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An outbreak of neonatal enteritis in buffalo calves associated with astrovirus

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ABSTRACT

Background: Enteritis of an infectious origin is a major cause of productivity and economic losses to cattle producers worldwide. Several pathogens are believed to cause or contribute to the development of calf diarrhea. Astroviruses (AstVs) are neglected enteric pathogens in ruminants, but they have recently gained attention because of their possible association with encephalitis in humans and various animal species, including cattle.
Objectives: This paper describes a large outbreak of neonatal diarrhea in buffalo calves (*Bubalus bubalis*), characterized by high mortality, which was associated with an AstV infection.
Methods: Following an enteritis outbreak characterized by high morbidity (100%) and mortality (46.2%) in a herd of Mediterranean buffaloes (*B. bubalis*) in Italy, 16 samples from buffalo calves were tested with the molecular tools for common and uncommon enteric pathogens, including AstV, kobuvirus, and torovirus.
Results: The samples tested negative for common enteric viral agents, including Rotavirus A, coronavirus, calicivirus, pestivirus, kobuvirus, and torovirus, while they tested positive for AstV. Overall, 62.5% (10/16) of the samples were positive in a single round reverse transcription polymerase chain reaction (PCR) assay for AstV, and 100% (16/16) were positive when nested PCR was performed. The strains identified in the outbreak showed a clonal origin and shared the closest genetic relationship with bovine AstVs (up to 85% amino acid identity in the capsid).
Conclusions: This report indicates that AstVs should be included in a differential diagnosis of infectious diarrhea in buffalo calves.

Keywords: Buffalo; enteritis; infection; *astroviridae*

INTRODUCTION

Calves enteritis is a major cause of economic losses to livestock farmers worldwide [1,2]. The clinical signs can range from mild to profuse diarrhea associated with dehydration, disturbance of acid-base and electrolyte balance, and, sometimes, sudden death [3,4] The

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Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

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prevalence of neonatal diarrhea is generally high in bovine and buffalo calves < 1 month of age. Various infectious agents (rotavirus, coronavirus, calicivirus, Salmonella, *Escherichia coli*, and *Cryptosporidium parvum*), either alone or in combination, may be associated with enteric disease in calves [2]. Several observational and experimental studies have examined the etiology of neonatal enteritis in calves [3,5,6]. On the other hand, determining the contribution of single viral species to calf neonatal diarrhea has been challenging because of virus-related factors, co-infections, and variations in the animal host [2,3,5,7]. Moreover, environmental, management, and nutritional factors may influence the outcome of the disease [1,4]. Starting from the 2000s, the development of sequence-independent nucleic acid amplification techniques combined with high throughput sequencing allowed an examination of the diversity of the enteric virome in several animal species, including livestock animals, leading to the discovery of novel viruses or rediscovery of neglected viruses [2]. Astroviruses (AstVs) were first discovered in calves with acute enteritis in England in 1978 [8]. On the other hand, the first genomic sequence data of a bovine AstV was published only in 2011 [9]. AstVs are non-enveloped spherical viruses with a positive-sense, single-stranded RNA of 6.4–7.3 kb in length. The genome contains 3 open reading frames (ORFs) and a 3′ poly-A tail (ICTV.https://talk.ictvonline.org/ictv-reports/ictv_9th_report/positive-sense-rna-viruses-2011/w/posrna_viruses/247/astroviridae, accessed 19 November 2019). AstVs are a major cause of acute gastroenteritis in young children [10]. In addition, AstVs have been detected in various domestic and wild animals in both terrestrial and aquatic environments [11,12]. Although AstVs are often associated with gastrointestinal tract infections [13,14], they have also been found in other organs. For example, AstVs have been described in association with encephalitis (cattle, pigs, mink, and humans), respiratory disease (deer), hepatitis and nephritis (birds) [11,15-19].

This paper describes a large outbreak of neonatal diarrhea associated with AstV infection and characterized by high mortality in a herd of Mediterranean buffaloes (*Bubalus bubalis*).

