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Infection dynamics of Porcine circovirus 3 (PCV-3) in longitudinally sampled pigs from four Spanish farms

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Abstract:	Porcine circovirus 3 (PCV-3) is a recently discovered virus in domestic pigs and wild boar. The virus has been described in pigs with different clinical/pathological presentations and healthy animals, but the dynamics of infection is currently unknown. The aim of this study was to longitudinally monitor PCV-3 infection in 152 pigs from 4 different healthy farms (A, B, C and D) by means of PCR in serum. The selected animals were sampled five (farm A) or six (farms B-D) times from weaning until the end of the fattening period. PCV-3 genome was found in pigs from all tested ages and farms; few animals had an apparent long-term infection (4 to 23 weeks). PCV-3 frequency of detection remained fairly uniform along tested ages within farms A and C, but was more variable among sampling times in farms B and D. Eight partial genome sequences were obtained from six different animals. Phylogenetic tree and pairwise distance analysis showed high similarity among sequences and with available genomes from different countries. This is the first study on PCV-3 infection dynamics in longitudinally sampled pigs. Most pigs got infection during their life, although PCV-3 did not appear to be linked with any specific age.

Infection dynamics of *Porcine circovirus 3* (PCV-3) in longitudinally sampled pigs from four Spanish farms

Running title: Infection dynamics of PCV-3

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Summary

Porcine circovirus 3 (PCV-3) is a recently discovered virus in domestic pigs and wild boar. The virus has been described in pigs with different clinical/pathological presentations and healthy animals, but the dynamics of infection is currently unknown. The aim of this study was to longitudinally monitor PCV-3 infection in 152 pigs from 4 different healthy farms (A, B, C and D) by means of PCR in serum. The selected animals were sampled five (farm A) or six (farms B-D) times from weaning until the end of the fattening period. PCV-3 genome was found in pigs from all tested ages and farms; few animals had an apparent long-term infection (4 to 23 weeks). PCV-3 frequency of detection remained fairly uniform along tested ages within farms A and C, but was more variable among sampling times in farms B and D. Eight partial genome sequences were obtained from six different animals. Phylogenetic tree and pairwise distance analysis showed high similarity among sequences and with available genomes from different countries. This is the first study on PCV-3 infection dynamics in longitudinally sampled pigs. Most pigs got infection during their life, although PCV-3 did not appear to be linked with any specific age.

Keywords

Porcine circovirus 3 (PCV-3); dynamics; longitudinal; PCR; domestic pigs

1| INTRODUCTION

Recently, an emerging circovirus species was discovered and named *Porcine circovirus 3* (PCV-3) [1,2]. The newly described virus belongs to the family *Circoviridae*, genus *Circovirus* [3]. Circovirus virions have a non-enveloped, icosahedral structure containing a circular single-stranded DNA (ssDNA) molecule. Viral DNA includes two major opening reading frames (ORFs), which encode for capsid and replicase proteins [4,5].

PCV-3 is the third member of this genus able to infect swine. PCV-1 was the first described member of this family and is considered non-pathogenic for pigs [6–8]. In contrast, PCV-2 is associated with several clinical/pathological conditions and considered one of the most important pathogen of the pig industry causing important economic losses [9].

Since the first description in North America [1,2], many reports have identified PCV-3 in Europe [10–12], Asia [13–16] and South America [17,18], suggesting a worldwide circulation. Moreover, retrospective studies have shown PCV-3 circulation at least since the 1990s [19–21] and, according to phylogenetic analyses, the common ancestor was dated around 50 years ago [18,22]. The virus has also been detected recently in wild boar with fairly high prevalence, suggesting a potential role as reservoir for the domestic swine [23,24].

The first metagenomics analyses revealed PCV-3 genome in sows with porcine dermatitis and nephropathy disease (PDNS) and chronic reproductive failure[1]. Subsequently, PCV-3 was found in tissue homogenates in pigs with a causally unexplained myocarditis [2]. Thereafter, reports identified PCV-3 genome in nursery and fattening pigs with different clinical/pathological presentations as respiratory disorders [20,25] and in neonatal piglets with congenital tremors [26]. In addition, the genome was detected in apparently healthy sows and fattening pigs as well as in stillborns [11,25,27].

