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Molecular investigations on the prevalence and viral load of enteric viruses in pigs from five European countries

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ABSTRACT

Enteric viral infections in pigs may cause diarrhea resulting in ill-thrift and substantial economic losses. This study reports the enteric infections with porcine astrovirus type 4 (PAstV4), porcine group A rotavirus (GARV), porcine group C rotavirus (GCRV), porcine circovirus type 2 (PCV2) and porcine kobuvirus (PKoV) in 419 pigs, comprising both healthy and diarrheic animals, from 49 farms in five European countries (Austria, Germany, Hungary, Spain and Sweden). Real-time RT-PCR assays were developed to test fecal samples and to compare the prevalence and viral load in relation to health status, farms of origin and age groups. The results showed that PAstV4 (70.4%) was the dominant virus species, followed by PKoV (56.7%), PCV2 (42.2%), GCRV (3%) and GARV (0.9%). Diarrheic pigs had a higher viral load of PAstV4 in the nursery and growing-finishing groups. Rotaviruses were mainly detected in diarrheic pigs, whereas PCV2 was more often detected in clinically healthy than in diarrheic pigs, suggesting that most PCV2 infections were subclinical. PAstV4, PCV2 and PKoV were considered ubiquitous in the European pig livestock and co-infections among them were frequent, independently of the disease status, in contrast to a low prevalence of classical rotavirus infections.

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1. Introduction

Pigs are an important source for food supply worldwide. Maintaining pig health and improving feed conversion rate are crucial to meet the increasing demand of food for a fast growing human population in the world. Certain highly contagious, transboundary diseases such as classical swine fever have been controlled and even eradicated from most European countries by different strategies including strict stamping-out policy, vaccination, and improved diagnosis and biosafety, resulting in a better health status of pigs in these countries. By contrast, endemic diseases are still present in spite of being managed by veterinarians and farmers. Among these pathological conditions, diarrhea in young pigs causes substantial economic losses due to increased mortality or morbidity with decreased growth and prolonged time for reaching market weight.

Pig diarrhea is a multi-factorial problem, also interpreted as a disease complex. Besides bacteria such as enterotoxigenic *Escherichia coli* (ETEC) and *Clostridium perfringens* types A and C, a number of viruses have been reported either causing or triggering the disease. Rotaviruses are one of the major classical causes of

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diarrhea in piglets (neonatal, nursery and post-weaning) as well as in many other species including humans. Rotaviruses are non-enveloped, double-stranded RNA viruses with a genome comprising 11 segments, and are divided into seven (A–G) antigenically distinct serogroups based on the serological characteristics of the membrane protein VP6. Serogroups A and C are the most frequent ones causing diarrhea in piglets. Group A rotavirus (GARV) is detected most frequently in pigs younger than 60 days of age (Chang et al., 2012), with the highest prevalence at 3–5 weeks of age (Bohl, 1979). By contrast, group C rotavirus (GCRV) is becoming dominant in diarrheic suckling piglets (less than 20 days of age) in the US (Marthaler et al., 2014). Rotaviruses are transmitted primarily via fecal-oral route and viral infection is widespread in swine herds. The widely accepted mechanism of rotavirus-induced diarrhea in pigs and humans is related to villous atrophy with loss of intestinal absorptive cells (Chang et al., 2012), which leads to malabsorption (Kapikian, 2001).

Several "emerging" viruses such as astroviruses and kobuvirus have been detected more often in diarrheic pigs as well as in healthy pigs (Khamrin et al., 2010; Park et al., 2010; Dufkova et al., 2013). Porcine astrovirus (PAstV) belongs to the genus *Mamastrovirus* of the family *Astroviridae*. Astroviruses are non-enveloped and contain a single-stranded RNA genome. The viruses infect a broad range of hosts such as humans, cattle, sheep/goats, pigs, mink and bats, and are phylogenetically grouped into different lineages. Porcine astroviruses can be divided into five types 1–5 (PAstV1–PAstV5) (Indik et al., 2006; Laurin et al., 2011; Luo et al., 2011; Shan et al., 2011). All five types of astroviruses have been reported in Europe (Brnić et al., 2013, 2014; Machnowska et al., 2014; Monini et al., 2015; Reuter et al., 2011). As astroviruses are found both in diarrheic and in healthy pigs, their role as a causative agent of pig diarrhea has not been unequivocally established. Similarly, a causal role of porcine kobuvirus (PKoV), a small picornavirus with a single-stranded RNA genome, in disease has yet to be defined despite a high prevalence of infection in diarrheic as well as in healthy pigs (Khamrin et al., 2009; Dufkova et al., 2013).