MATERIALS AND METHODS

Description of the outbreak

The outbreak occurred between February and March 2018 in a buffalo herd in Piana di Fondi (prefecture of Latina) in Central Italy (41°18'04.6"N 13°23'13.9"E). The herd consisted of approximately 800 animals, with nearly 350 births and 200–250 calves per year. In February 2018, when the enteritis outbreak started, there were 65 calves aged between 3 and 20 days. The calves were housed in separate individual boxes. The animals presented with severe enteric signs, profuse watery diarrhea, anorexia, depression, and weakness.

Laboratory investigations

The samples were screened for common enteric pathogens of calves [6,20-24] using molecular assays. Moreover, bacteriological and parasitological investigations were carried out using standard diagnostic procedures [2,3,5]. For a virological investigation, 10% of the small intestine and stool homogenates were prepared in phosphate-buffered saline (pH 7.3) and centrifuged at 10,000 × g for 3 min. A total of 200 μL of the supernatants were used for nucleic acid extraction using a QIAamp *cador* Pathogen Mini Kit (Qiagen GmbH, Germany). Reverse transcription (RT) and polymerase chain reaction (PCR) were performed using a One-Step RT-PCR kit (Invitrogen Carlsbad, USA).

As the samples tested negative by molecular investigations for common enteric viral agents, the samples were subjected to additional analyses, including molecular screening for AstV, kobuvirus, and torovirus [25-27]. **Table 1** provides information on the diagnostic panel for enteric viruses. Testing for AstV was performed using a pan-astrovirus assay, targeting the ORF1b region and amplifying the majority of human and animal AstVs [25] with a nested RT-PCR protocol. After RT of the RNA (50°C for 30 min and 95°C for 3 min), the thermal file of the first- and second-round PCRs included the activation of the DNA polymerase at 95°C for 3 min and an additional 25 cycles at 94°C for 30 sec, 50°C for 30 sec, and 68°C for 30 sec. Additional details regarding the protocols are available upon request.

PCR amplicons were purified using a PureLink Quick Gel Extraction Kit (Thermo Fisher Scientific,USA) and sequenced by Eurofins Genomics laboratories (Italy). Sequences of approximately 400 nucleotides (nt) in length were obtained. Upon sequence analysis, the AstV sequences generated from diagnostic RT-PCR in the 16 animals were virtually identical (100% nt), suggesting the circulation of a unique strain in the calves. The sequences were

Table 1. List of oligonucleotides used in this study

Pathogen	Target gene	Assay	Primer/probes	Sequence 5′-3′	Amplicon size (bp)	Reference (s)
AstV	ORF1b–ORF2	RT-PCR/3′RACE	First primer forward	GARTTYGATTGGRCKCGKTAYGA		[25]
			Second primer forward	GARTTYGATTGGRCKAGGTAYGA		
			Primer reverse	GGYTTKACCCACATNCCRAA		
		He-PCR/nested-3′RACE	First primer forward	CGKTAYGATGGKACKATHCC	422	
			Second primer forward	AGGTAYGATGGKACKATHCC		
Pestivirus	5′UTR region	qRealTime-PCR	Pesti-qF	GATGCCATGTGGACGAGGGC	NA	[22]
			BVDgen-R	TATGTTTTGTATAAAAGTTCA		
			BVDgen-Pb	FAM-CTCTGCTGTACATGGCACATG-TAMRA		
Calicivirus	RNA-polymerase	RT-PCR	289d	TGACGATTTCATCATCMCCRTA	330	[21]
			290d	GATTACTCCASSTGGGAYTCMAC		
			289h	TGACGATTTCATCATCACCATA		
			290h	GATTACTCCAGGTGGGACTCCAC		
CoV	Gene M	qRT-PCR	BCoV-F	CTGGAAGTTGGTGGAGTT	NA	[20]
			BCoV-R	ATTATCGGCCTAACATACATC		
			BCoV-Pb	FAM-CCTTCATATCTATACACATCAAGTTGTT-BHQ1		
RVA	VP2	qRT-PCR	VP2F1	TCTGCAGACAGTTGAACCTATTAA	NA	[23]
			VP2F2	CAGACACGGTTGAACCCATTAA		
			VP2F3	TCGGCTTGATACAGTAGAACCTATAAATG		
			VP2F4	TGTCAGCTGATACAGTAGAACCTATAAATG		
			VP2F5	TCAGCTGACACAGTAGAACCTATAAATG		
			VP2R1	GTTGGCGTTTACAGTTCGTTCAT		
			VP2R2	GTTGGCGTCTACAATTCGTTCAT		
			VP2-probe	FAM-ATGCGCATRTTRTCAAAHGCAA-MGB-NFQ		
Kobuvirus	RNA-polymerase	RT-PCR	UNIV-KOBU-F	TGGAYTACAAGRTGTTTTGATGC	216	[26]
			UNIV-KOBU-R	ATGTTGTTRATGATGGTGTTGA		
Torovirus	Spike	RT-PCR	F1	GTGTTAAGTTTGTGCAAAAAT	741	[27]
			R1	TGCATGAACTCTATATGGTGT		
		Nested PCR	F2	TGGATTAATTCAGGAGGTGCC	653	
			R2	CACTCTACATAGAGCGGTGTC		
Cryptosporidium parvum	SSU rRNA	PCR-RFLP	First primer forward	TTCTAGAGCTAAT ACATGCG	826–864	[24]
			First primer reverse	CCCATTTCCTTCGAAACAGGA		
			Second primer forward	GGAAGGGTTGTATTTATTAGATAAAG		
			Second primer reverse	AAGGAGTAAGGAACAACCTCCA		
-	-	3′RACE	QT	CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGCTTTTTTTTTTTTTTTT	3,200	[28]
			QO	CCAGTGAGCAGAGTGACG		
			QI	GAGGACTCGAGCTCAAGC		