Based on current published data, it is not demonstrated whether PCV-3 infection is linked to a particular pathological condition or any specific age [19].

Based on available literature, it looks evident that PCV-3 is present in almost all pig ages (from fetuses to adults). However, a comprehensive study of the infection dynamics of this virus in a healthy pig population has not been described so far. Therefore, the aim of the present study was to longitudinally assess the dynamics of PCV-3 infection in a set of pigs from four clinically healthy conventional farms from Spain.

2| MATERIAL AND METHODS

2.1| Study design

Serum samples corresponding to 152 pigs from four selected clinically healthy conventional farms from Spain were chosen for this study (Table 1). Samples were collected longitudinally (sampling the same individual repeatedly) during years 2012 and 2016 for different study purposes [28–30]. In the first farm (Farm A), 34 piglets were sampled at 2, 8, 13, 18 and 24 weeks of age. In farm B, 44 piglets were sampled at 2, 7, 12, 18, 22 and 25 weeks of age. From farm C, 28 animals were followed up at 2, 6, 10, 14, 18 and 25 weeks. Finally, 46 piglets were sampled at 4, 8, 12, 16, 21 and 25 weeks of age from farm D. The weeks were grouped according to the production phase (lactation, from 1 to 4 weeks of age; nursery, from 5 to 9 weeks of age; and growing/fattening; >10 weeks of age) (Figure 1).

2.2| DNA extraction and specific polymerase chain reaction (PCR) for PCV-3 detection and sequencing

DNA was extracted from 200 µL of serum using MagMAX™ Pathogen RNA/DNA Kit (Applied Biosystems®) according to the manufacturer's protocol. Double distilled water and a plasmid containing the full-length PCV-3 genome included into a PCV-3 negative serum [31] were used as negative and positive controls, respectively.

To detect the presence of PCV-3 DNA in tested samples, a conventional PCR assay was performed based on a previous protocol described by Franzo and colleagues [31], with slight modifications. Three µL of extracted DNA were added to a PCR mix and amplified using the below described thermal protocol. The reaction was carried out in a final volume of 50 µL mixture containing 1x PCR Buffer, 400 µM of dNTPs, 0.2 µM of forward primer located in genomic positions 233-255 (5'-AAAGCCCGAAACACAGGTGGTGT-3'), 0.2 µM of reverse primer placed between nucleotide positions 742 and 718 (5'- TTTTCCCGACATCCTGGAGGACCAAT- 3'), one Unit of DNA polymerase Platinum™ SuperFi™ (Invitrogen™) and double distilled water. The PCR thermic protocol was 98°C for 5 min followed by 40 cycles of 94°C for 30 s, 58°C for 15 s, and 72°C for 1 min, and a final elongation at 68ºC for 7 min.

For sequencing purposes, the extracted DNA from PCV-3 PCR positive samples was amplified as described above, using as forward primer 5'-CACCGTGTGAGTGGATATAC- 3' and reverse primer 5'-CACCCCAACGCAATAATTGTA- 3' (located in the genomic positions 74-94 and 1,144-1,123, respectively) under the thermal conditions described by Fux and collaborator[32]. In order to increase the amount of amplicon to be sequenced the PCR products were re-amplified with the same protocol. All PCR products were electrophoretically separated on 1.2% TAE agarose gel. The PCV-3 PCR-positive samples were purified using NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel)

according to the manufacturer's protocols and the quality and quantity of genomic DNA was analysed with BioDrop DUO (BioDrop Ltd).