Porcine circovirus type 2 (PCV2) is a single-stranded DNA virus belonging to the genus *Circovirus* in the family *Circoviridae* (Allan and Ellis, 2000; Segalés et al., 2005). There are two major genotypes (2a, 2b): PCV2b has replaced PCV2a and became predominant in swine farms (Segalés et al., 2013). In addition, a third genotype, PCV2c, is found in Danish archived samples (Dupont et al., 2008; Segalés et al., 2008). PCV2 infection is associated with several diseases or disease clusters with diarrhea as one of the clinical signs. Moreover, PCV2 modulates the immune response (Kekarainen et al., 2008); therefore, co-infection with PCV2 may contribute to severe clinical diseases.

The prevalence of rotaviral and emerging viral enteric infections in most European pig herds is poorly documented. Therefore, the objective of this study was to provide data on the prevalence of PAstV4, GARV and GCRV, PCV2 and PKoV infections in both clinically healthy and diarrheic pigs from farms in Austria, Germany, Hungary, Spain and Sweden.

2. Materials and methods

2.1. Sample collection

A total of 419 fecal samples from 49 pig farms were collected by veterinarians in five European countries during 2010–2013. The number of samples from each country was 136 (Austria), 44 (Germany), 50 (Hungary), 83 (Spain), and 106 (Sweden). These samples originated from pigs at different ages: suckling (0–5 weeks), nursery (6–10 weeks), growing-finishing (11–18 weeks) and unknown aged pigs. With the exception of the German samples, which included only diarrheic feces, both healthy and diarrheic pigs were sampled in each country. The samples were transported in cool containers to the laboratory and stored in a −20 °C freezer until analyzed. Specificity of the assays was evaluated by testing ten swine virus isolates, namely, EU and NA strains of porcine reproductive and respiratory syndrome virus (PRRSV), two strains of transmissible gastroenteritis coronavirus (TGEV), porcine epidemic diarrhea virus (PEDV), porcine parvovirus (PPV), GARV CN86 strain, PCV2, border disease virus 137/4 and classical swine fever virus (CSFV).

2.2. Nucleic acid extraction

Approximately 0.2 g of feces were resuspended in 500 μl of water containing 10^6 pfu of bacteriophage MS2 as extrinsic control. The mixture was centrifuged at 4000 × *g* for 20 min and 350 μl of

Table 1
Primers and probes designed for real-time PCR assays in this study.

