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ORIGINAL ARTICLE

Detection and genotyping of *Porcine circovirus 2* (PCV-2) and detection of *Porcine circovirus 3* (PCV-3) in sera from fattening pigs of different European countries

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Abstract

PCV-2 is considered one of the most economically important viral agents in swine worldwide. Recently, PCV-3 has been found in pigs affected by different disorders and in healthy animals. The objective of this epidemiological work was to describe the frequency of detection of PCV-2 and PCV-3 in pig farms of 9 European countries. Moreover, a second aim was to assess the most frequent PCV-2 genotypes found in the studied farms. Sera from 5 to 10 pigs per farm were collected from 2 to 11 farms per studied country. A total of 624 sera of fattening pigs (10–25 week old) from 64 farms from Spain (n = 11), Belgium (n = 10), France (n = 8), Germany (n = 8), Italy (n = 7), Denmark (n = 8), the Netherlands (n = 5), Ireland (n = 5) and Sweden (n = 2) were analysed by conventional PCR. In addition, one or two PCV-2-positive samples per farm were genotyped by sequencing the ORF2 gene. PCV-3 PCR-positive samples with relatively low Ct values were also sequenced and phylogenetically analysed. PCV-2 DNA was detected in pig sera from all European tested countries, but Sweden. A total of 132 out of 624 (21%) sera were positive for PCV-2 PCR, corresponding to 30 out of the 64 (47%) tested farms. PCV-3 DNA was detected in 52 out of 624 (8%) sera, corresponding also to 30 out of the 64 (47%) studied farms from all tested countries. A total of 48 PCV-2 PCR-positive samples were successfully sequenced and genotyped, being PCV-2d the most frequently genotype found (n = 28), followed by PCV-2b (n = 11) and PCV-2a (n = 9). These results pointed out PCV-2d as the most prevalent genotype currently in Europe. The PCV-3 phylogenetic analysis showed high identity (>98%) among sequences from all the analysed countries. The relatively low co-infection (3%), likely suggest an independent circulation patterns of PCV-2 and PCV-3.

KEYWORDS

genotype, phylogenetic analysis, porcine circovirus type 2 (PCV-2), porcine circovirus type 3 (PCV-3), swine

Segalés and Sibila equally contributed to this study.

1 | INTRODUCTION

Porcine circoviruses (PCVs) are small and non-enveloped viruses represented by three recognized species (PCV-1, PCV-2 and PCV-3), and a tentative novel one designated as PCV-4. The PCV-1 is considered non-pathogenic for swine and was firstly described in 1982 as a contaminant of a pig kidney cell line (Tischer, Gelderblom, Vettermann, & Koch, 1982). PCV-2 was discovered as the cause of a sporadic systemic disease in the 1990s in Canada (Clark, 1996; Harding, 1996), which were followed by severe outbreaks all over the world (Segalés, Kekarainen, & Cortey, 2013). PCV-2 is the causative agent of systemic and reproductive diseases (named collectively as porcine circovirus diseases, PCVDs), resulting in important losses to the pig production (Segalés, 2012). In 2015, PCV-3 was firstly described in pigs with different clinical–pathological outcomes (Palinski et al., 2017; Phan et al., 2016). Subsequently, this virus has been detected by molecular methods in pigs displaying a number of different conditions such as porcine dermatitis and nephropathy syndrome (PDNS) (Arruda et al., 2019; Palinski et al., 2017), respiratory disease (Kedkovid et al., 2018; Qi et al., 2019; Shen et al., 2018; Zhai et al., 2017), enteric disease (Qi et al., 2019; Zhai et al., 2017), reproductive problems (Arruda et al., 2019; Deim et al., 2019; Faccini et al., 2017), multisystemic affection (Phan et al., 2016) and in healthy animals (Klaumann, Franzo, et al., 2018; Stadejek, Woźniak, Miłek, & Biernacka, 2017). The PCV-4 is the most recently described PCV species, and it has been detected in animals with respiratory disease, diarrhoea and PDNS-like lesions (Zhang et al., 2019). PCV-1, PCV-2 and PCV-3 have been found infecting pigs all around the world, whereas PCV-4 has been only described in China so far.

Porcine circoviruses have a circular and single-stranded DNA genome, with a length around 1,759, 1,768 and 2,000 nt in PCV-1, PCV-2 and PCV-3 species, respectively. The genome of PCVs has three major open reading frames (ORFs). ORF1 encodes the replication proteins (*Rep and Rep'*), ORF2 encodes the structural protein (*cap*), and ORF3 encodes non-structural proteins with apoptotic capacity in PCV-1 and PCV-2 (Cheung, 2012). PCV-2 has also an ORF4, which codifies for a protein with anti-apoptotic effects (He et al., 2013; Lin et al., 2018). So far, ORF3 or ORF4 have not been described in the PCV-3 genome.

