.83 | 99.34 | 99.42 | 98.61 | 99.42 | 99.2 | 57.88 |
| 26730 | 93.35 | 93.69 | 93.67 | – | 98.98 | 98.98 | 99.42 | 98.98 | 98.76 | 57.88 |
| 26875 | 98.17 | 99.03 | 98.49 | 93.52 | – | 99.49 | 98.83 | 99.49 | 99.27 | 57.81 |
| 36716 | 98.39 | 99.1 | 98.61 | 93.64 | 98.71 | – | 98.76 | 99.56 | 99.34 | 57.73 |
| 42799 | 92.84 | 93.18 | 93.06 | 97.57 | 93.01 | 93.11 | – | 98.76 | 98.54 | 57.52 |
| 43660 | 98.25 | 98.9 | 98.37 | 93.45 | 98.47 | 98.73 | 93.06 | – | 99.34 | 57.88 |
| 43661 | 98.2 | 98.95 | 98.37 | 93.38 | 98.51 | 98.73 | 92.96 | 98.44 | – | 57.88 |
| BoAstV–CH15 | 59.46 | 59.46 | 59.51 | 59.66 | 59.44 | 59.44 | 59.44 | 59.46 | 59.46 | – |
| Capsid polyprotein / ORF 2 | | | | | | | | | | |
| BoAstV CH13 | – | 99.47 | 99.47 | 99.47 | 99.47 | 99.61 | 99.61 | 99.61 | 99.74 | 64.2 |
| 23871 | 98.47 | – | 99.47 | 99.47 | 99.47 | 99.61 | 99.61 | 99.61 | 99.74 | 64.07 |
| 23985 | 98.38 | 99.12 | – | 99.47 | 99.47 | 99.61 | 99.61 | 99.61 | 99.74 | 64.2 |
| 26730 | 95.18 | 95.53 | 95.4 | – | 99.47 | 99.61 | 99.61 | 99.61 | 99.74 | 64.2 |
| 26875 | 98.42 | 99.17 | 98.99 | 95.53 | – | 99.61 | 99.61 | 99.61 | 99.74 | 64.07 |
| 36716 | 98.47 | 99.04 | 98.95 | 95.36 | 98.9 | – | 99.74 | 99.74 | 99.87 | 64.2 |
| 42799 | 94.74 | 94.92 | 94.79 | 98.25 | 94.83 | 94.74 | – | 99.74 | 99.87 | 64.2 |
| 43660 | 98.47 | 98.95 | 98.86 | 95.58 | 98.82 | 99.04 | 94.96 | – | 99.87 | 64.2 |
| 43661 | 98.42 | 98.9 | 98.73 | 95.36 | 98.77 | 98.99 | 94.92 | 98.9 | – | 64.2 |
| BoAstV CH15 | 64.03 | 64.25 | 64.16 | 64.16 | 63.86 | 64.07 | 63.9 | 64.07 | 64.07 | – |

covered the almost entire genome (>99.8%) of Parainfluenza Virus 5 (GenBank accession number: NC_006430) with a mean read-depth of ~13.