Target virus and region	Primer or probe name	Sequence (5′–3′)[a]	5′end position (accession no.)	Reference
GRAV (VP6)	T207-F	GGAGGTTCTGTAYTCATTGTCAAAA	26 (X94617)	Logan et al., 2006
	T234-R	CCTATTCCTCCTGTTTGAAAATCAT	178 (U10031)	This study
	T223-ROX	AAT(+C)AAA(+T)GATAG(+T)CAC(+T)ATGA	120 (U10031)	This study
GCRV (VP6)	T148-F	CCGTGAAGAGAATGGTGATGTAGA	1187 (M94157)	Chun et al., 2010
	T243-R	CATAGTTCACATTTCATCCTCCTG	1348 (M94157)	This study
	T150-Quasar670	AACCAATCTCTATGTGGACTACATACCA	1225 (M94157)	Chun et al., 2010
PAstV1 (capsid)	T217-F	CCAAAACCAGCAATCCGTCAA	260 (Y15938)	This study
	T218-R	GCCCCTAAAGCAACGATCGG	420 (Y15938)	This study
	T219-Quasar705	TTCTTGTCAAGGATAATACGGGG	363 (Y15938)	This study
PAstV4 (RdRp)	T220-F	ACAGCGCTGCATGGGAAACTC	863 (GU562296)	This study
	T221-R	AGGCTTATGCTTTGGTCCGC	1045 (GU562296)	This study
	T222-FAM	AGGCAGATGGACAGGCTTTGGAG	1001 (GU562296)	This study
PKoV (5′UTR)	T248-F	TCTCTGACCTCTGAAGTGCACT	462 (JX401523.1)	This study
	T249-R	TGAAGAAGCCATGTGTCTTGTC	589 (JX401523.1)	This study
	T250-FAM	GGTTGCGTGGCTGGGAATCCAC	486 (JX401523.1)	This study
PCV2 (Rep)	T176-F	GGCCACCTGGGTGTGGTAAA	571 (JQ002672)	Gagnon et al., 2010
	T177-R	CCCACCACTTGTTTCTAGGTGGTT	660 (JQ002672)	Gagnon et al., 2010
	T178–FAM	TTTGCAGACCCGGAAACCACATACTGGA	609 (JQ002672)	Gagnon et al., 2010
MS2	T210-F	TGGCACTACCCCTCTCCGTATTCAC	289 (NC.001417)	Liu et al., 2011a
	T211-R	GTACGGGCGACCCCACGATGAC	387 (NC.001417)	Liu et al., 2011a
	T212-TET	CACATCGATAGATCAAGGTGCC	330 (NC.001417)	Liu et al., 2011a

Note: (+C) or (+T) indicates a locked nucleic acid (LNA).

the supernatant were used for nucleic acid extraction by Qiagen RNeasy Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

2.3. Development of the real-time RT-PCR assays and detection of viral infections

In order to detect enteric viral pathogens and quantify the corresponding viral load, one multiplex and two single-plex real-time RT-PCR assays were developed in this study. Primers and probes (Table 1) were designed based on the conserved regions of viral genomes. Path-ID™ Multiplex One-Step RT-PCR Kit (Applied Biosystems) was used in the multiplex assay for the detection of PAstV4, GARV and GCRV, as well as PAstV1 and MS2. The assay was carried out in a 25-μl reaction containing 12.5 μl of 2× buffer, 1 μl of primer mix (10 μM each virus specific primer and 1 μM MS2 primers), 0.5 μl of 10 μM probe mix, 2.5 μl of 10× Multiplex Enzyme Mix, 2 μl of extracted RNA, and 6.5 μl of water. The assay was performed in CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories), with the following cycling steps: 45 °C for 10 min; 95 °C for 10 min, 48 cycles of 95 °C for 15 s and 60 °C for 45 s. The quantification cycle (Cq) values were determined automatically by the software with manual adjustment. The values are defined as the number of cycles at which the fluorescence exceeded the threshold. The analytic sensitivity of the multiplex assay was determined by testing the 10-fold dilution series of the RNA standards. The two single-plex real-time PCR assays for detection of PCV2 and PKoV, respectively, employed AgPath-ID™ One-Step RT-PCR Reagents (Applied Biosystems). The 25-μl reaction contained 1 μl of the enzyme, 12.5 μl of 2 × buffer, 1 μl of 10 μM each primer, 0.5 μl of 10 μM probe, 2 μl of viral nucleic acids and 8 μl of water. The cycling steps were the same as in the multiplex assay.

2.4. Preparation of the RNA standards

Four sets of RNA standards were *in vitro* generated for PAstV1, PAstV4, GARV and GCRV, respectively. The procedures for preparation of the RNA standards were described previously (Liu et al., 2008). Briefly, the four target regions were amplified by RT-PCR, and cloned into a pCR™4-TOPO® Vector (Life technologies). RNA copies of the four targets were *in vitro* transcribed from the linearized plasmids, purified after DNase digestion of plasmid DNA, and quantified spectrophotometrically using a NanoDrop 1000 (Thermo Scientific). The standards were 10-fold diluted in water containing carrier RNA, and were used to evaluate the analytic sensitivity of the multiplex assay.

2.5. Statistical analysis

Statistical analyses were performed with SPSS for Windows 17.0 (SPSS, Chicago, IL). For comparison of the viral load in different age groups (suckling, nursery and growing-finishing), the ANOVA test was used. For comparison of the viral load between healthy and diarrheic groups, the Mann–Whitney (Wilcoxon) two-sample test was used. Differences in the proportion of viral infections between healthy and diarrheic pigs were tested in R version 3.0.1.