AstV, astrovirus; ORF, open reading frame; RT-PCR, reverse transcriptase polymerase chain reaction; RACE, rapid amplification of complementary DNA ends; He, eminested; qRT-PCR, quantitative real-time polymerase chain reaction; CoV, coronavirus; RVA, rotavirus A; SSU, small subunit; rRNA, ribosomal RNA; NA, not applicable.

also analyzed using the web-based tools Basic Logic Alignment Search Tool (Basic Logic Alignment Search Tool (BLAST), https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed 19 November 2019) and FASTA (https://www.ebi.ac.uk/Tools/sss/fasta/, accessed 19 November 2019), using the default values to find homologous hits, yielding the highest nt identity (90.4%) to the strain Ishikawa9728 (GenBank accession No. LC047788) [29].

Sequence analysis of the ORF1b and ORF2 genes

The 3′ end of the genome (3.2 kb) of AstVs was obtained by a 3′-Rapid Amplification of complementary DNA Ends (RACE) protocol [28] using the AstV ORF1b primers described by Chu et al. [25] and the reverse primer QT (**Table 1**). RT-PCR and PCR assays were conducted using SuperScript III First-Strand Synthesis SuperMix (Invitrogen Carlsbad) and LaTakara PCR kit version 2.1 (TaKaRa Bio Europe S.A.S, France), respectively. The amplicons were purified using PureLink Quick Gel Extraction Kit (Thermo Fisher Scientific), and genomic DNA libraries were obtained using the Nextera XT DNA Sample Prep Kit (Illumina, USA). The libraries were equimolarly pooled and pair-end sequenced (2 × 250 bp) using a 500 cycle MiSeq Reagent Kit v2 on Illumina MiSeq platform (Illumina). Upon the reference assembly, 84,122 reads with a mean coverage of 6,572 X were mapped to the strain Ishikawa9728 (GenBank accession No. LC047788) [29]. Genome annotation was carried out using FindORFs software in Geneious version 9.1.8. The GenBank accession number of the AstV sequence ITA/2018/122 was MN718861.

The deduced amino acid sequences of ORF1b (partial) and ORF2 (full-length) of the AstVs described in this study were aligned with the cognate sequences of Mamastroviruses and the Avastrovirus strain GA2011 (GenBank accession No. JF414802), retrieved from GenBank, using the plugin MAFFT version 1.3.6 implemented in the software Geneious v. 9.1.8 (Biomatters, New Zealand). The correct substitution model settings for the phylogenetic analysis and evaluation of the selection pressure on the coding sequences were carried out using a jModelTest, based on the least Bayesian Information Criterion scores. Phylogenetic analyses were conducted using Mr Bayes. The genetic distance (p-dist) was calculated using the MEGAX software.

