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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 ORF1 band 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) 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 cileum (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
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 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 (BLAST2 and FASTA3 with default values were used to find homologous hits.

### Table 1

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

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

2.3. 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.

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

See: FASTA. https://www.ebi.ac.uk/Tools/sss/fasta/. (accessed 19 November 2019).
Table 2

| 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 GYK TAY GA | Chu et al., 2008 |
|          |             |       | Second primer forward | GAR TTY GAT TGG RCK AGG TAY GA |              |
|          |             |       | Primer reverse | GGY TTK ACC CAC ATN CCR AA |              |
|          |             |       | First primer forward | GGY TTK ACC CAC ATN CCR AA |              |
|          |             |       | Second primer forward | GGY TTK ACC CAC ATN CCR AA |              |
|          |             | He-PCR/nested-3′RACE | First primer forward | GGY TTK ACC CAC ATN CCR AA |              |
|          |             |       | Second primer forward | GGY TTK ACC CAC ATN CCR AA |              |
|          |             |       | Primer reverse | GGY TTK ACC CAC ATN CCR AA |              |
|          |             |       | First primer forward | GGY TTK ACC CAC ATN CCR AA |              |
|          |             |       | Second primer forward | GGY TTK ACC CAC ATN CCR AA |              |
| Pestivirus| 5′UTR region | qRT-PCR-PCR | Pestis-qF | GATGCCATGTTAGAGGCGG | Lourdo et al., 2015 |
|          |             |       | BVDgen-R | TATGTTTTGTATAAAGGTCGA |              |
|          |             |       | BVDgen-Pb | TATGTTTTGTATAAAGGTCGA |              |
| Calicivirus| RNA- polymerase | RT-PCR | 2890d | TGAGAAGCTATCAGATCCTTA | Zintz et al., 2005 |
|          |             |       | 2890d | TGAGAAGCTATCAGATCCTTA |              |
| CoV      | Gene M      | qRT-PCR-PCR | BCoV-F | CTGAGAAGCTATCAGATCCTTA | Decaro et al., 2008 |
|          |             |       | BCoV-R | ATATTGCGCTAATACATATC |              |
|          |             |       | BCoV-Pb | FAM−CTTCATATCTTATACATCACAGATCCTTA−TAMRA |              |
| RVA      | VP2         | qRT-PCR-PCR | VP2F1 | TCT GCA GAC AGT TGA ACC TAA A | Gutiérrez-Aguirre et al., 2009 |
|          |             |       | VP2F2 | CAG ACA CGG TGG TGC ACC TAA A |              |
|          |             |       | VP2F3 | TGG GCT TCA TAC AGT AGA ACC TAT AAA TG |              |
|          |             |       | VP2F4 | TGT CAG CTG ATA CAG TAG AAT TAC TAA A |              |
|          |             |       | VP2F5 | TCA GGT GAC ACA GTC TAA A |              |
|          |             |       | VP2F6 | GGG GCT TAC AGT TAA A |              |
|          |             |       | VP2F7 | GGG GCT TAC AGT TAA A |              |
|          |             |       | VP2F8 | FAM − CTTCATATCTTATACATCACAGATCCTTA−TAMRA |              |
| Cryptosporidium parvum | SSU rRNA | PCR-RFLP | First Primer Forward | TTCTAGAGCTAAT GAC TAA A | Xiao et al., 2001 |
|          |             |       | First Primer Reverse | CCCATTTCTCTGAAACAGGA |              |
|          |             |       | Second Primer Forward | GGAAGGGGTGTATTTTATATAGTAAAG |              |
|          |             |       | Second Primer Reverse | AAGGAGGTAAAGAAACCAGGCTCA |              |
|          |             |       | – | – | Scotto-Lavino et al., 2006 |
|          |             |       | 3′RACE | QT | Scotto-Lavino et al., 2006 |
|          |             |       | QQ | CCA GTG AGC AGA GTG AGC |              |
|          |             |       | QI | GAG GAC TAG ACC TCA AGC |              |

AstV = Astrovirus; RT = Reverse transcriptase; 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).
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), calcivirus, 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, Marcyl’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), 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 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. The appropriate substitution model settings for the phylogenetic tree were derived using jModelTest, based on the least Bayesian Information neticanalysis and estimation of selection pressure on coding sequences implemented in the software Geneious v. 9.1.8 (Biomatters, New Zealand). The appropriate substitution model settings for the phylogenetic tree were derived using jModelTest, based on the least Bayesian Information neticanalysis and estimation of selection pressure on coding sequences implemented in the software Geneious v. 9.1.8 (Biomatters, New Zealand).