3. Results

3.1. Performance of the real-time RT-PCR assays

In order to have a powerful tool for simultaneous detection of the astroviruses and rotaviruses, different primers/probes for each target were extensively evaluated for their ability to be multiplexed without compromising sensitivity. The four sets of primers and probes (Table 1) were finally selected for the multiplex assay, which was further evaluated for its sensitivity and specificity for detecting the four target porcine viruses. The analytical sensitivity of the multiplex assay was determined by testing the 10-fold dilution serials of the RNA standards. The assay was found to be able to detect 10-100 genome copies per reaction, showing a high sensitivity. The assay had a good repeatability and linearity in three independent runs. When tested with a limited number of known positive samples, the four dually labeled probes hybridized specifically to their target swine virus species/genotypes, and no cross-reactivity was observed. The MS2 assay did not perform well likely due to a low primer concentration and therefore, could not be used as an extrinsic control as initially planned. The two TaqMan real-time PCR assays for the detection of PCV2 and kobuvirus were evaluated in a less extent in terms of analytical sensitivity. When evaluated with the ten swine virus isolates, the three assays were found highly specific. In a subsequent metagenomic investigation of 12 Spanish samples, PAstV4 was confirmed in all these positive samples whereas PAstV1 was found in one of the seven samples that were tested PAstV1 positive by the multiplex assay. The results suggested, despite carefully designing of primers and probe for PAstV1, the multiplex assay was still lacking specificity for accurate detection of PAstV1. Therefore, PAstV1 data were not included in this report. A total of 15 astrovirus sequences, together with seven

Table 2
Prevalence of enteric viral infections in both healthy and diarrheic stool samples from five EU countries. The number of positive samples is given followed by percentage in parentheses.

Country	Status	No. of samples (farms)	PAstV4	GARV	GCRV	PCV2[a]	PKoV[a]
Austria	Healthy	87 (1)	87 (100%)	0	1 (1%)	83 (95.4%)	5/10 (50%)
	Diarrhea	49 (1)	49 (100%)	0	0	39 (79.6%)**	7/16 (43.8%)
Germany	Healthy	0	–	–	–	–	–
	Diarrhea	44 (3)	17 (38.6%)	0	6 (13.6%)	5 (11.4%)	24 (54.5%)
Hungary	Healthy	13 (1)	13 (100%)	0	0	0	6/11 (54.5%)
	Diarrhea	37 (4)	29 (78.4%)	4 (10.8%)	0	2 (5.4%)	24/26 (92.3%)*
Spain	Healthy	40 (8)	40 (100%)	0	0	28 (70%)	19 (47.5%)
	Diarrhea	43 (18)	43 (100%)	0	6 (13.95%)	16 (37%)**	32 (74.4%)*
Sweden	Healthy	63 (8)	7 (11.1%)	0	0	3/62 (4.8%)	31/51 (60.8%)
	Diarrhea	43 (6)	10 (23.3%)	0	0	0/42	5/29 (17.4%)**
Summary	Healthy	203	72.4%	0	0.5%	56.4%	54.5% (61/112)
	Diarrhea[b]	216	68.5%	1.8%	6.0%	28.8%	58.2% (92/158)
Total	–	419	70.4%	0.9%	3%	42.2% (176/417)	56.7% (153/270)

[a] When partial samples were tested, the numbers of positive and the tested are given as "positive/tested" followed by percentage in parentheses.
[b] After excluding the German diarrheic samples, the summary prevalence in diarrheic samples is 76.1% (PAstV4), 2.3% (GARV), 3.5% (GCRV), 33.3% (PCV2), and 57.1% (PKoV).
* Indicates significant difference ($p < 0.05$) in the proportion test.
** Indicates significant difference at $p < 0.01$ in the proportion test.

kobuvirus sequences, generated in the meatgenomic investigation have been deposited in GenBank with accession numbers (KT892953- KT892974).

