Detection of porcine enteric viruses (Kobuvirus, Mamastrovirus and Sapelovirus) in domestic pigs in Corsica, France

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

The study was exempt from authorization because the molecular analysis was carried out on stool samples collected by technicians from live pigs (or from the ground). All the breeders gave their explicit agreement for the collection of the samples, and all the data were anonymized.

Abstract

Many enteric viruses are found in pig farms around the world and can cause death of animals or important production losses for breeders. Among the wide spectrum of enteric viral species, porcine Sapelovirus (PSV), porcine Kobuvirus (PKoV) and porcine Astrovirus (PAstV) are frequently found in pig feces. In this study we investigated sixteen pig farms in Corsica, France, to evaluate the circulation of three enteric viruses (PKoV, PAstV-1 and PSV). In addition to the three viruses studied by RT–qPCR (908 pig feces samples), 26 stool samples were tested using the Next Generation Sequencing method (NGS). Our results showed viral RNA detection rates (i) of 62.0% [58.7–65.1] (n = 563/908) for PSV, (ii) of 44.8% [41.5–48.1] (n = 407/908) for PKoV and (iii) of 8.6% [6.8–10.6] (n = 78/908) for PAstV-1. Significant differences were observed for all three viruses according to age (P-value = 2.4e–13 for PAstV-1; 2.4e–12 for PKoV and 0.005 for PSV). The type of breeding was significantly associated with RNA detection only for PAstV-1 (P-value = 9.6e–6). Among the 26 samples tested with NGS method, consensus sequences corresponding to 10 different species of virus were obtained This study provides first insight on the presence of three common porcine enteric viruses in France. We also showed that they are frequently encountered in pigs born and bred in Corsica, which demonstrates endemic local circulation.

Key words: Enteric viruses; epidemiology; domestic pigs; virology; molecular biology

Importance : This study provides important information in the comprehension of the epidemiology of different viruses circulating in swine farms. We have shown the great diversity of viruses that could be present in extensive farms. Moreover, to our knowledge, this is the first detection of these different viruses in France. So far, this study has to be considered as a first step in the study of enteric viruses in Corsican pig farms.

Introduction

Pig farms make an important contribution to the economy of world agriculture and are an important source of food. Porcine diarrhea can cause mortality in animals, especially in piglets, and cause economic losses to the pig farmers; many of the pathogens responsible can also infect humans. A very broad spectrum of viruses that can cause porcine diarrhea has been found in pig feces, including porcine Sapelovirus (PSV), porcine Kobuvirus (PKoV), porcine Sapovirus, porcine Astrovirus (PAstV), porcine Bocavirus and porcine Rotavirus (Estes *et al.*, 1983 ; Reuter *et al.*, 2008 ; Shan *et al.*, 2011a ; Liu *et al.*, 2012 ; Meng, 2012 ; Yu *et al.*, 2013 ; Zhang *et al.*, 2014). The most prevalent viruses detected in pig feces are PKoV, Mamastroviruses or Astrovirus 4 (PAstV), porcine Circovirus (PCV) and PSV (Zhou *et al.*, 2016 ; Chen *et al.*, 2018).

Kobuviruses belong to the *Picornaviridae* family. The genome is a single-stranded 8.2-8.3-kb RNA molecule that contains a large open reading frame coding for a polyprotein (Reuter *et al.*, 2009 ; Yu *et al.*, 2011). Different species of kobuviruses have been found around the world in diverse animal species (pigs, cattle, sheep, goats, bats, rodents, felines, canines, etc.) and humans. It is suspected to be a pathogen that causes digestive disorders, particularly diarrhea in humans and animals, with transmission occurring via the fecal–oral route (Khamrin *et al.*, 2014).

Porcine Sapelovirus (Family *Picornaviridae*, genus *Sapelovirus*) is a non-enveloped virus of 7.5–8.3 kb positive-polarity single-stranded RNA genome (Krumbholz *et al.*, 2002). *Sapelovirus* genus is closely related to the genus Enterovirus and consists of three species: *Avian Sapelovirus, Sapelovirus A* (Porcine Sapelovirus [PSV]) and *Sapelovirus B* (simian Sapelovirus), with a single serotype (ICTV, 2019). PSV is transmitted via the fecal–oral route, and infection of pigs can be asymptomatic or associated with diarrhea, respiratory distress, encephalitis, skin lesions and reproductive tract disorders (Lan *et al.*, 2011 ; Kim *et al.*, 2016b ; Ray *et al.*, 2018). PSV is circulating in China, India, Korea, the United States, Brazil and Europe (Germany, the United Kingdom and Spain) (Buitrago *et al.*, 2010 ; Lan *et al.*, 2011 ; Chen *et al.*, 2012 ; Donin *et al.*, 2014 ; Schock *et al.*, 2014 ; Son *et al.*, 2014 ; Arruda *et al.*, 2017 ; Ray *et al.*, 2018). Recently, PSV was detected and characterized for the first time in France, in Corsica. Importantly, the PSV-infected piglet from which the sequenced strain was isolated (PSV OPY-1-Corsica-2017; Genbank accession no. MH513612) was born and bred on the island of Corsica, suggesting local transmission (Piorkowski *et al.*, 2018).

