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Identification of astroviruses in bovine and buffalo calves with enteritis

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ABSTRACT

Astroviruses (AstVs) have been identified in the stools of calves with enteritis and in the brain tissues of bovines with encephalitis but their pathogenic role has not been clarified. In this study, we report the detection and characterization of bovine and water buffalo AstV strains identified in young bovine and buffalo calves with enteritis in Italy between 2012 and 2015. By negative staining transmission electron microscopy (TEM) observation, AstV-like particles were identified in the stools of the animals and AstV RNA was confirmed molecularly. The sequence (~3.2-kb) at the 3′ end of the genome was determined for two bovine and two buffalo AstVs. Sequence and phylogenetic analysis on the partial ORF1b and full-length ORF2 revealed a marked genetic diversity although the viruses were distantly related to other AstV identified from ruminants. Gathering sequence information on ruminant AstVs is important to understand the extent of inter-species circulation and for the development of reliable, specific diagnostic tools.

1. Introduction

Astroviruses (AstVs) are a group of small, non-enveloped RNA viruses with an icosahedral capsid of 27–30 nm in diameter (Matsui and Greenberg, 1996) with a peculiar five- or six-pointed star shaped appearance when observed by negative staining electron microscopy (Caul and Appleton, 1982). The family *Astroviridae* has been classified by International Committee on Taxonomy of Viruses (ICTV)[1] into two genera, namely *Mamastrovirus* and *Avastrovirus* known to infect mammalian and avian species, respectively. The genome length is 6.8 to 7.9 kb and includes a 5′ untranslated region (UTR), followed by three open reading frames (ORFs) namely ORF1a, ORF1b and ORF2, a 3' UTR and a poly-A tail. There is a frame shift between ORF1a and ORF1b. ORF1a and ORF1b encode nonstructural proteins, a serine protease, and an RNA-dependent RNA polymerase (RdRp). ORF2 is expressed from a subgenomic RNA and encodes the viral capsid protein (Mendez and Arias, 2007).

Since the first description of human AstV in children with diarrhea in 1975 (Appleton and Higgins, 1975), a wide variety of AstVs have been reported in multiple animals including cattle, pigs, sheep, minks, dogs, cats, mice, sea lions, bats, whales, chickens, and turkeys (De Benedictis et al., 2011). Common clinical signs caused by enteric AstV infection in humans include vomiting and diarrhea. These signs range from mild to severe and affect primarily children and immunocompromised individuals (Moser and Schultz-Cherry, 2005).

Bovine AstV was first described in England in 1978 from acute enteritis in calves (Woode and Bridger, 1978). The bovine AstV strain UK was initially considered to be avirulent, as experimental infection of two gnotobiotic calves with AstV-like virus did not cause diarrhea (Woode and Bridger, 1978). In 1984, two bovine AstV isolates from the USA, US1 and US2, antigenically related to the isolate obtained in England (UK), were shown to cause infection and cytopathology of M cells of the dome epithelium covering the Peyer's patches of the calf ileum (Woode et al., 1985). In addition, co-infection with other enteric pathogens, such as bovine rotavirus (RV) and bovine torovirus, was shown to increase the severity of AstV infection (Woode et al., 1984). Antigenic differences have been observed among bovine AstV strains, suggesting that multiple serotypes of bovine AstV may exist in nature (Woode et al., 1985).

Even though bovine AstV was one of the earliest mammalian AstV to be discovered and studied in detail, no further research has been performed on this candidate bovine enteric pathogen over the past three decades. The genomic sequence of bovine AstVs was determined only in 2011 (Tse et al., 2011). Recently, bovine and ovine AstVs have been associated with

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[1] See: International Committee on Taxonomy of Viruses (ICTV). https://talk.ictvonline.org/taxonomy/. (accessed 19 November 2019).

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Table 1
List of herds, data on morbidity and mortality in calves and pathogens detected in this study. The numbers and values are referred to the time of samples collection.

	Herds							
	Lecce 2012	Potenza 1 2012	Potenza 2 2012	Potenza 3 2013	Potenza 4 2013	Foggia 2015	Foggia 2 2013	Foggia 3 2013
	Bovine	Bovine	Bovine	Bovine	Bovine	Bovine	Buffalo	Buffalo
Species								
No animals	298	311	398	356	285	321	453	491
No calves	24	22	35	28	19	26	40	42
No calves with clinical signs	10	2	2	3	2	3	18	15
Morbidity in calves (%)	41.66	9.09	5.71	10.70	10.52	11.54	45.00	35.71
No deaths in calves	2	2	1	1	2	1	9	5
Mortality in calves (%)	8.33	9.09	2.86	3.57	10.52	3.85	22.50	11.90
No samples analysed	5	2	2	3	2	3	6	4
Pathogens								
AstV (TEM) (pooled samples)	+	−	−	−	−	+	+	+
AstV (PCR)	4	−	−	−	−	2	4	4
CoV	−	−	−	−	−	1	−	−
RVA	2	2	−	2	−	−	−	−
Calicivirus	−	−	−	−	−	−	−	−
Cryptosporidium parvum	−	−	1	1	−	−	−	−
Clostridium spp	−	−	−	−	−	−	−	−
Escherichia coli	−	1	−	−	−	−	1	−

AstV: astrovirus; TEM: transmission electron microscope; CoV: Coronavirus; RVA: Rotavirus A.

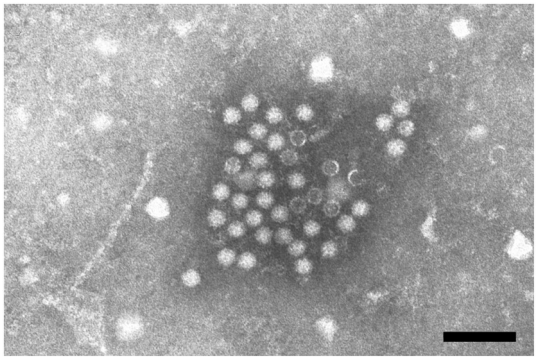

Fig. 1. Electron microscopy observation of AstV-positive. Negative staining microphotograph of 28–32 nm icosahedral particles showing the characteristic star-like surface, detected in the intestinal contents of a calf. Bar = 100 nm.

encephalitis in cattle and sheep (Li et al., 2013; Bouzalas et al., 2014; Schlottau et al., 2016; Boujon et al., 2017, 2019; Pfaff et al., 2017; Seuberlich et al., 2016), noting similar findings observed in humans (Quan et al., 2010; Vu et al., 2016) and in minks (Blomström et al., 2010). Altogether, these findings suggest that these enteric viruses may acquire neurotropism, although the mechanisms of this change in tissue tropism are not yet known (Selimovic-Hamza et al., 2017). In spite of the new perspectives and accumulating evidence on the biological plasticity of AstVs, there is still limited information on the epidemiology and genetic diversity of these viruses in ruminants (Tse et al., 2011; Oem and An, 2014; Alfred et al., 2015; Sharp et al., 2015), thus hindering to depict a complete portrait of their impact on large ruminants in terms of health and production. This gap may be an obstacle to the development of effective, specific diagnostic tools and unnecessarily delay the development/adoption of measures of prophylaxis. In this study, we report the characterization of bovine and buffalo AstV strains identified from different enteritis outbreaks occurred in Italy in 2012, 2013 and 2015.

