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Retrospective detection of *Porcine circovirus 3* (PCV-3) in pig serum samples from Spain

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Summary

Porcine circovirus 3 (PCV-3) is an emerging circovirus species that has recently been reported in different countries around the world, suggesting a widespread circulation. In this study, sera samples originating from 654 pigs of different production phases and clinical/pathological conditions, submitted for diagnostic purposes between 1996 and 2017, were randomly selected. Detection of PCV-3 genome in such samples was attempted with a previously described PCR method, and the partial genome sequence was obtained from selected PCV-3-positive samples from different years. Compiled data confirmed that PCV-3 has been circulating in the Spanish pig population since 1996. The overall frequency of PCV-3 PCR-positive samples in the study period was 11.47% (75 of 654). Phylogenetic analysis of twelve PCV-3 partial sequences obtained showed a high nucleotide identity with the already known PCV-3 sequences, with minor variations among years. No significant correlation was found between the detection of PCV-3 and any production phase nor clinical/pathological condition. These results confirm PCV-3 circulation at least since 1996 in the Spanish pig population with a low/moderate frequency. Although the information obtained was limited, PCV-3 did not appear to be linked to any specific pathological condition or age group.

KEYWORDS
polymerase chain reaction, *Porcine circovirus 3*, retrospective, sequencing, Spain

1 | INTRODUCTION

A new circovirus species able to infect the domestic pig has been recently described and named *Porcine circovirus 3* (PCV-3). It was initially found in 2016 in tissues of swine affected by porcine dermatitis and nephropathy syndrome (PDNS), reproductive failure, and cardiac and multisystemic inflammation (Palinski et al., 2017; Phan et al., 2016) in the USA. Subsequently, other reports from Europe (Collins, McKillen, & Allan, 2017; Faccini et al., 2017; Franzo, Legnardi, Hjulsager et al., 2018; Stadejek, Woźniak, Miłek, & Biernacka, 2017), Asia (Fan et al., 2017; Ku et al., 2017; Kwon, Yoo, Park, & Lyoo, 2017; Shen et al., 2017; Zhai et al., 2017; Zheng et al., 2017) and South America (Tochetto et al., 2017) described the presence of PCV-3 genome in tissue samples or sera from pigs with different clinical presentations as well as in healthy animals.

PCV-3 has a circular genome that contains 1,999–2,000 nucleotides with two major open reading frames (ORFs) oriented in opposite directions encoding capsid (Cap) and replicase (Rep) proteins (Palinski

et al., 2017). A number of complete genomes of PCV-3 are available so far, indicating a fairly high nucleotide identity ranging from 96% to 100% (Franzo, Legnardi, Hjulsager et al., 2018; Zheng et al., 2017). Based on phylogenetic analyses, PCV-3 has a close evolutionary relationship with *Fox circovirus*, *Canine circovirus* and *Bat circoviruses* (Palinski et al., 2017; Phan et al., 2016). PCV-3 evolution and origin are still unknown features of this newly discovered viral agent.

PCV-3 is the third circovirus type found in swine. PCV-1 was the first described member of the *Circoviridae* family around 40 years ago (Tischer, Gelderblom, Vettermann, & Koch, 1982; Tischer, Rasch, & Tochtermann, 1974), which has been historically considered non-pathogenic for swine (Allan et al., 1995). By mid-late 1990s, PCV-2 was discovered in association with the so-called post-weaning multi-systemic wasting syndrome (PMWS, nowadays known as PCV-2-systemic disease, PCV2-SD) (Segalés, 2012). PCV2-SD was considered a devastating disease during more than a decade (Segalés, Kekarainen, & Cortey, 2013), although it is nowadays under control as a result of vaccination (Segalés, 2015). In spite of being discovered by late 1990s, PCV-2 was circulating in the swine population long time before; retrospective studies have shown that PCV-2 was present in pigs as early as 1962s and PCV2-SD already existed by mid-1980s (Jacobsen et al., 2009). Taking into account the natural history of previously known porcine circoviruses, it is very likely that PCV-3 has been also present within the swine population before its initial detection in 2016. Therefore, the aim of this work was to perform a retrospective study to detect evidence of PCV-3 infection in Spanish serum samples of pigs collected between 1996 and 2017. Moreover, the genetic characterization of PCV-3 during the 22-year study period based on partial sequences of the viral genome is provided.