Astroviruses are nonenveloped single-stranded RNA viruses with positive polarity, with an icosahedral capsid (Monroe *et al.*, 1993), that belong to the family *Astroviridae*, which includes two genera: *Mamastrovirus* (mammals) and *Avastrovirus* (avian) (ICTV, *Astroviridae*, 2019). Astroviruses can infect a large spectrum of animal species (pigs, deer, marine mammals, rodents, birds, pets, etc.) as well as humans (Johnson *et al.*, 2017). Astrovirus infections are generally associated with more or less severe gastrointestinal signs in mammals (Mendez and Arias, 2007), but have also been detected in healthy individuals (Reuter *et al.*, 2011). In humans, they cause intestinal disorders, particularly in children and immunocompromised individuals (Holtz *et al.*, 2011 ; Wylie *et al.*, 2012).

The present study was conducted in Corsica, a French Mediterranean island, where livestock farming is a principal economic activity. In this region, more than 54,000 pigs, predominantly of the "Nustrale" breed, are bred using a traditional extensive farming system (Casabianca. F *et al.*, 2000 ; DRAAF., 2017). Traditional extensive (or semi-extensive) outdoor system of pig farming is the main method of breeding. It favors contact with wild animals, which could result in sharing of pathogens such as hepatitis E virus (HEV) and Aujesky's disease agent (Jori *et al.*, 2016 ; Charrier *et al.*, 2018). For HEV, we recently reported RNA detection in 9.2% of tested pig stool samples, with 75% of pig farms showing at least one positive sample (Capai *et al.*, 2019). Exploring the circulation of other enteric viruses in such pig farms could help to gain knowledge in the epidemiological cycle of HEV through the possible role of co-infection and super-infection. The aim of our study was to investigate the circulation of three enteric viruses (PKoV, PAstV-1 and PSV) in samples also analyzed for HEV.

Materials and methods

Study area, pig farms and sampling plan

Study area, samples/data collection, pig farms sampled, sampling plan and ethics statement are as described previously (Capai et al. 2019).

Briefly, **(i)** we collected fresh stool samples individually on the ground where pigs were pasturing and also intra-rectally using a glove with the help of a qualified technician; **(ii)** three types of breeding system operated in Corsica were included: seven outdoor extensive farms (E-farms), six outdoor semi-extensive farms (SE-farms) and three indoor closed farms (C-farms); **(iii)**. for each stool sample, the township, anonymous breeder code, breeding type,

age and breed of pig and nature of the sample ("rectal" or "on the ground" feces) were recorded; **(iv)**. Four age categories were defined among the young pigs: 1–3 months, 3–4 months, 4–6 months and adults (older than 6 months). Samples from plots where post-weaning pigs were held together with older pigs (age mixed) were classified as the "Herd" group.

RNA extraction and reverse transcription–quantitative polymerase chain reaction (RT–qPCR)

One gram of fecal sample was resuspended in 9 mL of phosphate-buffered saline and then centrifuged at 5,000 × g for 10 min. The resulting supernatant was collected and stored at −80°C until processed. Viral RNA was extracted from 200 µL of supernatant using QIAamp Cador Pathogen on a QIAcube HT (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Samples were spiked with an internal control (T4 and MS2 phages) before extraction, to monitor the extraction and subsequent steps, as described previously (Ninove et al., 2011). Nucleic acids were eluted in 100 µL of RE buffer and stored at –80°C.

Samples initially collected for the detection of HEV RNA were analyzed by RT–qPCR for PKoV, PSV and PAstV-1. Of the 919 samples initially collected, 908 were available for this study. Details of the three molecular assay (Kim *et al.*, 2016a ; Zhou *et al.*, 2016) are presented in Table 1 using an Applied Quant Studio 3 (Applied Biosystems, CA, USA). For PKoV RNA and PAstV-1 RNA detection, a RT–qPCR test was considered positive if negative controls were negative, positive controls were positive and an exponential curve was observed before a 35-Ct threshold.

For PSV RNA detection, the qPCR machine was programmed to perform a melt-curve analysis at the end of the run to ensure assay specificity; RT–qPCR results were considered positive if a melt curve was detected at between 83°C and 85°C and an amplification curve was observed before a 35-Ct threshold. The QuantiTect SYBR® Green PCR Kit was used for this biomolecular detection (Qiagen, Hilden, Germany).

Statistical analyses

The detection rate of RNA viruses (PKoV, PAstV-1 and PSV) in pig fecal samples was calculated at the individual level and the pig farm level. Positivity rate was also estimated in each subgroup, and a two-sided 95% confidence interval [95% CI] was calculated. Categorical variables were expressed as the number of cases (percentages). Frequencies were

compared using the χ^2 test or Fisher's exact test ($P < 0.05$). A bivariate analysis was carried out to identify the variables that were related to infection with each virus. The multivariate logistic regression analysis included variables that were related to outcome variables in the bivariate analysis with a *P*-value < 0.2 or a possible association. Odds ratios (ORs), including their 95% CIs, were calculated for the logistic regression models. As in Capai *et al.* (2019), samples with no associated age (Herd group) were excluded from the multivariate analysis, and previous results for the detection rate of HEV RNA among pig feces were included in the analysis to estimate a possible association between coinfections with different viruses (Capai et al. 2019). All statistical analyses were performed using the R program (http://www.r-project.org).