3.2. Prevalence of swine enteric viruses in pig farms from five European countries

The overall prevalence of the enteric viral infections is shown at the bottom line of Table 2. It was 70.4% for PAstV4, 0.9% for GARV, 3% for GCRV, 42.2% for PCV2 and 56.7% for PKoV. The results showed that PAstV4 was the dominant virus in the investigated European farms, but prevalence varied depending on the farm of origin. The prevalence of PAstV4 was 100% in pigs from the Austrian and Spanish farms ($n = 219$) regardless of health status. In the Hungarian farms, PAstV4 was demonstrated in each healthy pig ($n = 13$) and in 29 out of 37 diarrheic pigs (78.4%). PAstV4 was found in 38.6% (17 out of 44) diarrheic pigs in the German farms. The lowest incidence (16%) was recorded in the Swedish farms.

Out of 37 Hungarian diarrheic samples, GARV was detected in 4 (10.8%) pigs from one farm, but not in the other countries (Table 2). GCRV was found in 6 out of 44 (13.6%) 4–5 day-old diarrheic German pigs (one farm) and in 6 out of 43 (13.9%) pigs with diarrhea in Spain, as well as in one healthy Austrian pig. GCRV was not detected in Hungarian and Swedish pigs.

PCV2 was demonstrated by PCR in 114 out of 202 (56.4%) healthy pigs and in 62 out of 215 (28.8%) diarrheic pigs (Table 2). The Austrian farm had the highest infection rate, 95% (83 out of 87) of the healthy pigs and 80% (39 out of 49) of the diarrheic pigs were positive for PCV2. In the Spanish farms, the prevalence of PCV2 positive healthy pigs (70%) was almost twice as high as in diarrheic pigs (37%). The PCV2-positive pigs were lower in the farms from Germany (11%), Hungary (4%) and Sweden (3%).

The prevalence of PKoV ranged from 46% (12 out of 26) in the Austrian farm to 81% (30 out 37) in the Hungarian farms (Table 2). Overall, PKoV was demonstrated in more than 50% of the pigs (153 out of 270). Chi-square test revealed, that a higher prevalence of PKoV infection was significantly ($p < 0.05$) associated with diarrhea in the Hungarian and Spanish farms. In contrast, PKoV was more commonly demonstrated in healthy than in diarrheic pigs in the Swedish farms ($p < 0.05$).

With exception of the German samples, this study investigated enteric viral infections in both clinically healthy and diarrheic pigs. By excluding the German diarrhea-only data, the overall prevalence of infections in diarrheic pigs was 76.1% (PAstV4), 2.3% (GARV), 3.5% (GCRV), 33.3% (PCV2) and 57.1% (PKoV), which, except for PCV2 infection, was slightly higher than in healthy pigs (Table 2).

3.3. Viral load distribution between healthy and diarrheic pigs

The real-time RT-PCR assays allowed quantifying the viral load besides detecting the presence of viral infections. By using the prepared RNA standards, the astrovirus and rotavirus genome copy numbers per microliter of nucleic acids extracted from the samples were calculated (Table 3). PAstV4 had a high but similar viral load in both healthy pigs (1.93×10^6; range: 1.7×10^5 to 7.48×10^6) and diarrheic pigs (2.36×10^6; range: 1.09×10^3 to 2.82×10^6). It differed significantly ($p < 0.05$) in viral load between healthy and diarrheic pigs from the Austrian and Hungarian farms. The viral load was low for GARV (57.5; range: 0.4 to 2.26×10^2), and GCRV in one healthy (1.69×10^2) and in 12 diarrheic piglets (6.96×10^3; range: 2.48×10^1 to 1.82×10^4).

PCV2 and PKoV were relatively quantified by comparing Cq values. Comparison of Cq values of diarrheic and healthy samples from the same countries showed roughly similar Cq values of PCV2 and PKoV in both healthy and diarrheic pigs, but variations between countries were observed. Higher Cq values regarding PCV2 were observed in the diarrheic pigs, which suggested a 10-fold lower viral load in the diarrheic pigs than in the healthy pigs from Spanish farms. Similarly, higher PCV2 Cq values were observed in the diarrheic pigs from the Austrian farm. By contrast, PKoV infection in healthy pigs resulted in higher Cq values than in diarrheic pigs from both Hungarian and Spanish farms, indicating a higher amount of viral load in the diseased pigs (Table 3).