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 nt% | Bov/ITA/2015/954–1 nt% | Buf/ITA/2013/619 nt% | Bov/ITA/2013/750 nt% | Buf/JPN/2013/5/hikawa24–6 nt% | Buf/JPN/2015/2013/5/hikawa24–6 nt% | Buf/JPN/2011/5/hokkaido11–55 nt% | Cap/JPN/2017/2012/61 nt% | Buf/JPN/2015/2015/2013/5/hokkaido11–55 nt% | Buf/JPN/2015/2013/5/hokkaido11–55 nt% | Buf/JPN/2015/2015/2013/5/hokkaido11–55 nt% |
|---------------------|-----------------------|-----------------------|-----------------------|---------------------|-----------------------|---------------------------------|---------------------------------|---------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|
| Buf/ITA/2012/715    | KT963071              | 64.15                 | 63.00                 | 94.15               | 93.00                 | 51.35                           | 50.00                           | 52.35                           | 51.00                           | 52.35                           | 51.00                           | 52.35                           |
| Bov/ITA/2015/954–1  | KT96316               | 64.15                 | 63.00                 | 94.15               | 93.00                 | 51.35                           | 50.00                           | 52.35                           | 51.00                           | 52.35                           | 51.00                           | 52.35                           |
| Buf/JPN/2013/619    | KT963069              | 64.15                 | 63.00                 | 94.15               | 93.00                 | 51.35                           | 50.00                           | 52.35                           | 51.00                           | 52.35                           | 51.00                           | 52.35                           |
| Buf/JPN/2013/750    | KT963070              | 64.15                 | 63.00                 | 94.15               | 93.00                 | 51.35                           | 50.00                           | 52.35                           | 51.00                           | 52.35                           | 51.00                           | 52.35                           |

2.5. Cloning, sequencing and genome annotation

The PCR products were purified using TOPOXLI 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 aminoacid (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 tree were derived using jModelTest, based on the least Bayesian Information neticanalysis and estimation of selection pressure on coding sequences were derived using jModelTest, based on the least Bayesian Information neticanalysis and estimation of selection pressure on coding sequences.
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, 4/5 in Foggia 2013, 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 (Foggia 2012), Bov/ITA/2015/954–1 (Foggia 2015), Bov/ITA/2013/619 (Foggia 202-2013) and Bov/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, Bov/ITA/2013/619 and Bov/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,
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 Hokkaido 11–55 (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 adenine nucleotide in the highly conserved nt stretch upstream of ORF2, AATTGGAGGGGNACNANAGTGCN, which is believed to be part of a promoter region for synthesis of subgenomic RNAs (Walter et al., 2001). Also, the N5–8 stretch was significantly longer, 11 nt for the bovine strains and 14 nt for the buffalo strains.

The ORF2 of the strains Buf/ITA/2012/715, Bov/ITA/2015/954–1, Buf/ITA/2013/619 andBuf/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 Ishikawa 24–6 (83.54% nt, 81.79% aa) and Hokkaido 12–7 (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 AstVs sequences and were distinctly related to human, mink, ovine and bovine neurotropic AstVs (Figs. 3 and 4). Phylogenetic RdRp-based tree showed that strain Bov/ITA/2013/750 intermingled different clusters in which Bov/ITA/2013/619, and Bov/ITA/2013/750 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, babuline, ovine, deer, yak, AstV strains. In the first major clade Bov/ITA/2013/619 formed a separate cluster together with a Swiss strain VC34.375 (MK987101) (p-dist = 0.58) and Bov/ITA/2013/750 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 Bov/ITA/2013/750 was distantly related to other bovine, babuline, 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. 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
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-diarrheal samples (Oem and An, 2014). Screening of bovine and buffalo herds in China has identified AstV in 92/211 samples (43.6%) from diarrhoeal samples (Oem and An, 2014). Screening of bovine and/or of other related ruminants (Alfred et al., 2015). Eleven out of these 15 AstV-related RNA sequences from fecal samples from diarrheal calves (9 out 91, 9.9%) but not in 0/24 non-diarrheal samples from healthy or diarrheal calves. The study was founded with grants from Ricerca Corrente 2013 of Italian Ministry of Health “IZS PB 07/13 RC”.

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