Virus genome sequencing

Virus genome sequencing was performed for 26 stool samples as described previously (Stang *et al.*, 2005). A random RT–qPCR was performed using tagged random primers. A ProtoScript® II Reverse Transcriptase kit (New England Biolabs) was used for reverse transcription with random tagged primers, and Platinum® Taq High Fidelity polymerase enzyme (Thermo Fisher Scientific) with specific primers for amplification. After Qubit quantification using Qubit® dsDNA HS Assay Kit and Qubit 2.0 fluorometer (ThermoFisher Scientific), amplicons were fragmented (sonication) into fragments of 200 bp length. Libraries were built by adding barcodes for sample identification, and primers for for amplificationusing the AB Library Builder System (ThermoFisher Scientific). To pool equimolar amounts of the barcoded samples, a quantification by quantitative PCR using an Ion Library TaqMan™ Quantitation Kit (Thermo Fisher Scientific) was performed. An automated Ion Chef instrument (ThermoFisher) was used for emulsion PCR of the pools and loading them on a 520 chip. Sequencing was performed using S5 Ion torrent technology (Thermo Fisher Scientific) following the manufacturer's instructions. Reads were trimmed (reads with quality score < 0.99 and length < 100 bp were removed, and the 30 first and 30 last nucleotides were removed from the reads), and de novo contigs were produced. These contigs were submitted to Blastn to determine the best reference sequences(s). A consensus sequence was obtained after mapping of the reads on the previously determined reference using CLC genomics workbench software 20.0.4 (Qiagen). The de novo contig was compared with the consensus sequence to ensure that the reference sequence did not affect the

182 consensus sequence. Only sequences corresponding to enteric viruses found in pigs were
183 selected for analysis.

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Results

As suggested by Arya *et al.* (2012), we previously calculated the minimum sample size required to achieve the objectives related to the HEV (n = 176 stool specimens) (Capai et al. 2019). Overall, we collected 919 pig feces samples from 16 pig farms selected according to location and breeding system.

For PSV RNA detection, using the SYBR green A range of temperatures around 84°C (83–85°C) was tolerated. Indeed, a one-nucleotide difference within the amplified sequence can impact the melt-curve dissociation temperature (Huang *et al.*, 2011 ; Westerman *et al.*, 2012).

Viral RNA detection rate for the three viruses in feces from domestic pigs and univariate analysis

Our results showed viral RNA detection rates (i) of 62.0% [58.7–65.1] (n = 563/908) for PSV, (ii) of 44.8% [41.5–48.1] (n = 407/908) for PKoV and (iii) of 8.6% [6.8–10.6] (n = 78/908) for PAstV-1 (Table 2).

For PSV and PKoV, there was no statistical association with the type of breeding system; in contrast, PAstV-1 was detected more frequently in C-farms compared with SE- and E-farms (P-value = 9.6e–6) (Table 2).

Significant differences were observed for all three viruses according to age (P-value = 2.4e–13 for PAstV-1; 2.4e–12 for PKoV and 0.005 for PSV) (Table 2). RNA virus detection by age group showed a significant decrease in the rate of positive cases after three months for PAstV-1 (30.6% vs. 6.0%; P-value = 3.87e–7) and between 1 and 3 months (69.4%) and in adults (28.9%) for PKoV (P-value = 6.37e–12). For PSV, the detection rates by age group were between 61.0% and 77.5% (Figure 1). However, the positivity rate among pigs under six months of age was significantly lower than that in pigs older than 6 months (71.4% vs. 61.1%; P-value = 0.014; OR = 1.59, CI 95% 1.09–2.32).

Description of coinfections in samples of pig feces

Table 3 lists all infections and coinfections detected in pig feces samples. Of the 908 samples tested, 697 samples were positive for at least one virus (76.8%). A total of 344 samples contained at least two distinct viral RNA (37.9%), of which 259 specimens (28.5%)

were coinfected by two viruses, 78 specimens (8.6%) were coinfected by three viruses and seven specimens (0.8%) were positive for all four viruses (Table 3).

Detection rate of viral RNA at the farm level

Table 4 shows the detection rates for each farm. At least one pig was detected positive for PKoV RNA in each of the 16 pig farms sampled (100%), 62.5% for PAstV-1 infection (n = 10/16) and 93.8% for PSV infection (n = 15/16). The positivity rate at farm level ranged between 22.5% and 80.8%, 0.0% and 34.0%, and 0.0% and 87.5% for PKoV, PAstV-1 and PSV, respectively.

Multivariate analysis: associated factors identified for each viral infection

A multivariate logistic regression analysis was performed, and the results showed associations depending on the three viruses (Table 5). A strong association was observed between PKoV and PSV detection (OR = 3.36 [2.44–4.67]; *P*-value = 9.1e–15). PKoV was also associated with PAstV-1 codetection (OR = 2.16 [1.29–3.70]; *P*-value = 0.015) and young pigs under 4 months of age (OR = 3.11 [2.31–4.20]; *P*-value = 4.3e–10). PAstV-1 detection was also associated with age, with pigs over 3 months of age significantly less frequently infected than younger pigs (OR = 0.18 [0.11–0.29]; *P*-value = 2.8e–9). PSV infection was not associated with age or type of breeding.

Next-generation sequencing (NGS)

In addition to the three viruses studied by RT–qPCR, 26 stool samples were tested using the NGS method. Overall, consensus sequences corresponding to 10 different species of virus were obtained. PAstVs were detected in ten samples (38.5%), porcine stool/serum-associated circular virus in six (23.1%), Bocavirus in five (19.2%), Sapelovirus and Posavirus in four (15.4%), Circovirus in three (11.5%), Pasivirus in two (7.7%) and Rotavirus and porcine Enterovirus G in one (3.8%). Among these samples, 14 (54%) had consensus sequences corresponding to at least two different viruses.