Ethics statement

All procedures performed in studies were in accordance with the ethical standards of the institutional and national research committees. The intestinal content of the small intestine and stools from the rectum samples from the animals were collected from the breeding veterinarian with the farmers' permission in their premises. All applicable national and institutional guidelines for the care and use of animals were followed.

RESULTS

The outbreak

The animals showed severe enteric signs, profuse watery diarrhea, anorexia, depression, and weakness. The enteric disease affected 100% of the 65 calves of the herd, over a 20-day time span, with a case fatality rate of 46.2% (30/65). After 3–4 days, the animals either exhibited remission of the clinical signs or died from dehydration. Necropsies were carried out in the herd revealing the presence of moderate acute catarrhal inflammation in the small intestine with enlarged mesenteric lymph nodes in some animals. Of the 30 dead animals, samples (intestinal content of the small intestine and stools from the rectum) were collected from 16 animals for a microbiological investigation.

Screening for enteric pathogens

The samples were screened using molecular methods for rotavirus type A (RVA), coronavirus, calicivirus, pestivirus, and *C. parvum*, commonly associated with neonatal enteritis in calves [6,20-24] (**Table 1**). The samples collected from the buffalo calves tested negative by molecular investigations for common enteric viral agents, including RVA, coronavirus, calicivirus, pestivirus, kobuvirus, and torovirus, whilst they tested positive to AstV. In total, 10/16 (62.5%) samples were positive in a single round RT-PCR assay for AstV, and 16/16 (100%) were positive when nested PCR was utilized. Sequence analysis of the partial RNA-dependent RNA-polymerase (RdRp) sequences generated with the diagnostic primers showed that all the AstV sequences were quite similar to each other (> 99% nt).

The bacteriological investigations revealed the presence of enterotoxigenic *E. coli* in 4 animals.

Sequence analysis of the AstV strain

The sequence of the 3′end of the genome (3.2 kb) of the AstV strain Buf/ITA/2018/122 was amplified using a 3′ RACE protocol. The sequence included a 720 nt (239 aa) portion of the ORF1b (RdRp), the complete (2,232 nt, 743 aa) capsid precursor, the 3′ untranslated region (76 nt), and the poly-A tail. The nucleotide sequence of strain Buf/ITA/2018/122 was deposited in GenBank under accession No. MN718861.

The partial RdRp and full-length capsid phylogenetic trees showed that the strain Buf/ITA/2018/122 formed a separate cluster with deer, buffalo, ovine, bovine, porcine, and yak AstV sequences, and it was distantly related to mink, human, bovine neurotropic, and ovine AstVs (**Figs. 1** and **2**). In the RdRp-based tree, the strain Buf/ITA/2018/122 clustered with bovine AstVs identified in Japan from 2009 to 2015 and the Italian strain Bov/ITA/2015/954-1 (MN718860) (**Fig. 1**).

In the capsid-based tree, 2 major clades were observed among bubaline, deer, bovine, yak, and ovine AstV strains. Strain Buf/ITA/2018/122 appeared closer genetically to the Japanese strain, Bov/JPN/2013/Ishikawa9728 (LC047788) [28] (p-dist = 0.15) and formed a cluster together with other Italian and Japanese bovine AstVs strains (p-dist = 0.39–0.46) (**Fig. 2**).

DISCUSSION

Diarrhea in calves usually occurs as either sporadic cases or small clusters, scattered temporarily in the herd, and may present less frequently as large outbreaks [2,7]. A study in France estimated that 80% of herds and 20% of neonatal calves are affected by diarrhea, with a case fatality rate of 50% [30]. In the buffalo herd studied in this report, there were high morbidity (100%) and case fatality rates (46%) of diarrhea in calves, consistent with the definition of an outbreak, defined as “cases of disease in excess of what would normally be expected in a defined community, geographical area or season” [31]. The sudden spread of enteritis to all calves and the observed mortality rate, affecting nearly half of the animals, prompted the owner of the herd to seek a diagnosis.