3.4. Prevalence and viral load in different age groups

The prevalence of PAstV4 and PCV2 was significantly ($p < 0.05$) lower in the suckling group than in the other two age groups (Table 4). While PAstV4 was demonstrated in 48.0% of the diarrheic pigs compared to 26.3% of the healthy pigs ($p < 0.01$) in the suckling group, it was found in 100% of both the healthy and diarrheic pigs in the nursery and growing-finishing groups. PCV2 was more frequently ($p < 0.05$) demonstrated in healthy than in diarrheic pigs in the nursery and growing-finishing groups. By contrast, the prevalence of PKoV remained at a similar level regardless of age. Among the different age groups, GARV was detected only in the unknown age pigs (15.4%). Except for 1 out of 13 GCRV in the growing-finishing healthy pigs, 7 out of 13 GCRV were found in suckling diarrheic pigs, 1 out of 13 in nursery diarrheic pigs and 4 out of 13 in growing-finishing diarrheic pigs.

Co-infection with two or more viruses was found in 65.0% of the healthy and 55.1% of the diarrheic pigs (Table 4). In the suckling pigs, co-infection rates were significantly ($p < 0.05$) higher in diarrheic pigs than in healthy pigs. Co-infection was also more

Table 3
Enteric viral load (copies per microliter) of PAstV4 and Rotaviruses or Cq values of PCV2 and PKoV in healthy and diarrheic stool samples from 5 EU countries. The viral load values represent mean ± SD.

Country	Status	PAstV4	GARV	GCRV	PCV2	PKoV
Austria	Healthy	$1.56 \times 10^6 \pm 2.37 \times 10^6$		1.69×10^2	33.64	33.89
	Diarrhea	$2.82 \times 10^6 \pm 3.84 \times 10^6$*			35.28	34.48
Germany	Healthy	–	–	–	–	–
	Diarrhea	1.93×10^6		9.72×10^3	26.32	25.65
Hungary	Healthy	$7.48 \times 10^6 \pm 2.00 \times 10^7$			–	35.14
	Diarrhea	$2.86 \times 10^6 \pm 5.31 \times 10^6$*	5.75×10^1		38.38	24.22
Spain	Healthy	$1.24 \times 10^6 \pm 2.75 \times 10^6$			32.37	31.84
	Diarrhea	$2.20 \times 10^6 \pm 3.70 \times 10^6$		$4.20 \times 10^3 \pm 7.29 \times 10^3$	35.90	27.76
Sweden	Healthy	$1.70 \times 10^5 \pm 4.50 \times 10^5$			39.12	26.22
	Diarrhea	$1.09 \times 10^3 \pm 3.23 \times 10^3$			0	31.06

*Indicates significant difference ($p < 0.05$) between health and diarrhea within the age group.

Table 4
Prevalence of enteric viral infections in different age groups.

Age group	Status	No. of samples	PAstV4	GARV	GCRV	PCV2	PKoV	Co-infection[1]
Suckling pigs	Healthy	76	20 (26.3%)[a]	0	0	3 (3.9%)[a]	37/62 (59.7%)	14 (10.6%)[a]
(0–5 weeks)	Diarrhea	100	48 (48.0%)[**(a)]	0	7 (7.0%)	10 (10.0%)[(a)]	57/86 (66.3%)	44 (44.0%)[* (a)]
Nursery pigs	Healthy	96	96 (100%)[b]	0	0	81 (84.4%)[b]	22/47 (46.8%)	88 (91.7%)[b]
(6–10 weeks)	Diarrhea	29	29 (100%)[(b)]	0	1 (3.5%)	16 (55.2%)[**(b)]	12/19 (63.2%)	24 (82.8%)[(b)]
Growing-finishing pigs (11–18 weeks)	Healthy	31	31 (100%)[b]	0	1 (3.2%)	30 (96.8%)[b]	2/4 (50.0%)	30 (96.8%)[b]
	Diarrhea	61	61 (100%)[(b)]	0	4 (6.6%)	36 (59.0%)[**(b)]	21/38 (55.3%)	48 (78.7%)[(b)]
Unknown	Diarrhea	26	10 (38.5%)	4 (15.4%)	0	0	2/15 (13.3%)	3 (11.5%)

Different superscript letters within a column indicate significant differences ($p < 0.05$, *R* 3.0.1) among the age groups.
[1] Details of co-infection are presented in Supplementary Table S1.
[*] Indicates significant difference at $p < 0.05$ between health and diarrhea within the age group.
[**] Indicates significant difference at $p < 0.01$ between health and diarrhea within the age group.

frequently ($p < 0.05$) demonstrated in the nursery and growing-finishing pigs than in the suckling pigs.