2. Materials and methods

2.1. Sample collection

Between 2012 and 2015 a total of 27 fecal samples from 8 herds located in Southern Italy were submitted to our laboratories. The samples were collected from sporadic cases and small outbreaks of enteritis occurred in six bovine herds, located in Lecce (nr. 1), Potenza (nr. 4) and Foggia (nr. 1) and in two buffalo herds located in Foggia. The animals, aged 1–4 months, displayed severe enteric signs with profuse watery brown to yellow feces and mucus. The signs tended to disappear 5–7 days after their onset in the recovering calves. In a buffalo herd (Foggia 32,013) along with the with acute enteric disease, in some animals the enteric signs tended to configure as a chronic disease, with the calves showing marked delay in growth and hypotrichosis.

Morbidity in calves ranged from 5.7% (2/35) to 45% (18/40) (Table 1). Mortality ranged from 2.8% (1/35) to 22.5% (9/40). All the samples were stored at −80 °C until use.

By negative staining TEM microscopy, AstV-like particles were identified in four homogenates of pooled enteric specimens from the four different herds (two from buffalo and two from bovine herds), on the basis of their typical morphologic characteristics (Fig. 1).

2.2. RNA extraction and screening for AstV by reverse transcription PCR

RNA extracts were prepared from 10% fecal homogenates in phosphate-buffered saline (PBS), pH 7.3, after clarification by centrifugation at 10,000 ×*g* for 3 min. Two-hundred microliters of the supernatants were used for RNA and DNA extraction with the QIAamp *cador* Pathogen Mini Kit (Qiagen GmbH, Hilden, Germany), following the manufacturer's protocol.

Screening for AstV was accomplished by One Step Reverse Transcription (RT)-PCR and nested PCR assays using a broadly reactive set of primers targeting the ORF1b region of AstV (Table 2). The specificity of the amplification was confirmed by direct sequencing of the PCR products after gel-excision and elution. Analyses of the sequences with web-based tools (BLAST[2]) and FASTA[3] with default values were used to find homologous hits.

[2] See: Basic Logic Alignment Search Tool (BLAST). https://blast.ncbi.nlm.nih.gov/Blast.cgi (accessed 19 November 2019).

[3] See: FASTA. https://www.ebi.ac.uk/Tools/sss/fasta/. (accessed 19 November 2019).

Table 2
List of oligonucleotides used in this study.

Pathogen	Target gene	Assay	Primer/Probes	Sequence 5′-3′	Reference(s)
AstV	ORF1b-ORF2	RT-PCR /3'RACE	First primer forward	GAR TTY GAT TGG RCK CGK TAY GA	Chu et al., 2008
			Second primer	GAR TTY GAT TGG RCK AGG TAY GA	
		He-PCR/nested-3'	forward	GGY TTK ACC CAC ATN CCR AA	
		RACE	Primer reverse	CGK TAY GAT GGK ACK ATH CC	
			First primer forward	AGG TAY GAT GGK ACK ATH CC	
			Second primer forward		
Pestivirus	5'UTR region	qRealTime-PCR	Pesti-qF	GATGCCATGTGGACGAGGGC	Losurdo et al., 2015
			BVDgen-R	TATGTTTTGTATAAAAGTTCA	
			BVDgen-Pb	FAM- CTCTGCTGTACATGGCACATG-TAMRA	
Calicivirus		RT-PCR	289d	TGACGATTTCATCATCMCCRTA	Zintz et al., 2005
	RNA-polymerase		290d	GATTACTCCASSTGGGAYTCMAC	
			289 h	TGACGATTTCATCATCACCATA	
			290 h	GATTACTCCAGGTGGGACTCCAC	
CoV	Gene M	qRealTime-PCR	BCoV-F	CTGGAAGTTGGTGGAGTT	
			BCoV-R	ATTATCGGCCTAACATACATC	
			BCoV-Pb	FAM¯CCTTCATATCTATACACATCAAGTTGTT-BHQ1	Decaro et al., 2008
RVA	VP2	qRealTime-PCR	VP2F1	TCT GCA GAC AGT TGA ACC TAT TAA	Gutiérrez-Aguirre et al., 2009
			VP2F2	CAG ACA CGG TTG AAC CCA TTA A	
			VP2F3	TCG GCT TGA TAC AGT AGA ACC TAT AAA TG	
			VP2F4	TGT CAG CTG ATA CAG TAG AAC CTA TAA ATG	
			VP2F5	TCA GCT GAC ACA GTA GAA CCTATA AAT G	
			VP2R1	GTT GGC GTT TAC AGT TCG TTC AT	
			VP2R2	GTT GGC GTC TAC AAT TCG TTC AT	
			VP2-probe	FAM- ATG CGC ATR TTR TCA AAH GCA A-MGB-NFQ	
Cryptosporidium parvum	SSU rRNA	PCR-RFLP	First Primer Forward	TTCTAGAGCTAAT ACATGCG	Xiao et al., 2001
			First Primer Reverse	CCCATTTCCTTCGAAACAGGA	
			Second Primer	GGAAGGGTTGTATTTATTAGATAAAG	
			Forward	AAGGAGTAAGGAACAACCTCCA	
			Second Primer Reverse		
–	–	3'RACE	QT	CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGCTTTTTTTTTTTTTTTT	Scotto-Lavino et al., 2006
			QO	CCA GTG AGC AGA GTG ACG	
			QI	GAG GAC TCG AGC TCA AGC	

AstV = Astrovirus; RT = Reverse trascriptase; RACE = Rapid amplification of cDNA ends; He = eminested; qRT-PCR = Quantitative Real Time PCR; CoV = Coronavirus; RVA = Rotavirus A; SSU = Small subunit.

