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Multiple novel and prevalent astroviruses in pigs☆

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ABSTRACT

Knowledge of porcine astrovirus diversity and epidemiology remains limited. We used a broad range PCR approach to investigate the presence and diversity of astroviruses in healthy pigs of different ages on 20 farms and in 3 slaughterhouses situated in the province of Quebec, Canada between 2005 and 2007. Our study unexpectedly revealed remarkable levels of genetic diversity and high prevalence of astroviruses in pigs of this province. Astroviruses were detected on every farm investigated and in all age groups of pigs, from suckling piglets to adults. In addition, we found that nearly 80% of healthy finisher pigs harbour astroviruses in their intestine at slaughter. Phylogenetic evidence based on partial polymerase and complete capsid sequences, suggests that porcine astroviruses do not form a monophyletic group but are rather found on separate branches across the mamastrovirus tree. In addition to type species strains, we found highly divergent strains that form two additional lineages, one of which falls outside existing taxonomic groups. The presence of diverse astroviruses in a majority of healthy pigs likely represents a continuous source of infection to piglets and possibly to other animal species including humans. Porcine astrovirus strains appeared phylogenetically related not only to prototypical human astroviruses, as was already known, but also to novel human strains recently discovered suggesting multiple cross species transmission events between these hosts and other animal species. Overall, the findings reported in this study suggest an active role of pigs in the evolution and ecology of the *Astroviridae*.

1. Introduction

The family *Astroviridae* consists of small (28–30 nm), non-enveloped, single-stranded positive sense RNA viruses of 6.4–7.3 kb in size. These viruses generally exhibit a distinctive five or six pointed star shape appearance under EM which led Madeley and Cosgrove (1975) to coin the term *astrovirus* to describe them. The AstV genome includes three open reading frames (ORFs) designated ORF1a, ORF1b and ORF2. ORF1a and ORF1b situated at the 5′ end of the genome encode non-structural polyproteins including a protease and a RNA dependent RNA polymerase (RdRp). ORF2 situated at the 3′ end of the genome encodes the structural capsid protein and is transcribed as a subgenomic mRNA (Mendez and Arias, 2007; Monroe, 2005).

Astroviruses are enteric viruses that have been isolated from a number of host species including humans, mink, sheep, pigs, chicken, ducks, turkeys and more recently from marine mammals, dogs, cheetahs and bats (Monroe, 2005; Jonassen et al., 2001; Zhu et al., 2009; Rivera et al., 2010; Atkins et al., 2009; Toffan et al., 2009). The family *Astroviridae* is currently divided in two genera; strains of the genus *Mamastrovirus* are found in infected mammals and strains of the genus *Avastrovirus* are found in avian hosts (Monroe, 2005). According to the latest ICTV report,

☆ GenBank accession numbers of all novel sequences described in this study are from HM756258 to HM756273.

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the *Avastrovirus* genus contains 3 species while the *Mamastrovirus* genus contains 6 species, all of which are defined based on the species of origin (Monroe, 2005).

Astroviruses are generally associated with either mild or severe signs of enteric disease such as diarrhoea and vomiting in a number of mammalian species (Mendez and Arias, 2007). Astroviruses have also been found to cause no detectable disease or to cause disease only occasionally (Bridger et al., 1984; Marshall et al., 1987; Chu et al., 2008). Human astroviruses (HuAstVs) have been the most extensively studied group of astroviruses and have been repeatedly shown to be a common cause of diarrhoea in young children, the elderly and the immunocompromised (Dennehy et al., 2001; Gallimore et al., 2006; Gray et al., 1987; Akihara et al., 2005). AstV also appear second to rotaviruses as gastroenteritis agents responsible for children hospitalization (Glass et al., 1996). There are presently 8 recognized serotypes of closely related HuAstVs, all of which belong to a single species (Monroe, 2005). Phylogenetic groupings (genotypes) of HuAstV strains were found to be fully congruent with serotyping analyses since 8 well defined clusters were formed regardless of the genomic region analysed (Noel et al., 1995; Lukashov and Goudsmit, 2002). In recent years, a number of genetically distinct strains of HuAstV have been identified, characterized and proposed to represent novel AstV species based on a genetic distance criteria (Finkbeiner et al., 2008; Kapoor et al., 2009) although this proposal is still awaiting ICTV's approval.

Little is known about the presence, clinical significance and diversity of porcine astroviruses. Porcine AstVs were first detected by EM in faeces of a diarrheic piglet (Bridger, 1980). It was then successfully propagated in porcine embryonic kidney cells and shown to cause mild diarrhoea in piglets under experimental conditions (Shimizu et al., 1990). Molecular characterization and phylogenetic analysis of the capsid gene from this isolate (PAstV-1) followed and helped determine that past spill over events between cats, pigs and humans were likely to have happened (Jonassen et al., 2001; Wang et al., 2001). A closely related strain was also more recently isolated from a pig diarrhoeal sample and successfully propagated in vitro (Indik et al., 2006). Apart from these few reports, there is to knowledge no available data on the diversity of porcine astroviruses. We used a PCR approach to investigate the presence and diversity of porcine astroviruses using both specific and broad range (pan-astrovirus) primers. Our study reveals the presence in swine of prototypical strains as well as two lineages of previously unknown astroviruses. In addition, we found that astroviruses were present on all swine farms investigated independent of age group and in nearly 80% of slaughtered healthy pigs.