TABLE 1 Number of PCV-3 in serum samples from 1996 to 2017 tested by conventional PCR

Year	Number of examined cases (*n*)	PCV-3 PCR-positive cases	Percentage (%)
1996	13	1	7.69
1997	20	2	10.00
1998	20	1	5.00
1999	19	1	5.26
2000	19	1	5.26
2001	20	2	10.00
2002	20	3	15.00
2003	20	1	5.00
2004	20	1	5.00
2005	17	0	0.00
2006	39	2	5.13
2007	39	6	15.38
2008	40	7	17.50
2009	26	0	0.00
2010	40	6	15.00
2011	39	3	7.69
2012	37	9	24.32
2013	22	3	13.64
2014	42	7	16.67
2015	41	8	19.51
2016	27	1	3.70
2017	74	10	13.51
Total	654	75	11.47

2 | MATERIAL AND METHODS

2.1 | Retrospective swine sera

Serum samples stored at −20°C from 654 pigs submitted for diagnostic purposes at the Veterinary Pathology Diagnostic Service of the *Universitat Autònoma de Barcelona* (Barcelona, Spain) between 1996 and 2017 were used for this study. From 1996 to 2005, 17 to 20 sera were randomly selected from the serum bank, except for the year 1996, where only 13 serum samples were available; between 2006 and 2017, 26–74 sera were chosen. Sera corresponded to animals from different, non-related diagnostic studies performed across years, and information about the overall production phase and clinical/pathological status of tested pigs is summarized in Tables 1 and 2, respectively.

2.2 | DNA extraction and polymerase chain reactions (PCR) to detect and sequence PCV-3

DNA from 200 μl of serum samples was extracted using MagMAx™ Pathogen RNA/DNA Kit (Applied Biosystems®) according to the manufacturer's protocol. A plasmid containing the full-length PCV-3 sequence (Franzo, Legnardi, Centelleghe et al., 2018) and double distilled water were used as positive and negative controls, respectively. To demonstrate the presence of PCV-3 DNA in studied samples, a conventional PCR assay was performed; according to a previous protocol described (Franzo, Legnardi, Hjulsager et al., 2018), three μl of extracted DNA was added to a PCR mix and amplified using the same thermal protocol. The reaction was carried out in a final volume of 50 μl mixture containing 10 μl of 5× PCR Buffer, 2 μl of 10 μM dNTPs, 1 μl of 10 pmol forward primer (located in genomic positions 233-255), 5′-AAAGCCCGAAACA CAGGTGGTGT-3′, 1 μl of 10 pmol of a reverse primer 5′-TTTTCC CGCATCCTGGAGGACCAAT- 3′ (placed between nucleotide positions 718–742), 1 Units of DNA polymerase Platinum™ SuperFi™ (Invitrogen™) and 32.5 μl of water. For the partial genome sequence amplification, two specific primer pairs able to detect two overlapping amplicons were used (Palinski et al., 2017): a forward 5′-CAC CGTGTGAGTGGATATAC-3′ and reverse primer 5′-CAAACCCACC CTTAACAG-3′ (located in the genomic positions 74–93 and 909–927, respectively), and a forward primer 5′-GTCGTCTTGGAGC CAAGTG-3′ and reverse 5′-CGACCAAATCCGGGTAAGC-3′ (placed between positions 1612–1627 and 415–433, respectively). The extracted DNA was added to a same quantity of mix as reported above applying as well the same PCR conditions. The PCR products were run on 1.8% TAE agarose gel and purified using NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel) according to the

TABLE 2 Frequencies of PCV-3-positive and -negative samples grouped according to different clinical/pathological presentations and production phase