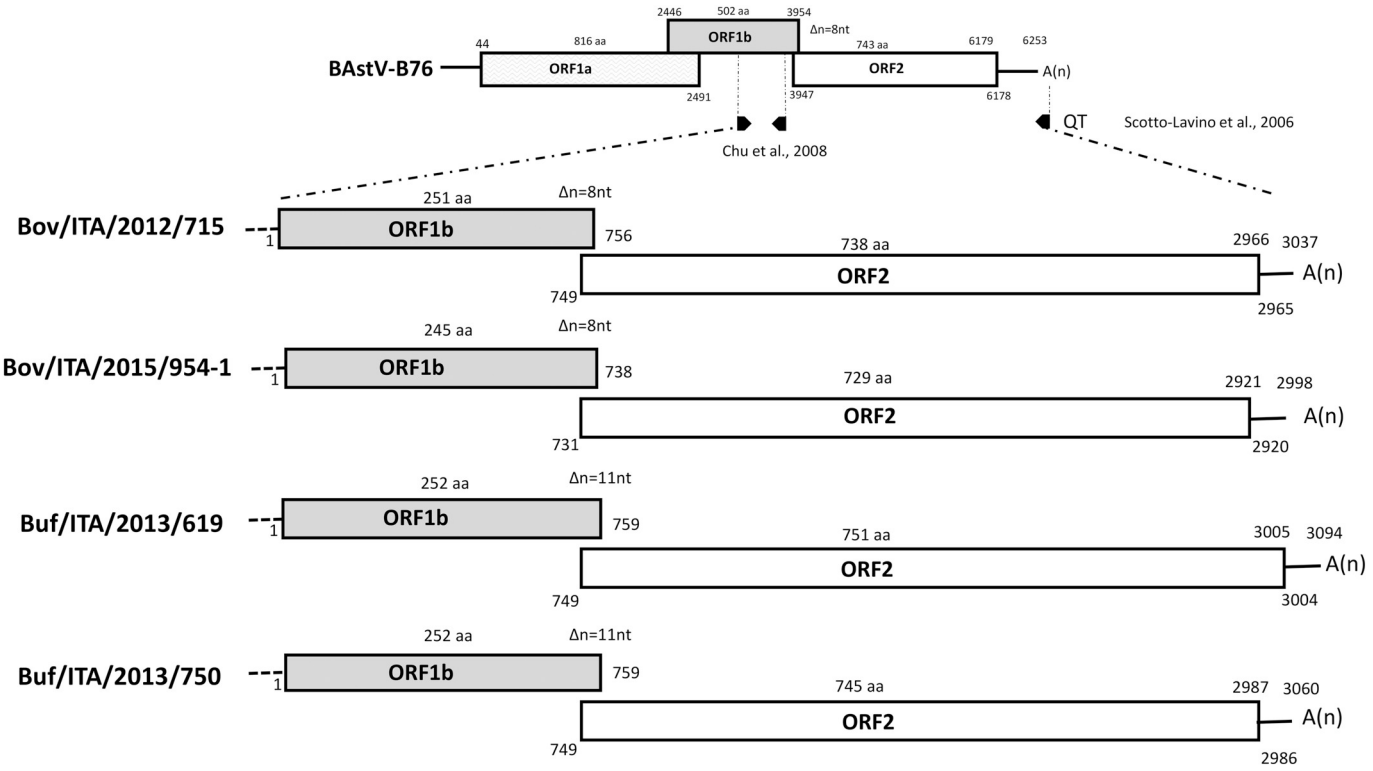

Fig. 2. Strategy used for characterization of the AstV strains. Primer sequences and positions are referred to the sequence of the astrovirus strain BAstV-B76 (GenBank accession no. HQ916316).

Table 3
Comparison of the nt and aa % identities of partial (642 nt. 214 aa) ORF1b sequences of AstVs detected in this study and closely related AstV strains retrieved from GenBank. Highest nt and aa identities are indicated in bold.

Strain name	GenBank accession no.	Bov/ITA/2012/715		Bov/ITA/2015/954–1		Buf/ITA/2013/619		Buf/ITA/2013/750	
		nt%	aa%	nt%	aa%	nt%	aa%	nt%	aa%
Bov/ITA/2012/715	KT963071	–	–	64.15	63.00	94.15	93.00	53.35	52.00
Bov/ITA/2015/954–1	MN718860	64.15	63.00	–	–	62.40	61.00	61.35	60.00
Buf/ITA/2013/619	KT963069	94.15	93.00	62.40	61.00	–	–	51.35	50.00
Buf/ITA/2013/750	KT963070	53.35	52.00	61.40	60.00	51.35	50.00	–	–
Bov//JPN/2013/Ishikawa24–6	LC047787	63.24	62.09	86.73	85.33	59.89	58.39	64.10	62.75
Bov/CHN/2011/B34	HQ916315	65.02	63.87	87.96	86.56	61.76	60.26	62.28	60.93
Bov/JPN/2015/Kagoshima2–3-1	LC047797	64.14	62.99	88.57	87.17	60.83	59.33	62.28	60.93
Bov/CHE/2016/VC34.375	MK987101	97.15	96.00	60.73	59.33	93.50	92.00	52.35	51.00
Bov/JPN/2009/Hokkaido11–55	LC047790	97.15	96.00	64.39	62.99	**96.50**	**95.00**	52.35	51.00
Cap/CHE/2017/G2.1	MK404645	95.12	93.97	59.79	58.39	91.60	90.10	51.35	50.00
Bov/JPN/2015/Kagoshima2–3-2	LC047798	**98.15**	**97.00**	62.58	61.18	95.50	94.00	53.35	52.00
Bov/JPN/2015/Kagoshima2–38	LC047800	97.21	96.06	63.49	62.09	93.50	92.00	49.35	48.00
Bov/USA/2013/BSRI-1	KP264970	96.15	95.00	65.27	63.87	93.50	92.00	52.35	51.00
Bov/CHN/2011/B170	HQ916314	65.89	64.74	86.10	84.70	65.37	63.87	**66.73**	**65.38**
Bov/CHN/2011/B76	HQ916316	61.41	60.26	88.57	87.17	61.76	60.26	61.35	60.00
Bov/JPN/2009/Hokkaido12–7	LC047791	63.24	62.09	96.94	95.54	63.59	62.09	63.19	61.84
Bov/JPN/2009/Hokkaido12–18	LC047792	62.33	61.18	96.42	95.02	62.68	61.18	62.28	60.93
BovJPN/2013/Ishikawa9728	LC047788	65.89	64.74	98.47	97.07	66.24	64.74	62.28	60.93
Bov/JPN/2009/Kagoshima2–24	LC047799	65.02	63.87	98.97	97.57	65.37	63.87	61.35	60.00
Bov/JPN/2009/Hokkaido11–7	LC047789	62.33	61.18	95.90	94.50	62.68	61.18	59.46	58.11
Bov/JPN/2014/Kagoshima1–2	LC047795	62.33	61.18	94.83	93.43	60.83	59.33	62.28	60.93
Bov//JPN/2015/Kagoshima2–52	LC047801	64.14	62.99	**99.47**	**98.07**	64.49	62.99	62.28	60.93
Bov/JPN/2009/Hokkaido12–27	LC047794	65.02	63.87	96.94	95.54	61.76	60.26	64.10	62.75
Bov/CHN/2011/B18	HQ916313	65.02	63.87	90.35	88.95	61.76	60.26	63.19	61.84
Bov/CHN/2011/B76–2	HQ916317	61.41	60.26	86.10	84.70	57.96	56.46	61.35	60.00
Buf/CHN/2013/BufAstGX-M541	KJ476837	61.41	60.26	86.10	84.70	58.93	57.43	61.35	60.00
Buf/CHN/2013/BufAstGX-M552	KJ476838	62.33	61.18	88.57	87.17	59.89	58.39	63.19	61.84
Bov/CHN/2013/G1	KJ476833	65.89	64.74	87.35	85.95	62.68	61.18	61.35	60.00
Deer/DNK/2010/1	HM447045	63.24	62.09	88.57	87.17	60.83	59.33	63.19	61.84
Bov/CHN/2013/Guangxi J27	KJ476832	63.24	62.09	88.57	87.17	60.83	59.33	63.19	61.84
Bov/CHN/2014/GX27	KJ620980	64.14	62.99	89.17	87.77	60.83	59.33	64.10	62.75
Bov/CHN/2013/J7	KJ476834	63.24	62.09	88.57	87.17	59.89	58.39	63.19	61.84
Bov/CHN/2014/GX7	KJ620979	64.14	62.99	89.17	87.77	60.83	59.33	64.10	62.75
Bov/CHN/2013/J8	KJ476836	64.14	62.99	89.17	87.77	60.83	59.33	64.10	62.75
Bov/CHN/2013/J22	KJ476835	65.02	63.87	88.57	87.17	61.76	60.26	61.35	60.00
Deer/DNK/2010/2	HM447046	63.24	62.09	88.57	87.17	60.83	59.33	61.35	60.00
Yak/CHN/2013/S8	KM822593	63.24	62.09	86.73	85.33	59.89	58.39	64.10	62.75
Po/USA/2010/51	JF713712	60.15	59.00	85.40	84.00	58.93	57.43	61.35	60.00
Ov/CHE/2017/S5.1	MK404648	63.15	62.00	85.40	84.00	62.50	61.00	62.35	61.00
Ov/CHE/2017/S6.1	MK404649	66.15	65.00	85.40	84.00	63.50	62.00	66.35	65.00
Bov/CHE/2012/CH13	KM035759	51.15	50.00	44.40	43.00	48.50	47.00	47.35	46.00
Bov/USA/2011/NeuroS1	KF233994	51.15	50.00	42.40	41.00	48.50	47.00	47.35	46.00
Po/CHN/2013/GX1	NC_025379	44.15	43.00	48.40	47.00	43.50	42.00	41.35	40.00
Ov/GBR/1997	NC_002469	39.15	38.00	44.40	43.00	36.50	35.00	50.35	49.00