### 3.2. Molecular bovine astrovirus characteristics

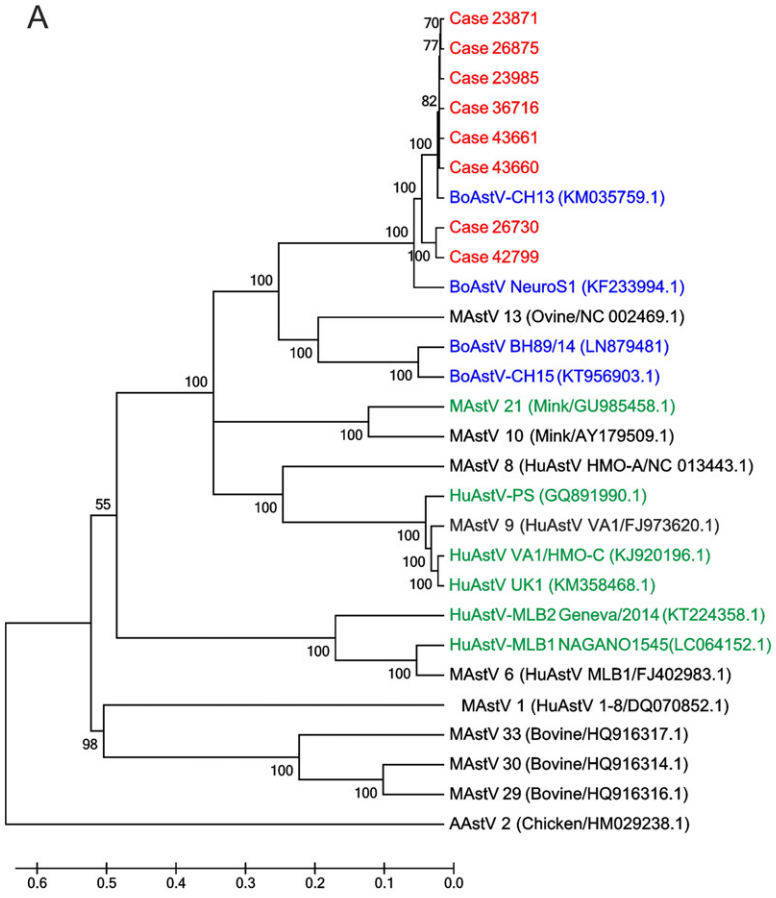
The genomic organization of the eight astrovirus strains was strikingly similar and in-line with the characteristic features of the *Astroviridae*, i.e., it revealed three ORFs (ORF 1a,b and 2) with a ribosomal slippery sequences (5′-AAAAAAC-3′) and a conserved stem-loop of 23 nt between ORF 1a and ORF 1b (Fig. 2 A). ORF 1a (2586 nt), ORF 1ab (4112 nt), ORF 2 (2286 nt) and the 3′ UTR (88 nt, excluding the poly-A tail) were exactly the same size as in the BoAstV CH13 reference sequence. Only in two strains (26730 and 42799) the 3′ UTR was composed of 87 nt (Fig. 2B). At the nucleotide level, the similarity between the consensus sequences and the BoAstV CH13 reference genome was >98% for 6 strains (23871, 23985, 26875, 36716, 43660 and 43661) and slightly lower (~93%) for 26730 and 42799 (Table 2). Pairwise comparison of protein sequences of nsp 1ab and of the capsid polyprotein revealed similarities of >98% and >99%, respectively, for all strains when compared to those in BoAstV CH13 (Table 2). Similar to the coding parts of the genome, the UTRs were remarkably conserved between strains. We found a stem-loop structure composed of 40 nt in the 5′ UTR (Fig. 2C) and a large stem-loop formed by 65–66 nt in the 3′ UTR (Fig. 2B). However, depending on individual nucleotide sequences, the shape and energetic stability of these predicted RNA structures varied slightly.

