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Porcine astrovirus viremia and high genetic variability in pigs on large holdings in Croatia

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ABSTRACT

Astroviruses are emerging viral agents, primarily enteropathogenic in mammals, but recently have been acknowledged to have extra-intestinal implications in humans and mink. Porcine astrovirus is thought to be widely distributed and highly prevalent among pigs, nevertheless its clinical significance remains doubtful as it can be detected in diarrheic as well as in healthy pigs. Recent reports imply the immense genetic variability among porcine astrovirus strains with five distinct lineages being characterized so far. Herein, we report porcine astrovirus circulation in the blood of healthy pigs in different age categories bred on two large industrial holdings in Croatia, with viral RNA seroprevalence of 3.89%. These are the first extra-intestinal findings of astrovirus in pigs, indicating a more complex pathogenesis than previously thought. Partial polymerase sequences of serum-derived strains provisionally clustered into porcine astrovirus lineages 2 and 4, sharing high genetic identity with previously described porcine astrovirus strains. The results were supported by detecting porcine astrovirus strains in composite fecal samples, regardless of pig category or holding tested. Phylogenetic analysis of derived strains suggested the presence of porcine astrovirus lineages previously detected in pig sera with an additional highly genetically divergent lineage 5, reported for the first time in Europe. Moreover, the existence of possible sub lineages should not be excluded. The results obtained in the present study, contribute to knowledge of porcine astrovirus pathogenesis; even though it's possible clinical significance remains unclear. High fecal prevalence accompanied with vast genetic diversity on a relatively confined area, underscores the importance of pigs as porcine astrovirus reservoirs with eventual recombination events as a possible outcome.

1. Introduction

Astroviruses are emerging, primarily enteropathogenic viral agents that have been comprehensively investigated in the past few years. They belong to the family *Astroviridae*, which represents a group of small, non-enveloped viruses with star-shaped surface appearance under electron microscopy (EM), hence the name (Madeley and Cosgrove, 1975). Astrovirus has a single-stranded positive-sense RNA genome, 6.4–7.3 kb in size, forming three open reading frames; ORF1a, ORF1b and ORF2 in a 5′-to-3′ direction, respectively. ORF1a and ORF1b encode for non-structural polyproteins 1a and 1ab that include the serine protease and RNA-dependent RNA polymerase (RdRp); ORF2, on the other hand, encodes for a capsid structural polyprotein (Mendez and Arias, 2007). Astroviruses are divided into two genera; members of the genus *Mamastrovirus* and *Avastrovirus* infect mammalian and avian hosts, respectively. According to the current Virus Taxonomy, the genus *Mamastrovirus* is comprised of six officially recognized species, however phylogenetic analysis based on the entire ORF2 sequence suggests subdivision into two genogroups (Bosch et al., 2011). Furthermore, recent taxonomic proposals (Adams and Carstens, 2012) indicate 19 species within *Mamastrovirus* genus (*Mamastrovirus* 1–19) based on the genetic criteria, in contrary to previous host-based criterion.

Astroviruses are able to infect a wide variety of host species, including humans, pigs, wild boars, sheep, cattle, roe deer, dogs, cats, cheetahs, mink, bats, marine mammals, rats, mice, rabbits, chicken, ducks, turkeys, guinea fowls and pigeons (Bosch et al., 2011; Martella et al., 2011; Phan et al., 2011; Reuter et al., 2012; Zhao et al., 2011). Usually, astrovirus infections are associated with mild or severe symptoms of gastroenteritis in mammals (Mendez and Arias, 2007), even so astroviruses can be detected in healthy individuals (Chu et al., 2008; Luo et al., 2011; Martella et al., 2011; Reuter et al., 2011). Apart from being primarily enteropathogens, astroviruses can be found extra-intestinally in poultry (Gough et al., 1984; Imada et al., 2000; Koci et al., 2000; Koci et al., 2003) and more recently in brain tissue of mink as possible

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causative agents of "shaking mink syndrome" (Blomstrom et al., 2010). More interestingly, human astrovirus strains have caused systemic infections in younger and immunocompromised individuals, having more implications besides usual gastrointestinal illness (Holtz et al., 2011; Quan et al., 2010; Wunderli et al., 2011; Wylie et al., 2012).

In addition to classical *Human astrovirus* (HuAstV) genotypes 1–8 (Bosch et al., 2011), several other distinct HuAstV strains (MLB and VA/HMO), have been detected so far (Bosch et al., 2011; Finkbeiner et al., 2008, 2009a,b; Kapoor et al., 2009).

Pigs were recognized as astrovirus hosts in the 1980's (Bridger, 1980). The official Taxonomy (Bosch et al., 2011) recognizes *Porcine astrovirus* (PoAstV) as the only species in the genus *Mamastrovirus*; however recent reports indicate at least five distinct PoAstV lineages (Laurin et al., 2011; Shan et al., 2011). Porcine astrovirus is thought to be widely distributed (De Benedictis et al., 2011). It has been detected in diarrhoeic (Bridger, 1980; Indik et al., 2006) and healthy pigs (Luo et al., 2011; Reuter et al., 2011), however clinical significance needs to be further clarified (De Benedictis et al., 2011). These diverse astrovirus strains harbored by pigs, possibly reflect different origins and potential past interspecies transmission and recombination events, some of them presumably with human strains (Indik et al., 2006; Jonassen et al., 2001; Luo et al., 2011; Ulloa and Gutierrez, 2010).