2.3. Detection of other pathogens

The enteric samples were also screened molecularly for other bovine enteric pathogens, including RV type A (RVA), coronavirus (CoV), calicivirus, pestivirus and *Cryptosporidium parvum* (Table 2). Bacteriological examination of the fecal samples and presumptive identification was performed by using conventional standard culture methods with commercially available media (Oxoid, Milan, Italy). Bacterial identifications were achieved by evaluating the biochemical characteristics with the API system (bioMerieux, Marcy l'Etoile, France).

2.4. 3′ rapid amplification of cDNA ends (3'RACE) of AstVs

The 3′ end of the genome (3.2 kb) of AstVs was amplified with a 3′-RACE protocol (Scotto-Lavino et al., 2006) using the AstV RdRp universal forward primers described by Chu et al. (2008) and the reverse primer QT (Table 2). One step RT-PCR and PCR assays were performed using SuperScript® III First-Strand Synthesis SuperMix (Invitrogen Carlsbad, United States) and LaTakara PCR kit version 2.1 (TaKaRa Bio Europe S.A.S, Saint-Germain-en-Laye, France), respectively.

2.5. Cloning, sequencing and genome annotation

The PCR products were purified using TOPOXL Gel Purification kit (Invitrogen, Carlsbad, United States), cloned into the PCR XL-TOPO vector (Invitrogen, Carlsbad, United States) and sequenced by Eurofins Genomics laboratories (Milano, Italy). The genome annotation was performed by using FindORFs software in Geneious version 9.1.8. The AstV sequences have been submitted in GenBank with accession numbers KT963069–71 and MN718860.

2.6. Sequence and phylogenetic analyses

Deduced aminoacidic (aa) sequences of partial ORF1b and complete ORF2 the AstVs detected in the study were aligned with cognate sequences of Mamastroviruses (MAstVs) and the Avastrovirus (AvAstV) strain GA2011 (GenBank accession no. JF414802), retrieved from GenBank, using the plugin MAFFT version 1.3.6 (Katoh, 2002) implemented in the software Geneious v. 9.1.8 (Biomatters, New Zealand). The appropriate substitution model settings for the phylogenetic analysis and estimation of selection pressure on coding sequences were derived using jModelTest, based on the least Bayesian Information

Table 4
Comparison of the nt and aa % identities of complete (2943 nt, 981 aa) ORF2 sequences of AstVs detected in this study and closely related AstV strains retrieved from GenBank. Highest nt and aa identities are indicated in bold.

