First whole genome characterization of porcine astrovirus detected in swine faeces in Italy

Luca Tassoni[1#], Gianpiero Zamperin[2#], Eliana Schiavon[1], Vera Vendramin[3], Lara Cavicchio[1], Monica Mion[1], Francesco Tonon[4], Isabella Monne[2] and Maria Serena Beato[1*]

[#]*These authors contribute equally to this work.*
[1]*Diagnostic Virology Laboratory, Department of Animal Health, Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe), Viale dell'Università 10, 35020 Legnaro (PD), Italy.*
[2]*OIE/FAO, National Reference Laboratory for Avian Influenza and Newcastle Disease (IZSVe), Viale dell'Università 10, 35020 Legnaro (PD), Italy.*
[3]*IGA Technology Services, Via Linussio 51, 33100 Udine, Italy.*
[4]*Veterinary Consultant, Suivet, Italy.*

[*]*Corresponding author at: Diagnostic Virology Laboratory, Department of Animal Health, Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe), Viale dell'Università 10, 35020 Legnaro (PD), Italy. Tel.: +39 049 8084461, e-mail: msbeato@izsvenezie.it.*

Veterinaria Italiana 2019, **55** (3), 221-229. doi: 10.12834/VetIt.1873.9956.1
Accepted: 15.07.2019 | Available on line: 30.09.2019

Keywords
Porcine astroviruses, Metagenomics, Whole genome.

Summary
Porcine astroviruses (PoAstV) are found in the gastrointestinal tract of healthy and diseased pigs worldwide. However, their role in causing enteric disease in pigs and other animals has not been elucidated. In the present report, we describe for the first time in Italy, the identification and genetic characterization, through whole genome sequencing, of a PoAstV2 in pigs in Northeast Italy in 2015. This instance is the first detection of PoAstV2 in pigs in Italy. The phylogenetic analysis of the complete ORF2 segment highlights the high similarity of this virus to those circulating that same year in Japan. There are very few full astrovirus genomes available, and the present data represent an important contribution towards a better understanding of the characteristics and evolution of these viruses.

Prima caratterizzazione dell'intero genoma di porcine astrovirus individuato nelle feci di un suino in Italia

Parole chiave
Astrovirus suino, Metagenomica, Intero genoma.

Riassunto
Gli astrovirus sono virus identificati nel tratto gastrointestinale dei suini con e senza manifestazioni cliniche. La loro presenza nella popolazione suina è segnalata a livello globale. Il loro ruolo come agenti causali di patologia gastroenterica è tuttora poco chiaro. Nel presente lavoro si descrive, per la prima volta in Italia, l'identificazione e la caratterizzazione genetica, attraverso sequenziamento dell'intero genoma, di un Porcine Astrovirus tipo 2 (PoAstV2) in Veneto nel 2015. L'analisi filogenetica dell'ORF2 evidenzia una alta similarità del virus con altri PoAstV circolanti lo stesso anno in Giappone. Poche sono le sequenze complete di astrovirus, e il presente dato rappresenta un importante contributo verso una migliore comprensione delle caratteristiche dei PoAstV.

Introduction

Astroviruses (AstVs) are emerging viruses in the family *Astroviridae* that infect a wide range of mammalian and avian species. They are non-enveloped viruses with a radius of 28-30 nm and a single-stranded positive sense RNA genome (Cortez *et al.* 2017, Boujon *et al.* 2017). The genomes of AstVs range in size from 6.4 to 7.7 kb, are polyadenylated at their 3' end and contain three ORFs, namely, ORF1a, ORF1b and ORF2 (Cortez *et al.* 2017, Boujon *et al.* 2017). ORF1a encodes the nonstructural polyprotein 1a, whilst the entire ORF1 sequence encodes both a protease and an RNA-dependent RNA polymerase, with a ribosomal

frame shift at the ORF1a/1b (ORF1ab) junction (De Benedictis *et al.* 2011). ORF2 encodes the viral capsid structural polyprotein required for virion formation (De Benedictis *et al.* 2011).

Based on the host, two genera have been identified: *Mamastrovirus* and *Avastrovirus* (Boujon *et al.* 2017). Thirty-three species of *Mamastrovirus* and 7 species of *Avastrovirus* have been identified based on the genetic differences in the complete ORF2 amino acid sequence (Guiz *et al.* 2013). Astroviruses have been detected in the intestines and faeces of animals and are responsible for gastrointestinal disorders mainly in young individuals (Cortez *et al.* 2017, Boujon *et al.* 2017, De Benedictis *et al.* 2011). However, astrovirus infections have been described in diseased animals with extra-intestinal manifestations, including respiratory and neurological signs (Cortez *et al.* 2017, Boujon *et al.* 2017, De Benedictis *et al.* 2011). Additionally, astroviruses have been detected in clinically healthy mammalian and avian hosts, suggesting that infections in the absence of clinical signs are likely (Cortez *et al.* 2017, De Benedictis *et al.* 2011).

Porcine astroviruses (PoAstVs) belong to the *Mamastrovirus* genus and are distributed worldwide. Five genotypes of PoAstVs have been identified (Xiao *et al.* 2013); these genotypes belong to seven *Mamastrovirus* clades recognized by the International Committee on Taxonomy of Viruses (ICTV), namely, clades 3, 22, 24, 26, 27, 31 and 32 (Boujon *et al.* 2017). This classification possibly reflects the different origins of PoAstVs along with interspecies transmission and recombination events, some of which presumably occurred with human strains (Cortez *et al.* 2017).