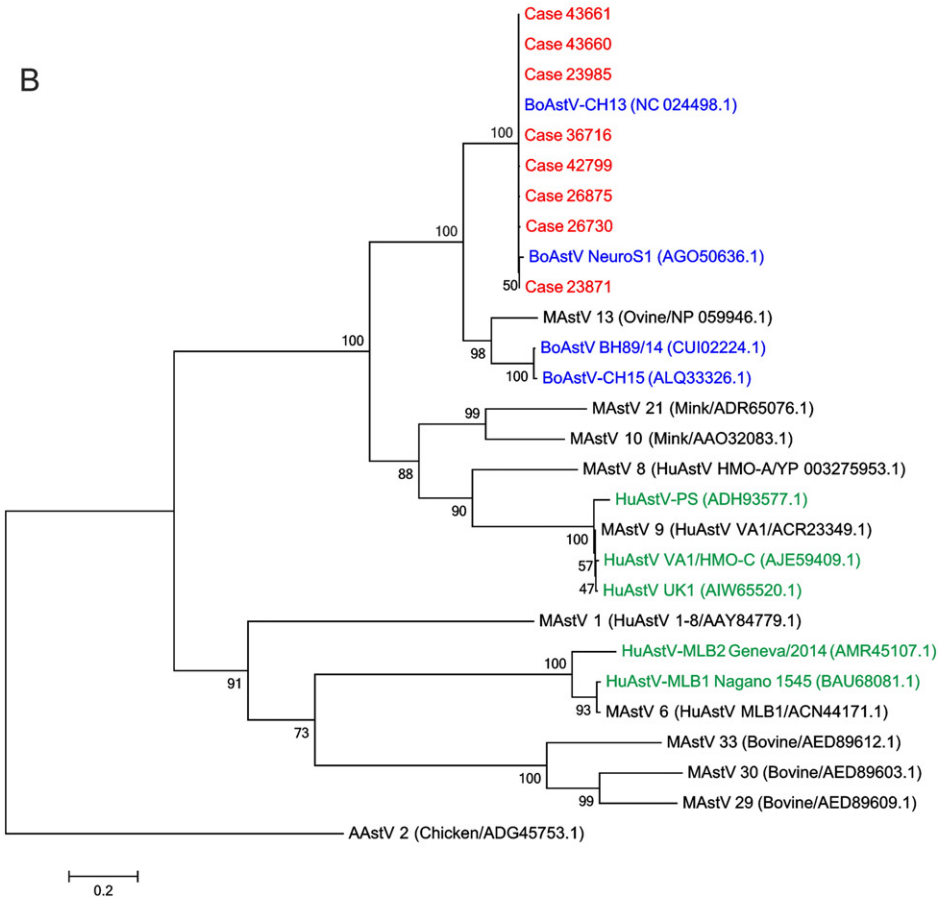
### 3.3. Phylogenetic analysis

The observed similarities between the bovine neurotropic strains were also evident by phylogenetic comparison on the nucleotide and protein level. All the strains under investigation were phylogenetically very close to the reference strain BoAstV CH13 and to BoAstV NeuroS1 with a maximum p-distance of 0.0108 on the protein level (capsid protein), and they clustered distant from classical bovine and human strains derived from feces as well as BoAstV CH15 and BoAstV BH89/14 (Fig. 3).

## 4. Discussion

This study focused on cases of bovine encephalitis in which BoAstV CH13 infection was suspected based on RT-PCR or ISH results. We were able to obtain full astrovirus genome sequences from brain tissues of eight animals. The identification of Parainfluenza virus 5 in one of these cattle was unexpected, but not unprecedented. We also recently found this virus by NGS in two other cases of bovine encephalitis; however its role in disease pathogenesis for the moment remains unknown (Wuthrich et al., 2016).


A
Case 23871
Case 26875
Case 23985
Case 36716
Case 43661
Case 43660
BoAstV-CH13 (KM035759.1)
Case 26730
Case 42799
BoAstV NeuroS1 (KF233994.1)
MAstV 13 (Ovine/NC 002469.1)
BoAstV BH89/14 (LN879481)
BoAstV-CH15 (KT956903.1)
MAstV 21 (Mink/GU985458.1)
MAstV 10 (Mink/AY179509.1)
MAstV 8 (HuAstV HMO-A/NC 013443.1)
HuAstV-PS (GQ891990.1)
MAstV 9 (HuAstV VA1/FJ973620.1)
HuAstV VA1/HMO-C (KJ920196.1)
HuAstV UK1 (KM358468.1)
HuAstV-MLB2 Geneva/2014 (KT224358.1)
HuAstV-MLB1 NAGANO1545(LC064152.1)
MAstV 6 (HuAstV MLB1/FJ402983.1)
MAstV 1 (HuAstV 1-8/DQ070852.1)
MAstV 33 (Bovine/HQ916317.1)
MAstV 30 (Bovine/HQ916314.1)
MAstV 29 (Bovine/HQ916316.1)
AAstV 2 (Chicken/HM029238.1)
0.6 0.5 0.4 0.3 0.2 0.1 0.0