As the number of rotavirus positive samples was limited, statistical analysis of viral load in relation to different age groups was mainly focused on PAstV4. The healthy pigs from the suckling group had the highest PAstV4 viral load (4.92×10^6 genome copies per microliter), which differed significantly ($p < 0.05$) from the genome copies in diarrheic pigs in the other two age groups, but not to diarrheic pigs in the same age group. The PAstV4 viral load was significantly higher in diarrheic samples in the nursery ($p < 0.01$) and growing-finishing ($p < 0.05$) groups.

4. Discussion

This study investigated the prevalence of five enteric viral infections in 419 pigs of different ages, comprising both healthy and diarrheic animals, from farms in five European countries. A multiplex quantitative real-time RT-PCR assay was initially developed in order to detect PAstV1, PAstV4, GARV and GCRV; and RNA standards were prepared for quantifying viral load in terms of genome equivalent copies. In addition, two single-plex real-time PCR assays were developed to detect PCV2 and PKoV. With a limited number of sequences available for designing primers and probes specific for the two types of astroviruses, it was hard to ensure that the assay initially developed would be able to detect "specifically" the target virus in an expanding range of new samples. Lack of type-specific virus isolates would make it even harder, which is opposite to the development of a real-time RT-PCR assay for detection of CSFV where reference pestiviral RNA panel and virus isolates of different genotype are available for validation (Liu et al., 2011b). It is likely that the PAstV1 primers and/or probe shared high sequence similarity with other astroviruses, resulting in a low specificity, which was only revealed by a metagenomic approach. Therefore, despite the inclusion of PAstV1 primers/probe in the multiplex assay, the data regarding PAstV1 were excluded from this report.

Among the five types of PAstV, the main reasons for targeting PAstV4 in this study included: (a) a wide distribution of PAstV4 in 13 US states investigated for pig diarrhea (Mor et al., 2012); (b) presence of PAstV4 in both domestic pigs and wild boar in Hungary (Reuter et al., 2011, 2012) and in pigs from Czech Republic (Dufkova et al., 2013); and more importantly, (c) preliminary detection of PAstV4 in Swedish piglets (Wallgren et al., 2014). The multiplex assay successfully detected PAstV4 in the five countries. Moreover, earlier studies have shown varying incidences of PAstV in pigs. In the USA, one study indicated a prevalence of 64% ($n = 509$) in pigs mainly coming from farms with a history of diarrhea (Xiao et al., 2013) and another reported a similar prevalence of 62% ($n = 269$) (Mor et al., 2012). In contrast, only 19.4% ($n = 129$) of the domestic pigs from South Korea were positive for PAstV (Lee et al., 2013). The observed difference might reflect different livestock density and farms' biosecurity measures. Despite high prevalence, there has been no strong evidence that PAstV can cause enteric disease associated with clinical signs. Only an oral infection of 4 day-old piglets with a cell culture grown PAstV1 resulted in mild diarrhea (Shimizu et al., 1990).

Most of the piglets at the Swedish farms were younger than 15 days of age, and had median viral load of about 20 copies of PAstV4 genome. It is assumable that newborn piglets receive colostrum derived immunity but at different levels. Hence, individuals with a low colostrum intake may have a higher amount of virus. On the other hand, high viral load (up to 10^6 copies of PAstV4) was frequently detected in the 3-week-old Spanish piglets, resulting in high mean viral load in the suckling pig group (0–5 weeks). With the increasing age, pigs from the nursery and growing-finishing groups were exposed to astroviruses more often, but to a limited viral load probably reflecting the effect of an adaptive immunity.