PoAstV was first detected in the faeces of diarrheal pigs in 1980 using electron microscopy (Bridger 1980, Shirai *et al.* 1985). PoAstV1 was the first lineage to be characterized, and it has been identified in US (Xiao *et al.* 2013), Canada (Luo *et al.* 2011), the Czech Republic (Indik *et al.* 2006) and Colombia (Ulloa and Gutierrez 2010). The other genotypes have been identified in the USA, Canada, China, and Hungary at varying frequencies and have been detected in healthy and diseased animals (Laurin *et al.* 2011, Shan *et al.* 2012, Reuter *et al.* 2011, Reuter *et al.* 2012, Lan *et al.* 2011, Shan *et al.* 2011, Chen *et al.* 2018, Karlsson *et al.* 2016, Zhang *et al.* 2014). Intriguingly, PoAstV has also been shown to be associated with respiratory disease (Padmanabhan and Hause 2016), and it was detected in the brains of piglets suffering from congenital tremors (Blomstrom *et al.* 2014). Pigs infected with PoAstV may or may not present clinical signs. Therefore, the economic impact of swine astrovirus infections is poorly understood.

Here, we applied a metagenomics approach to sequence and analyse the whole genome of PoAstV from faeces collected from piglets suffering from acute gastroenteritis in Italy. The paucity of whole genome data renders the full characterization and comparison with extant strains difficult and, importantly, limits the analysis studies upon the origin and evolution of these viruses.

Materials and methods

Sample collection

In November 2015, an outbreak of acute gastroenteritis was observed on a pig farm located in the Province of Treviso in the Veneto region of Northeastern Italy. This was an open cycle farm with approximately 460 sows and 5,000 piglets. Eighty percent of the piglets presented diarrhoea during the early post-weaning phase along with a reduction in food intake; morbidity was high, but mortality was low (approximately 10%). Faecal samples were collected from three diseased piglets by a field veterinarian as part of the routine veterinary care animals; in addition, one succumbed piglet was submitted for necropsy. The faeces and the piglet were submitted to the diagnostic laboratory at Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe). Microscopic test for parasites was conducted on the intestinal tract, bacteriology tests were performed on the faeces, intestine and liver and virological tests on faeces.

Virus detection

As first screening method, all faecal samples, including one from the dead piglet, were analysed by negative staining electron microscopy (EM) for detection of virus particles. Briefly, faeces were 10-fold diluted in phosphate buffered saline (PBS), repeatedly frozen and thawed and clarified by a two-step centrifugation (2,500 x g) at 4 °C for 30 min and 7,000 x g at 4 °C for 30 min. An aliquot of 85 µL of the supernatant was ultracentrifuged for 15 min in a Beckman Airfuge, using an A-100 rotor, at 20 psi (125,000 x g). The grids were stained using a 2% sodium phosphotungstate solution in distilled water (pH 6.8) for at least 3 min. The dried grids were observed using a TEM Philips operating at 80 kV, at a magnification of 19,000-45,000. Morphometric measurements were performed on astrovirus-like positive samples. A minimum of 20 viral particles were measured at a magnification of 36,000 and statistically analysed with Soft-imaging software analySIS 2.1 (GmbH_ 1996). Presence of Porcine Epidemic Diarrhea Virus (PEDV) was excluded by real time RT-PCR (IDEXX RealPCR PEDV/PDCoV Multiplex RNA Test). In addition a pan-Mamastrovirus RT-PCR analysis was performed. One gram of faeces was

diluted 1:5 (w/v) in 4 ml of phosphate-buffered saline solution (PBS). Each sample was mixed by vortexing and centrifuged at 14,000 × g for 5 minutes. The obtained supernatant was used for RNA extraction. The QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) was used according to the manufacturer's instructions, and the RNA was stored at < -70 °C until use. The SuperScript III One-Step RT-PCR System with Platinum Taq (Invitrogen, 12574-026) was used for the pan-Mamastrovirus RT-PCR analysis. The primer set (MamAstroF: 5' GGMATGTGGGTNAARCCTG 3'; MamAstroR: 5' TWTGGAGGGGMGGACCAAA 3') was designed to amplify ORF2, generating a 355 bp amplicon. The PCR conditions were as follows: 55 °C for 30 minutes (min); 94 °C for 2 min, followed by 35 cycles at 94 °C for 30 seconds (sec), 55 °C for 30 sec, and 68 °C for 20 sec; and a final elongation step at 68 °C for 5 min.

Virus isolation was attempted in newborn swine kidney (NSK) and porcine kidney-15 (PK15) cell lines using trypsin for three blind passages. Results of virus isolation were confirmed by the observation of cytopathic effect (CPE) and by pan-*Mamastrovirus* RT-PCR analysis of the cell culture after the third passage. The faecal samples were stored and maintained at < 70 °C throughout the period of the study.

Nucleic acid extraction and library preparation

Sequencing was conducted for one of the three faecal samples with a sufficient amount of DNA target. The faecal sample was thawed and mixed by vortexing, and approximately 250 mg of previously defrosted and mixed faeces was sampled using a sterile spatula in a 0.1 mm glass bead tube and immediately suspended in 650 µl of warmed PM1 buffer containing β-mercaptoethanol following the manufacturer's instructions for the PowerMicrobiome RNA Isolation Kit (MoBio - Qiagen, Carlsbad, CA, USA). The glass bead tube was quickly vortexed and loaded in a TissueLyser instrument (Qiagen, Carlsbad, CA, USA) for 2 min of homogenization at 30 beats/sec. The RNA was extracted following the manufacturer's instructions.

The integrity and quantity of the extracted RNA was checked on a Caliper GX (PerkinElmer, Waltham, MA). Fifty nanograms of RNA was used as input for library preparation with the TruSeq Stranded TotalRNA Sample Prep Kit (Illumina, San Diego, CA) directly from the 'Synthesize First Strand cDNA' step following the manufacturer's instructions. The final library was quantified using a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA), and the DNA quality was tested with the Agilent 2100 Bioanalyzer High Sensitivity DNA assay (Agilent Technologies, Santa Clara, CA).