Strain name	GenBank accession no.	Bov/ITA/2012/715		Bov/ITA/2015/954–1		Buf/ITA/2013/619		Buf/ITA/2013/750	
		nt%	aa%	nt%	aa%	nt%	aa%	nt%	aa%
Bov/ITA/2012/715	KT963071	–	–	53.56	52.31	78.81	77.16	51.40	49.55
Bov/ITA/2015/954–1	MN718860	53.56	52.31	–	–	52.06	50.81	64.62	63.77
Buf/ITA/2013/619	KT963069	78.81	77.16	52.06	50.81	–	–	50.12	49.27
Buf/ITA/2013/750	KT963070	51.40	49.55	64.62	63.77	50.12	49.27	–	–
Bov//JPN/2013/Ishikawa24–6	LC047787	**83.54**	**81.79**	57.46	56.21	74.18	72.53	52.37	51.52
Bov/CHN/2011/B34	HQ916315	82.43	80.68	56.60	55.35	73.67	72.02	53.37	52.52
Bov/JPN/2015/Kagoshima2–3-1	LC047797	80.93	79.18	58.12	56.87	72.91	71.26	52.37	51.52
Bov/CHE/2016/VC34.375	MK987101	79.24	77.49	54.63	53.38	**84.20**	**82.55**	51.87	50.02
Bov/JPN/2009/Hokkaido11–55	LC047790	73.10	71.35	49.68	48.43	70.06	68.41	51.37	50.52
Cap/CHE/2017/G2.1	MK404645	74.42	72.67	54.72	53.47	72.23	70.58	53.12	51.27
Bov/JPN/2015/Kagoshima2–3-2	LC047798	73.10	71.35	51.30	50.05	71.38	69.73	51.62	50.77
Bov/JPN/2015/Kagoshima2–38	LC047800	74.20	72.45	55.22	53.97	73.30	71.65	51.62	49.77
Bov/USA/2013/BSRI-1	KP264970	73.69	71.94	52.28	51.03	71.92	70.27	50.12	48.27
Bov/CHN/2011/B170	HQ916314	54.38	52.63	73.63	72.38	53.57	51.92	67.28	66.43
Bov/CHN/2011/B76	HQ916316	53.88	52.13	73.48	72.23	55.77	54.12	67.57	66.72
Bov/JPN/2009/Hokkaido12–7	LC047791	54.38	52.63	**93.25**	**92.00**	53.21	51.56	64.00	62.15
Bov/JPN/2009/Hokkaido12–18	LC047792	54.09	52.34	92.29	91.04	54.41	52.76	63.34	61.49
BovJPN/2013/Ishikawa9728	LC047788	57.24	55.49	87.69	86.44	54.96	53.31	60.49	58.64
Bov/JPN/2009/Kagoshima2–24	LC047799	51.77	50.02	87.25	86.00	52.60	50.95	61.86	60.01
Bov/JPN/2009/Hokkaido11–7	LC047789	51.77	50.02	87.49	86.24	52.60	50.95	61.39	59.54
Bov/JPN/2014/Kagoshima1–2	LC047795	51.77	50.02	87.49	86.24	52.29	50.64	61.62	59.77
Bov//JPN/2015/Kagoshima2–52	LC047801	51.46	49.71	87.41	86.16	52.60	50.95	62.09	60.24
Bov/JPN/2009/Hokkaido12–27	LC047794	52.39	50.64	87.56	86.31	52.60	50.95	62.09	60.24
Bov/CHN/2011/B18	HQ916313	55.54	53.79	69.31	68.06	58.37	56.72	68.02	66.17
Bov/CHN/2011/B76–2	HQ916317	56.88	55.13	69.48	68.23	59.37	57.72	67.65	65.80
Buf/CHN/2013/BufAstGX-M541	KJ476837	54.51	52.76	68.77	67.52	58.37	56.72	68.64	66.79
Buf/CHN/2013/BufAstGX-M552	KJ476838	54.79	53.04	68.59	67.34	58.62	56.97	68.99	67.14
Bov/CHN/2013/G1	KJ476833	55.98	54.23	70.27	69.02	58.43	56.78	**71.55**	**69.70**
Deer/DNK/2010/1	HM447045	54.91	53.16	70.76	69.51	58.47	56.82	70.65	68.80
Bov/CHN/2013/Guangxi J27	KJ476832	54.63	52.88	69.23	67.98	55.74	54.09	70.32	68.47
Bov/CHN/2014/GX27	KJ620980	54.63	52.88	69.23	67.98	55.74	54.09	70.32	68.47
Bov/CHN/2013/J7	KJ476834	54.91	53.16	69.40	68.15	56.01	54.36	70.48	68.63
Bov/CHN/2014/GX7	KJ620979	54.91	53.16	69.40	68.15	56.01	54.36	70.48	68.63
Bov/CHN/2013/J8	KJ476836	54.91	53.16	69.23	67.98	55.74	54.09	70.32	68.47
Bov/CHN/2013/J22	KJ476835	54.63	52.88	69.05	67.80	55.74	54.09	70.48	68.63
Deer/DNK/2010/2	HM447046	52.33	50.58	70.24	69.99	55.90	54.25	69.41	68.56
Yak/CHN/2013/S8	KM822593	54.47	52.72	70.62	70.05	56.55	54.90	68.71	67.86
Po/USA/2010/51	JF713712	53.20	51.45	71.36	70.11	55.04	53.39	69.11	68.26
Ov/CHE/2017/S5.1	MK404648	61.84	60.09	71.59	70.34	59.80	58.15	68.26	67.41
Ov/CHE/2017/S6.1	MK404649	59.28	57.53	71.91	70.66	60.80	59.15	68.35	67.50
Bov/CHE/2012/CH13	KM035759	21.34	19.59	16.01	14.76	17.31	15.66	14.87	13.02
Bov/USA/2011/NeuroS1	KF233994	20.59	18.84	14.52	13.27	18.02	16.37	14.12	12.27
Po/CHN/2013/GX1	NC_025379	22.34	20.59	26.50	25.25	22.81	21.16	21.87	20.02
Ov/GBR/1997	NC_002469	14.34	12.59	13.50	12.25	22.81	21.16	13.37	11.52

Criterion (BIC) scores (Posada, 2009). Phylogenetic analyses were conducted using Mr. Bayes plugin (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) implemented in the software Geneious version 9.1.8 (Biomatters, New Zealand). P-distance (p-dist), defined as the proportion (p) of aa sites at which the two sequences to be compared are different, was obtained by dividing the number of aa differences by the total number of sites. The p-dist was calculated using the software MEGAX (Kumar et al., 2018).

3. Results

3.1. Diagnostic investigations

A total of 4 herds tested positive to AstV in TEM and RT-PCR. In detail, 4/5 samples tested positive to AstV in RT-PCR in Lecce 2012, 2/3 in Foggia 2015, 4/6 in Foggia 22,013 and 4/4 in Foggia 32,013. BLAST and FASTA analyses of the sequences confirmed the highest nt identity to the AstV strains belonging to the genus *Mamastrovirus*.

The fecal samples were also screened for other bovine enteric pathogens. Samples of Lecce 2012 also tested positive for RVA (2/5), with sample Bov/ITA/2012/715 being a co-infection AstV + RVA. In the herd Foggia 2015, 1/3 samples also tested positive for bovine CoV, with sample Bov/ITA/2015/954–1 being a co-infection AstV + CoV. In the herd Foggia 22,013, bacteriological investigations revealed the presence of *Escherichia coli*, whilst in Foggia 32,013 the samples were only positive to AstV. RVA was identified in three herds, *Cryptosporidium parvum* and *Escherichia coli* in 2 different herds each (Table 1).

3.2. Sequence and phylogenetic analysis of bovine and buffalo AstVs

The sequence of ~3.2-kb in length at the 3′ end of the AstV genome was determined for bovine and buffalo strains, including strain Bov/ITA/2012/715 (Lecce 2012), Bov/ITA/2015/954–1 (Foggia 2015), Buf/ITA/2013/619 (Foggia 2-2013) and Buf/ITA/2013/750 (Foggia 3-2013). The sequences spanned the 3′ end of ORF1b, the full-length ORF2 and the 3' UTR through the poly-A tail.