Based on the diagnostic investigations, AstV appeared as the possible causative agent of the enteritis outbreak. The virus was detected in all the tested samples. Upon sequencing of the short RdRp region targeted in the diagnostic, all the AstV strains were of clonal origin, suggesting that a unique AstV strain sustained the infection in the animals. In some

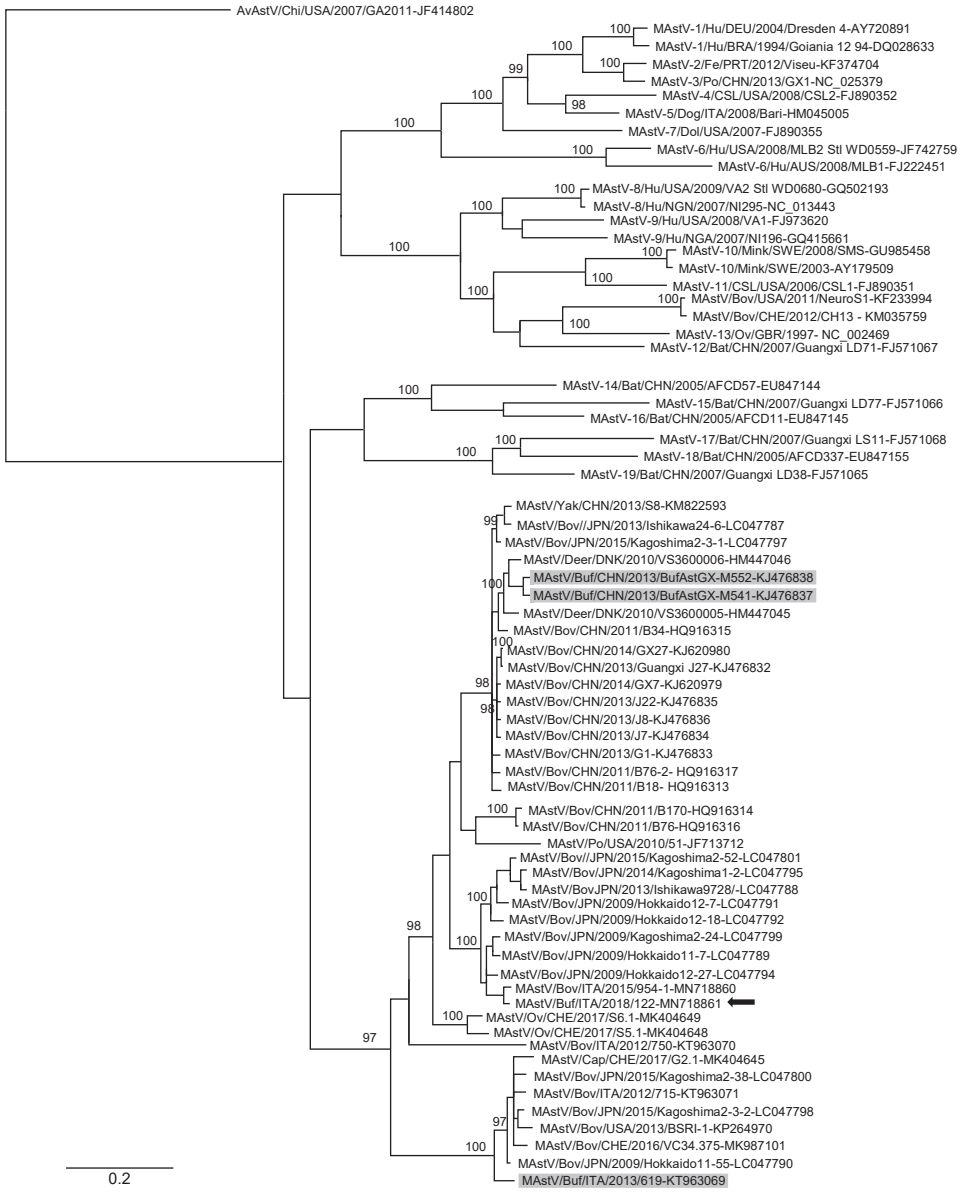

Fig. 1. Bayesian RNA-dependent RNA-polymerase-based phylogenetic tree of AstVs. The tree was constructed using a partial (238 aa) portion at the COOH end of the ORF1b-translated product. The posterior probability values > 95% are indicated at the tree nodes. The black arrow indicates the buffalo AstV strain Buf/ITA/2018/122, while grey highlights the evident buffalo strains retrieved from the NCBI database. The scale bar indicates the number of aa substitutions per site. MAstV, mammalian AstV; AvAstV, avian AstV; AstV, astrovirus.