The faecal samples were deposited in the IZSVe Biobank (IZSVe VIR SCT3 LVD 15).

Sequencing and data preprocessing

The cDNA library was processed with the Illumina cBot for cluster generation on the flow cell following the manufacturer's instructions and was sequenced in paired-end mode on a HiSeq 2500 (Illumina, San Diego, CA). The CASAVA 1.8.2 version of the Illumina pipeline was used to convert the sequencing data from .BCL to FASTQ format.

The Illumina read quality was assessed using FastQC v0.11.2 [Andrews S. FastQC, A quality control tool for high throughput sequence data (http://www.bioinformatics.babraham.ac.uk/projects/fastqc. 2014)]. The raw data were filtered by removing the following reads: a) reads with more than 10% undetermined ('N') bases, b) reads with more than 100 bases with a Q score below 7, and c) duplicated paired-end reads. The remaining reads were clipped from the Illumina TruSeq adapters with Scythe v0.991 [(https://github.com/vsbuffalo/scythe) and trimmed with Sickle v1.33 (https://github.com/najoshi/sickle)]. Reads shorter than 80 bases or reads that remained unpaired after the previous filtering were discarded.

The high-quality reads were further filtered to remove host contamination by alignment with the *Sus scrofa* transcriptome (ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCF/000/003/025/GCF_000003025.5_Sscrofa10.2) using BWA v0.7.12-r1039 (Li and Durbin 2010), and the mapped reads were discarded.

Metagenomic analysis

Taxonomic assignment of the high-quality host-free reads was carried out using BLASTN 2.6.0+ (Altschul *et al.* 1990) for alignment with the integrated NT database (version 8th February 2017) and Diamond v0.8.36 (Buchfink *et al.* 2015) for alignment with the integrated NR database (version 8th February 2017). Alignment hits with e-values larger than 1×10^{-3} were discarded; this threshold value was selected because it was more conservative than the default threshold (1×10^{-2}) used in MEGAN.

The taxonomic classification of each read was determined by the lowest common ancestor (LCA)-based algorithm that was implemented in MEGAN v6.7.0 (Huson *et al.* 2016).

De novo assembly

For the reconstruction of the astrovirus consensus sequence, reads taxonomically classified as belonging to the family *Astroviridae* were selected. These reads were assembled *de novo*

using IDBA-UD v1.1.1 (Peng *et al.* 2012) with the multi-kmer approach using a minimum value of 24, a maximum value of 124 and an inner increment of 10. An assembled sequence of a length comparable to that of the astrovirus genome (6-8 kb) was selected as the astrovirus consensus sequence. To assure that the selected sequence was truly representative of the astrovirus genome present in the sample, we aligned all reads classified as belonging to the family *Astroviridae* against the astrovirus consensus sequence with BWA. A visual inspection of the alignment was performed with tablet v1.14.10.21 (Milne *et al.* 2010), and the consensus sequence was manually revised based on this alignment. We also checked whether all positions in the astrovirus consensus sequence were filled with the consensus nucleotide for that position by calling variants with LoFreq v2.1.2 (Wilm *et al.* 2012). According to the recommendations for LoFreq use, the alignment was first processed with Picard-tools v2.1.0 (http://picard.sourceforge.net) and GATK v3.5 (McKenna *et al.* 2010) [The Genome Analysis Toolkit: a MapReduce framework for analysing next-generation DNA sequencing data (DePristo *et al.* 2011, Van der Auwera *et al.* 2013)] in order to correct potential errors, realign reads around indels and recalibrate base quality. LoFreq was then run with fixed alignment with the option 'call-indels' to produce a VCF file containing both SNPs and indels. Variant indels and SNPs with a frequency lower than 50% were discarded from the final set. If needed, the remaining variants were used to change the astrovirus consensus sequence accordingly.

Phylogenetic analysis

To classify the virus correctly, the ORF2 amino acid sequence was deduced from the nucleotide sequence, and phylogenetic analysis was performed. Sequences representative of each PoAstV clade were included in the dataset along with target sequences obtained from a BLASTp search (Tamura *et al.* 2013).

Moreover, a phylogenetic analysis of the ORF1ab and ORF1a amino acid sequences was conducted on only the PoAstV2 samples to identify the differences between the phylogenetic tree topologies.

The best substitution models were identified for each dataset using ModelFinder in MEGA version 6 (Altschulz *et al.* 1997), and the selected models were applied in the phylogenetic analyses performed using the maximum likelihood method implemented in PhyML 3.0 (Guindon *et al.* 2010). The branch support of the tree was measured by non-parametric bootstrap analysis with 100 replicates.

Results

At necropsy the dead piglet showed peritonitis, oedema of the colon and hyperaemia of the pyloric region of the stomach, and severe catarrhal enteritis with foam and loss of tone of the intestinal tract was observed. The presence of the foam resembled the typical presentation of enteric disease caused by astrovirus in turkeys (Toffan *et al.* 2012). Microscopic examination of intestinal tract did not show presence of parasites. Bacteriology test highlighted the presence of *Escherichia coli* not belonging to neither O138, O139, O147, O149, O8 serogroups nor O141K85AB and O141K85AC serotypes in intestine and faeces. The presence of *Salmonella* spp. and *Clostridium perfringens* was excluded. The EM examination evidenced only astroviridae virus particles. All faecal samples tested positive for *Astroviridae* by EM and by pan-*Mamastrovirus* RT-PCR (Figures 1, 2).