2.3| Sequence analyses

PCV-3 positive samples were selected and submitted to Sanger-sequencing, which was performed with BigDye® Terminator v3.1 Cycle Sequencing Kit, following the manufacturer's protocol at the Genomic and Bioinformatics Service of the *Universitat Autònoma de Barcelona* (Barcelona, Spain). The sequencing reactions were analysed using an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystem®)**.**

Sequences and chromatograms were manually explored to trim bad-quality bases with BioEdit 7.2 [33]. The assembly of the consensus sequences extracted from different fragments was attempted using DNASTAR Lasergene software [34]. The partial genomes obtained were aligned using Clustal Omega [35] with 74 complete genome sequences available at the GenBank (Supplementary Table 1) and trimmed accordingly for comparison purposes. A phylogenetic tree was constructed with the Maximum-Likelihood (ML) method based on the best predicted-substitution model (lowest BIC score) by means of the Tamura-Nei plus Gamma substitution model [36] using MEGA software version 7 [37]. The robustness of the clade was evaluated with 1,000 bootstrap replicates. The obtained sequences were deposited at the GenBank (references MH780665- MH780672).

2.4| Statistical analyses

Statistical analyses were performed using XLSTAT 365 Microsoft Excel 2016. To test for significant differences between weeks of age in each tested farm, the Fisher's exact test was performed. The significance level was set as 0.05.

3| RESULTS

3.1| PCV-3 detection by PCR

PCV-3 genome was detected in all tested farms and sampling points during the study period.

Overall, PCV-3 PCR positivity was found in 28 out of 34 (82.35%), 32 out of 44 (72.72%), 22 out of 28 (78.57%) and 34 out of 46 (71.74%) pigs in farms A, B, C and D, respectively. Results of the PCV-3 frequency of detection obtained by PCR in each age-group are summarised in Figure 1. Individual PCR results for each pig from each farm are displayed in Supplementary Table 2.

Globally, the PCV-3 positive percentage was fairly uniform within each tested farm (Figure 1). In farm A, PCV-3 DNA detection frequency ranged from 23.53% (8 out of 34 pigs) at the second sampling to 32.35% (11 out of 34 animals) at the last one. In farm B, PCV-3 genome presence varied from 9.09% (4 out of 44, first sampling) to 36.37% (15 out of 44, fifth sampling). Such frequency ranged from 10.71% (3 out of 28, fifth sampling) to 34.71% (10 out of 28, fourth sampling) in farm C, and from 6.52% (3 out of 46, third sampling) to 34.78% (16 out of 46, second sampling) in farm D. No statistically significant differences were found across the tested weeks of age ($p>0.05$) in farms A and C; however, differences in PCV-3 frequency were detected among tested ages in farms B and D (Figure 1).

In most of the cases, the detection of PCV-3 was either intermittent or found once in life (Supplementary Table 1). In farm A, 3 out of 28 (10.7%) animals showed infection intermittently and 10 animals (35.71%) had a continuous PCR-positive result during a period ranging from 5 to 22 weeks; only one pig was positive at all sampling times. In farm B, intermittent detection of PCV-3 was observed in 10 out of 44 animals (22.7%); 8 more pigs (18.18%) showed continuous PCR positivity during a period of 4 to 23 weeks; again, one of them was PCV-3 PCR positive at all sampling points. In farm C, 8 out of 28 (28.6%) animals had PCV-3 DNA in serum intermittently and only two more animals (7.14%) were positive during two consecutive samplings. Finally, in farm D, most pigs were PCV-3 PCR positive once during the study period (26 out of 46; 56.52%), 5 out of 46 (10.87%) had an intermittent detection of PCV-3 during a period from 5 to 17 weeks, and, finally, 3 more had continuous PCR PCV-3 detection ranging from 4 to 9 weeks. The numbers of animals PCV-3 PCR positive in more than one sampling are depicted in Table 2.

3.2| Sequence alignment and phylogenetic analysis

In total, 8 PCV-3 partial sequences were finally obtained across three tested farms (Farms B and C) corresponding to six different animals; from two of them, sequences at two sampling points were obtained. Sequences were retrieved from four farm B pigs at 12, 18, 22 and 18 plus 22 weeks of age, respectively, one farm C animal at 10 and 18 weeks and another at 25 weeks of age. The obtained sequences comprised part of the rep protein gene (954 nucleotides). The phylogenetic tree and pairwise distance demonstrated high similarity among obtained PCV-3 partial sequences and also with the corresponding sequence fragment of the complete Spanish genome from a domestic pig available at GenBank (>99%) (Figure 2). In fact, most sequences obtained from farm B (4 out of 5)

were identical to the one obtained from a 25 week-old pig from farm C, and clustered close to USA and China sequences. The two sequences from the same pig (10 and 18 weeks of age) of farm C were identical, and very close (99.9%) to the existing Spanish complete genome sequence from the GenBank from a domestic pig. One sequence from farm B clustered together with a German sequence, although nucleotide identity was >99% as well.