animals, it was possible to identify *E. coli*, a common enteric pathogen in calves [2]. The role of AstVs as causative agents of enteritis in calves is unclear [2]. An experimental infection of gnotobiotic calves with the bovine AstV strain UK did not cause diarrhea, suggesting that the virus was avirulent [8]. Upon experimental challenge, 2 bovine AstV strains, antigenically related to the UK strain, could induce an infection and pathological changes in the intestinal cells (M cells) of the calf ileum [32]. Moreover, a co-infection with bovine RVA and bovine torovirus increased the severity of AstV infection [33]. Overall, the causative role of AstVs in calf enteritis has been evaluated in epidemiological studies, but the results are not yet conclusive [13,28,34,35]. In contrast to literature data, these findings appear to support the notion that some AstV strains can, under some conditions, trigger severe acute enteric

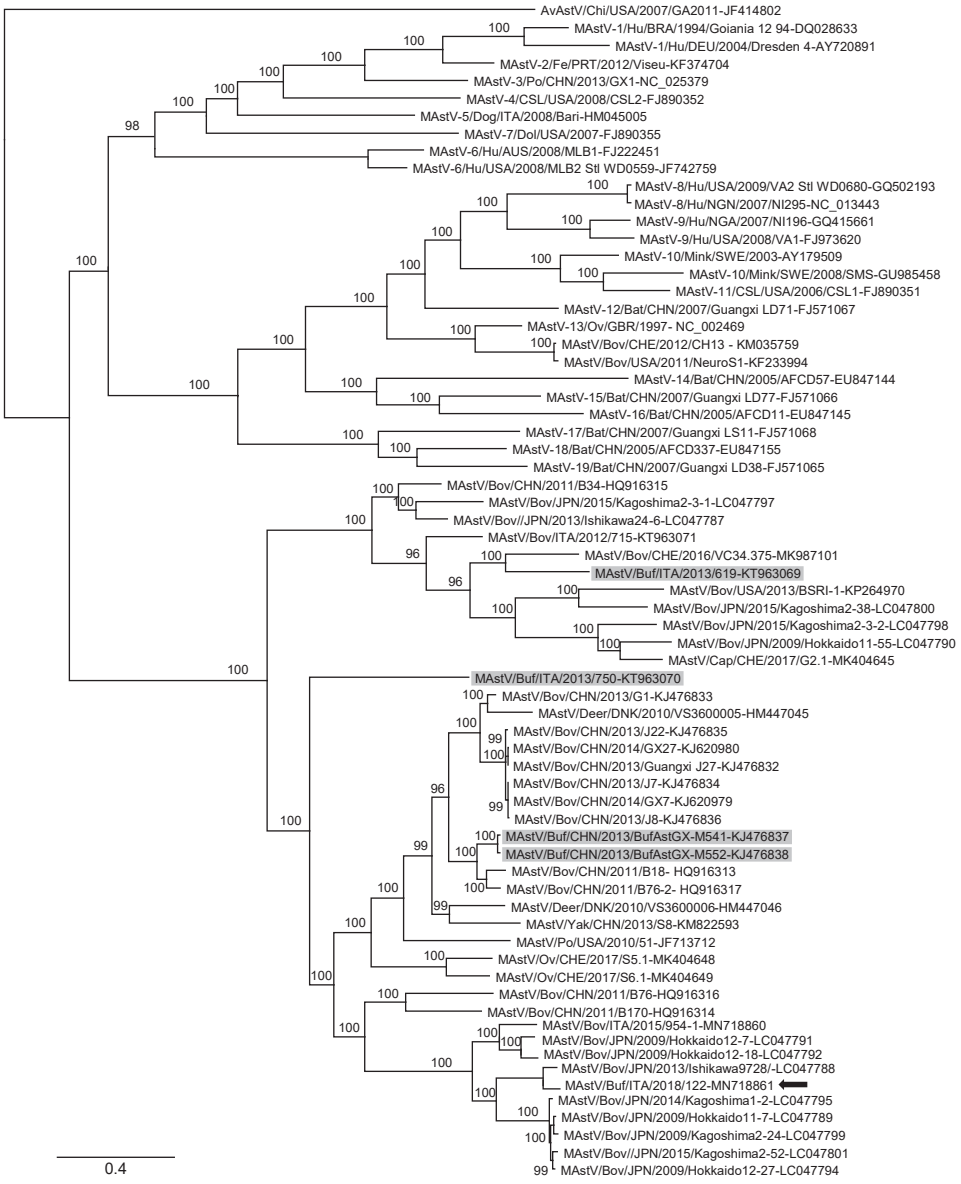

Fig. 2. Bayesian capsid-based phylogenetic tree of AstVs. The tree was elaborated using a 714 aa long alignment of the capsid precursor sequence. Posterior probability values > 95% are indicated at the tree nodes. The black arrow indicates the buffalo AstV strain Buf/ITA/2018/122, whilst grey highlights evidence buffalo strains retrieved from NCBI database. The scale bar indicates the number of aa substitutions per site.
MAstV, mammalian AstV; AvAstV, avian AstV; AstV, astrovirus.

disease in calves. AstVs are genetically heterogeneous viruses, and they can generate major phenotype and pathotype changes. Some AstV strains can acquire extra-intestinal tropism, causing encephalitis [16,36] and respiratory disease [15], or they can cross species barriers [11,12]. Finally, experiments carried out in animal models should be interpreted with caution because some AstV strains can show unexpected, non-predictable phenotypes under field conditions. More importantly, the experiments were carried out with a few of the multitude of ruminant AstV strains.

Sequence and phylogenetic analysis on RdRp and capsid showed that the buffalo AstV detected in this study was distantly related to AstVs from other animal species and from