Virus isolation attempts were not successful as CPE was not observed for any samples and absence of astrovirus particles was confirmed by EM and RT-PCR.

Following library preparation and Illumina sequencing, we generated 53,586,949 paired-end reads (2 × 125 bp), which allowed the recovery of 15,299 high-quality host-free reads truly belonging to the family *Astroviridae* (a ratio of 0.15% PoAstV2 DNA to exogenous DNA). No other reads belonging to other virus families were identified. The PoAstV2 reads represented a 590-fold sequence coverage of base pairs and a 1165-fold physical coverage of

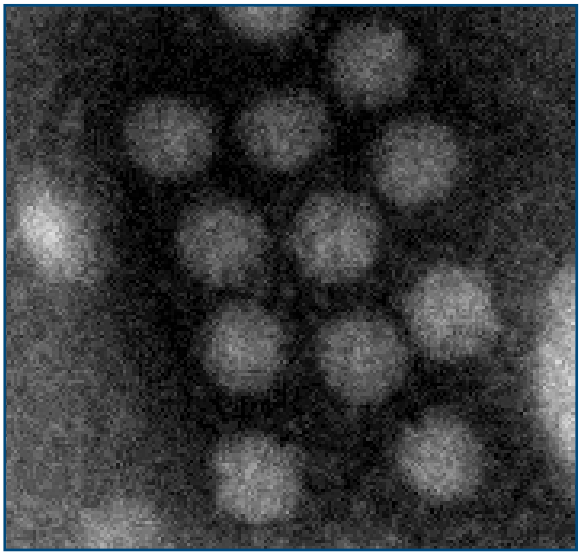

Figure 1. *Electron microscopy picture of astrovirus (larger particles of 18-20 nm) and parvovirus (smaller particles of 27-30 nm) observed directly in faecal sample of puppy no. 6 (36,000 Kx).* The characteristic 'star-like appearance' of astrovirus particles is evident compared to the round shaped morphology of parvovirus. Negative staining was obtained with 2% sodium phosphotungstate solution.

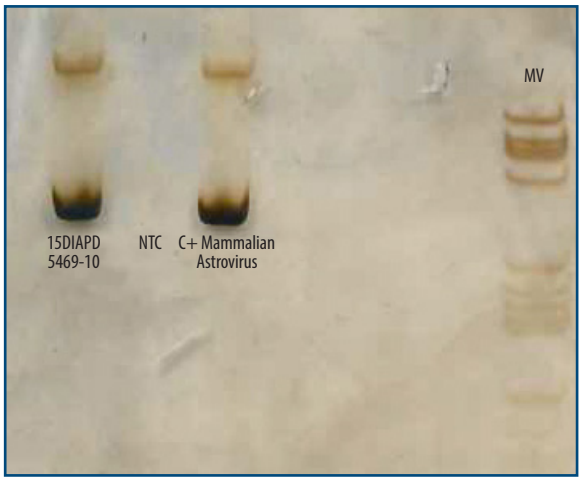

Figure 2. *Acrilammide gel of the positive sample submitted to metagenomics analysis.*

the PoAstV2 genome, which allowed us to perform a successful *de novo* assembly of the detected astrovirus.

The phylogenetic tree obtained from the deduced ORF2 amino acid sequence, which includes the five genotypes of PoAstV, allows the classification of the virus PoAst/Italy/DIAPD5469-10/2015 as PoAstV2 (Figure 3).

According to a recent classification, two PoAstV2 lineages, namely, lineages 1 and 2, were clearly delineated, with the tree supported by high bootstrap values (Ito *et al.* 2017).

Lineage 1 is characterized by PoAstV2 sequences originating primarily in Asia, with the exception of two PoAstV2 sequences originating in Belgium, and one bovine astrovirus (BoAstV) (Figure 3). Lineage