4| DISCUSSION

Several epidemiological reports have detected PCV-3 genome in pigs from all production phases, associated or not with pathological disorders [1,2,25–27]. However, the lack of an existing comprehensive approach on the dynamics of infection justified to carry out specific research on longitudinally sampled animals and assess how the virus is circulating in conventional healthy farms. Moreover, already published studies testing PCV-3 frequency in different age-groups are fragmented and comparisons are not possible since information came from different sources, farms and countries. Therefore, the present study represents the first approach to investigate the PCV-3 infection dynamics in the same subset of animals.

Obtained results confirmed that this virus is apparently widespread (at least in the four selected farms), able to infect pigs at all tested ages and to cause long-term infection in few animals. In fact, there was not a particular PCV-3 infection dynamics pattern that could be inferred from the frequency of detection in the four studied farms. The higher frequency of PCV-3 genome detection occurred at different time-points in the studied herds, which might be linked with the potential existence of maternally derived immunity or its duration.

However, while this might be the case for farms B, C and D (lower frequency of PCV-3 infection at early ages), a different situation was found in farm A, where a moderate percentage of infected piglets was already detected at 2 weeks of age (around 26%). It is possible that such amount of PCV-3 PCR positive pigs at early ages is related with intrauterine infections, but the fact that a low-moderate percentage of pigs were found PCV-3 infected at all tested ages poses certain discussion elements on how the pig immune system reacts against this virus. Definitively, further studies are needed to assess the circulation patterns of PCV-3 as well as to develop techniques to monitor the immune response against the virus, still lacking at present.

The most obvious comparison of PCV-3 infection dynamics is with that of PCV-2, another member of the *Circoviridae* family. In the specific case of this latter infectious agent, the virus is considered of ubiquitous nature [38] and can be found in different age groups. However, a distinct pattern of dynamics of infection is seen for PCV-2 in non-vaccinated farms, with usual low or very low prevalence during the lactating period, loss of maternally derived immunity between 6-10 weeks of age and subsequent peak of infection during the late nursery or early fattening period [39–41]. In general, the prevalence at the peak of infection can be rather high, being close to 90-100% of infected pigs in some cases [40,41], which is fairly different from current observations for PCV-3. An interesting point would have been the study of the infection status of sows, since at least for PCV-2 is known that infection at early ages is correlated with the percentage of infection in sows [41]. Sow sera were not available for the present study, but PCV-3 has already been detected in 29% of the tested serum from sows in farms located in Poland and 47.37% in Thailand [12,42].

In the present study a quantitative PCR described by Franzo and colleagues [31] was attempted in some of the PCV-3 positive samples (data not shown). High Ct values were

obtained in most of the cases, and the viral load was below the quantification limit of the PCR (10 copies of DNA/µL). These results are in agreement with studies that detected low amount of PCV-3 DNA in serum samples [12,25,32], which would suggest a subclinical infection. Moreover, this was probably the main reason why only a few number of PCV-3 sequences were obtained.

Phylogenetic analyses and pairwise distance estimation with the eight PCV-3 partial sequences obtained throughout this study demonstrated high similarity with the corresponding sequences available at GenBank. Moreover, the sequences from the same animal (farm C) at 10 and 18 weeks of age were identical, as well as the sequences from the animal (farm B) analyzed at 18 and 22 weeks. These results would suggest possible long-lasting or persistent infections of PCV-3 in some animals with the same viral variant. Taking into account the low number of sequences obtained, it was not possible to assess if more than one PCV-3 strain was circulating in the same animal over time. However, at least two different strains were detected in both farms B and C taking into account the phylogenetic distribution of obtained sequences, indicating that the potential circulation of more than one strain in the same farm and, eventually in the same animal, is feasible. In any case, all sequences obtained were very closely phylogenetically related, indicating the low variability found so far with PCV-3 in comparison with PCV-2, and further suggesting a much lower mutation rate of the novel virus compared with other circoviruses [19]. Importantly, the potential long-lasting or persistent infections seem to be relatively frequent based on obtained results; a variable percentage ranging from 6.5% (farm D) to 25% (farm B) of analyzed pigs were PCR positive during 3 or more samplings. Long duration of infection is rather typical of ssDNA viruses infecting swine such as PCV-2 [39,40] and Torque teno sus viruses [43,44].