The 3′ end of ORF1b for the Bov/ITA/2012/715, Bov/ITA/2015/954–1, Buf/ITA/2013/619 and Buf/ITA/2013/750 strains comprised of 756, 738, 759 and 759 nt and encoded for 251, 245, 252 and 252 aa at the C-terminus of the RdRp, respectively (Fig. 2). Sequence comparison in the ORF1b (RdRp) was calculated using a 642 nt (214 aa) long fragment located at the very 3'end of the gene. Upon sequence comparison,

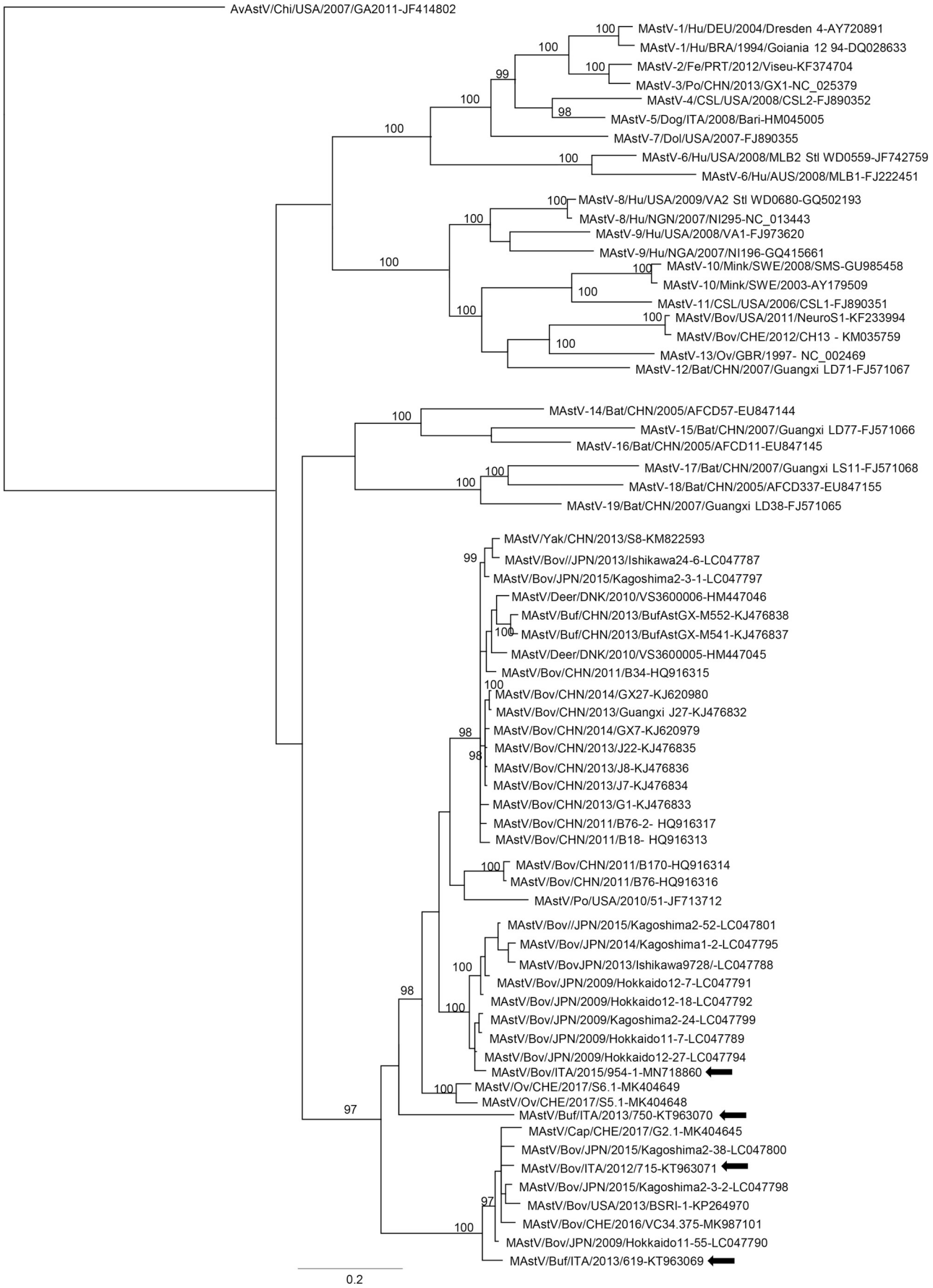

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Fig. 3. Phylogenetic tree based on partial (214aa) RdRp of astroviruses retrieved from GenBank database and bovine and bubaline astroviruses detected in this study. Posterior output of the tree was derived from Bayesian inference using 4 chains run for > 1 million generations, a general time-reversible model (6-character states), a proportion of invariable sites, a gamma distribution of rate variation across sites and a subsampling frequency of 1000. Posterior probability values > 95% are indicated on the tree nodes. Black arrows indicate the AstV strains detected in this study. Avian astrovirus strain GA2011 (GenBank accession nr JF414802) was used as an outgroup. Genus, host, country, year of detection, strain name, and GenBank accession numbers are indicated for each strain. The scale bar indicates the number of nt substitutions per site. MAstV Mammalian Astrovirus, AvAstV avian astrovirus.

strains Bov/ITA/2012/715 and Bov/ITA/2015/954–1 displayed 64.15% nt and 63.00% aa identity to each other and the highest identity to the Japanese strains Kagoshima2–3-2 (98.15% nt, 97.00% aa) and Kagoshima 2–52 (99.47% nt, 98.07% aa), respectively (Table 3). Strains Buf/ITA/2013/619 and Buf/ITA/2013/750 showed 51.35% nt and 50.00% aa identity to each other and the highest identity to the Japanese strain Hokkaido11–55 (96.50% nt and 95.00% aa) and to the Chinese strain B170 (66.73%nt and 65.38% aa), respectively (Table 3). The similarity of the bovine with buffalo strains retrieved in this study ranged from 53.35 to 94.15% nt and from 52.00 to 93.00% aa.

An 8-nt overlap, typical of bovine AstV strains, occurred between the termination codon of ORF1b and the initiation codon of ORF2 for the strains Bov/ITA/2012/715 and Bov/ITA/2015/954–1, whilst the strains Buf/ITA/2013/619 and Buf/ITA/2013/750 displayed a longer overlap of 11-nt. In the four strains, like other bovine AstVs, a cytosine replaced the initial adenosine nucleotide in the highly conserved nt stretch upstream of ORF2, ATTTGGAGNGGNGGACCNAAN$_{5–8}$ATGNC, which is believed to be part of a promoter region for synthesis of subgenomic RNAs (Walter et al., 2001). Also, the N$_{5–8}$ stretch was significantly longer, 11 nt for the bovine strains and 14 nt for the buffalo strains.