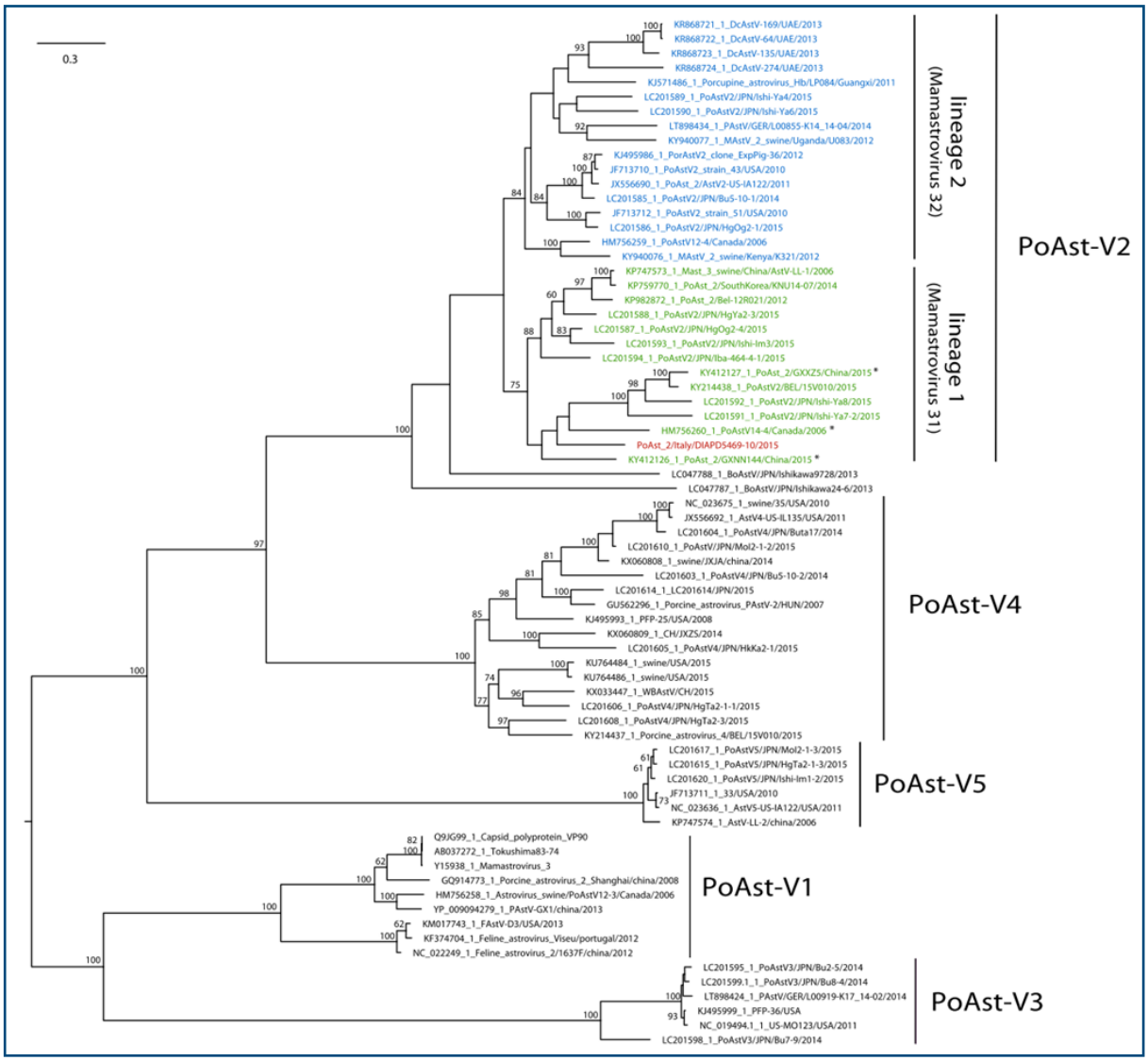

Figure 3. *Phylogenetic tree based on deduced amino acid sequences of ORF2 of PoAstV, including PoAstV of 5 different genotypes.* The tree was obtained using the maximum likelihood method implemented in PhyML3.0. A non-parametric bootstrap analysis with 100 replicates was conducted to obtain branch supports. The substitution model used was LG+G+I+F, and it was selected using MEGA6 model finder.
*PoAstV for which only the ORF2 sequence is available.

2 is characterized by higher heterogeneity than lineage 1 in terms of country and species of origin. Phylogenetic analysis including the sequences of PoAstVs 1-5, revealed that the Italian PoAstV2 clusters within lineage 1 and shows the highest similarity (%) with strains from Japan and China detected in 2015 (Monini *et al.* 2015). The paucity of PoAstV sequences from Europe may bias such findings.

The different topologies observed in the ORF2, ORF1ab and ORF1a phylogenetic trees (Figures 4, 5, 6) confirm the results previously reported in the literature (Ito *et al.* 2017). Color coding allows a simple comparison to be made between the topology obtained for the ORF2 (Figures 3, 4), ORF1ab and ORF1a phylogenetic trees (Figures 5, 6). Recombination events in the evolutionary history of these viruses might explain the differences observed.

In addition, the ORF1ab and ORF1a trees are characterized by lower branch supports and smaller distances between taxa in comparison with the ORF2 tree (Figures 4, 5, 6).

The deduced amino acid sequence of PoAst2/Italy/DIAPD5469-10/2015 shows higher similarity with that of Bel12R021/2012 (73.15%) for ORF2 and with that of Bel15V01/2015 for ORF1ab and ORF1a (96.6% and 97.82%, respectively).

Discussion

This study reports for the first time the full-length genome of a PoAstV in Italy belonging to genotype 2. Only 303 whole-genome sequences of Mammalian Astrovirus are publicly available, 79 sequences from swine. In recent years, the number of PoAstV whole genome sequences has increased; however, the majority of the available Mammalian astrovirus whole genome sequences are from humans (90) (data retrieved from Genbank, 20 February 2019). A recent study conducted in Japan (Ito *et al.* 2017) has contributed in the increase of PoAstV sequences, but data from Europe are limited. The lack of adequate of genome-level data for PoAstV hampers the determination of a correct classification of PoAstV and limits our ability to monitor the diversity and evolution of PoAstV.

With reference to data available on PoAstV in Italy, one study investigated the prevalence of PoAstV in Italian swine herds (Monini *et al.* 2015). Faecal samples were collected from 2012 to 2014 in the northern, central and southern regions of Italy. Nineteen PoAstVs were detected and all were classified by using the ORF1b-ORF2 sequence as PoAstV4 (Monini *et al.* 2015). In this study, no significant association was identified between astrovirus infection and the presence of diarrhoea (Monini *et al.* 2015). Outside Italy, PoAstV2 was detected in healthy pigs in Croatia in 2013 (Brnic *et al.* 2013), with a high prevalence