Obtained partial sequences were very close each other although a broad mixing among sequences from Spain and different countries were found. However, in all cases the nucleotide identity among them was very high (>99%), suggesting that minimal variation does currently exist among PCV-3 strains. Of course, the complete genome would have been more accurate in order to distinguish potential different variants infecting the studied farms.

In summary, this is the first longitudinal study to assess the infection dynamics of PCV-3 in commercial healthy farms. Although a particular general infection dynamics pattern was not able to be ascertained, the obtained data confirmed that PCV-3 circulated in the chosen clinically healthy farms at all tested ages and most pigs got infection during their lifetime.

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CONFLICT OF INTEREST STATEMENT

All authors have declared no conflict of interest.

REFERENCES

1. PALINSKI R, PIÑEYRO P, SHANG P *et al*. A Novel Porcine Circovirus Distantly Related to Known Circoviruses Is Associated with Porcine Dermatitis

and Nephropathy Syndrome and Reproductive Failure. McFadden G, ed. *J Virol* 2017;91(1):e01879-16; doi:10.1128/JVI.01879-16

2. PHAN TG, GIANNITTI F, ROSSOW S *et al*. Detection of a novel circovirus PCV3 in pigs with cardiac and multi-systemic inflammation. *Virol J* 2016:13;184; doi:10.1186/s12985-016-0642-z

3. International Committee for the Taxonomy of Viruses, ICTV 2017. www.ncbi.nlm.nih.gov/ICTVdb/ (acessed 5 August 2018)

4. RITCHIE BW, NIAGRO FD, LATIMER KS *et al*. Ultrastructural, protein composition, and antigenic comparison of psittacine beak and feather disease virus purified from four genera of psittacine birdS. *J Wildl Dis* 1990;26(2):196-203; doi:10.7589/0090-3558-26.2.196

5. ROSARIO K, BREITBART M, HARRACH B *et al*. Revisiting the taxonomy of the family Circoviridae: establishment of the genus Cyclovirus and removal of the genus Gyrovirus. *Arch Virol* 2017;162; doi:10.1007/s00705-017-3247-y

6. TISCHER I, GELDERBLOM H, VETTERMANN W *et al*. A very small porcine virus with circular single-stranded DNA. *Nature* 1982;295(5844):64-66; doi:10.1038/295064a0.

7. TISCHER I, RASCH R, TOCHTERMANN G. Characterization of papovavirus- and picornavirus-like particles in permanent pig kidney cell lines. *Zentralbl Bakteriol Orig A* 1974;226(2):153—167

8. ALLAN GM, MCNEILLY F, CASSIDY JP *et al*. Pathogenesis of porcine circovirus; experimental infections of colostrum deprived piglets and examination of pig foetal material. *Vet Microbiol* 1995;44(1):49-64; doi:10.1016/0378-1135(94)00136-K

9. SEGALÉS J. Porcine circovirus type 2 (PCV2) infections: Clinical signs, pathology and laboratory diagnosis. *Virus Res* 2012;164(1):10-19; doi: 10.1016/j.virusres.2011.10.007

10. FACCINI S, BARBIERI I, GILIOLI A *et al*. Detection and genetic characterization of Porcine circovirus type 3 in Italy. *Transbound Emerg Dis* 2017;64(6):1661-1664; doi:10.1111/tbed.12714

11. FRANZO G, LEGNARDI M, HJULSAGER CK *et al*. Full-genome sequencing of porcine circovirus 3 field strains from Denmark, Italy and Spain demonstrates a high within-Europe genetic heterogeneity. *Transbound Emerg Dis* 2018;65(3):602-606; doi:10.1111/tbed.12836