The highest genome variation among PCVs has been found in PCV-2, which prompted for different proposals of sub-classifications beyond the species level (Segalés et al., 2008). The most accepted PCV-2 classification includes four different genotypes (from PCV-2a to PCV-2d) (Segalés et al., 2008). Taking into account that PCV-2 has a high mutation rate, close to single-stranded RNA viruses (Firth, Charleston, Duffy, Shapiro, & Holmes, 2009), it was expectable to find new sequences representing novel genotypes, and thus, PCV-2e (Davies, Wang, Dvorak, Marthaler, & Murtaugh, 2016; Harmon et al., 2015) and PCV-2f (Bao et al., 2018) have also been suggested. Moreover, a recently new proposed classification re-arranged the classification into eight genotypes in total, from PCV-2a to PCV-2h based on ORF2 gene analysis with maximum p-distance of 13% between genotypes, bootstrap higher than 70% and at least 15 sequences in each cluster (Franzo & Segalés, 2018). In contrast, PCV-3 shares high similarity among available sequences, which makes difficult to establish further classification of this virus species into genotypes (Klaumann, Correa-Fiz, et al., 2018). Despite it is too early for a definitive classification, two (PCV-3a and PCV-3b) (Fux et al., 2018; Li et al., 2018) or three main clades (PCV-3a, PCV-3b and PCV-3c) (Fu et al., 2018) have been proposed for this virus. Even though there are some different proposed groups, most authors agreed that the marker positions that determine the clade divisions are located in the amino acids (aa) sites S122A (ORF1), and A24V and R27K (ORF2) (Chen et al., 2017; Fux et al., 2018; Li et al., 2018).

PCV-2 is frequently found in pigs co-infected with other pathogens, such as *Porcine reproductive and respiratory syndrome virus* (PRRSV), *Porcine parvovirus* (PPV), *Influenza A virus* (IAV) and *Mycoplasma hyopneumoniae*, among others (Opriessnig & Halbur, 2012; Segalés et al., 2013). These co-infections and the interaction of the mentioned pathogens with PCV-2 have been widely studied (Allan et al., 1999; Grau-Roma, Fraile, & Segalés, 2011; Opriessnig, Fenaux, et al., 2004; Opriessnig, Thacker, et al., 2004). On the contrary, PCV-2 and PCV-3 co-infections have been only assessed in studies from Asia (Kim et al., 2018; Xia et al., 2019) and only one in Europe (Woźniak, Miłek, Matyba, & Stadejek, 2019).

Taking into account the abovementioned premises, the present work aimed to assess the frequency of detection of PCV-2 and PCV-3, as well as their co-infection in serum samples of fattening pigs coming from 64 farms located in 9 European countries. In addition, the PCV-2 genotype dominance in these herds and the phylogenetic variability of PCV-3 were also investigated.

2 | MATERIAL AND METHODS

2.1 | Samples

A total of 624 serum samples from pigs ranging from 10 to 25 weeks of age were collected from randomly selected clinically healthy animals. All available information regarding the sampled animals and farms are provided in Table S1. Sera from 5 to 10 pigs per farm were collected from 2 to 11 farms per country. The farms under study were from Spain (n = 11), Belgium (n = 10) France (n = 8), Germany (n = 8), Denmark (n = 8), Italy (n = 7), the Netherlands (n = 5), Ireland (n = 5) and Sweden (n = 2).

2.2 | DNA extraction, and conventional and quantitative PCR methods

DNA was extracted from 200 μl of serum using MagMAx™ Pathogen RNA/DNA Kit (Applied Biosystems®) according to the manufacturer's protocol. A PCV-2 field isolate was used as positive extraction control. A PCR-negative serum sample for both PCV-2 and PCV-3 was used as negative extraction control.

The PCV-2 PCR targeted the *cap* gene region (ORF-2) and was performed using the primers PCV2all_F and PCV2all_R previously described by Oliver-Ferrando et al. (2016) with slight modifications. The PCR mix included 2.5 µl of extracted DNA, 1.25 µl of each mentioned primer at 10 µM, 5 µl of 5 × PCR buffer, 2.5 µl of $MgCl_2$ at 25 mM, 0.75 U of GoTaq® Flexi DNA Polymerase (Promega), 1 µl of dNTP stock solution at 5 mM, and DEPC-treated water up to 25 µl of final volume. The thermal conditions initiated with one cycle at 94°C for 5 min, followed by 35 cycles at 95°C for 30 s, 53°C for 30 s and 72°C for 40 s. The final elongation was at 72°C for 7 min.

The PCV-3 PCR targeted the *rep* gene region (ORF1) of the virus and was performed as previously described (Saporiti et al., 2019). Briefly, 2.5 µl of extracted DNA, 12.5 µl of Go Taq® G2 Green Master Mix (Promega) and 0.4 µM of each primer were added to the reaction, as well as sterile water to reach final volume of 25 µl. Amplification was also performed at the same thermal conditions as previous described (Saporiti et al., 2019).

The PCR products of PCV-2 and PCV-3 were checked by electrophoresis on 1.5% TAE agarose gel. Considering that PCV-3 sequencing was demonstrated to be possible only at low Ct values (Fux et al., 2018; Saporiti et al., 2019), a real-time qPCR was performed (Saporiti et al., 2019) in order to select those samples with the lowest Ct values. The qPCR results were expressed in log10 of PCV-3 DNA copies/µl of serum.