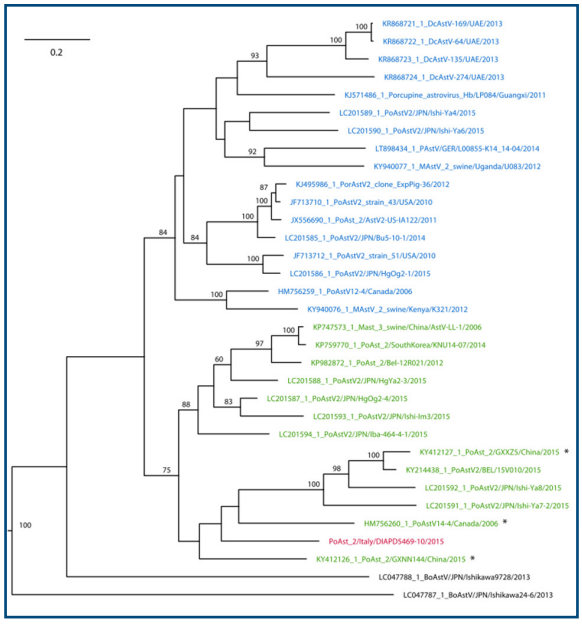

Figure 4. *Detail of ORF2 phylogenetic tree showing PoAstV2 only.* This figure is an enlargement of the data illustrated in Figure 1, focusing on PoAstV2. Each colour identifies further genetic groups within PoAstv2. Only bootstrap values ≥ 60 are shown. Lineage 1 samples are coloured in green while lineage 2 are in light blue. Whole genome was not available for sequences marked by an asterisk.
*PoAstV for which only the ORF2 sequence is available.

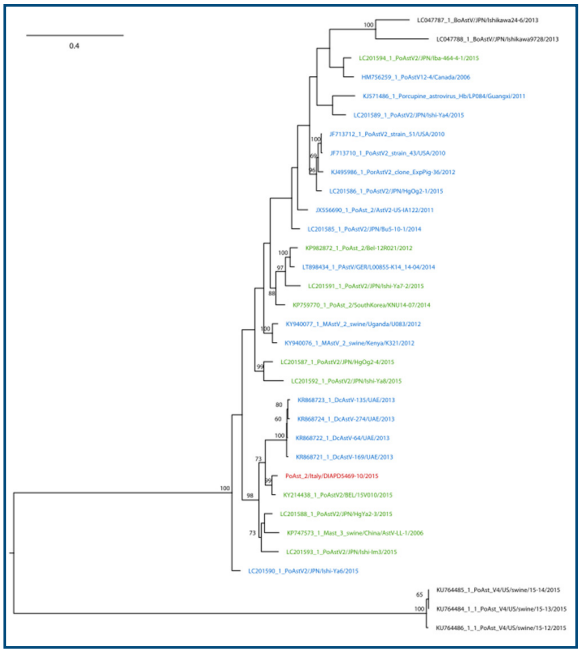

Figure 5. *Phylogenetic tree of the deduced amino acid sequences of ORF1ab of PoAstV2 only.* The tree was obtained using ML method (LG + G) implemented in PhyML3.0, performing a 100 replicates non parametric bootstrap analysis for the branch supports. Only bootstrap values ≥ 60 are shown in the figure. Lineage 1 viruses are coloured in green while lineage 2 are in light blue.

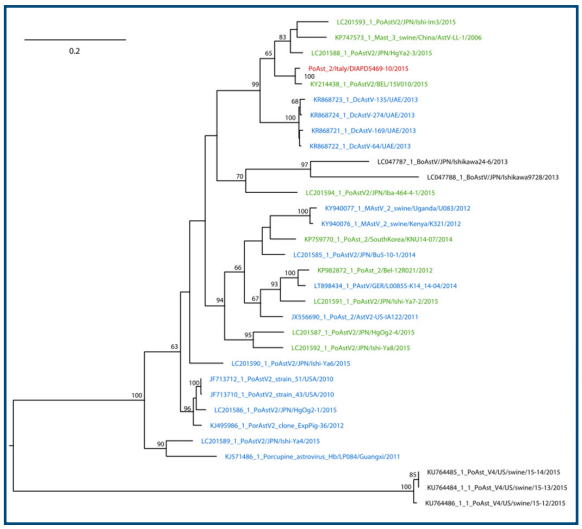

Figure 6. *Phylogenetic tree of the deduced amino acid sequences of ORF1a of PoAstV2 only.* The tree was obtained using ML method (LG + G) implemented in PhyML3.0, performing a 100 replicates non parametric bootstrap analysis for the branch supports. Only bootstrap values above 60 are shown in the figure. Lineage 1 samples are coloured in green while lineage 2 are in light blue.

compared to that of other PoAstV lineages (Brnic *et al.* 2014), and in clinically healthy pigs in the Czech Republic between 2010 and 2011 (Dufkova *et al.* 2013). Outside Europe, PoAstV2 has been detected in healthy young piglets in China and Japan (Ito *et al.* 2017, Li *et al.* 2015, Cai *et al.* 2016), in the USA (Xiao *et al.* 2013) and for the first time in East Africa by Amimo and colleagues (Amimo *et al.* 2014). Unfortunately, isolation attempts in continuous cell lines failed, confirming the difficulties to replicate astroviruses *in vitro*, as already reported in the literature (De Benedictis *et al.* 2011).

Regarding the role of PoAstV in multifactorial enteric disorders of pigs only scarce information data is available.

Better surveillance and diagnosis in the field regarding the involvement of PoAstV during enteric disorders in pigs may shed light on its prevalence and role. PoAstV is not considered taken into consideration in differential diagnosis with other pathogens responsible for gastroenteric disorders in pigs; accordingly, its prevalence might be underestimated and other genotypes are probably circulating in Italy and Europe. The absence of a cell culture systems and animal models hinder the opportunity to further characterize and study the pathogenic role of PoAstV (Cortez *et al.* 2017). In fact, it remains difficult to associate PoAstV infections to disease. In this context, the piece of information provided in this study, represent an achievement in this field. This complete PoAstV sequence can facilitate porcine astrovirus diagnostics and further molecular epidemiology analysis of PoAstV in Italy.

Notwithstanding the potential for interspecies transmission of astroviruses, resources are still lacking for improve knowledge upon animal astrovirus infections.

In addition, further efforts are warranted in order to understand the evolution of astroviruses, with emphasis on their ability to recombine and to cross the species barrier.