12. STADEJEK T, WOŹNIAK A, MIŁEK D *et al*. First detection of porcine circovirus type 3 on commercial pig farms in Poland. *Transbound Emerg Dis* 2017;64(5):1350-1353; doi:10.1111/tbed.12672

13. KU X, CHEN F, LI P, *et al*. Identification and genetic characterization of porcine circovirus type 3 in China. *Transbound Emerg Dis* 2017;64(3):703-708; doi:10.1111/tbed.12638

14. KWON T, YOO SJ, PARK CK *et al*. Prevalence of novel porcine circovirus 3 in Korean pig populations. *Vet Microbiol* 2017;207:178-180; doi:10.1016/j.vetmic.2017.06.013

15. SHEN H, LIU X, ZHANG P, *et al*. Genome characterization of a porcine circovirus type 3 in South China. *Transbound Emerg Dis* 2017; doi:10.1111/tbed.12639

16. HAYASHI S, OHSHIMA Y, FURUYA Y *et al*. First detection of porcine circovirus type 3 in Japan. *J Vet Med Sci* 2018;80(9):1468-1472; doi:10.1292/jvms.18-0079

17. TOCHETTO C, LIMA DA, VARELA APM *et al*. Full-Genome Sequence of Porcine Circovirus type 3 recovered from serum of sows with stillbirths in Brazil. *Transbound Emerg Dis* 2017; doi:10.1111/tbed.12735

18. SARAIVA GL, VIDIGAL PMP, FIETTO JLR *et al*. Evolutionary analysis of Porcine circovirus 3 (PCV3) indicates an ancient origin for its current strains and a worldwide dispersion. *Virus Genes* 2018;54(3):376-384; doi:10.1007/s11262-018-1545-4

19. KLAUMANN F, FRANZO G, SOHRMANN M *et al*. Retrospective detection of Porcine circovirus 3 (PCV-3) in pig serum samples from Spain. *Transbound Emerg Dis* 2018;0(0); doi:10.1111/tbed.12876

20. SUN J, WEI L, LU Z *et al*. Retrospective study of porcine circovirus 3 infection in China. *Transbound Emerg Dis* 2018;65(3):607-613; doi:10.1111/tbed.12853

21. YE X, BERG M, FOSSUM C *et al*. Detection and genetic characterisation of porcine circovirus 3 from pigs in Sweden. *Virus Genes* 2018;54(3):466-469; doi:10.1007/s11262-018-1553-4

22. FU X, FANG B, MA J *et al*. Insights into the epidemic characteristics and evolutionary history of the novel porcine circovirus type 3 in southern China. *Transbound Emerg Dis* 2017; doi:10.1111/tbed.12752

23. FRANZO G, TUCCIARONE CM, DRIGO M *et al*. First report of wild boar susceptibility to Porcine circovirus type 3: High prevalence in the Colli Euganei Regional Park (Italy) in the absence of clinical signs. *Transbound Emerg Dis* 2018;0(0); doi:10.1111/tbed.12905

24. KLAUMANN F, DIAS-ALVES A, CABEZÓN O *et al*. Porcine circovirus 3 is highly prevalent in serum and tissues and may persistently infect wild boar (Sus scrofa scrofa). *Transbound Emerg Dis* 2018;0(0); doi:10.1111/tbed.12988

25. ZHAI S-L, ZHOU X, ZHANG H *et al*. Comparative epidemiology of porcine circovirus type 3 in pigs with different clinical presentations. *Virol J* 2017;14(1):222; doi:10.1186/s12985-017-0892-4

26. CHEN GH, MAI KJ, ZHOU L *et al*. Detection and genome sequencing of porcine circovirus 3 in neonatal pigs with congenital tremors in South China. *Transbound Emerg Dis* 2017;64(6):1650-1654; doi:10.1111/tbed.12702

27. ZHENG S, WU X, ZHANG L *et al*. The occurrence of porcine circovirus 3 without clinical infection signs in Shandong Province. *Transbound Emerg Dis* 2017;64(5):1337-1341; doi:10.1111/tbed.12667