2. Materials and methods

2.1. Stool samples and viral RNA extraction

A total of 200 composite faecal samples were collected from 20 farms in the province of Quebec, Canada between 2005 and 2007. Each sample consisted of a mix made up of 5 sub samples collected in different parts of each pen. 60 samples were from nursing pigs (less than 4 weeks of age), 60 samples were from post-weaning pigs (between 4 and 12 weeks of age) and 80 samples were from finisher pigs (over 12 weeks of age). A total of 96 faecal samples originating from individual caecal content of slaughtered adult pigs in 3 different abattoirs in the province of Quebec were also collected during the same period. Viral RNA was extracted from the supernatants of 20% (w/v) faecal suspensions in Eagle's minimum essential medium (MEM, Invitrogen, Mississauga, ON, Canada) using the viral RNA mini kit (Qiagen, Mississauga, ON, Canada). 140 μl of the suspension was treated following the manufacturer's protocol. The RNA was stored at −70 °C until needed.

2.2. Astrovirus sequence amplification

All primers used in this study are listed in Table 1 and were synthesized at Integrated DNA Technologies (IDT,

Table 1
Oligonucleotides used in this study.

Primer name	Primer sequence 5′–3′	Reference
AST248F	GTG TCA CAG GTC CAA AAC CAG CAA T	Indik et al. (2006)
AST665R	TGG TGT TCG TCA ACC ACC AGC C	Indik et al. (2006)
ASTneF	CTC GAG GCA TGC ATC CTC AC	Indik et al. (2006)
ASTneR	AAG AGA AGC ACG GAC AAC TG	Indik et al. (2006)
panAV-F11	GARTTYGATTGGRCKCGKTAYGA	Chu et al. (2008)
panAV-F12	GARTTYGATTGGRCKAGGTAYGA	Chu et al. (2008)
panAV-F21	CGKTAYGATGGKACKATICC	Chu et al. (2008)
panAV-F22	AGGTAYGATGGKACKATICC	Chu et al. (2008)
panAV-R1	GGYTTKACCCACATICCRAA	Chu et al. (2008)
AST16-2F1	GCG TGT CCA AGA AGC TTA CC	This study
AST16-2F2	GGA CAG ATC TCC ACC ACC AT	This study
AST14-4F1	CCT TCG TTG GGA GAA GAT GA	This study
AST14-4F2	GGT ATG TGC TCA TGC CCT CT	This study
AST12-4F1	GCG GTG GGA GAA GAT GAA TA	This study
AST12-4F2	TCC TAA CCC GCT ATG TGC TC	This study
AST12-3F1	TTG CCA TCT GGG GAA GTA AC	This study
AST12-3F2	TGC CTA CAT CAA TGG TCC AA	This study
QT	CCA GTG AGC AGA GTG ACG AGG ACT CGA GCT CAA GCT $(T)_{16}$	Scotto-Lavino et al. (2006)
QO	CCA GTG AGC AGA GTG ACG	Scotto-Lavino et al. (2006)
QI	AGG ACT CGA GCT CAA GCT	Scotto-Lavino et al. (2006)

Coralville, IA, USA). Preliminary screenings were performed separately with two sets of primers targeting different regions of the AstV genome using the one-step RT-PCR kit from Qiagen (Mississauga, ON, Canada).

2.2.1. Nested RT-PCR 1

AST248F/AST665R primer pair was originally designed based on a conserved region at the 5′ end of open reading frame 2 (ORF2) encoding the capsid protein of a porcine astrovirus strain (Indik et al., 2006). A volume of 1 μl of the RNA extract was mixed with 5 μl of 5× buffer, 400 nM of each deoxinucleotide triphosphate (dNTP), 400 nM of each primer and 1 μl of the enzyme mix in a final volume of 25 μl. Following the initial RT reaction at 42 °C for 30 min, the reaction was denatured at 95 °C for 15 min and amplified for 40 cycles of the following conditions: 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. A final extension was done at 72 °C for 5 min. Semi-nested PCR was performed using 1 U of *Platinum Taq* DNA polymerase (Invitrogen, Mississauga, ON, Canada) in a final reaction volume of 50 μl containing 1 μl of a 1/1000 diluted cDNA, 3 mM $MgCl_2$, 200 nM each dNTP, 200 nM of forward (ASTneF) and reverse primer (ASTneR). The reaction mix was initially denatured at 94 °C for 2 min and amplified for 25 cycles with the following conditions: 94 °C for 15 s, 55 °C for 30 s, 72 °C for 30 s and a final extension at 72 °C for 7 min.

