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

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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

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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).

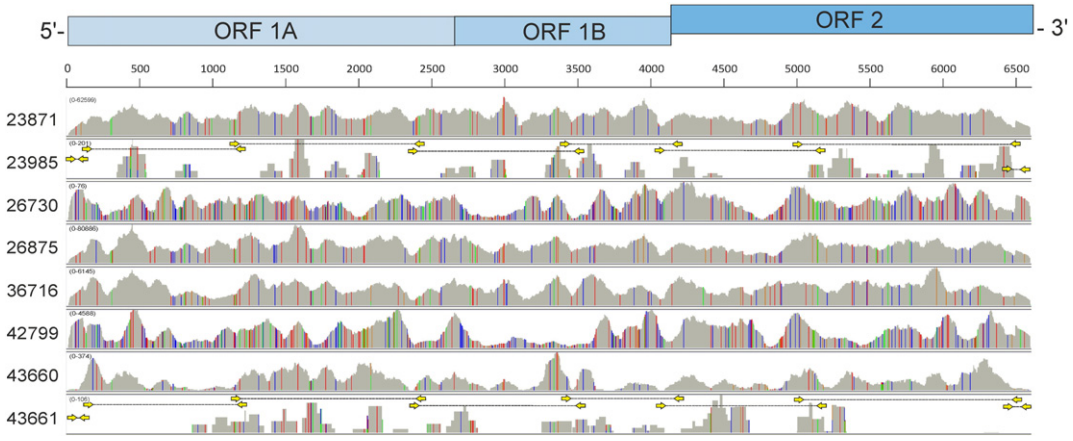

Fig. 1. Next Generation Sequencing coverage plots of bovine astrovirus-positive animals. For each animal the read depth (y-axis) is presented over the entire lengths of the BoAstV CH13 reference sequence (x-axis). The corresponding genome organization is shown on top. Grey colors indicate sequences identical 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.