Porcine astroviruses strains detected among our samples were closed to pig strains from different countries: Hungary (JQ340310 90.81-92% of homology); United States (KJ495987 93.36% of homology) and New Zealand (KJ495990 92.8% of homology). The main astroviruses found, were *Mammastrovirus* 3, *Porcine Astrovirus* 2 and 4. Concerning Bocaviruses strains detected, homology of 97.08% was determined with pigs from: a Hungrian domestic pig (KF206167), 95-97.65% with a Chinese pigs (KX017193; KM402139

and HM053693), 94.13-96.48% with an American pigs (KF025394 and KF025484) and 95.28% with a Croatian pig (KF206161). Concerning, the sequences obtained for the Sapelovirus, the strongest homologies were observed when compared with strain MH513612 also isolated in Corsica (92-99.91% of homology) (Piorkowski *et al.*, 2018).

For the other results, Table 6 summarizes all the sequences obtained, their length, the reference sequence to which each corresponds and the percentage of homology with this sequence.

Discussion

This study investigated the detection rate of three enteric viral infections, PKoV, Astrovirus-1 and Sapelovirus, in 908 pigs of different ages and breeding systems. To our knowledge, this work is the first to study factors associated with presence of viral RNA of PSV, PKoV and PAstV-1 in stools of domestic pigs in France.

RNA detection rate of the three enteric viruses

PSV was the most prevalent (62%) and observed rates are higher than those described in the US, Brazil and India (7-31%) (Donin *et al.*, 2014 ; Chen *et al.*, 2018 ; Ray *et al.*, 2018). One PSV strain (OPY-1-Corsica-2017) had been previously isolated and sequenced (GenBank acc. no. MH513612) (Piorkowski *et al.*, 2018). Phylogenetic analysis shows that OPY-1-Corsica-2017 is closely related to Indian (KY053835) and German (LT900497, NC_003987 and AF406813) strains also from pig feces.

PKoV RNA was detected in almost half of samples, which is similar to data reported from pig farms in China (45.7%) (Yang *et al.*, 2014) and Japan (45.4%) (Khamrin *et al.*, 2010). In five European countries, PKoV infections have been described previously as highly prevalent in both diarrheic and healthy pigs, 54.5% and 58.2%, respectively (Zhou *et al.*, 2016).

PAstV-1 RNA was detected in less than 10% of our samples, but comparison is difficult because other studies were performed at the genus level (*Mamastrovirus*: 52% positive) (Chen *et al.*, 2018) or for all PAstVs combined (Thailand: 6.5%; MN, USA: 62%; Slovakia: 93.2%) (Mor *et al.*, 2012 ; Kumthip *et al.*, 2018 ; Salamunova *et al.*, 2018).

Very early exposure of pigs

The analysis of the detection of viral RNA according to pig age showed that for all viruses tested, pigs < 4 months old were consistently the age group exhibiting the highest rate. This association between age and rate of infection was confirmed for PKoV and PAstV-1 in the multivariate analysis. It may correlate with high exposure after weaning (about 2 months after birth) but also suggest persisting presence of viruses in the breeding environment and loss of maternal immunity. Piglet passive immunity is derived from colostrum and not from breastfeeding (Bourne et Curtis, 1973), with a decrease in immunoglobulins A, G and M after farrowing (Klobasa et Butler, 1987). This trend was also observed during our previous study of HEV) (Capai *et al.*, 2019). Correlation of decreasing positivity rates and increasing age of pigs is described for Kobuviruses in Italy (Di Profio *et al.*, 2013) and East Africa (Amimo *et al.*, 2014). A higher PKoV detection rate in young piglets has also been reported in other studies (Reuter *et al.*, 2009 ; Park *et al.*, 2010 ; Barry *et al.*, 2011). However, the kinetics of infection were different in each study and may depend on the organization of each farm and other environmental characteristics

In traditional Corsican farms, the average slaughter age is higher (12-18 months) compared with industrial farms. Therefore, at the time of slaughtering the majority of *Nustrale* Corsican pigs will have cleared replicative viral infection. The same finding was already reported for hepatitis E virus (Capai *et al.*, 2019). In contrast, the situation is different for Sapeloviruses with rates of replicating infection at 60% in adult pigs. Farming type does not seem to influence the detection rates of studied viruses but still need confirmation from future studies.

A potentially very broad spectrum of viruses in pig feces

In this study, 37.9% of pigs were coinfected with at least two different viruses (including HEV). Strong trends were observed in the multivariate analysis regarding the associations between different viral infections (PKoV/PSV, PKoV/PAstV-1, PSV/HEV and PSV/PKoV). This wide variety of viruses in the feces was confirmed with the NGS method, which showed the presence of other viruses frequently found in pigs: Posavirus, other Astroviruses, Bocavirus, Enterovirus G, Circovirus, Pasivirus and Rotavirus. Coinfections were also evidenced in the NGS results, with more than half of the samples containing consensus sequences corresponding to at least two different viruses. These results are in line with previous studies reporting multiple coinfections of farmed pigs with porcine enteric

305 viruses (Xiao *et al.*, 2013 ; Zhang *et al.*, 2014), especially in pigs with diarrhea (Shan *et al.*,
306 2011b ; Sachsenroder *et al.*, 2012 ; Zhang *et al.*, 2013).