Clinical/pathological disorders																
Production phase	PCV-3 PCR Results	Total	Gastrointestinal		Neurological		Respiratory		Reproductive		Systemic		Others		Asymptomatic	
		n	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
Foetuses	Positive	3	0	0.00	0	0	0	0.00	3	100	0	0.00	0	0.00	0	0.00
	Negative	3	0	0.00	0	0.00	0	0.00	3	100	0	0.00	0	0.00	0	0.00
Lactation	Positive	10	1	10.00	2	20.00	5	50.00	0	0	1	10.00	1	10.00	0	0.00
	Negative	113	29	25.66	22	19.47	6	5.31	0	0	19	16.81	37	32.74	0	0.00
Nursery	Positive	36	10	27.78	2	5.56	9	25.00	0	0	10	27.78	2	5.56	3	8.33
	Negative	262	49	18.70	16	6.11	67	25.57	0	0	74	28.24	30	11.45	26	9.92
Fattening	Positive	21	1	4.76	2	9.52	3	14.29	0	0	10	47.62	1	4.76	4	19.05
	Negative	172	13	7.56	7	4.07	35	20.35	0	0	67	38.95	10	5.81	40	23.26
Sows	Positive	0	0	0.00	0	0.00	0	0.00	0	0	0	0.00	0	0.00	0	0.00
	Negative	5	0	0.00	0	0	1	20.00	0	0	1	20.00	3	60.00	0	0.00
Total[a]	Positive	70	12	17.14	6	8.57	17	24.29	3	4.29	21	30.00	4	5.71	7	10.00
	Negative	555	91	16.40	45	8.11	109	19.64	3	0.54	161	29.01	80	14.41	66	11.89
	Total	625	103	16.48	51	8.16	126	20.16	6	0.96	182	29.12	84	13.44	73	11.68

[a]No information was available for five out 75 PCV-3 PCR-positive animals and 24 of 579 for PCV-3-negative animals.

manufacturer's protocol. The quality and quantity of genomic DNA was analysed with BioDrop DUO (BioDrop Ltd).

Twelve samples from different years were then Sanger sequenced for verification at the Genomic and Bioinformatics Service of the *Universitat Autònoma de Barcelona* (Barcelona, Spain), which was performed with BigDye® Terminator v3.1 Cycle Sequencing Kit, following the manufacturer's protocol. The sequencing reactions were analysed using an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystem®).

2.3 | Sequence alignment and phylogenetic analysis

Sequence analysis was performed with Finch TV program 1.4.1 (http://www.geospiza.com), the consensus was obtained utilizing ChromasPro (CromasPro Version 1.5) and sequences were aligned with 51 PCV-3 complete genomes available at the GenBank (retrieved on November 2017) using MAFFT (Kumar, Stecher, & Tamura, 2016). A phylogenetic tree was conducted using the maximum-likelihood (ML) method implemented in PhyML (Guindon et al., 2010); the robustness of the ML tree was evaluated by bootstrap analysis with 1,000 boostrap replicates. The raw genetic distance among strains was calculating using MEGA7 software (Kumar et al., 2016). Sequences obtained were deposited at the GenBank (references MG807066 to MG807089).

2.4 | Association between the presence of PCV-3 and production phase and clinical/pathological conditions

All studied animals were classified according to their respective production phases (lactation, from 1 to 3 weeks of age; nursery, from 4 to 9 weeks of age; fatteners, >10 weeks of age; sows and foetuses were also included). Animals were still classified according to their different clinical/pathological presentations (gastrointestinal, systemic, neurological, reproductive, respiratory and other conditions such as cutaneous, musculoskeletal or inconclusive clinical signs). A number of asymptomatic animals were included as well. Table 2 summarizes the number of pigs in each production phase as well as their clinical/pathological presentation. Specifically, as previous studies reported coinfections of PCV-3 with PCV-2 (Fu et al., 2017; Ku et al., 2017) and *Porcine reproductive and respiratory syndrome virus* (PRRSV) (Fu et al., 2017), two of the most important pathogens affecting pigs worldwide, the concurrent presence PCV-2 and PRRSV was also assessed. These two latter viruses were investigated for diagnostic purposes (immunohistochemistry and/or in situ hybridization) in just some of the studied animals, attending to the demand of the submitting veterinarian.