2.2.2. Nested RT-PCR 2

The primers were originally designed based on a conserved region in the RNA-dependent-RNA polymerase (RdRp) gene of AstVs (Chu et al., 2008). PCR primers panAV-F11 (forward), panAV-F12 (forward) and panAV-R1 (reverse) were used in the initial RT-PCR; while primers panAV-F21 (forward), panAV-F22 (forward) and panAV-R1 (reverse) were used in the semi nested PCR. 1 μl of RNA extract was mixed with 5 μl of 5× buffer, 400 nM of each deoxinucleotide triphosphates (dNTP), 400 nM of each primer and 1 μl of enzyme mix in a final volume of 25 μl. Following the initial RT reaction at 50 °C for 60 min, the reaction was denatured at 95 °C for 15 min and amplified for 40 cycles of the following conditions: 94 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s with a final extension at 72 °C for 5 min. Semi-nested PCR was performed using 1 U of *Platinum Taq* DNA polymerase (Invitrogen, Mississauga, ON, Canada) in a final reaction volume of 50 μl containing 1 μl of a 1/1000 diluted cDNA, 3 mM $MgCl_2$, 200 nM each dNTP and 200 nM of each primer. The reaction was initially denatured at 94 °C for 1 min and amplified in 25 cycles with the following conditions: 94 °C for 30 s, 50 °C for 30 s and at 72 °C for 30 s and a final extension at 72 °C for 5 min.

PCR products were either processed on standard 1% agarose gels and stained with SYBR safe (Invitrogen, Mississauga, ON, Canada) or analysed using a Qiaxcel instrument (Qiagen, Mississauga, ON, Canada). Expected amplicon sizes were approximately 180 bp using nested

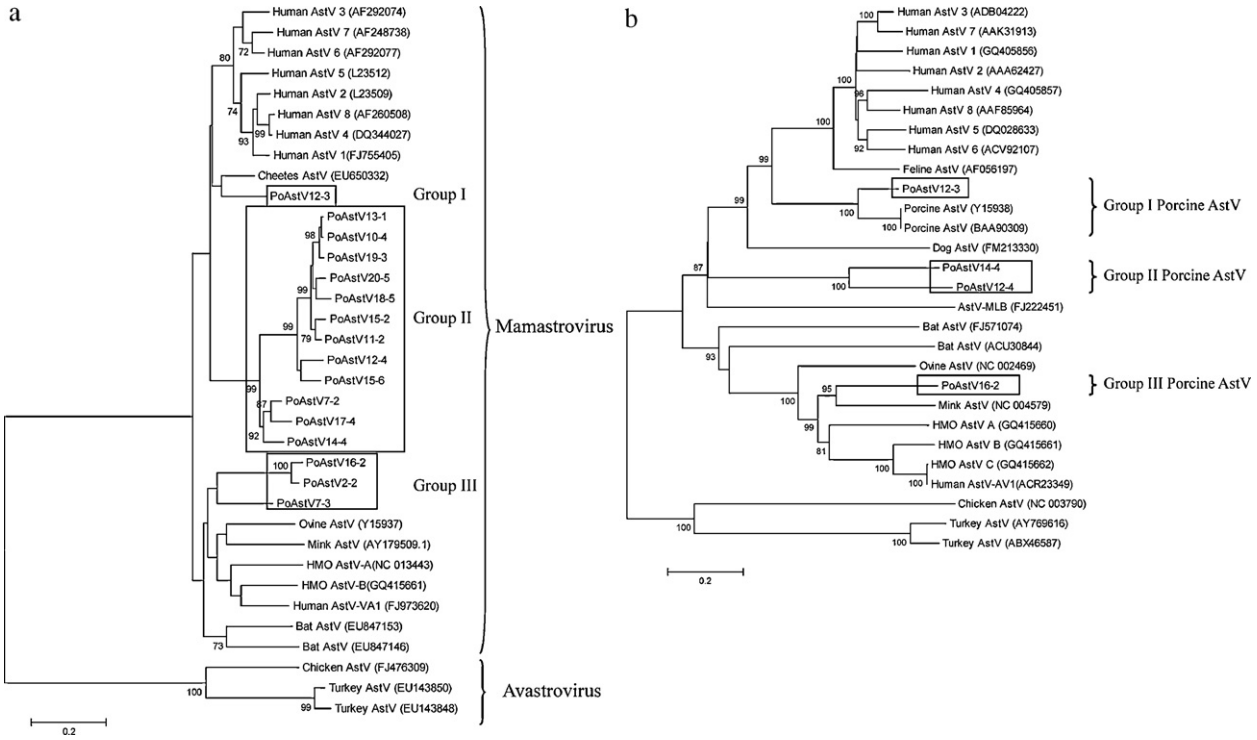

Fig. 1. Phylogenetic relationship of Canadian porcine astroviruses (boxed) and prototypical astrovirus species. (a) Tree based on partial RdRp coding region (~300 nt) amplified using pan-astrovirus primers. (b) Tree based on complete ORF2 coding region (~800 aa). Phylogenetic trees were generated using the neighbour-joining method implemented in the program MEGA4. Bootstrap values are expressed in percentage based on 1000 replications and indicated at the node. Bootstrap values of ≤70% were hidden. Scale bar represents distance expressed as nucleotide or amino acid substitutions per site. Accession numbers for the AstV strains characterized in this study are from HM756258 to HM756273.