2.3 | Sequencing and genotyping/phylogenetic studies

2.3.1 | PCV-2

The PCR-positive samples per farm showing the strongest band intensity in the gel (one or two per farm) were selected for genotyping. PCV-2 genotyping was based on the ORF2 sequence (Franzo et al., 2015). Amplification was performed by PCR using the same primers targeting the ORF2 region mentioned above (Oliver-Ferrando et al., 2016) with slight modifications. The reaction was carried out on a mixture containing 3 µl of extracted DNA, 1× PCR buffer, 0.25 mM of dNTPs, 0.2 µM of each mentioned primers, one unit of DNA polymerase Platinum™ SuperFi™ (Invitrogen™) kit and sterile water to final volume of 50 µl. The PCR thermal conditions were 98°C for five minutes followed by 40 cycles of 94°C for 30 s, 53°C for 30 s and 72°C for 1 min, and a final elongation at 72°C for 7 min.

PCV-2 amplicons were purified with ExoSAP-IT™ (Thermo Fisher Scientific) kit and sequenced by the Sanger method (BigDye® Terminator v3.1 Cycle Sequencing Kit). The sequencing reactions were analysed using an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystem®) at Servei de Genòmica, Universitat Autònoma de Barcelona (Spain). The quality of the sequences was analysed by the Finch TV programme and trimmed in BioEdit software 7.2.6 (Hall, 1999). When double or bad quality sequences were reported, the sample was discarded to avoid error in genotyping.

The PCV-2 sequences obtained were phylogenetically analysed following the proposed classification by Franzo and Segalés (2018). The amplicons of PCV-2 ORF2 gene obtained herein were aligned against the representative strains of the proposed PCV-2 genotypes using MAFFT software (Katoh, Rozewicki, & Yamada, 2019). A neighbour joining method using the p-distance model was used to build the phylogenetic tree with 1,000 bootstraps. The phylogenetic tree was further edited using the iTOL software (Letunic & Bork, 2019).

The consensus sequences of PCV-2 ORF2 were deposited at the NCBI GenBank with accession numbers MN653165 - MN653212.

2.3.2 | PCV-3

For PCV-3 phylogenetic analyses, the full genome of the virus was attempted from those samples with the lowest qPCR Ct values per country. Three PCRs aiming the complete genome were performed using previously described primers and thermal conditions (Fux et al., 2018) with slight modifications. Briefly, 2 µl of extracted DNA, 1 µl of each primer (10 µM), 10 µl of 5× SuperFi Buffer, 2 µl of dNTP Mix (10 µM, Roche), 1 Units of DNA polymerase Platinum SuperFi (Invitrogen) and sterile water were mixed to bring the final volume up to 50 µl. Thermal conditions were at 98°C for 5 min, 40 cycles of 98°C for 30 s, 55°C for 1 min and 72°C for 2 min, followed by a final extension at 72°C for 7 min. The amplicons were purified with a NucleoSpin® Gel extraction (Macherey-Nagel) Kit according to the manufacturer's protocol and sequenced by the Sanger method (ABI 3730XL - Macrogen Europe, Madrid, Spain). The quality of the sequences was analysed by the Finch TV software and trimmed in BioEdit versus 7.2.6 (Hall, 1999). After eliminating low-quality sequences, the three different amplicons obtained per sample were assembled using the reference mapped-based strategy (Li & Durbin, 2010) to achieve PCV-3 genomes with a maximum length of 1.902 bp. The Integrative Genomics Viewer software (Robinson, Thorvaldsdóttir, Wenger, Zehir, & Mesirov, 2017) was used for visualizing the assembly.

For the PCV-3 genome analysis, the sequences were then trimmed and a gap was included in the nucleotide positions missing (1,056–1,153). The partial genome sequences obtained were aligned against the 44 sequences within the same genomic region previously used and classified by Fux et al. (2018). The obtained sequences were translated using MEGA 7 (Kumar, Stecher, & Tamura, 2016), and the amino acids were inspected manually to assess the previously described group-specific motifs (Fux et al., 2018). A neighbour joining (NJ) tree was constructed using the p-distance model. The tree was evaluated with 1,000 bootstrap replicates to analyse the robustness of the clustering (MEGA7). The nucleotide identity matrix comparing the obtained sequences was done using Clustal Omega (Sievers & Higgins, 2014). The phylogenetic trees were edited using the iTOL software (Letunic & Bork, 2019).

FIGURE 1 Neighbour joining tree with p-distance model and bootstrapping at 1,000 replicates. Tree constructed based on ORF-2 gene of 48 PCV-2-positive samples following the Franzo and Segalés (2018) genotyping method. The samples were labelled using the 'Country_Farm_Sample' code and coloured by genotype (PCV-2a in purple, PCV-2b in orange, PCV-2c in blue, PCV-2d in green, PCV-2e in pink, PCV-2f in yellow, PCV-2g in red and PCV-2h in light blue)

Sequences of PCV-3 were deposited at the NCBI GenBank with the accession numbers MN583564 - MN583578.

3 | RESULTS

3.1 | PCV-2 and PCV-3 detection

PCV-2 DNA was detected in 132 out of 624 analysed sera (21%), corresponding to 30 out of 64 farms (47%). The virus genome was detected in a variable proportion of pigs from all tested countries, ranging from 3 positive samples out of 50 (6%) in the Netherlands to 47 out of 67 (70%) in France. Sweden was the only country where PCV-2 DNA was not detected in any of the two analysed farms (Table 1).

PCV-3 DNA was found in 52 out of 624 studied sera (8%) coming also from 30 farms out of the 64 tested farms (47%); the frequency of PCV-3 PCR positivity ranged from 4% in Ireland (2/50) and Italy (3/67) to 14% (7/50) and 15% (3/20) in the Netherlands and Sweden, respectively (Table 1).