2.5 | Statistical analyses

The statistical analyses were performed using XLSTAT 365 Microsoft Excel 2016 Statistics Software. To test for significant differences between production phases, the corresponding clinical/pathological status and the PCV-3 PCR positivity frequencies across the 22 years tested in this study, a Fisher's exact test was performed. p values <.05 were considered to be statistically significant.

3 | RESULTS

3.1 | PCV-3 detection by PCR

PCV-3 PCR positivity was found in 75 of 654 (11.46%) serum samples. The first PCV-3 PCR-positive sample dated back to 1996, and

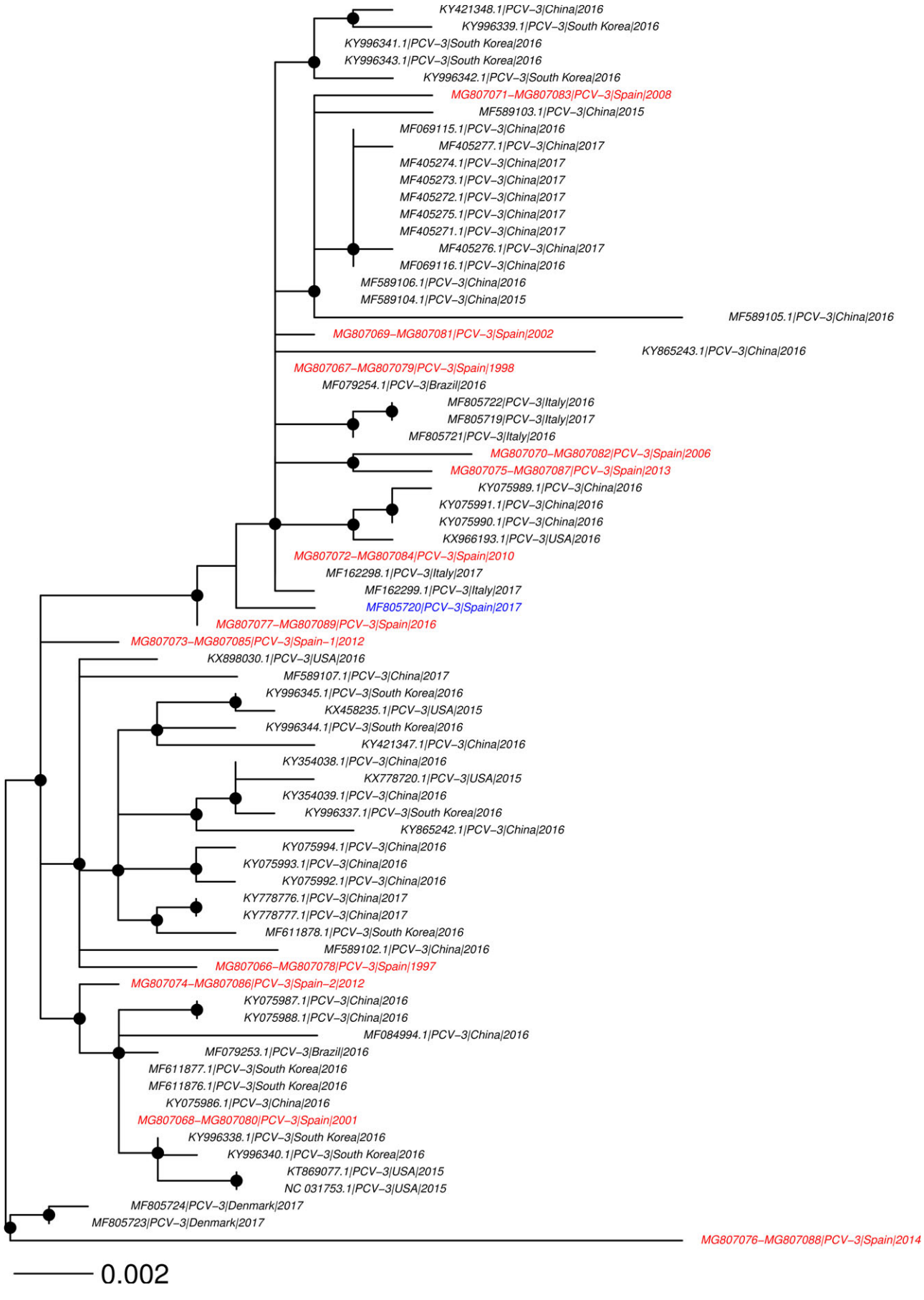
KY421348.1|PCV–3|China|2016
KY996339.1|PCV–3|South Korea|2016
KY996341.1|PCV–3|South Korea|2016
KY996343.1|PCV–3|South Korea|2016
KY996342.1|PCV–3|South Korea|2016
MG807071–MG807083|PCV–3|Spain|2008
MF589103.1|PCV–3|China|2015
MF069115.1|PCV–3|China|2016
MF405277.1|PCV–3|China|2017
MF405274.1|PCV–3|China|2017
MF405273.1|PCV–3|China|2017
MF405272.1|PCV–3|China|2017
MF405275.1|PCV–3|China|2017
MF405271.1|PCV–3|China|2017
MF405276.1|PCV–3|China|2017
MF069116.1|PCV–3|China|2016
MF589106.1|PCV–3|China|2016
MF589104.1|PCV–3|China|2015
MF589105.1|PCV–3|China|2016
MG807069–MG807081|PCV–3|Spain|2002
KY865243.1|PCV–3|China|2016
MG807067–MG807079|PCV–3|Spain|1998
MF079254.1|PCV–3|Brazil|2016
MF805722|PCV–3|Italy|2016
MF805719|PCV–3|Italy|2017
MF805721|PCV–3|Italy|2016