Accession number(s)

The raw MiSeq data were submitted to the NCBI Sequence Read Archive (SRA; http://www.ncbi.nlm.nih.gov/Traces/sra/) under accession number SRR6297822. The astrovirus consensus sequences were submitted to GenBank under the accession number MG930777.

Grant support

We thank the Italian Ministry of Health for supporting the work of G. Zamperin through the RC IZS VE 05/14 Project: "Sviluppo ed applicazione di strategie di sequenziamento di nuova generazione (NGS) per la caratterizzazione del viroma in volatili e conigli"; project leader: Isabella Monne.

References

Amimo J.O., Okoth E., Junga J.O., Ogara W.O., Njahira M.N., Wang Q., Vlasova A.N., Saif L.J. & Djikeng A. Molecular detection and genetic characterization of kobuviruses and astroviruses in asymptomatic local pigs in East Africa. *Arch Virol*, **159**, 1313-1319.

Altschul S.F., Gish W., Miller W., Myers E.W. & Lipman D.J. 1990. Basic local alignment search tool. *J Mol Biol*, **215**, 403-410.

Altschul S.F., Madden T.L., Schaffer A.A., Zhang J., Zhang Z., Miller W. & Lipman D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res*, **25**, 3389-3402.

Blomstrom A.L., Ley C. & Jacobson M. 2014. Astrovirus as a possible cause of congenital tremor type AII in piglets? *Acta Vet Scand*, **56**, 82-014-0082-y.

Boujon C.L., Koch M.C. & Seuberlich T. 2017. The expanding field of mammalian astroviruses: opportunities and challenges in clinical virology. *Adv Virus Res*, **99**, 109-137.

Bridger J.C. 1980. Detection by electron microscopy of caliciviruses, astroviruses and rotavirus-like particles in the faeces of piglets with diarrhoea. *Vet Rec*, **107**, 532-533.

Brnic D., Prpic J., Keros T., Roic B., Staresina V. & Jemersic L. 2013. Porcine astrovirus viremia and high genetic variability in pigs on large holdings in Croatia. *Infect Genet Evol*, **14**, 258-264.

Brnic D., Jemersic L., Keros T. & Prpic J. 2014. High prevalence and genetic heterogeneity of porcine astroviruses in domestic pigs. *Vet J*, **202**, 390-392.

Buchfink B., Xie C. & Huson D.H. 2015. Fast and sensitive protein alignment using DIAMOND. *Nat Methods*, **12**, 59-60.

Cai Y., Yin W., Zhou Y., Li B., Ai L., Pan M. & Guo W. 2016. Molecular detection of porcine astrovirus in Sichuan Province, China. *Virol J*, **13**, 6-015-0462-6.

Chen Q., Wang L., Zheng Y., Zhang J., Guo B., Yoon K.J., Gauger P.C., Harmon K.M., Main R.G. & Li G. 2018. Metagenomic analysis of the RNA fraction of the fecal virome indicates high diversity in pigs infected by porcine endemic diarrhea virus in the United States. *Virol J*, **15**, 95-018-1001-z.

Cortez V., Meliopoulos V.A., Karlsson E.A., Hargest,V., Johnson C. & Schultz-Cherry S. 2017. Astrovirus biology and pathogenesis. *Annu Rev Virol*, **4**, 327-348.

De Benedictis P., Schultz-Cherry S., Burnham A. & Cattoli G. 2011. Astrovirus infections in humans and animals - Molecular biology, genetic diversity, and interspecies transmissions. *Infect Genet Evol*, **11**, 1529-1544.

DePristo M.A., Banks E., Poplin R., Garimella K.V., Maguire J.R., Hartl C., Philippakis A.A., del Angel G., Rivas M.A., Hanna M., McKenna A., Fennell T.J., Kernytsky A.M., Sivachenko A.Y., Cibulskis K., Gabriel S.B., Altshuler D. & Daly M.J. 2011. A framework for variation discovery and genotyping using next-generation DNA sequencing data. *Nat Genet*, **43**, 491-498.

Dufkova L., Scigalkova I., Moutelikova R., Malenovska H. & Prodelalova J. 2013. Genetic diversity of porcine sapoviruses, kobuviruses, and astroviruses in asymptomatic pigs: an emerging new sapovirus GIII genotype. *Arch Virol*, **158**, 549-558.

Guindon S., Dufayard J.F., Lefort V., Anisimova M., Hordijk W. & Gascuel O. 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst Biol*, **59**, 307-321.

Guix S., Bosch A. & Pintó R. 2013. Astrovirus taxonomy. *In* Astrovirus research essential ideas, everyday impacts, future directions (Schultz-Cherry S., ed). Springer, New York, NY, 97-118.

Huson D.H., Beier S., Flade I., Gorska A., El-Hadidi M., Mitra S., Ruscheweyh H.J. & Tappu R. 2016. MEGAN community edition - Interactive exploration and analysis of large-scale microbiome sequencing data. *PLoS Comput Biol*, **12**, e1004957.

Indik S., Valicek L., Smid B., Dvorakova H. & Rodak L. 2006. Isolation and partial characterization of a novel porcine astrovirus. *Vet Microbiol*, **117**, 276-283.

Ito M., Kuroda M., Masuda T., Akagami M., Haga K., Tsuchiaka S., Kishimoto M., Naoi Y., Sano K. & Omatsu T. 2017. Whole genome analysis of porcine astroviruses detected in Japanese pigs reveals genetic diversity and possible intra-genotypic recombination. *Infect Genet Evol*, **50**, 38-48.