307 Our study has several limitations. First, it was not initially designed to study these
308 three viruses but to determine the prevalence of the HEV in pig farms in Corsica. Differences
309 in detection could exist depending on criteria such as sampling time and place, age of pigs and
310 clinical background of the tested animal population (diarrheic or healthy pigs). The lack of
311 collection of clinical information (diarrhea or other symptoms) for the pigs included may have
312 led to a bias in the analysis of the data; these data could have been essential for the
313 improvement of knowledge concerning the studied viruses. The number of viruses studied
314 could also have been larger to better assess the presence of major enteric viruses in Corsican
315 pig farms. Finally, the lack of phylogenetic analysis of the different strains found is also a
316 major limitation of our study. Indeed, the sequences obtained being in very varied zones of
317 the genome of the various viruses, the phylogenetic analyses could not be realized.

318 In future studies, information about possible symptoms in pigs should be collected. It
319 would also be useful to evaluate the phylogeny of the different strains found and to set up an
320 RT–qPCR primer system to distinguish the different strains of Sapelovirus. Moreover, in view
321 of the large variety of viruses present in the pig feces and the availability of microfluidic PCR
322 technology, it would be helpful to set up microarrays that can detect all the principal known
323 porcine enteric viruses. Moreover, the real impact of Kobuvirus, Astroviruses, Sapelovirus
324 and other enteric viruses on animal health and breeding systems remains largely unknown and
325 needs further epidemiological studies.

326 In conclusion, this study provides first insight on the presence of three common
327 porcine enteric viruses in France and showed that they are frequently encountered in Corsica
328 in pig farms using the traditional extensive breeding. The three viruses studied were found on
329 almost all the farms, indicating widespread distribution. Moreover, the pigs tested were born
330 and bred in Corsica, which demonstrates endemic local circulation. Whether such infections
331 and co-infections can affect the productivity, impact the growth of pigs or cause immune
332 weakness remains to be established. So far, this study has to be considered as a first step in
333 the study of enteric viruses in Corsican pig farms.

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Table 1: RT–qPCR detection assays used in the study

Viruses	Name of primers and probes	Sequences	References
PSV	FW: SYBR-PSV1 primer REV: SYBR-PSV2 primer	GGCAGTAGCGTGGCGAGC CTACTCTCCTGTAACCAGT	(Kim *et al.*, 2016)
PKoV	FW: T-248-F-PKoV REV: T-249-R-PKoV Probe: T-250-PKoV-FAM	TCTCTGACCTCTGAAGTGCACT TGAAGAAGCCATGTGTCTTGTC GGTTGCGTGGCTGGGAATCCAC	(Zhou *et al.*, 2016)
PAstV-1	FW: T217-F-PAstV-1 REV: T218-R-PAstV-1 Probe: T-219-PAstV-1-VIC	CCAAAACCAGCAATCCGTCAA GCCCCTAAAGCAACGATCGG TTCTTGTCAAGGATAATACGGGG	

Table 2: Viral RNA in stools of domestic pigs stratified by breeding system and age

Factor	Condition	Number of samples		positive (n)	PKoV RNA detection (%)		*P*-value	positive (n)	PAstV-1 RNA detection (%)		*P*-value	positive (n)	PSV RNA detection (%)		*P*-value
		N	%		%	[95% CI]			%	[95% CI]			%	[95% CI]	
Breeding systems	E-farm	310	34.1	136	43.9	[38.3–49.6]		17	5.5	[38.3–49.6]		186	60.0	[54.3–65.5]	
	SE-farm	396	43.6	180	45.5	[40.5–50.5]	0.91	27	6.8	[4.5–9.8]	**9.6e–6**	233	58.8	[53.8–63.7]	**0.0094**
	C-farm	201	22.1	91	45.3	[38.2–52.4]		34	16.9	[12–22.8]		143	71.1	[64.3–77.3]	
Age	1–3 months	111	12.2	77	69.4	[59.9–77.7]		34	30.6	[22.2–40.1]		86	77.5	[68.6–84.9]	
	3–4 months	143	15.7	84	58.7	[50.2–66.9]		9	6.3	[2.9–11.6]		91	63.6	[55.2–71.5]	
	4–6 months	162	17.8	71	43.8	[36.0–51.8]	**2.4e–12**	4	2.5	[0.7–6.2]	**2.4e–13**	120	74.1	[66.6–80.6]	**0.005**
	Adults (>6 months)*	190	20.9	54	28.4	[22.1–35.4]		17	8.9	[5.3–13.9]		116	61.1	[53.7–68.0]	
	Herds (age mixed)	302	33.3	121	40.1	[34.5–45.8]		14	4.6	[2.6–7.7]		150	49.7	[43.9–55.4]	
All pigs		**908**	**100.0**	**407**	**44.8**	**[41.5–48.1]**		**78**	**8.6**	**[6.8–10.6]**		**563**	**62.0**	**[58.7–65.1]**	

*6–8 months old (n = 47); Sow/Boar (n = 30); at least 6 months old (n = 108)

E-farm: Extensive farm; SE-farm: semi-extensive farm; C-farm: closed farm; Herds: pigs without associated age

Table 3: Coinfections in pig feces samples

	N						
No infection	211	**Number for each coinfection**					
Single infection	353	**PKoV**	**PSV**	**PAstV-1**	**HEV**		
		102	229	9	13		
Double infection	259	**PKoV/PSV**	**PKoV/PAstV-1**	**PKoV/HEV**	**PSV/HEV**	**PAstV-1/PSV**	**PAstV-1/HEV**
		216	4	3	21	14	1
Triple infection	78	**PKoV/PAstV-1/PSV**	**PKoV/HEV/PSV**	**PKoV/HEV/PAstV-1**	**HEV/PAstV-1/PSV**		
		39	35	2	2		
All four viruses	7						