PCR 1 or 400 bp using nested PCR 2. PCR products of the expected size were either directly sequenced in both directions using the big dye v3.1 chemistry on a 3730xl instrument from Applied Biosystems (Foster, CA) or cloned using the TOPO 2.1 (T/A) cloning (Invitrogen, Mississauga, ON). A minimum of three clones from each strain was sequenced in the latter case.

2.3. Amplification of the 3′ end of selected AstV samples using 3′ RACE-PCR

Based on consensus nucleotide sequences of the RdRp fragments obtained from strains of the 3 different groups of porcine astroviruses, group specific primers were designed using Primer3 software (http://frodo.wi.mit.edu/cgibin/primer3/primer3_www.cgi). Specific strains were selected as representatives of each of the 3 porcine AstV groups: PoAstV12-3 for group I, PoAstV12-4 and PoAstV14-4 for group II and PoAstV16-2 for group III (see Fig. 1). The group-specific primers listed in Table 1 were used in combination with primers (QT), (QO) and (QI) (Scotto-Lavino et al., 2006) as previously described (L'Homme et al., 2009) for amplification of the 3′ ends of the selected porcine AstV strains. cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen, Mississauga, ON, Canada) and primer QT at 50 °C for 60 min following the manufacturer's instructions. A first PCR was performed with primers QO and strain-specific F1 primers using 1 U of *Platinum Taq* (Invitrogen, Mississauga, ON, Canada). A final reaction volume of 50 μl contained 1 μl of cDNA, 3 mM $MgCl_2$, 200 nM each dNTP and 200 nM of each primer. The reaction was initially denatured at 94 °C for 10 min, followed by a first cycle of 30 s at 58 °C and 40 min at 72 °C and 35 cycles at 94 °C for 30 s, 58 °C for 30 s and 72 °C for 5 min. A final extension was done at 72 °C for 15 min. Nested PCR was performed using primers QI and strain-specific F2 primers in a final reaction volume of 50 μl containing 1 μl of the product from the first PCR, 3 mM $MgCl_2$, 200 nM each dNTP, 200 nM of each primer and 1 U of *Platinum Taq* DNA polymerase. The reaction was initially denatured at 94 °C for 3 min, followed by a first cycle of 30 s at 58 °C and 40 min at 72 °C and 35 cycles at 94 °C for 30 s, 58 °C for 30 s and 72 °C for 5 min. A final extension was done at 72 °C for 15 min.

The RACE-PCR products were deposited on 1% agarose gels, stained with SYBR safe (Invitrogen, Mississauga, ON, Canada) and purified on Qiagen mini spin columns (Qiagen, Mississauga, ON, Canada). Gel purified amplicons were cloned using the PCR TOPO 2.1 (T/A) cloning kit (Invitrogen, Mississauga, ON, Canada). A minimum of three clones were selected for each strain, amplified in liquid broth followed by plasmid DNA extraction using an alkaline lysis method (QIAprep Spin Miniprep, Qiagen). Each clone was then completely sequenced in both directions using a primer walking strategy.

2.4. Sequence editing, assembly and phylogenetic analysis

Sequence editing, assembly and analysis were performed using BioEdit version 7.0.9.0 (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Multiple sequence alignments were done with CLUSTAL W (version 1.6). Phylogenetic analyses were carried out using the neighbour-joining (NJ) method with the Poisson distance correction calculation for amino acids and maximum composite likelihood model for nucleotides using the Molecular Evolutionary Genetic Analysis (MEGA 4.0) software. Percentage identity of the capsid gene was calculated using amino acid p-distance implemented in MEGA 4.0. Confidence values at the nodes were obtained by performing 1000 bootstrap analyses.

3. Results

3.1. Detection and preliminary phylogenetic grouping of porcine astroviruses

In our preliminary screening of farm composite faecal samples, we used two different sets of nested primers. The first set (primer set 1) targets a conserved region at the 5′ end of ORF2 and was specifically designed for and reported to successfully amplify porcine astroviruses (Indik et al., 2006). To maximize the likelihood of detecting a wide range of variable AstV strains, a second set (primer set 2) of degenerated primers was used (pan-astrovirus primers) which was designed based on conserved motifs from the AstV RdRp gene (Chu et al., 2008). This pan-astrovirus primer set has been shown to successfully detect a wide array of variable astroviruses in humans (Kapoor et al., 2009) and bats (Chu et al., 2008; Zhu et al., 2009). Selected amplicons were either sequenced directly in both directions or cloned and sequenced depending on the amount of DNA amplified and/or on the presence of multiple amplicons of similar sizes on agarose gels.