Only 3% (16/624) of the tested sera was positive to both viruses by PCR, which corresponded to 20% (13/64) of the studied farms. There was no evidence of co-infection in samples from three countries: Germany, the Netherlands and Sweden (Table 1).

The amount of virus in the PCV-3-positive samples was low and ranged from 0.97–3 Log10 copies/μl, being 18 out of 51 positive tested samples (one positive sample was not able to be tested quantitatively as no more serum sample nor DNA was available) below the quantification limit of the technique (between one and ten copies of DNA/μl).

3.2 | Sequencing and phylogenetic analysis

The *cap* gene region was sequenced and genotyped from 48 out of the 60 PCV-2 PCR-positive selected samples. Among the 12 non-genotyped cases, 2 of them corresponded to two samples showing double sequences and the remaining 10 displayed bad quality. Globally, 9 sequences corresponded to genotype PCV-2a (18.7%), 11 to PCV-2b (22.9%) and 28 to PCV-2d (58.3%) (Figure 1 and Table 2). Remarkably, one French farm (FR4) had co-infection with two different genotypes, PCV-2a and PCV-2b.

Regarding PCV-3, a qPCR was performed as a screening to select the samples (n = 16) with the highest viral load from the different selected countries. The Ct values obtained were in the range 32–27 which corresponded to 1.6–3 Log 10 PCV-3 DNA copies/μl, respectively. Due to the low viral load in the analysed samples, it was not possible to obtain the complete genome sequence; however, 1,902 nt length sequences were recovered from 15 out of the 16 selected samples and used to perform the phylogenetic analysis. The analysed sequences were from all countries but Denmark and Ireland, because all the positive samples from these countries had a very low PCV-3 load (from 0.1 to 1.3 Log10 copies/μl and one sample has the amount below the quantification limit). The identity matrix between the PCV-3 sequences obtained in this study ranged from 98.9% to 100%, and when compared with all the 255 available references at GenBank (July 2019) with the same length, the similarity ranged from 96.7% to 100%. The NJ phylogenetic tree was built including the references from Fux et al. (2018). Based in the proposed clade classification and amino acid marker positions described by Fux (2018), eight samples clustered in the proposed

TABLE 1 Results of PCV-2 and PCV-3 PCR positivity per country, farm and sample levels

	FARMS†				SAMPLES‡			
Countries	*n*	PCV-2 (%)	PCV-3 (%)	Co-infection (%)	*n*	PCV-2 (%)	PCV-3 (%)	Co-infection (%)
Spain (SP)	11	4 (36.6)	4 (36.4)	1 (9.0)	110	18 (16.3)	7 (6.4)	1 (0.9)
Belgium (BG)	10	8 (80.0)	7 (70.0)	4 (40.0)	100	29 (29.0)	13 (13.0)	4 (4.0)
France (FR)	8	7 (87.5)	5 (62.5)	5 (62.5)	67	47 (70.1)	7 (10.4)	7 (10.4)
Germany (GER)	8	1 (12.5)	3 (37.5)	0 (0.0)	80	8 (10.0)	5 (6.3)	0 (0.0)
Italy (IT)	7	3 (42.8)	1 (14.3)	1 (14.2)	67	10 (14.9)	3 (4.5)	1 (1.5)
Denmark (DK)	8	3 (37.5)	3 (37.5)	1 (12.5)	80	11 (13.7)	5 (6.3)	2 (2.5)
The Netherlands (NE)	5	1 (20.0)	3 (60.0)	0 (0.0)	50	3 (6.0)	7 (14.0)	0 (0.0)
Ireland (IR)	5	3(60.0)	2 (40.0)	1 (20.0)	50	6 (12.0)	2 (4.0)	1 (2.0)
Sweden (SW)	2	0 (0.0)	2 (100.0)	0 (0.0)	20	0 (0.0)	3 (15.0)	0 (0.0)
TOTAL	64	30 (46.8)	30 (46.9)	13 (20.3)	624	132 (21.1)	52 (8.3)	16 (2.6)

†Number of farms positive by PCV-2 and/or PCV-3 PCR (percentage of positive farms among total investigated ones).

‡Number of serum samples positive for PCV-2 and/or PCV-3 PCR (percentage of positive samples among total investigated ones).