MG807070–MG807082|PCV–3|Spain|2006
MG807075–MG807087|PCV–3|Spain|2013
KY075989.1|PCV–3|China|2016
KY075991.1|PCV–3|China|2016
KY075990.1|PCV–3|China|2016
KX966193.1|PCV–3|USA|2016
MG807072–MG807084|PCV–3|Spain|2010
MF162298.1|PCV–3|Italy|2017
MF162299.1|PCV–3|Italy|2017
MF805720|PCV–3|Spain|2017
MG807077–MG807089|PCV–3|Spain|2016
MG807073–MG807085|PCV–3|Spain–1|2012
KX898030.1|PCV–3|USA|2016
MF589107.1|PCV–3|China|2017
KY996345.1|PCV–3|South Korea|2016
KX458235.1|PCV–3|USA|2015
KY996344.1|PCV–3|South Korea|2016
KY421347.1|PCV–3|China|2016
KY354038.1|PCV–3|China|2016
KX778720.1|PCV–3|USA|2015
KY354039.1|PCV–3|China|2016
KY996337.1|PCV–3|South Korea|2016
KY865242.1|PCV–3|China|2016
KY075994.1|PCV–3|China|2016
KY075993.1|PCV–3|China|2016
KY075992.1|PCV–3|China|2016
KY778776.1|PCV–3|China|2017
KY778777.1|PCV–3|China|2017
MF611878.1|PCV–3|South Korea|2016
MF589102.1|PCV–3|China|2016
MG807066–MG807078|PCV–3|Spain|1997
MG807074–MG807086|PCV–3|Spain–2|2012
KY075987.1|PCV–3|China|2016
KY075988.1|PCV–3|China|2016
MF084994.1|PCV–3|China|2016
MF079253.1|PCV–3|Brazil|2016
MF611877.1|PCV–3|South Korea|2016
MF611876.1|PCV–3|South Korea|2016
KY075986.1|PCV–3|China|2016
MG807068–MG807080|PCV–3|Spain|2001
KY996338.1|PCV–3|South Korea|2016
KY996340.1|PCV–3|South Korea|2016
KT869077.1|PCV–3|USA|2015
NC 031753.1|PCV–3|USA|2015
MF805724|PCV–3|Denmark|2017
MF805723|PCV–3|Denmark|2017
MG807076–MG807088|PCV–3|Spain|2014
0.002