Pairwise identity comparisons of deduced amino acid sequences amplified using primer set 1 revealed that all porcine AstV sequences were highly similar to each other and to prototypical porcine strain PAstV-1 (Genbank accession # Y15938) (>97% amino acid identity). Phylogenetic analyses using amino acid or nucleotide sequences formed a monophyletic group (not shown). Alignment of the deduced amino acid sequences amplified using the pan-astrovirus primers (primer set 2) revealed that all porcine strains amplified contained the characteristic "YGDD" motif located near the C-terminal region of the RdRp (not shown). This motif is found in the RdRp protein of all astroviruses characterized so far as well as in the RdRp of other RNA virus families such as the *Caliciviridae* (Green, 2007). Pairwise identity comparisons of deduced amino acid sequences amplified using primer set 2 revealed more heterogeneity among strains (51–98% amino acid identity) than sequences generated using primer set 1 even though the former are situated in the more highly conserved polymerase region. Phylogenetic analyses using these partial polymerase sequences from selected strains in addition to prototypical animal AstV strains clustered Canadian porcine strains in three distinct lineages (Fig. 1a – group I, II, III). Group I strains were related to PAstV-1 strain and to typical human and feline strains. Group II strains were isolated on a separate branch from other known AstVs with no apparent closely related strains. Finally, group III strains were related to mink, ovine and most interestingly to novel human AstV strains.