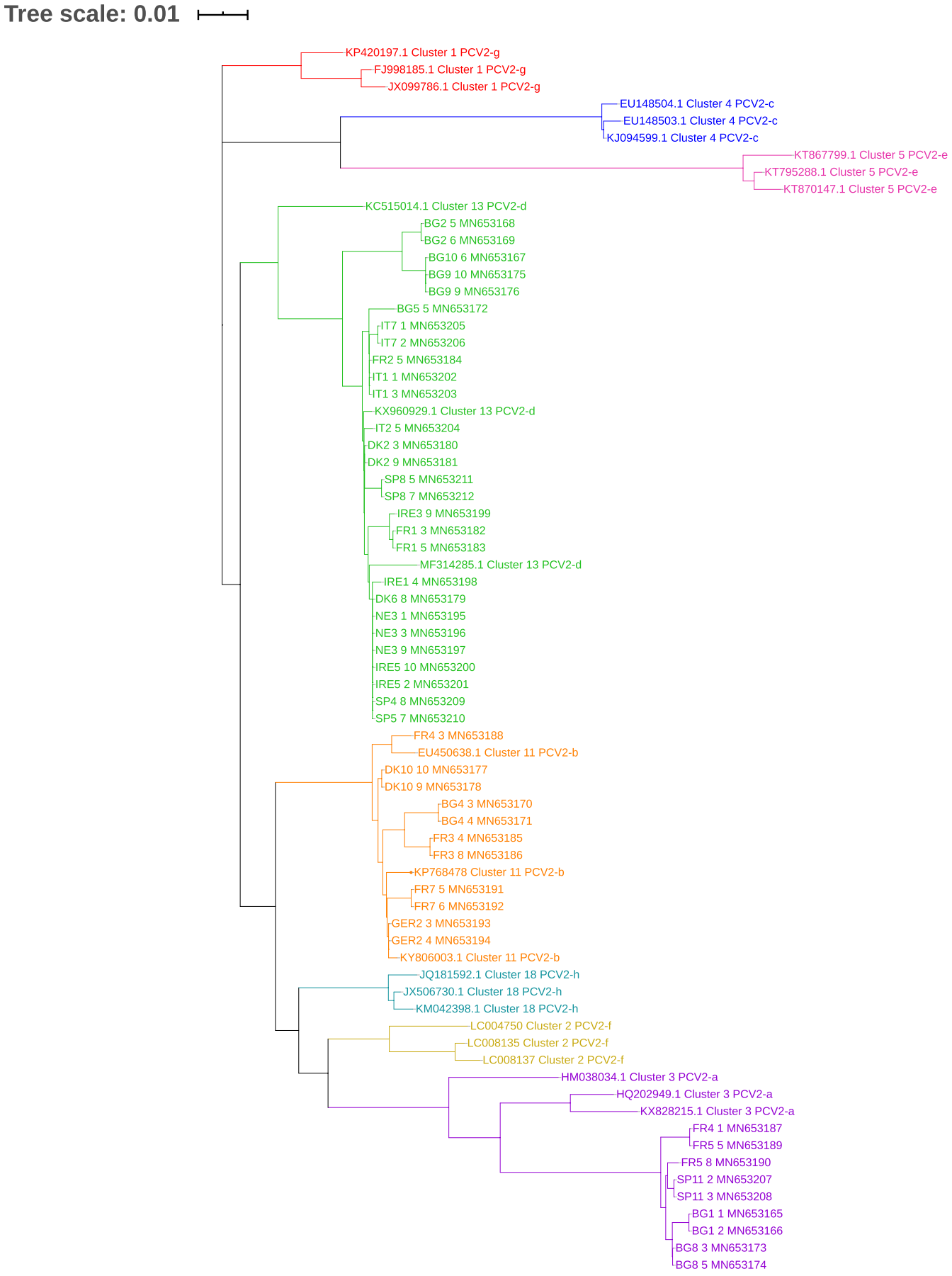
Tree scale: 0.01
KP420197.1 Cluster 1 PCV2-g
FJ998185.1 Cluster 1 PCV2-g
JX099786.1 Cluster 1 PCV2-g
EU148504.1 Cluster 4 PCV2-c
EU148503.1 Cluster 4 PCV2-c
KJ094599.1 Cluster 4 PCV2-c
KT867799.1 Cluster 5 PCV2-e
KT795288.1 Cluster 5 PCV2-e
KT870147.1 Cluster 5 PCV2-e
KC515014.1 Cluster 13 PCV2-d
BG2 5 MN653168
BG2 6 MN653169
BG10 6 MN653167
BG9 10 MN653175
BG9 9 MN653176
BG5 5 MN653172
IT7 1 MN653205
IT7 2 MN653206
FR2 5 MN653184
IT1 1 MN653202
IT1 3 MN653203
KX960929.1 Cluster 13 PCV2-d
IT2 5 MN653204
DK2 3 MN653180
DK2 9 MN653181
SP8 5 MN653211
SP8 7 MN653212
IRE3 9 MN653199
FR1 3 MN653182
FR1 5 MN653183
MF314285.1 Cluster 13 PCV2-d
IRE1 4 MN653198
DK6 8 MN653179
NE3 1 MN653195
NE3 3 MN653196
NE3 9 MN653197
IRE5 10 MN653200
IRE5 2 MN653201
SP4 8 MN653209
SP5 7 MN653210
FR4 3 MN653188
EU450638.1 Cluster 11 PCV2-b
DK10 10 MN653177
DK10 9 MN653178
BG4 3 MN653170
BG4 4 MN653171
FR3 4 MN653185
FR3 8 MN653186
KP768478 Cluster 11 PCV2-b
FR7 5 MN653191
FR7 6 MN653192
GER2 3 MN653193
GER2 4 MN653194
KY806003.1 Cluster 11 PCV2-b
JQ181592.1 Cluster 18 PCV2-h
JX506730.1 Cluster 18 PCV2-h
KM042398.1 Cluster 18 PCV2-h
LC004750 Cluster 2 PCV2-f
LC008135 Cluster 2 PCV2-f
LC008137 Cluster 2 PCV2-f
HM038034.1 Cluster 3 PCV2-a
HQ202949.1 Cluster 3 PCV2-a
KX828215.1 Cluster 3 PCV2-a
FR4 1 MN653187
FR5 5 MN653189
FR5 8 MN653190
SP11 2 MN653207
SP11 3 MN653208
BG1 1 MN653165
BG1 2 MN653166
BG8 3 MN653173
BG8 5 MN653174