FIGURE 1 Phylogenetic tree based on the partial genomes (1,001 nucleotide sequences) of PCV-3 Spanish strains and PCV-3 freely available sequences at Genbank. The phylogenetic tree was constructed using the maximum-likelihood algorithm of PhyML software with 1.000 bootstrap replicates. Spanish sequences obtained in the present study and Franzo, Legnardi, Hjulsager et al. (2018) have been coloured in red and blue, respectively. Nodes demonstrating a branch support higher than 70% have been marked with a full circle [Colour figure can be viewed at wileyonlinelibrary.com]

the viral genome was subsequently detected in sera from all tested years except 2005 and 2009. The frequency in the positive years ranged from 3.70% (one of 27) in 2016 to 24.32% (nine of 37) in 2012 (Table 1). No statistical differences in frequency of PCV-3 PCR animals were observed across the years tested ($p > .05$).

3.2 | Sequence alignment and phylogenetic analysis

Partial sequences were obtained from 12 PCV-3 PCR-positive samples from different years and comprised part of the rep (sequence length ranging from 848 to 949 nucleotide positions) and cap (sequence length ranging from 338 to 387 nucleotide positions) genes. The phylogenetic analysis was performed on the concatenation of the partial rep and cap sequences (covering a total of 1,001 nucleotides of the whole PCV-3 genome) (Figure 1). The phylogenetic tree and pairwise distance analysis showed more than 98% of identity among the Spanish strains and also between the Spanish strains and other already published PCV-3 sequences. The oldest PCV-3 partial sequences obtained were from 1997 (GenBank references MG807066 for Rep gene and MG807078 for Cap gene), which shared a 98% nucleotide identity with the first PCV-3 sequence described in the USA in 2016 (KX898030.1). Although some Spanish clusters were identified (Figure 1), the sequences were globally scattered along the phylogenetic tree.

3.3 | Association between the presence of PCV-3, production phase and clinical/pathological conditions

The detection of PCV-3 genome split by production phase and clinical/pathological conditions is summarized in Table 2. No information about production phase and clinical/pathological condition was available for five of 75 PCV-3 PCR-positive pigs and 24 of 579 negative ones.

Overall, 36 of 298 nursery pigs (12.08%), 21 of 193 (10.88%) fatteners and 10 of 123 (8.13%) lactating pigs were PCV-3 PCR positive, and no statistically significant differences were observed among production phases ($p > .05$). None of the five sows tested were PCV-3 PCR positive; in contrast, three of six (50%) foetuses were positive.

According to the different clinical/pathological conditions, 17 of 126 (13.49%) pigs with respiratory disorders were PCV-3 PCR positive as well as 21 of 182 pigs (11.53%) with systemic problems and 12 of 103 pigs (11.65%) with gastrointestinal disorders. Likewise, the positivity rate was identified in six of 51 pigs (11.76%) with neurological and three of six (50%) with aborted foetuses. Also, four of 84 (4.76%) pigs included in the group of other conditions and seven of 73 (9.58%) asymptomatic animals were PCV-3 PCR positive. No statistically significant differences were observed among clinical/pathological conditions ($p > .05$).

Among PCV-3 PCR-positive animals, 37 were originally tested for PCV-2 and 45 for PRRSV. In total, nine of 37 (24.32%) and seven of 45 (15.55%) of the PCV-3 PCR-positive cases were also positive for PCV-2 and PRRSV, respectively.

4 | DISCUSSION

PCV-3 was first reported in 2016. Since then, many investigations have shown the presence of its genome in pig samples from different production phases, affected by a variety of pathological disorders, and also distributed in different countries worldwide. A recent study indicated the presence of PCV-3 genome as early as 2002 in tissue and faecal samples in Northern Ireland (Collins et al., 2017), suggesting that PCV-3 is not a new virus and has probably been circulating for a relatively extended period in pig populations around the world. The present retrospective investigation further confirms such a hypothesis; PCV-3 has been circulating in the Spanish pig population at least since 1996, the earliest year of detection of PCV-3 worldwide to date.