The ORF2 of the strains Bov/ITA/2012/715, Bov/ITA/2015/954–1, Buf/ITA/2013/619 and Buf/ITA/2013/750 was 2217, 2190, 2256 and 2238 in length and encoded for a capsid precursor protein of 738, 729, 751 and 745 aa, respectively. Sequence comparison in the ORF2 (capsid) was calculated using a 2943 nt (981 aa) long fragment. Upon sequence comparison, strains Bov/ITA/2012/715 and Bov/ITA/2015/954–1 displayed 53.56% nt (52.31% aa) identity to each other and the highest identity to Japanese strains Ishikawa24–6 (83.54% nt, 81.79% aa) and Hokkaido12–7 (93.25% nt, 92.00% aa), respectively (Table 4). Strains Buf/ITA/2013/619 and Buf/ITA/2013/750 showed 50.12% nt (49.27% aa) identity to each other and the highest identity to Swiss strain VC34.375 (84.20% nt, 82.55% aa) and to Chinese strain G1 (71.55% nt, 69.70% aa), respectively (Table 4). Identity among the Italian ruminant AstVs ranged from 51.40 to 78.81% nt and from 49.55 to 77.16% aa. The highly conserved motive SRGHAE at the C-terminus of capsid protein was not present (Jonassen et al., 1998).

The 3' UTR of the strains Bov/ITA/2012/715, Bov/ITA/2015/954–1, Buf/ITA/2013/619 and Buf/ITA/2013/750 was 72, 78, 90 and 74 nt long, respectively. Also, the conserved stem-loop II motive (s2m) conserved in several AstVs, CoVs and picornaviruses (Tengs et al., 2013), was not present in the bovine and buffalo AstVs detected in this study.

Phylogenetic tree based on the partial RdRp and complete capsid sequences showed that the four AstV strains formed distinctive cluster together with bovine, deer, buffalo, yak, ovine and porcine AstV sequences and were distantly related to human, mink, ovine and bovine neurotropic AstVs (Figs. 3 and 4). Phylogenetic RdRp-based tree showed that strain Buf/ITA/2013/750 intermingled different clusters in which Buf/ITA/2013/619, and Bov/ITA/2013/715 segregated with bovine AstVs retrieved in Japan, USA and Switzerland between 2009 and 2017 and Bov/ITA/2015/954 segregated with Japanese bovine AstVs identified between 2009 and 2015 (Fig. 3). Phylogenetic capsid-based tree also revealed the presence of two major clades among bovine, bubaline, ovine, deer, yak, AstV strains. In the first major clade Buf/ITA/2013/619 formed a separate cluster together with a Swiss strain VC34.375 (MK987101) (p-dist = 0.58) and Bov/ITA/2013/715 belonged to a well-defined cluster together with Japanese (Kagoshima2–3-1, LC047797 and Ishikawa24–6, LC047787) and Chinese (B34, HQ916315) AstV strains (p dist = 0.61–0.72). Buf/ITA/2013/750 strain was basal to the second major clade (p-dist = 1.10–1.50) in which Bov/ITA/2015/954 formed a separate cluster with Japanese bovine AstVs (p-dist = 0.24–0.47) (Fig. 4).

4. Discussion

In this study, we report the identification and genomic characterization of AstV strains identified from distinct outbreaks of enteritis in bovine and buffalo calves. AstVs were initially considered to be avirulent in experimental infections in calves (Woode and Bridger, 1978). In subsequent studies, AstVs were found to elicit histological lesions in the first part of the intestine in experimental infections (Woode et al., 1985). However, sequence data and diagnostic systems were not available until recently, thus hampering a diagnosis of AstV infection in most laboratories. Likewise, in this study the identification of AstVs was achieved by observation in TEM, even if a specific hyperimmune serum was not used to increase the sensitivity of the assay, as immunological reagents are not available for these viruses. The development of consensus primer sets for AstVs (Chu et al., 2008) finally provided researchers with a new tool for identification of diverse strains of AstVs from different animal species.

Sequence and phylogenetic analysis revealed a marked genetic diversity among the four Italian ruminant AstVs that were distantly related to other AstV species officially recognized by ICTV. In the phylogenetic tree based on the partial RdRp, the four strains detected in this study formed an independent cluster together with other bovine and buffalo AstVs although strain Buf/ITA/2013/750 appeared more distantly related. Upon phylogenetic analysis based on the complete capsid region, strain Buf/ITA/2013/619 and Bov/ITA/2012/715 formed a well-defined clade with other bovine and caprine AstVs detected in Asian and European countries. In the second clade Buf/ITA/2013/750 was distantly related to other bovine, bubaline, yak, deer and porcine AstVs whilst Bov/ITA/2015/954–1 clustered together with bovine AstVs retrieved in Japan. In addition, all the strains sequenced in this study were highly divergent from the bovine AstV NeuroS1 and CH13 strains previously associated with neurologic disease in cattle (Li et al., 2013).

Classification of AstVs is cumbersome, due to the massive number of genetically heterogeneous strains identified from different animal species. Accumulation of AstV sequences from several animal hosts, generated in metagenomics studies, is unveiling a vast genetic diversity. The identification of animal-like AstVs in humans (De Benedictis et al., 2011; Janowski et al., 2019) and of neurotropic strains in humans and several animal species (Reuter et al., 2018) has markedly propelled the research on AstV. A 2011 revision of the ICTV classification (2009) recognized that classification based on genetic criteria is more appropriate. Based on phylogenetic analysis of MAstVs inferred on the aa sequence of the full length ORF2, 19 groups or species were proposed and the mean aa genetic distances (p-dist) range was calculated as 0.378–0.750, and as 0.006–0.312 between and within groups, respectively[1]. It is likely that with the accumulation of AstVs genome sequences, the criteria for species demarcation will be modified. Based on the aforementioned criteria, the four strains reported in this study, for instance, could represent each a candidate new species.

The role of AstVs in the etiology of calves enteritis has been addressed in a limited number of epidemiological studies (Alfred et al., 2015; Nagai et al., 2015; Sharp et al., 2015). A high prevalence of genetically diverse AstVs was reported in fecal samples from both healthy