In contrast to PAstV4, porcine rotaviruses were found in a low prevalence, which is in agreement with other European reports that showed a rotavirus prevalence of 4% in 169 piglets with diarrhea on 24 farms in Southern Germany (Wieler et al., 2001), and of 22.4% in 147 pigs (<16 weeks old) in Spain (Halaihel et al., 2010). However, high incidences of rotavirus have been reported from USA, where rotaviruses A, B and C were demonstrated in 83% of 7508 diarrheic samples submitted to University of Minnesota, USA (Marthaler et al., 2014), as well as in Sweden where rotavirus was demonstrated from each of 72 herds when pooled fecal samples were repeatedly collected from 10 litters per herd at 2, 4 and 6 weeks of age (Hestad et al., 2004). Thus, differing from astroviruses, rotaviruses are considered to be major pathogens causing diarrhea in pigs despite the low incidence found in this study. Indeed, GCRV, which mainly affects piglets younger than 7 days of age, was found significantly associated with diarrhea ($p < 0.05$), with a 40-fold higher viral load in diarrheic pigs compared to healthy ones. It is likely to be the case for the six rotavirus-positive piglets (4–5 days old) in one German farm, but less likely for the six Spanish diarrheic pigs, which were 7–12 weeks old and co-infected with PAstV4.

Kobuvirus infections have been considered ubiquitous and highly prevalent in European pig farms (Reuter et al., 2011), but its association with diarrhea seems controversial. A significantly higher prevalence of PKoV with larger amount of viruses was found in diarrheic pigs from Hungarian and Spanish farms ($p < 0.05$). In contrast, healthy pigs at the Austrian and Swedish farms had higher incidences of PKoV infection and these pigs also had a somewhat higher viral load than the diarrheic pigs. The high prevalence of PKoV infections was similar with previous reports presenting a prevalence of 75.3% in 985 diarrhea samples collected from 134 farms in 11 Chinese provinces (Zhang et al., 2013), and of 45.7% in 396 diarrheic samples from 37 farms in one Chinese province (Yang et al., 2014). Khamrin et al. (2009) reported an

exceptionally high prevalence of 99% (97 of 98 diarrheic samples) in Thailand, but the authors also described the association of PKoV with enteric diseases in pigs as unclear since no healthy pigs from the farms in the same areas were tested for PKoV infection. Indeed, a study demonstrating a causal role of PKoV in enteric disease in young pigs has not been published so far. Therefore, one might argue that PKoV could contribute to enteric disease only under certain conditions, which have yet to be defined. Still, the influence of PKoV should not be totally neglected. In the present study PKoV was demonstrated at rather equal incidences regardless of age, despite that a decrease of prevalence by age has been recorded in East Africa (Amimo et al., 2014) and in Italy, where the PKoV was detected only in 3.85% out of 280 fecal samples collected from healthy pigs aged between 6 and 10 months (Di Profio et al., 2013).

Unlike PKoV, it is established that PCV2 can contribute to several clinical diseases, including potential participation to enteric clinical disorders in nursery and growing-finishing pigs (Baró et al., 2015). Despite significantly higher prevalence of PCV2-positive pigs in the nursery and growing-finishing groups ($p < 0.05$), PCV2 was more frequently demonstrated in healthy pigs than in pigs with diarrhea. This suggests that most PCV2 infections were probably subclinical. Yet it would be interesting to elucidate the exact role of PCV2 infection in the apparently clinically healthy pigs.

5. Conclusion

This report established and applied quantitative real-time RT-PCR tools for the investigation of prevalence and viral load in 419 fecal samples collected from both healthy and diarrheic pigs in five European countries. While GARV and GCRV were found in a small percentage of enteric infections, PAstV4 became the dominant virus species with high viral load in the investigated European farms, but the prevalence varied greatly depending on the country of farm origin. PKoV was found ubiquitous in all five countries and PCV2 was highly prevalent, especially in the Austrian and Spanish farms. Moreover, the prevalence of PAstV4 and PCV2 increased from the suckling age group to nursery and growing-finishing groups. The study provided valuable data for further clinical research particularly in the context of co-infections.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetmic.2015.10.019.

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