Table 4: Positivity rates for the three viruses by pig farm

No. of pig farms	PKoV		PAstV-1		PSV		Total
	N	%	N	%	N	%	
1	27	65.9	0	0.0	13	31.7	41
2	28	27.2	13	12.6	70	68.0	103
3	25	29.4	3	3.5	56	65.9	85
4	61	48.8	3	2.4	88	70.4	125
5	25	56.8	11	25.0	38	86.4	44
6	9	45.0	1	5.0	9	45.0	20
7	20	22.5	1	1.1	62	69.7	89
8	10	27.8	0	0.0	21	58.3	36
9	37	59.7	4	6.4	41	66.1	62
10	25	41.0	9	14.8	37	60.7	61
11	13	32.5	8	20.0	17	42.5	40
12	59	80.8	25	34.2	65	89.0	73
13	24	55.8	0	0.0	0	0.0	43
14	32	71.1	0	0.0	16	35.6	45
15	9	36.0	0	0.0	16	64.0	25
16	4	25.0	0	0.0	14	87.5	16

Table 5: Statistical analysis of factors associated with each virus (multivariate logistic regression model with random effect at the farm level).

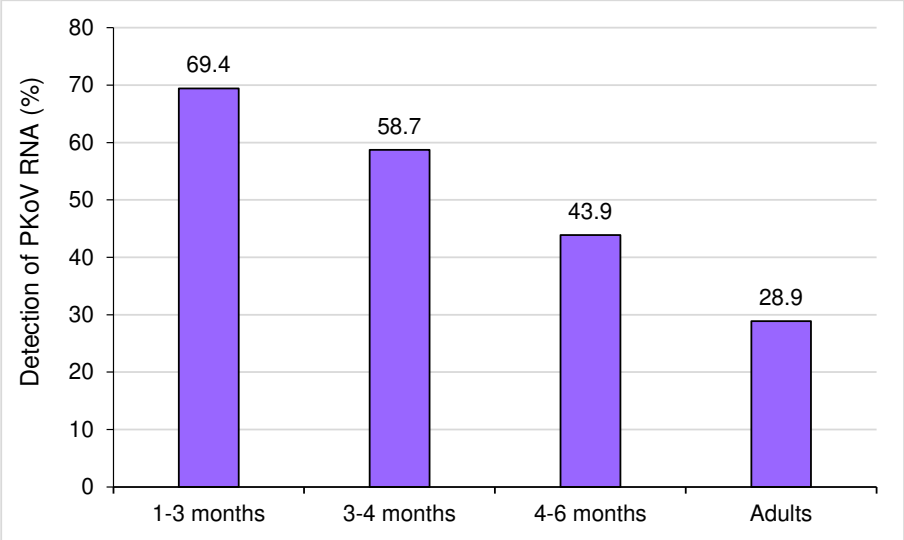
Viruses	Factor	Condition	OR	[95% CI]	P-value adjusted
PKoV	**Coinfection**	PSV	3.36	[2.44–4.67]	9.1e–15
	Coinfection	PAstV-1	2.16	[1.29–3.70]	0.015
	Age group	0–4 months	3.11	[2.31–4.20]	4.3e–10
PAstV-1	**Coinfection**	PKoV	2.25	[1.32–3.90]	0.014
	Age group	>3 months	0.18	[0.11–0.29]	2.8e–9
PSV	**Coinfection**	HEV	2.01	[1.17–3.64]	0.041
	Coinfection	PKoV	2.92	[2.13–4.04]	3.7e–8

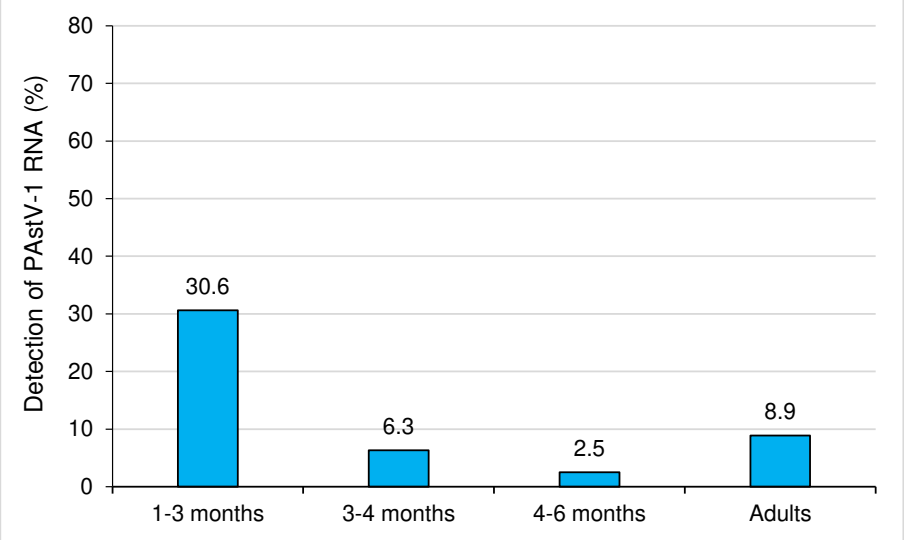
Only factors with *P*-values < 0.05 are included

Table 6:

Samples	Viruses	Sequence of reference	Accession number	Consensus length	% Cover	% Identity
1	/	/	/	/	/	/
2	Sapelovirus	Sapelovirus A strain OPY-1-Corsica-2017 polyprotein gene, complete cds	MH513612	5,927	96	92.46
	Bocavirus	Porcine bocavirus strain G85-1AT-HU, complete genome	KF206167	3,507	100	97.08
	Bocavirus	Porcine bocavirus isolate GD11, complete genome	KM402139	3,507	100	91.82
	Bocavirus	Porcine bocavirus 3C isolate pig/ZJD/China/2006, complete genome	JN681175	3,683	99	91.61
	Porcine Astrovirus	Mamastrovirus 3 isolate PAstV_GER_L00855-K14_14-04_2014 genome assembly, complete genome: monopartite	LT898434	1,508	52	90.88
3	Sapelovirus	Sapelovirus A strain OPY-1-Corsica-2017 polyprotein gene, complete cds	MH513612	492	100	93.09
4	Bocavirus	Porcine bocavirus strain CH/HNZM, complete genome	KX017193	2,105	99	97.22
	Porcine Astrovirus	Astrovirus wild boar/WBAstV-1/2011/HUN, complete genome	JQ340310	428	100	91.59
	Porcine serum-associated circular virus	Porcine serum-associated circular virus isolate BR3, complete genome	KU203353	531	86	86.33
	Enterovirus G	Enterovirus G isolate GER/F9-6/12-02-2013 polyprotein gene, partial cds	MF113376	956	100	86.72
5	Sapelovirus	Porcine Sapelovirus isolate PSV_P1-3-3_Contig(g12h12) polyprotein gene, partial cds	KF705647	498	98	92.77
	Sapelovirus	Sapelovirus A strain OPY-1-Corsica-2017 polyprotein gene, complete cds	MH513612	1,687	100	92.12
	Sapelovirus	Sapelovirus A isolate HuN21 polyprotein gene, complete cds	MF440649	1,230	100	86.59
	Sapelovirus	Sapelovirus A isolate PSV_GER_L00798-K11_14-02_2014 genome assembly, complete genome: monopartite	LT900497	1,894	100	87.08
	Bocavirus	Porcine bocavirus strain 644-1DI-HR, complete genome	KF206161	4,580	100	95.28
	Bocavirus	Porcine bocavirus isolate GD11, complete genome	KM402139	4,580	100	95.09
	Porcine Astrovirus	Porcine astrovirus 2 clone KDC-6 ORF1ab gene, partial cds; and ORF2 gene, complete cds	KJ495987	466	100	93.36
	Porcine Astrovirus	Porcine astrovirus 2 genes for ORF1ab, ORF1a, ORF2, complete cds, strain: PoAstV2/JPN/HgYa2-3/2015	LC201588	422	100	87
	Porcine Astrovirus	Astrovirus wild boar/WBAstV-1/2011/HUN, complete genome	JQ340310	533	100	90.81
	Porcine stool-associated circular virus	Porcine stool-associated circular virus 7 isolate EP2-B, complete genome	KJ577813	1,191	99	82.59
6	Posavirus	Posavirus 1 isolate PsaV_GER_L01017-K01_15-07_2015 genome assembly, complete genome: monopartite	LT898419	741	100	96.63
	Posavirus	Posavirus sp. isolate 12144_61, complete genome	KX673217	559	100	97.5
	Bocavirus	Porcine bocavirus 3 isolate IA159-3 NS1 and NP1 genes, complete cds; and VP1/VP2 gene, partial cds	KF025387	491	100	84.85
	Porcine serum-associated circular virus	Porcine serum-associated circular virus isolate BR3, complete genome	KU203353	929	99	86.03
7	/	/	/	/	/	/
8	/	/	/	/	/	/
9	Circovirus	Circovirus sp. isolate PoCirV_VIRES_JL01_C5 capsid protein gene, partial cds	MK377643	1,038	100	85.17
	Circovirus	Circovirus sp. isolate PoCirV_VIRES_GX05_C4 replicase gene, partial cds	MK377558	639	64	89.54
	Porcine Astrovirus	Porcine astrovirus 2 genes for ORF1ab, ORF1a, ORF2, complete cds, strain: PoAstV2/JPN/Ishi-Ya4/2015	LC201589	879	78	82.61
	Porcine Astrovirus	Porcine astrovirus 4 genes for ORF1ab, ORF1a, ORF2, complete cds, strain: PoAstV4/JPN/Bu5-10-2/2014	LC201603	989	100	83.64
10	Bocavirus	Porcine bocavirus 1 pig/ZJD/China/2006, complete genome	HM053693	383	100	97.65
11	Porcine Astrovirus	Porcine astrovirus 4 isolate 15-12, complete genome	KU764486	1,624	99	87.98
	Porcine Astrovirus	Porcine astrovirus 4 isolate 15-13, complete genome	KU764484	1,624	99	88.21
	Porcine stool-associated circular virus	Porcine stool-associated circular virus 7 isolate EP3-C, complete genome	KJ577814	719	100	86.77
12	Posavirus	Posavirus sp. isolate 17668_12, complete genome	KX673279	1,606	93	85.53
	Posavirus	Posavirus sp. isolate 17668_13_2, complete genome	KX673281	1,546	100	85.52
13	Porcine Astrovirus	Porcine astrovirus 4 strain JXJA, complete genome	KX060808	1,187	100	89.9