TABLE 2 Number of PCV-2 genotypes per country and percentage of each genotype found among the sequenced samples

Countries	No of sequences	PCV-2a	PCV-2b	PCV-2d
Spain (SP)	6	SP11-2, SP11-3 *n* = 2		SP4-8, SP5-7, SP8-5, SP8-7 *n* = 4
Belgium (BG)	12	BG1-1, BG1-2, BG8-3, BG8-5 *n* = 4	BG4-3, BG4-4 *n* = 2	BG2-5, BG2-6, BG5-5, BG9-9, BG9-10, BG10-6 *n* = 6
France (FR)	11	FR4-1, FR5-5, FR5-8 *n* = 3	FR3-4, FR3-8, FR4-3, FR7-5, FR7-6 *n* = 5	FR1-3, FR1-5, FR2-5 *n* = 3
Germany (GER)	2		GER2-3, GER2-4 *n* = 2	
Italy (IT)	5			IT1-1, IT1-3 IT2-5, I7-1, IT7-2 *n* = 5
Denmark (DK)	5		DK10-9- DK10-10 *n* = 2	DK2-3, DK2-9, Dk6-8 *n* = 3
The Netherlands (NE)	3			NE3-1, NE3-3, NE3-9 *n* = 3
Ireland (IR)	4			IR3-9, IR1-4, IR5-2, IR5-10 *n* = 4
Sweden (SW)	0			
Total	48	9 (18.7%)	11 (22.9%)	28 (58.3%)

Note: Each sequence genotype is coded using the country followed by the number of farm and the number of animal of the corresponding farm ('Country_Farm_Sample').

subgroup *a1*, two in the subgroup *a2*, and five that were classified as *b1* (Figure 2).

4 | DISCUSSION

Porcine circoviruses are considered ubiquitous in the swine production worldwide as indicated in many studies (Klaumann, Franzo, et al., 2018; Segalés et al., 2013). Therefore, it was expected to find PCV-2 and PCV-3 in most, if not all studied European countries (PCV-2 was not found in the two investigated Swedish herds), even the sampling per country was limited to 10 fattening pigs per farm. In consequence, it is likely that the present study underestimates the real prevalence of both viruses in these countries, but provides further evidence of PCV-3 distribution in Europe, the frequency of PCV-2/PCV-3 co-infections in fattening pigs and an updated PCV-2 genotyping in this geographic area. By consulting at the GenBank database (October 2nd, 2019), among the last 1,000 PCV-2 sequences uploaded, only 47 were from Europe, indicating the need of continuous surveillance and update current genotypes circulation in this continent.

In spite of ubiquity, the prevalence of PCV-2 in Europe can vary between countries. A recent study from Belgium indicated a 15.7% (50/319) frequency of PCV-2 detection in pigs displaying PCVD-like disease (Wei, Xie, Theuns, & Nauwynck, 2019), while in the present study in the same country was slightly higher, showing a frequency of 29% (29/100) in farms without major clinical diseases. In France, the difference was even higher, with a 70% (47/67) of positivity herein and 14.3% and 40.2% of pigs at 10 and 16 weeks old, respectively in a study conducted in 2011 by Fablet et al. (2011). In the island of Sardinia, in Italy, a 10% (12/120) of PCV-2 frequency was found (Dei Giudici et al., 2019). Although the mentioned study just referred to a specific region of Italy, it was close to the prevalence found in the present study (14.9%, 10/67) in the same country. Pigs from Poland were not screened in the present study, but two different studies recently described a higher prevalence of PCV-2 in serum from animals from non-vaccinated farms (58.3%, 35/60 and 63,5%, 33/52) compared to vaccinated ones (2.8%, 3/106 and 17,6%, 54/307) (Woźniak, Miłek, Bąska, Miłek, Bąska, & Stadejek, 2019; Woźniak, Miłek, Matyba, et al., 2019). Unfortunately, the information on vaccination status was not available for 5 tested farms in this study; however, PCV-2 vaccines were used in most of them (*n* = 44), and hence, it was expectable to find a relatively low percentage of infected animals overall. The case of Sweden was interesting since both farms were negative for PCV-2 detection. In fact, a previous study of PCV-2 detection in Sweden performed on stool samples yielded also a low prevalence (2.9%, 3/104) (Zhou et al., 2016). Anyway, the most likely reason for the low prevalence detected could be the low number of herds screened, as well as the sample matrix used (Woźniak, Miłek, Matyba, et al., 2019). In the study of Zhou and collaborators (2016), five European Countries (Austria, Germany, Hungary, Spain and Sweden) were screened and showed a higher percentage of PCV-2 DNA (42.2%, 176/417) (Zhou et al., 2016) when compared to the present work (21.1%, 132/624). It is very likely that different countries involved in each study, sampling ages, number of age groups studied, epidemiological situation at each time, health status,