Karlsson O.E., Larsson J., Hayer J., Berg M. & Jacobson M. 2016. The intestinal eukaryotic virome in healthy and diarrhoeic neonatal piglets. *PLoS One*, **11**, e0151481.

Lan D., Ji W., Shan T., Cui L., Yang Z., Yuan C. & Hua X. 2011. Molecular characterization of a porcine astrovirus strain in China. *Arch Virol*, **156**, 1869-1875.

Laurin M.A., Dastor M. & L'Homme Y. 2011. Detection and genetic characterization of a novel pig astrovirus: relationship to other astroviruses. *Arch Virol*, **156**, 2095-2099.

Li H. & Durbin R. 2010. Fast and accurate long-read alignment with Burrows-Wheeler transform. *Bioinformatics*, **26**, 589-595.

Li J.S., Li M.Z., Zheng L.S., Liu N., Li D.D & Duan Z.J. 2015. Identification and genetic characterization of two porcine astroviruses from domestic piglets in China. *Arch Virol*, **160**, 3079-3084.

Luo Z., Roi S., Dastor M., Gallice E., Laurin M.A. & L'Homme Y. 2011. Multiple novel and prevalent astroviruses in pigs. *Vet Microbiol*, **149**, 316-323.

McKenna A., Hanna M., Banks E., Sivachenko A., Cibulskis K., Kernytsky A., Garimella K., Altshuler D., Gabriel S., Daly M. & DePristo M. A.2010. The genome analysis toolkit: a mapreduce framework for analyzing next-generation DNA sequencing data. *Genome Res*, **20**, 1297-1303.

Milne I., Bayer M., Cardle L., Shaw P., Stephen G., Wright F. & Marshall D. 2010. Tablet-next generation sequence assembly visualization. *Bioinformatics*, **26**, 401-402.

Monini M., Di Bartolo I., Ianiro G., Angeloni G., Magistrali C.F., Ostanello F. & Ruggeri F.M. 2015. Detection and

molecular characterization of zoonotic viruses in swine fecal samples in Italian pig herds. *Arch Virol*, **160**, 2547-2556.

Padmanabhan A. & Hause B.M. 2016. Detection and characterization of a novel genotype of porcine astrovirus 4 from nasal swabs from pigs with acute respiratory disease. *Arch Virol*, **161**, 2575-2579.

Peng Y., Leung H.C., Yiu S.M. & Chin F.Y. 2012. IDBA-UD: a *de novo* assembler for single-cell and metagenomic sequencing data with highly uneven depth. *Bioinformatics*, **28**, 1420-1428.

Reuter G., Pankovics P. & Boros A. 2011. Identification of a novel astrovirus in a domestic pig in Hungary. *Arch Virol*, **156**, 125-128.

Reuter G., Nemes C., Boros A., Kapusinszky B., Delwart E. & Pankovics P. 2012. Astrovirus in wild boars (*Sus scrofa*) in Hungary. *Arch Virol*, **157**, 1143-1147.

Shan T., Li L., Simmonds P., Wang C., Moeser A. & Delwart E. 2011. The fecal virome of pigs on a high-density farm. *J Virol*, **85**, 11697-11708.

Shan T., Wang C., Tong W., Zheng H., Hua X., Yang S., Guo Y., Zhang W. & Tong G. 2012. Complete genome of a novel porcine astrovirus. *J Virol*, **86**, 13820-13821.

Shirai J., Shimizu M. & Fukusho A. 1985. Coronavirus-, calicivirus-, and astrovirus-like particles associated with acute porcine gastroenteritis. *Nihon Juigaku Zasshi*, **47**, 1023-1026.

Tamura K., Stecher G., Peterson D., Filipski A. & Kumar S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol*, **30**, 2725-2729.

Toffan A., Catania S., Salviato A., De Battisti C., Vascellari M., Toson M., Capua I. & Cattoli G. 2012. Experimental infection of poults and guinea fowl with genetically distinct avian astroviruses. *Avian Pathol*, **41**, 429-435.

Ulloa J.C. & Gutierrez M.F. 2010. Genomic analysis of two ORF2 segments of new porcine astrovirus isolates and their close relationship with human astroviruses. *Can J Microbiol*, **56**, 569-577.

Van der Auwera G.A., Carneiro M.O., Hartl C., Poplin R., Del Angel G., Levy-Moonshine A., Jordan T., Shakir K., Roazen D., Thibault J., Banks E., Garimella K.V., Altshuler D., Gabriel S. & DePristo M.A. 2013. From FastQ data to high confidence variant calls: the genome analysis toolkit best practices pipeline. *Curr Protoc Bioinformatics*, **43**, 11.10.1-33.

Wilm A., Aw P.P., Bertrand D., Yeo G.H., Ong S.H., Wong C.H., Khor C.C., Petric R., Hibberd M.L. & Nagarajan N. 2012. LoFreq: a sequence-quality aware, ultra-sensitive variant caller for uncovering cell-population heterogeneity from high-throughput sequencing datasets. *Nucleic Acids Res*, **40**, 11189-11201.

Wohlgemuth N., Honce R. & Schultz-Cherry S. 2019. Astrovirus evolution and emergence. *Infect Genet Evol*, **69**, 30-37.

Xiao C.T., Gimenez-Lirola L.G., Gerber P.F., Jiang Y.H., Halbur P.G. & Opriessnig T. 2013. Identification and characterization of novel porcine astroviruses (PAstVs) with high prevalence and frequent co-infection of individual pigs with multiple PAstV types. *J Gen Virol*, **94**, 570-582.

Zhang B., Tang C., Yue H., Ren Y. & Song Z. 2014. Viral metagenomics analysis demonstrates the diversity of viral flora in piglet diarrhoeic faeces in China. *J Gen Virol*, **95**, 1603-1611.