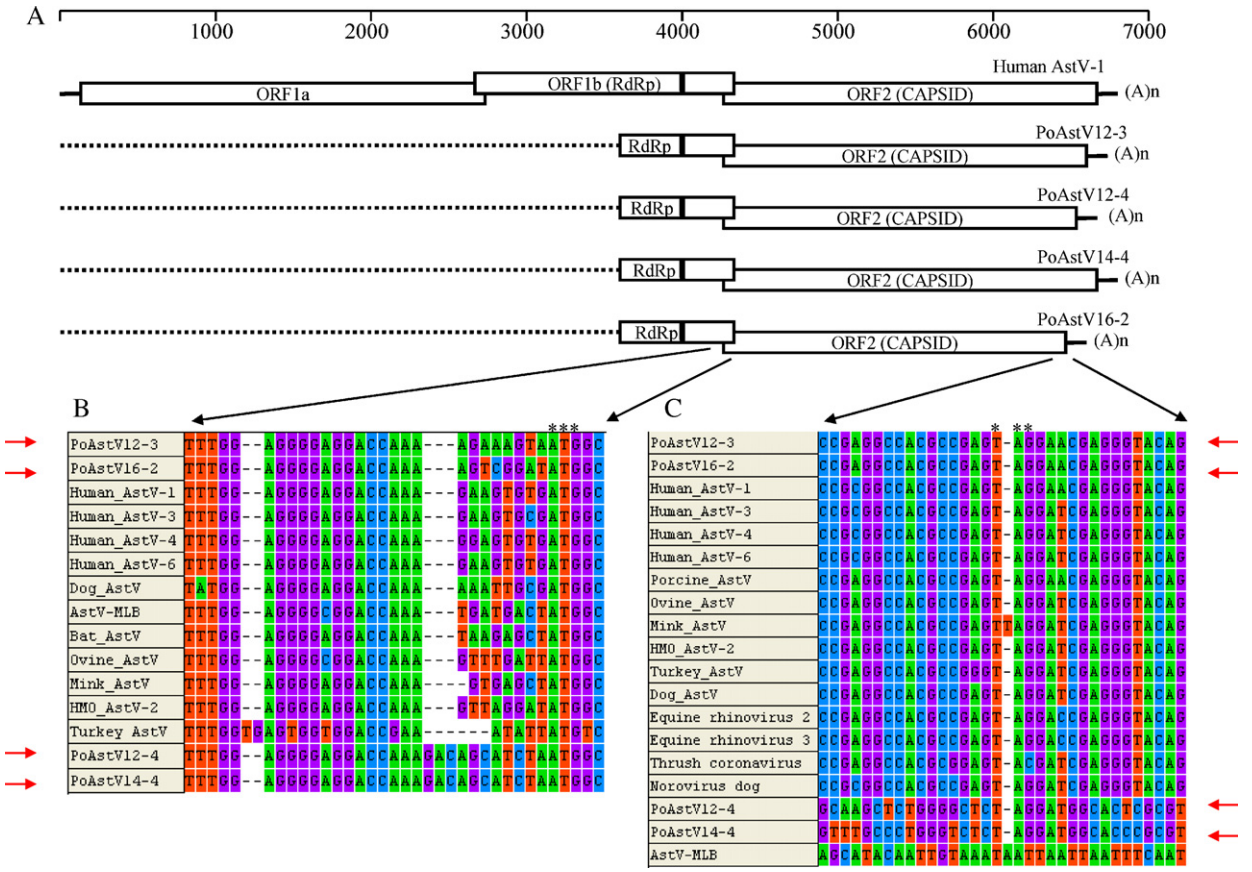

Fig. 2. Schematic representation of the complete human AstV-1 genome and partial novel porcine AstV genomes from our study (A). All 3 ORFs, 5′ UTR, 3′ UTR and polyA tail are shown. Dotted lines represent uncharacterized regions. The dark vertical lines inside the ORF1b boxes indicate the YGDD conserved motif of the RdRp present in all AstVs. (B) Nucleotide alignment of the conserved sequence at the ORF1b/ORF2 junction, the ATG initiation codon is indicated by asterisks. (C) Nucleotide alignment of the conserved sequence motif at the 3′ end of astroviruses and other RNA viruses. The ORF2 stop codons are indicated by asterisks. Red arrows indicate AstV strains characterized in this study. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.2. Genome features and phylogenetic analysis of the 3′ end from selected PoAstV strains

A total of 4 strains (PoAstV12-3, PoAstV12-4, PoAstV14-4, and PoAstV16-2) were selected as representatives of each of the three clusters of PoAstVs to permit in depth genomic investigation and strengthen their taxonomic grouping. Using 3′ RACE-PCR, we were able to amplify and characterize approximately 3 kb of nucleotide sequence from each strain which included the 3′ end of the RdRp gene, the complete capsid gene and the 3′ UTR (Fig. 2a). Astroviruses contain a conserved sequence (*UUUG-GAGNGGNGGACCNAAN*$_{5-8}$ *AUGNC*) located at the junction between ORF1b and ORF2 which has been proposed to be a regulatory element serving as a promoter for subgenomic RNA transcription (following the convention of HuAstV which places the initiation AUG codon – underlined – for ORF2 immediately upstream of the ORF1b stop codon) (Mendez and Arias, 2007). Alignment of the corresponding region from the 4 representative PoAstV strains characterized here along with prototypical AstV strains revealed that it is highly conserved in all strains (shown for ~30 nt in Fig. 2b). Interestingly, strains PoAstV12-4 and PoAstV14-4 contain a unique insertion of 3 nucleotides upstream of the ORF2 initiation codon which is not present in any of the other AstV strains. The ORF2 start codon (bold) of strains PoAstV12-3 and PoAstV16-2 appeared in an optimal Kozak context for translation initiation (RNN**AUG**G, where R = A/G and N = A/T/G/C) (Kozak, 1991), whereas those of strains PoAstV12-4 and PoAstV14-4 appeared in a less favourable context with a C residue at position -3 (Fig. 2b). The ORF2 genes are predicted to encode capsid proteins of between 755 and 783 amino acids in length which is comparable to all mamastrovirus known to date (Table 2). Molecular masses (Mr) are predicted to be between 82.1 kDa and 85.6 kDa. The length of the 3′UTR of the four PoAstV strains varied from 76 to 86 nucleotides which is comparable to most mamastrovirus known to date (Table 2). All mamastrovirus strains except human strain MLB-1 contain a highly conserved stem-loop II-like motif (s2m) in the 3′ UTR. This motif has also been reported in some picornaviruses and coronaviruses (Jonassen et al., 1998). The exact role of this motif is unclear, but due to its presence in many viral families, it has been suggested to have an important function in viral RNA replication. As is shown in Fig. 2c,

Table 2
Genome features comparison of novel porcine AstV from this study and selected reference strains.

Strain	ORF1b-ORF2 overlap (nt)	ORF2 length (aa)	3′ UTR length (nt)	Accession number
PoAstV12-3	8	779	84	HM756258
PoAstV12-4	8	769	76	HM756259
PoAstV14-4	8	783	81	HM756260
PoAstV16-2	11	755	86	HM756261
Human AstV-1	8	787	80	FJ755404
Human AstV-3	8	794	85	GU732187
Human AstV-4	8	771	84	DQ344027
Human AstV-6	8	778	84	GQ495608
Porcine AstV	N/A	783	75	Y15938
Dog AstV	11	774	83	FM213330
AstV-MLB	8	756	58	FJ222451
Bat AstV	11	760	65	FJ571067
Ovine AstV	8	763	59	NC_002469
Mink AstV	5	776	108	AY179509
HMO AstV-2	11	755	48	GQ415661
Turkey AstV-1	N/A	672	130	NC_002470

alignment of this region reveals that strains PoAstV12-3 and PoAstV16-2 contain a highly conserved s2m motif whereas the corresponding sequence of strains PoAstV12-4 and PoAstV14-4 appear highly divergent, which is reminiscent of human strain Ast-MLB-1 (Finkbeiner et al., 2008).