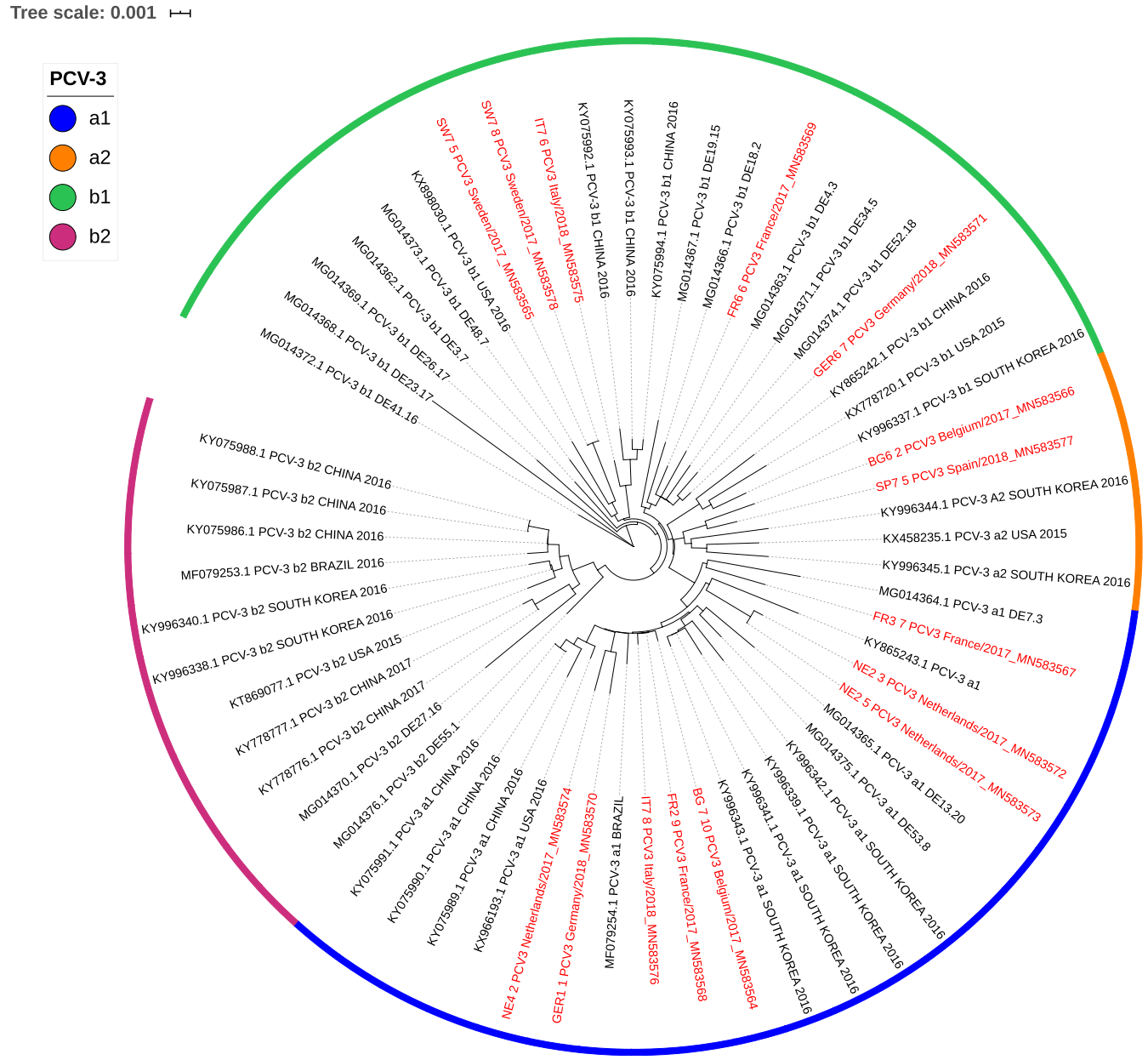

FIGURE 2 PCV-3 phylogenetic tree constructed using the neighbour joining (NJ) method and p-distance model. The 15 PCV-3 sequenced samples are labelled in red, and following the 'Country_Farm_Sample' code. The genomes used as reference for the comparison analysis are labelled in black. The blue, orange, green and pink colours indicate the subtypes proposed by Fux et al. (2018)

sample matrix, PCR method used and number of animals per farm may account for the differences among published studies.

Regarding PCV-3, several studies have explored the prevalence of infection in European countries (Denmark, Germany, Ireland, Italy, Poland, Spain and Sweden), obtaining relatively comparable results ranging from 10% to 30% (Collins, McKillen, & Allan, 2017; Franzo, Legnardi, et al., 2018; Fux et al., 2018; Klaumann, Franzo, et al., 2018; Stadejek et al., 2017; Ye, Berg, Fossum, Wallgren, & Blomström, 2018). The results of the present study offered slightly lower frequency of detection (8.3% overall), but this was expectable since from each farm only one age group was investigated. In all cases, the belief that PCV-3 is a ubiquitous virus across Europe was fully supported since it was found in farms from all studied countries. Also, the present work reports the presence of PCV-3 in the Netherlands, France and Belgium for the first time.