ruminants. The buffalo strain formed a separate cluster in the phylogenetic trees with other bovine AstVs identified in Japan and Italy. Based on the phylogenetic analysis and the genetic distance calculated on the aa sequence of the capsid region, the novel strain could represent a candidate new AstV species together with the Japanese strain Ishikawa 9728 [28], according to the International Committee on Taxonomy of Viruses (ICTV https://talk.ictvonline.org/ictv-reports/ictv_9th_report/positive-sense-rna-viruses-2011/w/posrna_viruses/247/astroviridae, accessed 19 November 2019) criteria for AstV species demarcation. In the capsid, the p-dist between the buffalo virus and the Japanese strain Ishikawa 9728 was 0.15, far beyond the cutoff (p-dist < 0.312) observed within members of the same species, whilst the p-dist to the closest relatives in the tree was 0.39–0.46, i.e., above the mean p-dist cut-off of 0.378 defined between different AstV species.

Recent discoveries of new AstVs and genetic and evolutionary studies have suggested their potential ability to cross species barriers and eventually adapt to new host species [11,28]. This includes, for example, several animal-like AstVs discovered in humans after 2010 [10-12,17]. Strain Buf/ITA/2018/122 was found to cluster with BoAstVs (MAstV/Bov/ITA/2015/954-1-MN718860; MAstV/Bov/JPN/2009/Hokkaido12-27-LC047794), rather than with other BuAstVs (**Figs. 1** and **2**), suggesting possible cross-species transmission, likely more common in closely related host species.

In the buffalo herd, after the 2018 outbreak, no additional cases of AstV-related enteritis were reported. The owner modified some procedures in the whole production workflow, including calf feeding with colostrum and milk from the dams for longer periods (up to 10 days of life) instead of 2 or 3 days, before introducing a reconstituted milk replacer in their diet [6]. This procedure is more expensive in the short term because buffalo milk is 4 to 5 times as expensive as milk replacer but was more effective in protecting calves from infectious diarrhea than other management strategies [4,6]. Immunoglobulins received with colostrum/milk may positively modulate the severity of diarrhea in calves. Immunoglobulins act against invading pathogens in the intestine in the period of colostrum ingestion subsequently because relevant amounts of bloodstream immunoglobulins are slowly transferred to the intestine [2,6,37].

In conclusion, although AstVs are not regarded as major enteric pathogens in ruminants, under some conditions, they can affect the health of calves significantly and cause severe economic losses.

Animal experiments are required to assess in more depth the pathogenic role of AstVs in ruminants. Furthermore, obtaining information on AstV genetic diversity in ruminants will help improve diagnostics and generate more reliable data in surveillance studies.

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