Phylogenetic and pair-wise amino-acid identity analyses of the complete capsid coding region confirmed the close relationship of strain PoAstV12-3 with prototypical PAstV-1 strain (80% aa identity) (Fig. 1b and Table 3). Strain PoAstV16-2 appeared divergent from prototypical porcine strains (28% aa identity) and closest to mink AstV (58% aa identity) and human HMO strain (54% aa identity) (Table 3 and Fig. 1b). Most interestingly, phylogenetic and distance analyses revealed that strains PoAstV12-4 and PoAstV14-4 were highly divergent from known AstV strains and formed a novel group of mammastrovirus distantly related to the human/feline/porcine AstV group (Fig. 1b and Table 3). Altogether, these analyses confirmed that swine harbour phylogenetically diverse AstV strains most likely derived from distinct ancestors. Phylogenetic analyses performed with either the larger polymerase fragment (~250 aa) (not shown) or the complete capsid coding sequence from the 4 novel porcine strains did not reveal any differences in the branching patterns of the threes indicating that these strains are unlikely to represent recombinants.

3.3. Prevalence of PoAstVs in slaughtered pigs and in farm samples

Using both primer sets 1 and 2, we were able to detect and confirm the presence of astroviruses on every farm and in all age groups from suckling piglets to finisher pigs. Since the pan-astrovirus primers detected a large number of AstV samples that were undetected by primer set 1, we used the former to screen a sample set from ceacal contents of 96 individual slaughtered finisher pigs. 76

Table 3
Pairwise amino acid (aa) sequence identity between selected AstV strains.

	PoAstV 12-3	PoAstV 12-4	PoAstV 14-4	PoAstV 16-2	Human AstV-1	Human AstV-3	Human AstV-4	Human AstV-6	Porcine AstV	Dog AstV	AstV-MLB	Bat AstV	Ovine AstV	Mink AstV	HMO AstV2	Turkey AstV-1
PoAstV 12-3		53	53	47	87	83	87	85	NA	81	64	52	47	49	52	33
PoAstV 12-4	30		94	48	52	51	52	49	NA	58	54	45	49	53	57	36
PoAstV 14-4	31	60		45	52	51	52	51	NA	59	52	47	46	44	54	35
PoAstV 16-2	29	23	24		46	48	46	45	NA	44	46	65	62	59	68	32
Human AstV-1	50	30	32	27		95	100	97	NA	80	62	51	46	51	52	30
Human AstV-3	52	32	33	28	81			93	NA	77	62	49	45	48	52	31
Human AstV-4	47	30	32	27	73	74		97	NA	80	62	51	46	51	52	30
Human AstV-6	48	30	32	28	78	80	74		NA	78	62	49	45	51	51	30
Porcine AstV	**80**	**30**	**31**	**28**	50	51	46	48		NA	NA	NA	NA	NA	NA	NA
Dog AstV	42	29	28	30	40	41	40	39	42		65	47	45	52	49	38
AstV-MLB	31	27	28	25	32	30	30	29	32	29		46	52	47	47	33
Bat AstV	30	25	25	34	30	30	28	30	29	28	26		63	64	67	31
Ovine AstV	31	25	26	50	29	29	29	29	30	31	25	34		62	64	33
Mink AstV	28	23	26	58	27	27	27	28	28	32	26	33	52		68	30
HMO AstV-2	28	25	25	54	28	28	27	27	27	29	26	33	51	51		38
Turkey AstV-1	20	18	18	21	19	20	20	19	20	22	21	18	28	28	28	

Values above and below the diagonal indicate partial ORF1b (~250 aa) identity and complete ORF2 identity respectively. Values underlined indicate inter-strain identities between the 4 porcine strains characterized in this study and values in boldface indicate identities between the 4 newly characterized porcine strains and the prototypical porcine strain (Y15938).
NA: not available.

samples (79%) were confirmed positive by sequencing of the nested PCR product. Phylogenetic analyses clustered 1% with group I, 99% with group II and none with group III. Strains from group III were only detected in farm samples from suckling piglets. We were also able to confirm a double infection with two different AstV strains in a single pig at slaughter.

Attempts in culturing strains from the three PoAstV groups in PK-15 cells with various concentrations of trypsin (2–9 μg/ml) were unsuccessful. Also, direct examination of faecal samples by EM did not permit visualization of typical star shape virions of the expected size although viral particles of the expected sizes were frequently observed (25–30 nm) (not shown).

4. Discussion

This report presents the first comprehensive investigation on the presence and diversity of AstVs in pigs. A remarkable and surprising diversity of AstVs was disclosed in these hosts. Based on the data presented, which includes genomic and phylogenetic analyses, we conclude in the existence of three distinct lineages of porcine astroviruses, two of which are unprecedented. Pairwise amino acid identities between strains within each of these lineages range from only 23% to 31%, which is comparable to distances between established AstV species. Porcine strains belonging to two of the lineages (group I and III) were more closely related to other animal's AstV strains than they were to each other suggesting different ancestral origin of porcine AstVs and past interspecies transmission involving sheep, mink, felines, humans and possibly additional yet unknown hosts. Likewise, a number of novel human AstV strains were recently shown to be genetically closer to animal AstVs strains and more distantly related to type HuAstV species suggesting different origins (Finkbeiner et al., 2008; Kapoor et al., 2009). In addition, Chu et al. (2008) and Zhu et al. (2009) have also shown that AstVs isolated from the same species of bats cluster in different groups following phylogenetic reconstruction. Taken together, data from these three mammalian hosts suggest that AstVs found within a single animal species can have distinct origins most likely reflecting past interspecies transmission which underscores the zoonotic potential of these viruses.