B
Case 43661
Case 43660
Case 23985
BoAstV-CH13 (NC 024498.1)
Case 36716
Case 42799
Case 26875
Case 26730
BoAstV NeuroS1 (AGO50636.1)
Case 23871
MAstV 13 (Ovine/NP 059946.1)
BoAstV BH89/14 (CUI02224.1)
BoAstV-CH15 (ALQ33326.1)
MAstV 21 (Mink/ADR65076.1)
MAstV 10 (Mink/AAO32083.1)
MAstV 8 (HuAstV HMO-A/YP 003275953.1)
HuAstV-PS (ADH93577.1)
MAstV 9 (HuAstV VA1/ACR23349.1)
HuAstV VA1/HMO-C (AJE59409.1)
HuAstV UK1 (AIW65520.1)
MAstV 1 (HuAstV 1-8/AAY84779.1)
HuAstV-MLB2 Geneva/2014 (AMR45107.1)
HuAstV-MLB1 Nagano 1545 (BAU68081.1)
MAstV 6 (HuAstV MLB1/ACN44171.1)
MAstV 33 (Bovine/AED89612.1)
MAstV 30 (Bovine/AED89603.1)
MAstV 29 (Bovine/AED89609.1)
AAstV 2 (Chicken/ADG45753.1)
0.2

To increase the efficiency of NGS for encapsidated viral RNA, host nucleic acids were depleted by centrifugation, filtration and nuclease treatment of brain tissue homogenates before viral RNA extraction. The efficacy of the pretreatment had been previously evaluated for two of the samples (23871 and 26875) showing that treated samples could give at least 100-fold higher mean coverage compared to untreated samples in the NGS (Supplementary Fig. 1). This procedure appeared to be very effective, since in six animals almost complete virus genomes could be assembled from the NGS data with very high read depth in some instances. However, in two animals, only parts of the viral genome were covered with relative low read depths and conventional resequencing was required to fill sequence gaps. We have previously seen that BoAstV CH13 RNA is abundant in neuron cells, i.e. in the grey matter of the CNS, and not equally distributed throughout the brain (unpublished results). It is likely that the variability in read depth reflects different levels of virus particle loads in the samples.

The viral genomes of all eight strains were very similar to each other and to BoAstV CH13 with regard to the genomic organization, the nucleotide sequences and the predicted amino acid sequences of the non-structural and structural polyproteins.

Current classification of astrovirus species relies on the sequence diversity of the capsid protein and strains with differences higher than 30%–35% have to be regarded as individual genotype species (Bosch et al., 2014). All the strains investigated in this study belong to the same astrovirus genotype species together with BoAstV CH13 and BoAstV NeuroS1 and differed from the next closely related branches of the phylogenetic tree (Fig. 3) by at least 33%. Therefore, we propose to tentatively term this virus BoAstV CH13/NeuroS1 until an official classification of the ICTV is available. A formal application for recognition of a new MAstV species will be made to the ICTV.

We observed a high similarity of the primary and predicted secondary structures of the UTRs between BoAstV CH13/NeuroS1 strains. The 5′ UTR of astroviruses varies between 11 and 85 nt in length and little is known about its structure and function. In AAstV a conserved stem-loop structure of 8–18 nt within the 5′ UTR has been proposed to function as a promoter element (Jonassen et al., 2003), however, to our knowledge a similar structure has so far not been reported for MAstV. In the BoAstV CH13/NeuroS1 strains of the present study, a prominent stem-loop structure was predicted for the first 41 nt of the genome. Comparative analyses of the corresponding sequences of other MAstV strains presented in Fig. 3 did not reveal a similar structure, except for BoAstV BH89/14, in which a 30 nt stem-loop was identified (unpublished results). In addition, we predicted a conserved stem-loop of 87–88 nt for the 3′ UTR. However, this was different in sequence and structure from the previously proposed stem-loop II motif in some other astrovirus species (Jonassen et al., 1998; Monceyron et al., 1997). Our findings support the notion that these RNA structures play a role in viral RNA replication and/or protein translation. It would be interesting to address the function of these structures experimentally, e.g., by mutagenesis studies in a reverse genetic system (Geigenmuller et al., 1997).