Compiled data along 22 years offered a mean PCV-3 PCR-positive rate of 11.47%. These data agree with the detection frequency found in Spanish serum samples (14 of 94; 15%) from a previous study, whilst they are slightly lower than those obtained in Italy (18.18%), Poland (25%), Denmark (30%), South Korea (44.20%) and China (59.46%) (Franzo, Legnardi, Hjulsager et al., 2018; Stadejek et al., 2017; Zheng et al., 2017). The study design of the mentioned works was different in relation to both the studied age categories and the PCR methodologies used, implying that a direct comparison of positivity frequency is not feasible. Therefore, the prevalence of the virus in the different geographical regions cannot be accurately stated at present.

The phylogenetic analysis of obtained sequences in the present retrospective study demonstrates a close distance between them and with the PCV-3 genomes available at the GenBank. Even if the genetic variability was globally low, the Spanish strains were intermingled amongst all currently available PCV-3 sequences in the phylogenetic tree. This result would suggest that PCV-3 has not shown a differentiated independent molecular evolution in the particular areas of the world where it has been detected so far. Moreover, the low genetic variability found in PCV-3 would point to a recent emergence (and consequently, a relatively short time to evolve) or, alternatively, to a slow evolution rate as has been found for PCV-1 (Cortey & Segalés, 2011). Further phylogenetic and evolutionary studies with higher numbers of PCV-3 sequences are needed to ascertain those hypotheses.

Some studies identified the presence of PCV-3 in several tissue samples or sera from pigs with different clinical presentations (Palinski et al., 2017; Tochetto et al., 2017; Zhai et al., 2017), as well as in healthy animals (Franzo, Legnardi, Hjulsager et al., 2018; Franzo, Legnardi, Centelleghe et al., 2018; Zheng et al., 2017). The data compiled in this study suggest a homogeneous PCV-3 frequency in the different production phases and clinical/pathological conditions, as no statistically significant differences were found among tested groups. Previous studies reported coinfections of PCV-3 with PCV-2 (Fu et al., 2017; Ku et al., 2017) and *Porcine reproductive and respiratory syndrome virus* (PRRSV) (Fu et al., 2017), two of the most important pathogens affecting pigs worldwide. In the studied animals, PCV-2 and PRRSV were investigated for diagnostic purposes attending to the demand of the submitting veterinarian. In total, 24.32% and 15.55% of the PCV-3 PCR-positive cases were also positive for PCV-2 and PRRSV, respectively. It is very likely that those percentages of coinfections reflect the relatively widespread nature of all these three viruses in Spain, rather than a potential synergism or association.

Noteworthy, at least for PCV-2, the link between clinical disease and the virus is associated with the viral load; the higher the viral load, the higher the probability of the animal being sick (Grau-Roma et al., 2009). Specifically, some reports have shown significant differences in PCV-2 load in serum samples, as well as in different tissues, between healthy and sick PCV-2-SD pigs; loads above 10^7 PCV2 genome copies/ml or higher have been usually detected in diseased animals (Brungborg, Moldal, & Jonassen, 2004; Olvera, Sibila, Calsamiglia, Segalés, & Domingo, 2004). Recently, Zhai et al. (2017) found lower qPCR Ct values in samples from animals with respiratory signs and diarrhoea compared to those of asymptomatic animals, suggesting that PCV-3 may be potentially associated with these clinical conditions. A recently described qPCR for PCV-3 detection (Franzo, Legnardi, Centelleghe et al., 2018) was attempted in the present study with a limited number of positive samples as an exploratory approach (data not shown). Results showed Ct values ranging from 24.23 to 39.04 (equivalent to $10^7$and 10^2 PCV-3 genome copies/ml of serum), but no apparent correlation with the clinical/pathological conditions was found. Definitively, more studies trying to confirm or rule out a potential relationship of PCV-3 or certain PCV-3 loads with disease in swine are needed.

In conclusion, the current study confirms PCV-3 circulation in the Spanish pig population with a low/moderate frequency, at least since 1996. The high identity among PCV-3 partial genome sequences indicates that this virus has remained relatively stable across the years. Preliminarily, PCV-3 infection did not appear to be linked to any specific pathological condition nor particular pig age group or production phase.

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CONFLICT OF INTEREST

All authors have declared no conflict of interest.

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