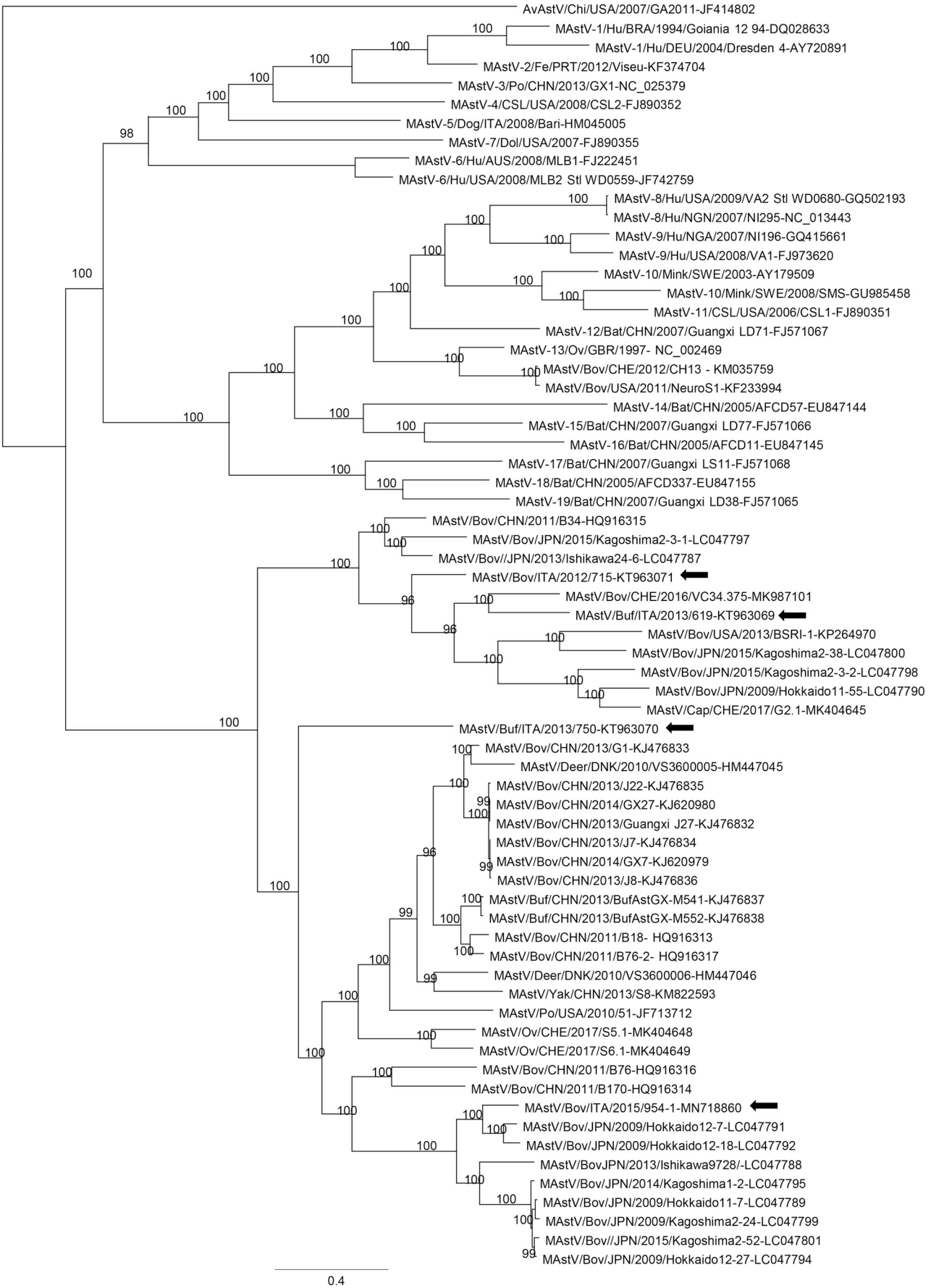

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Fig. 4. Phylogenetic tree based on complete (981aa) capsid of astroviruses retrieved from GenBank database and bovine and bubaline astroviruses detected in this study. Posterior output of the tree was derived from Bayesian inference using 4 chains run for > 1 million generations, a general time-reversible model (6-character states), a proportion of invariable sites, a gamma distribution of rate variation across sites and a subsampling frequency of 1000. Posterior probability values > 95% are indicated on the tree nodes. Black arrows indicate the AstV strains detected in this study. Avian astrovirus strain GA2011 (GenBank accession nr JF414802) was used as an outgroup. Genus, host, country, year of detection, strain name, and GenBank accession numbers are indicated for each strain. The scale bar indicates the number of nt substitutions per site. MAstV Mammalian Astrovirus, AvAstV avian astrovirus.

and diarrhoeic calves but no significant association between diarrhea and AstV was established (Sharp et al., 2015). A viral metagenomics study identified 15 AstV-related RNA sequences from fecal samples from either healthy or diarrheal calves. Eleven out of these 15 AstV-related RNA sequences were from calves with diarrhea (Nagai et al., 2015). Screening of bovine samples from Korean cattle identified AstVs in samples from diarrheal calves (9 out 91, 9.9%) but not in 0/24 non-diarrhoeal samples (Oem and An, 2014). Screening of bovine and buffalo herds in China has identified AstV in 92/211 samples (43.6%) (Alfred et al., 2015).

The AstV strains Bov/ITA/2012/715 and Bov/ITA/2015/954–1 were present in co-infection with bovine RVA and with CoV, respectively. Previous studies reported that bovine AstVs are not directly associated with severe diarrhea in calves under natural condition (Woode and Bridger, 1978; Bridger et al., 1984). Eventually, infections by bovine AstV could trigger severe diarrhea in co-infections with other enteric pathogens (Woode et al., 1984; Oem and An, 2014; Nagai et al., 2015). Overall, the discovery of a repertoire of genetically distantly related viruses from ruminants, in some case with different phenotypes (Reuter et al., 2018), would require interpreting the literature/experiments with caution.

Interestingly, in one of the water buffalo AstV-confirmed outbreak from our study, along with acute severe enteric disease, in some animals the enteric signs tended to configure as a chronic disease, with the calves showing marked delay in growth and hypotrichosis (matted and thinned fur). AstV was the only enteric pathogen detected in the buffalo herd and the problems were resolved by enacting stricter measure of prophylaxis, i.e. repeated disinfections of the calving areas and delaying the weaning phase.

5. Conclusions

The bovine and buffalo AstV strains in the present study revealed marked genetic differences in the RdRp and capsid regions with respect to other ruminant AstVs, although the data generated in this study cover a relatively restricted geographical area and time span. It may be hypothesized that a vast repertoire of ruminant AstV strains exist, coupled eventually with inter-species circulation across different ruminant species. Recent discoveries and genetic and evolutionary studies of novel AstVs have suggested their potential capability to cross species barriers and adapt to new host species (Nagai et al., 2015). Circulation of AstV among ruminants should not be uncommon, even for neurovirulent AstV strains (Boujon et al., 2017). Our results support a scenario in which water buffalo may be one of the hosts of the AstV of bovines and/or of other related ruminants (Alfred et al., 2015; Nagai et al., 2015).

Finally, the impressive genetic diversity of ruminant AstVs pose challenges for the development of reliable and effective diagnostic algorithms for these viruses. Gathering sequence data is pivotal to refine the diagnostic tool. Also, structured surveillance studies could help decipher more precisely the overall impact of AstVs on livestock animals and conceive measure of prophylaxis.

Declaration of Competing Interest

The authors declare no conflict of interest.

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