Besides BoAstV CH13/NeuroS1 two additional AstV strains have been detected in brain tissues of individual cattle with encephalitis: BoAstV CH15 by our group (Seuberlich et al., 2016) and BoAstV BH89/14 in Germany (Schlottau et al., 2016). Both are genetically very similar and clustered in the same branch of the phylogenetic trees (Fig. 3). Still little is known on the frequency and geographical distribution of neuronal infections with these strains. However, these findings point to the possibility that the spectrum of astroviruses associated with encephalitis may be much broader than currently known.

Collectively, our data underpins the hypothesis that BoAstV CH13/NeuroS1 strains from cattle brains are remarkably conserved and clearly different from previously described astrovirus species, in particular from those detected in bovine feces samples (MAstV 28–30, 33). This suggests that BoAstV CH13/NeuroS1 evolved towards a specific ecological niche, characterized by a high level tropism of these viruses to the nervous system. However, the importance of BoAstV CH13/NeuroS1 strains in gastrointestinal diseases still remains unknown. Future research should aim at investigate the disease pathogenesis and identify virus- and host factors that contribute to neuroinvasion and CNS pathology.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.meegid.2016.06.052.

## Funding

This work was funded by the Federal Food Safety and Veterinary Office (T. Seuberlich, grant MON-108) and by the Swiss National Science Foundation (T. Seuberlich and R. Bruggmann, grant 31003A_163438).

## GenBank accession numbers

BoAstV CH13/NeuroS1_23871: KX266901
BoAstV CH13/NeuroS1_23985: KX266905
BoAstV CH13/NeuroS1_26730: KX266902
BoAstV CH13/NeuroS1_26875: KX266903
BoAstV CH13/NeuroS1_36716: KX266904
BoAstV CH13/NeuroS1_42799: KX266906
BoAstV CH13/NeuroS1_43660: KX266908
BoAstV CH13/NeuroS1_43661: KX266907

## Acknowledgements

We thank the Next Generation Sequencing Platform of the University of Bern for performing the high-throughput sequencing as well as Muriel Fragnière and Ramona Lüthi for excellent technical support.

## References

Blomstrom, A.L., Widen, F., Hammer, A.S., Belak, S., Berg, M., 2010. Detection of a novel astrovirus in brain tissue of mink suffering from shaking mink syndrome by use of viral metagenomics. J. Clin. Microbiol. 48, 4392–4396.
Blomstrom, A.L., Ley, C., Jacobson, M., 2014. Astrovirus as a possible cause of congenital tremor type AII in piglets? Acta Vet. Scand. 56, 82.
Bosch, A., Pinto, R.M., Guix, S., 2014. Human astroviruses. Clin. Microbiol. Rev. 27, 1048–1074.
Bouzalas, I.G., Wuthrich, D., Walland, J., Drogemuller, C., Zurbriggen, A., Vandevelde, M., Oevermann, A., Bruggmann, R., Seuberlich, T., 2014. Neurotropic astrovirus in cattle with nonsuppurative encephalitis in Europe. J. Clin. Microbiol. 52, 3318–3324.
Brown, J.R., Morfopoulou, S., Hubb, J., Emmett, W.A., Ip, W., Shah, D., Brooks, T., Paine, S.M., Anderson, G., Virasami, A., Tong, C.Y., Clark, D.A., Plagnol, V., Jacques, T.S., Qasim, W., Hubank, M., Breuer, J., 2015. Astrovirus VA1/HMO-C: an increasingly recognized neurotropic pathogen in immunocompromised patients. Clin. Infect. Dis. 60, 881–888.
Cordey, S., Vu, D.L., Schibler, M., L'Huillier, A.G., Brito, F., Docquier, M., Posfay-Barbe, K.M., Petty, T.J., Turin, L., Zdobnov, E.M., Kaiser, L., 2016. Astrovirus MLB2, a new gastroenteric virus associated with meningitis and disseminated infection. Emerg. Infect. Dis. 22, 846–853.
De Benedictis, P., Schultz-Cherry, S., Burnham, A., Cattoli, G., 2011. Astrovirus infections in humans and animals - molecular biology, genetic diversity, and interspecies transmissions. Infect. Genet. Evol. 11, 1529–1544.
Finkbeiner, S.R., Kirkwood, C.D., Wang, D., 2008. Complete genome sequence of a highly divergent astrovirus isolated from a child with acute diarrhea. Virol. J. 5, 117.
Finkbeiner, S.R., Li, Y., Ruone, S., Conrardy, C., Gregoricus, N., Toney, D., Virgin, H.W., Anderson, L.J., Vinje, J., Wang, D., Tong, S.X., 2009. Identification of a novel astrovirus (astrovirus VA1) associated with an outbreak of acute gastroenteritis. J. Virol. 83, 10836–10839.