According to the latest ICTV guidelines, AstV species are defined based on the host of origin (Monroe, 2005). Hence, reminiscent of a number of AstV strains recently recovered from humans and bats (Kapoor et al., 2009; Chu et al., 2008), the PoAstV strains characterized in this study should be classified as a single viral species. However, it is now clear that multiple AstV lineages can infect the same animal species and there is also growing evidence that AstVs can cross the species barrier (Chu et al., 2008; Zhu et al., 2009; Kapoor et al., 2009). For these reasons, it has been suggested that the classification of AstV should not be solely based on the species of origin but should also rely on a distance criteria of either the RdRp or the capsid genes (Kapoor et al., 2009). The data presented in the present study supports this contention.

Porcine strains from one of the lineages (group I) were closely related to typical swine strains which have previously been characterized and associated with diarrhoea in pigs (Jonassen et al., 2001; Indik et al., 2006). Since all samples from this study, including those from group I, were from apparently healthy pigs, our results shed a new light on the pathogen/host relationship between pigs and astroviruses. It is well known that AstVs can cause enteric disease in numerous animal species (Mendez and Arias, 2007; Toffan et al., 2009; Atkins et al., 2009). However, it has also been reported that asymptomatic carriers of AstV exist (Rivera et al., 2010; Kapoor et al., 2009; Chu et al., 2008). AstV infections in swine may express their pathogenicity in only a small percentage of cases, in conjunction with other pathogens or more severely in young animals like has often been observed in other animal species such as humans (Madeley and Cosgrove, 1975; Bridger, 1980; Woode and Bridger, 1978; Snodgrass and Gray, 1977). It should be noted that only two strains of group I AstVs were detected in our study and that mild diarrhoea could have been overlooked in those cases. Alternatively, as mentioned before, there is a possibility that AstVs might express their pathogenicity in only a small percentage of cases.

Porcine strains belonging to group II formed a lineage that appeared phylogenetically isolated from previously characterized AstV strains. Strains of this group (PoAstV12-4 and PoAstV14-4) also contained divergent nucleotide sequences in both the ORF1b/ORF2 consensus region and in the 3′ UTR region (s2m motif) that are usually highly conserved among the *Astroviridae* which emphasizes the distinctness of strains belonging to this lineage. This situation is reminiscent of some human, bat and turkey strains (Jonassen et al., 1998; Chu et al., 2008; Finkbeiner et al., 2009). These findings bring into question the biological role of the s2m motif which is thought to be important in the viral life cycle (Jonassen et al., 1998, 2001). Perhaps these divergent strains are able to compensate by forming stable secondary structures with a similar role to the s2m in different parts of their 3′ UTR. Unlike strains from group I and III, strains from group II were not closely related to other animal's strains known to date and might represent an AstV group restricted to swine.

A third lineage of porcine strains (group III) appeared phylogenetically related to mink, ovine and novel human AstV strains, again suggesting past interspecies transmission events between these animals. Interestingly, strains from group III were only detected in suckling piglets which might suggest an age restricted pattern of infection for these strains.

We found high infection rates of AstVs in finisher pigs. According to our nested PCR results, 80% of the animals harbour astroviruses in their intestine. Our data corroborates previous reports where seroprevalence to AstV in pigs as high as 83% were reported in some herds (Shimizu et al., 1990). Group II PoAstVs were by far the most commonly detected AstV strains in the sampled population using the pan-AstV approach suggesting it was the dominant group of AstV in the studied population. Such high virus detection rates could imply that pigs are persistently infected with various strains of AstVs similar

to what has been reported in bats (Chu et al., 2008). Further studies to determine the pathogenic potential of porcine AstVs belonging to the different lineages in pigs of different ages are warranted.

5. Conclusion

This work unveiled extensive diversity and high prevalence of AstVs in porcine hosts. Genetic and phylogenetic characterization of the complete 3′ end from selected porcine AstV strains revealed the existence of 3 distinct lineages, most likely of different origins, with genetic distances between these being consistent with distances found between established species in the family *Astroviridae*. The fact that two lineages of porcine AstVs are phylogenetically related to human strains underscores the zoonotic potential of theses viruses. In addition, the work also showed high prevalence of AstVs in healthy pigs of different ages bringing into light new data about the relationship between porcine AstVs and their host. The combination of a large and diverse gene pool in pigs makes these animals an important and pivotal reservoir for the evolution and ecology of the *Astroviridae*.

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