	Bocavirus	Porcine bocavirus 3 isolate IL330 NS1 and NP1 genes, complete cds; and VP1/VP2 gene, partial cds	KF025394	483	100	96.48
14	Porcine Astrovirus	Porcine astrovirus 4 strain 35/USA, complete genome	JF713713	1,798	100	90.12
	Porcine Astrovirus	Porcine astrovirus 4 strain CH/JXZS/2014, complete genome	KX060809	1813	100	90.13
15	Porcine Astrovirus	Mamastrovirus 3 isolate PoAstV_VIRES_HeB02_C4 ORF1ab and ORF1a genes, partial cds	MK378508	2,192	100	90.81
	Circovirus	Circovirus sp. isolate PoCirV_VIRES_SD02_C2 replicase gene, partial cds	MK377699	557	100	88.6
16	Circovirus	Circovirus sp. isolate PoCirV_VIRES_HeB04_C3 capsid protein gene, partial cds	MK377607	777	100	90.27
17	Porcine Astrovirus	Porcine astrovirus 4 genes for ORF1ab, ORF1a, ORF2, complete cds, strain: PoAstV/JPN/MoI2-1-2/2015	LC201610	869	100	89.31
	Porcine Astrovirus	Porcine astrovirus 2 clone NZP-93_Subtype_2 ORF1ab gene, partial cds	KJ495990	375	100	92.8
	Bocavirus	Porcine bocavirus 3 isolate IA13-1 VP1/VP2 gene, complete cds	KF025484	931	100	94.13
	Porcine Astrovirus	Mamastrovirus 2 isolate U083, complete genome	KY940077	2,043	100	80.33
	Porcine Astrovirus	Mamastrovirus 3 isolate PoAstV_VIRES_GZ04_C10 ORF1ab and ORF1a genes, partial cds	MK378502	869	100	89.77
18	Porcine Astrovirus	Porcine astrovirus 4 genes for ORF1ab, ORF1a, ORF2, complete cds, strain: PoAstV4/JPN/Ishi-Ya7-1/2015	LC201613	1,970	91	89.22
	Porcine Astrovirus	Mamastrovirus 2 isolate U083, complete genome	KY940077	5,230	85	87.06
	Porcine Astrovirus	Mamastrovirus 3 isolate PAstV_GER_L00855-K14_14-04_2014 genome assembly, complete genome: monopartite	LT898434	4,050	100	85.87
19	/	/	/	/	/	/
20	Sapelovirus	Sapelovirus A strain OPY-1-Corsica-2017 polyprotein gene, complete cds	MH513612	2,360	100	99.91
	Pasivirus	Swine pasivirus SPaV1/US/17-50816IA60467-1/2001 polyprotein gene, complete cds	MG674090	1,186	100	77.88
	Porcine stool-associated circular virus	Porcine serum-associated circular virus isolate BR2, complete genome	KU203352	1,168	99	92.76
21	/	/	/	/	/	/
22	Rotavirus	Porcine rotavirus B isolate PoRVB_VP2_VIRES_NM01_C2 VP2 gene, partial cds	MK379346	55	100	98.18
23	/	/	/	/	/	/
24	Posavirus	Posavirus sp. isolate 17668_12, complete genome	KX673279	3,003	100	88.71
	Pasivirus	Pasivirus A isolate SPaV-A GER L01061-K07 15-03 2015 genome assembly, complete genome: monopartite	LT898422	451	100	83.44
	Porcine stool-associated circular virus	Porcine stool-associated circular virus 7 isolate EP3-C, complete genome	KJ577814	702	100	83.25
25	/	/	/	/	/	/
26	Posavirus	Posavirus sp. isolate 17668_13_2, complete genome	KX673281	1,282	99	89.31
	Posavirus	Posavirus 3 strain 10611 polyprotein gene, complete cds	KT833079	860	100	94.07
	Posavirus	Posavirus strain 7048 polyprotein gene, partial cds	KT833076	1.121	99	87.92





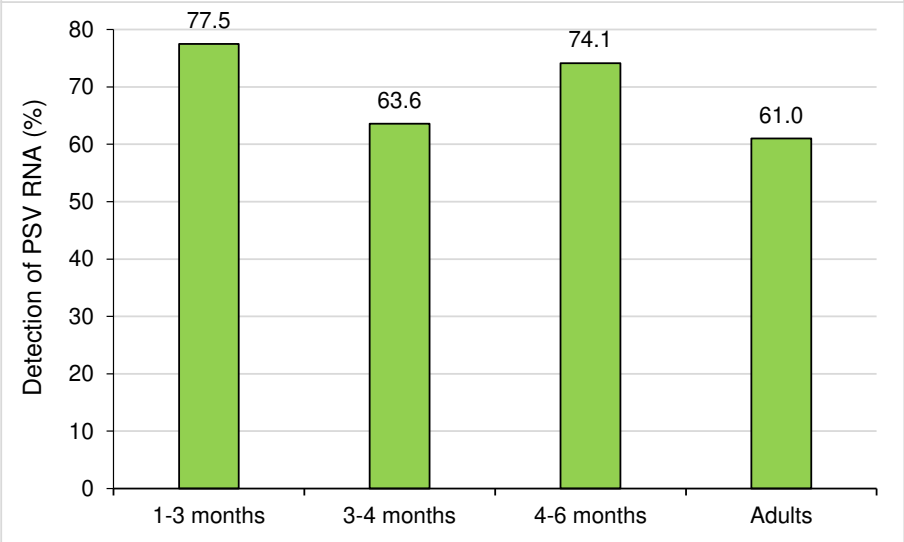


Figure 1 Detection rate of each RNA virus by age group