**Fig. 3.** Phylogenetic comparison of astrovirus strains of bovine encephalitis cases and representative astrovirus species. (A) Neighbor-joining tree based on the full genome nucleotide sequencing. (B) Maximum-likelihood tree based on the full-length capsid polyprotein amino acid sequences. Red, astrovirus strains under investigation in the present study; dark blue bovine astrovirus (BoAstV) strains of previously described neurologically diseased cattle; green, strains of encephalitis cases in other species; MAstV, mammalian astrovirus; HuAstV, human astrovirus; AAstV, avastrovirus. GeneBank accession numbers are given in brackets and p-distances are indicated by scales at the bottom.

Fremond, M.L., Perot, P., Muth, E., Cros, G., Dumarest, M., Mahlaoui, N., Seilhean, D., Desguerre, I., Hebert, C., Corre-Catelin, N., Neven, B., Lecuit, M., Blanche, S., Picard, C., Eloit, M., 2015. Next-generation sequencing for diagnosis and tailored therapy: a case report of astrovirus-associated progressive encephalitis. J. Pediatric. Infect. Dis. Soc. 4, e53–e57.

Geigenmuller, U., Ginzton, N.H., Matsui, S.M., 1997. Construction of a genome-length cDNA clone for human astrovirus serotype 1 and synthesis of infectious RNA transcripts. J. Virol. 71, 1713–1717.

Guix, S., Bosch, A., Pintó, R., 2013. Astrovirus taxonomy. In: Schultz-Cherry, S. (Ed.), Astrovirus Research Essential Ideas, Everyday Impacts, Future Directions. Springer, New York, NY, pp. 97–118.

Jonassen, C.M., Jonassen, T.O., Grinde, B., 1998. A common RNA motif in the 3′ end of the genomes of astroviruses, avian infectious bronchitis virus and an equine rhinovirus. J. Gen. Virol. 79 (Pt 4), 715–718.

Jonassen, C.M., Jonassen, T.T., Sveen, T.M., Grinde, B., 2003. Complete genomic sequences of astroviruses from sheep and turkey: comparison with related viruses. Virus Res. 91, 195–201.

Kiang, D., Matsui, S.M., 2002. Proteolytic processing of a human astrovirus nonstructural protein. J. Gen. Virol. 83, 25–34.

Langmead, B., Salzberg, S.L., 2012. Fast gapped-read alignment with bowtie 2. Nat. Methods 9, 357–U359.