Previous reports, generally being addressed to the feces-derived strains (De Benedictis et al., 2011), suggest that porcine astrovirus might be limited to the enteric system of pigs. Apart from the recent studies confirming astrovirus presence in blood, brain and several other tissues in humans and mink (Blomstrom et al., 2010; Holtz et al., 2011; Quan et al., 2010; Wunderli et al., 2011; Wylie et al., 2012), there is to our knowledge, no available data regarding extra-intestinal findings of porcine astrovirus in domestic pigs. In the present report we demonstrate, for the first time, the presence of porcine astrovirus in blood of pigs bred on large industrial holdings in Croatia. In relation to a possible high prevalence and genetic variability, we examined porcine fecal samples collected from the same farms exhibiting porcine astrovirus viremia. Therefore we notify three porcine astrovirus lineages circulating within the large industrial holdings, in all tested age groups and more interestingly, the first evidence of a newly described lineage 5, in Europe.

2. Materials and methods

2.1. Specimen collection

A total of 180 serum samples were collected in 2011 from pigs bred on three large industrial holdings (average 1200 saws per holding) located in Zagreb County (one holding, designated as holding-A) and Osijek–Baranja County (two holdings, designated as holding-B and holding-C) in Croatia. We sought to detect an estimated 20% astrovirus RNA seroprevalence (referred to porcine kobuvirus RNA seroprevalence (Reuter et al., 2010)) with 95% confidence interval (>1000 pigs/category) to assess the minimum sample size ($n = 14$) in each age category. To improve detection rates and eventual statistical significance we collected larger number of samples which were distributed over four age groups, more precisely 75, 50, 25 and 30 serum samples originated from post-weaning pigs, finisher pigs, gilts and sows, respectively. Blood samples were taken from the jugular vein by closed vacuum sets into sterile tubes without anticoagulants, transported and stored upon receiving in a cold environment (+4 °C). Additionally, 11 composite fecal samples were collected from two holdings that had tested positive for astrovirus in serum samples (holding-A and holding-B). Each composite sample consisted of at least five subsamples taken from different parts of the randomly chosen unit where pigs were kept in a group of up to 20 animals depending on pig category. These samples were also distributed by age; therefore they were collected from nursing, post-weaning and finisher pigs, gilts, sows and boars (the last category was bred solely on holding-B in Osijek–Baranja County). Individual pigs that had tested positive for astrovirus RNA in their sera were part of the groups from which composite fecal samples were collected. Fecal samples were collected in sterile containers, transported and stored as blood samples or deep frozen if not processed immediately. The pigs included in the present study did not exhibit gastrointestinal disease or any general signs common to many infectious diseases (healthy pigs, later in the text).

2.2. Sample preparation and viral RNA purification

Sera were separated from cellular elements by centrifuging coagulated blood (the blood clots were rimmed with a sterile glass stick to facilitate separation) for 15 min at 1000*g*. An amount of 140 μl of each serum sample was used for viral RNA purification by using the QIAamp viral RNA extraction kit (Qiagen, USA) according to the manufacturer's instructions. Fecal samples were resuspended in phosphate-buffered saline (PBS; pH 7.4) in order to obtain 20% w/v fecal suspensions which were then vortexed for 1 min and centrifuged for 15 min at 1000*g*. Supernatants were further centrifuged for 3 min at 18,000*g* and used for subsequent viral RNA purification as described above. The RNA samples purified from serum or fecal samples were stored at −80 °C until needed.

2.3. RT-PCR procedure for astrovirus detection

RNA samples were reverse transcribed by SuperScript III reverse transcriptase and random hexamers (Invitrogen Life Technologies, USA), following the manufacturer's instructions. The generated cDNA was used immediately for PCR amplification or stored at −20 °C.

The generic primers described previously (Chu et al., 2008) were used for astrovirus detection as they reportedly amplify the conserved RNA-dependent RNA polymerase (RdRp) gene fragment (422 bp in size) of numerous astrovirus species. Briefly, primers panAV-F11, panAV-F12 (both forward) and panAV-R1 (reverse) were used in the first PCR reaction, while primers panAV-F21, panAV-F22 (both forward) and panAV-R1 (reverse) were used in the hemi-nested PCR reaction. Each reaction mixture of 50 μl contained 10 μl of 5 × buffer, 1.5 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphates (dNTP), 400 nM of each primer, 1.25 U of GoTaq DNA polymerase (Promega, USA), nuclease free water and 6 μl of cDNA sample or initial PCR product. The PCR cycling conditions were as described by Luo et al. (2011), with minor modification in starting denaturation temperature. PCR products were separated by agarose gel electrophoresis in 1.5% agarose gel stained with ethidium bromide and visualized by UV transillumination. Results with amplicon sizes of around 420 bp were tentatively considered positive.

2.4. Sequencing, alignment and phylogenetic analysis

In order to confirm that astrovirus RdRp fragment was amplified and to obtain nucleotide sequences for subsequent phylogenetic analysis, we purified PCR products using Wizard SV Gel and PCR Clean-Up System (Promega, USA). Purified samples were sent for direct sequencing in both directions to Macrogen Inc., Amsterdam, The Netherlands (using the BigDyeTM terminator kit and run on ABI 3730XL). The first comparisons of sequence data with astrovirus reference strains in GenBank were performed by BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences characterized