The percentage of co-infection of PCV-2 and PCV-3 in the pig serum samples was fairly low (3%, 16/624), suggesting an independent circulation pattern of both viruses. The results are in accordance with the low frequency of co-infection found in Poland, in pigs vaccinated (0.9%, 1/106) and non-vaccinated (11.7%, 7/60) against PCV-2 (Woźniak, Miłek, Bąska, et al., 2019), which is so far the only study investigating the concomitant presence of both viruses in Europe. Such difference among PCV-2 vaccinated and non-vaccinated farms is logical, since the vaccine is very effective in decreasing the prevalence of infection of this virus (Segalés, 2015). Therefore, PCV-2 vaccinated farms should have lower percentage

of co-infected animals. On the other hand, studies performed in Asia have described the presence of both viruses to a variable frequency such as 1.5% (3/200) (Sun et al., 2018), 10.3% (28/272) (Zhao et al., 2018), 12.5% (1/8) (Kedkovid et al., 2018), 15.7% (35/222) (Ku et al., 2017) and 28.3% (13/46) (Kim et al., 2017). Again, differences among countries and studies may depend on the epidemiology of each region, but many other factors as sampling strategy and PCR methods used may account for variability.

Since PCV-2 has a high mutation rate (Firth et al., 2009), variation in its genome is constant and the generation of novel sequences classified in novel genotypes is likely (Franzo & Segalés, 2018). Although PCV-2a seemed to be the most frequent genotype before the advent of PCV-2-systemic disease outbreaks over the world, genotype PCV-2b was the most spread one during them and afterwards (Segalés et al., 2013). Curiously, in the last 5–7 years, PCV-2d has become the most prevalent genotype worldwide (Dei Giudici et al., 2019; Franzo & Segalés, 2018; Grierson, Werling, Bidewell, & Williamson, 2018; Xiao, Halbur, & Opriessnig, 2015). This finding has been recognized mainly in Asia (Franzo & Segalés, 2018), where most of the sequences available on GenBank comes from in the last 5 years. In the same line, the present study reinforces the domination of PCV-2d genotype also in Europe, since it was found in 58.3% (28/48) of the genotyped samples. In contrast, PCV-2-b and PCV-2a accounted for 22.9% (11/48) and 18.7% (9/48) of the sequenced cases. PCV-2a genotype was detected in three countries (Spain, Belgium and France). Noteworthy, these countries had the highest frequency of PCV-2 detection in general, with 16% in Spain, 29% in Belgium and 70% in France, which could increase the probability to detect this PCV-2a genotype in spite of the decreasing trend globally (Dei Giudici et al., 2019; Franzo & Segalés, 2018; Grierson et al., 2018; Xiao et al., 2015). Taken altogether, all three genotypes seem to be circulating in Europe nowadays, although the real prevalence of each of them should be elucidated by means of larger epidemiological studies in each country. Curiously, PCV-2 sequences from all genotypes tended to cluster within a same country, which probably reflects that most isolates are circulating at a local level rather than at a continental level. Almost all samples from the same farm clustered close one to another, which they also belonged to the same genotype, except for two samples from France (FR_4_1 and FR4_3), which were genotyped as PCV-2a and PCV-2b, respectively. Infection by more than one genotype in the same farm and even in the same pig has already been widely documented (Grau-Roma et al., 2008; Hesse, Kerrigan, & Rowland, 2008). Although the genotypes obtained in the present study were subjected to the new classification proposed by Franzo and Segalés (2018), with eight different genotypes, only the three major ones were detected.

It must be emphasized that obtained results may prompt the future interest to investigate the phylogeography of PCV-3 to determine its epidemiology at a global level, as it has been performed for other single-stranded DNA pig viruses (Cortey, Pileri, Segalés, & Kekarainen, 2012; Vidigal et al., 2012). The high identity of PCV-3 between available sequences (>98%) makes difficult to establish a potential genotype classification (Klaumann, Correa-Fiz, et al., 2018). However, taking into account the proposed marker positions determining clade divisions located in the amino acid (aa) sites S122A for ORF1 and, A24V and R27K for ORF2 (Fux et al., 2018), the 15 obtained sequences clustered in 3 different subgroups. At this stage, it is difficult to elucidate any potential clade distribution by country, since PCV-3 sequences found in different farms from some countries (Belgium, France, Germany and Italy) were represented in more than one clade, while the sequences from the Netherlands were found in one single group. Despite previous classification proposals, the high identity among different PCV-3 sequences obtained here fits with the very recent publication suggesting that one single PCV-3 genotype does exist so far (Franzo et al., 2020). Moreover, since sera samples were obtained from farms with no overt disease, it cannot be stated any apparent relationship between PCV-3 classification and virus pathogenicity or any specific disease.

In conclusion, PCV-2 and PCV-3 are spread all over Europe, but with a very low rate of co-infection, suggesting independent circulation patterns of both viruses. The most common genotype of PCV-2 found in this study was PCV-2d. This finding would reinforce the global trend shift from PCV-2a to PCV-2b first and then from PCV-2b to PCV-2d (Franzo & Segalés, 2018). The obtained PCV-3 sequences were closely related, with >98% of identity, further suggesting the existence of one single genotype circulating in Europe.

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CONFLICT OF INTEREST

All authors have declared no conflict of interest.

ETHICAL APPROVAL

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as this is a research data from general samples obtained in particular surveillance programmes performed in the different countries.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in the GenBank repository (https://www.ncbi.nlm.nih.gov/genbank/) with Accession numbers: from MN653165 to MN653212 for PCV-2 sequences, and from MN583564 to MN583578 for PCV-3 sequences.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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