Li, L., Diab, S., McGraw, S., Barr, B., Traslavina, R., Higgins, R., Talbot, T., Blanchard, P., Rimoldi, G., Fahsbender, E., Page, B., Phan, T.G., Wang, C., Deng, X., Pesavento, P., Delwart, E., 2013. Divergent astrovirus associated with neurologic disease in cattle. Emerg. Infect. Dis. 19, 1385–1392.

Mendez, E., Fernandez-Luna, T., Lopez, S., Mendez-Toss, M., Arias, C.F., 2002. Proteolytic processing of a serotype 8 human astrovirus ORF2 polyprotein. J. Virol. 76, 7996–8002.

Monceyron, C., Grinde, B., Jonassen, T.O., 1997. Molecular characterisation of the 3′-end of the astrovirus genome. Arch. Virol. 142, 699–706.

Naccache, S.N., Peggs, K.S., Mattes, F.M., Phadke, R., Garson, J.A., Grant, P., Samayoa, E., Federman, S., Miller, S., Lunn, M.P., Gant, V., Chiu, C.Y., 2015. Diagnosis of neuroinvasive astrovirus infection in an immunocompromised adult with encephalitis by unbiased next-generation sequencing. Clin. Infect. Dis. 60, 919–923.

Quan, P.L., Wagner, T.A., Briese, T., Torgerson, T.R., Hornig, M., Tashmukhamedova, A., Firth, C., Palacios, G., Baisre-De-Leon, A., Paddock, C.D., Hutchison, S.K., Egholm, M., Zaki, S.R., Goldman, J.E., Ochs, H.D., Lipkin, W.I., 2010. Astrovirus encephalitis in boy with X-linked agammaglobulinemia. Emerg. Infect. Dis. 16, 918–925.

Robinson, J.T., Thorvaldsdottir, H., Winckler, W., Guttman, M., Lander, E.S., Getz, G., Mesirov, J.P., 2011. Integrative genomics viewer. Nat. Biotechnol. 29, 24–26.

Sato, M., Kuroda, M., Kasai, M., Matsui, H., Fukuyama, T., Katano, H., Tanaka-Taya, K., 2016. Acute encephalopathy in an immunocompromised boy with astrovirus-MLB1 infection detected by next generation sequencing. J. Clin. Virol. 78, 66–70.

Schlottau, K., Schulze, C., Bilk, S., Hanke, D., Hoper, D., Beer, M., Hoffmann, B., 2016. Detection of a novel bovine astrovirus in a cow with encephalitis. Transbound. Emerg. Dis. 63, 253–259.

Seuberlich, T., Wuthrich, D., Selimovic-Hamza, S., Drogemuller, C., Oevermann, A., Bruggmann, R., Bouzalas, I., 2016. Identification of a second encephalitis-associated astrovirus in cattle. Emerg. Microbes Infect. 5, e5.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731–2739.

Theil, D., Fatzer, R., Schiller, I., Caplazi, P., Zurbriggen, A., Vandevelde, M., 1998. Neuropathological and aetiological studies of sporadic non-suppurative meningoencephalomyelitis of cattle. Vet. Rec. 143, 244–249.

Wilhelmi, I., Roman, E., Sanchez-Fauquier, A., 2003. Viruses causing gastroenteritis. Clin. Microbiol. Infect. 9, 247–262.

Wunderli, W., Meerbach, A., Gungor, T., Berger, C., Greiner, O., Caduff, R., Trkola, A., Bossart, W., Gerlach, D., Schibler, M., Cordey, S., McKee, T.A., Van Belle, S., Kaiser, L., Tapparel, C., 2011. Astrovirus infection in hospitalized infants with severe combined immunodeficiency after allogeneic hematopoietic stem cell transplantation. PLoS One 6, e27483.

Wuthrich, D., Boujon, C.L., Truchet, L., Selimovic-Hamza, S., Oevermann, A., Bouzalas, I.G., Bruggmann, R., Seuberlich, T., 2016. Exploring the virome of cattle with non-suppurative encephalitis of unknown etiology by metagenomics. Virology 493, 22–30.

Zuker, M., 2003. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. 31, 3406–3415.