28. FRAILE L, SIBILA M, NOFRARÍAS M *et al*. Effect of sow and piglet porcine circovirus type 2 (PCV2) vaccination on piglet mortality, viraemia, antibody titre and production parameters. *Vet Microbiol* 2012;161(1):229-234; doi:

10.1016/j.vetmic.2012.07.021

29. OLIVER-FERRANDO S, SEGALÉS J, LÓPEZ-SORIA S *et al*. Evaluation of natural porcine circovirus type 2 (PCV2) subclinical infection and seroconversion dynamics in piglets vaccinated at different ages. *Vet Res* 2016;47:121; doi:10.1186/s13567-016-0405-2

30. FENG H, SEGALÉS J, FRAILE L *et al*. Effect of high and low levels of maternally derived antibodies on porcine circovirus type 2 (PCV2) infection dynamics and production parameters in PCV2 vaccinated pigs under field conditions. *Vaccine* 2016;34(27):3044-3050; doi: 10.1016/j.vaccine.2016.04.088

31. FRANZO G, LEGNARDI M, CENTELLEGHE C *et al*. Development and validation of direct PCR and quantitative PCR assays for the rapid, sensitive, and economical detection of porcine circovirus 3. *J Vet Diagnostic Investig* 2018:1040638718770495; doi:10.1177/1040638718770495

32. FUX R, SÖCKLER C, LINK EK *et al*. Full genome characterization of porcine circovirus type 3 isolates reveals the existence of two distinct groups of virus strains. *Virol J*. 2018;15:25; doi:10.1186/s12985-018-0929-3

33. HALL TA. Bioedit: A User-Friendly Biological Sequence Alignment Editor and Analysis Program for Windows 95/98/NT. *Nucleic Acids Symp Ser* 1999;41:95-98.

34. BURLAND TG. DNASTAR's Lasergene Sequence Analysis Software BT - Bioinformatics Methods and Protocols. In: Misener S, Krawetz SA, eds. Totowa, NJ: Humana Press; 1999:71-91; doi:10.1385/1-59259-192-2:71

35. THOMPSON JD, GIBSON TJ, PLEWNIAK F *et al*. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 1997;25(24):4876-4882.

36. TAMURA K, NEI M. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol* 1993;10(3):512-526; doi: 10.1093/oxfordjournals.molbev.a040023.

37. KUMAR S, STECHER G, TAMURA K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Mol Biol Evol* 2016;33(7):1870-1874; doi:10.1093/molbev/msw054.

38. SEGALÉS J, ALLAN GM, DOMINGO M. Porcine circovirus diseases. *Anim Heal Res Rev* 2005;6. doi:10.1079/AHR2005106

39. LAROCHELLE R, MAGAR R, D'ALLAIRE S. Comparative serologic and virologic study of commercial swine herds with and without postweaning multisystemic wasting syndrome. *Can J Vet Res* 2003;67

40. SIBILA M, CALSAMIGLIA M, SEGALES J *et al*. Use of a polymerase chain reaction assay and an ELISA to monitor porcine circovirus type 2 infection in pigs from farms with and without postweaning multisystemic wasting syndrome. *Am J Vet Res* 2004;65; doi:10.2460/ajvr.2004.65.88

41. GRAU-ROMA L, HJULSAGER CK, SIBILA M *et al*. Infection, excretion and seroconversion dynamics of porcine circovirus type 2 (PCV2) in pigs from post-weaning multisystemic wasting syndrome (PMWS) affected farms in Spain and

Denmark. *Vet Microbiol* 2009;135; doi:10.1016/j.vetmic.2008.10.007

42. KEDKOVID R, WOONWONG Y, ARUNORAT J *et al*. Porcine circovirus type 3 (PCV3) shedding in sow colostrum. *Vet Microbiol* 2018;220:12-17; doi: 10.1016/j.vetmic.2018.04.032

43. SIBILA M, MARTÍNEZ-GUINÓ L, HUERTA E *et al*. Torque teno virus (TTV) infection in sows and suckling piglets. *Vet Microbiol* 2009;137(3):354-358; doi: 10.1016/j.vetmic.2009.01.008

44. NIETO D, ARAMOUNI M, SIBILA M *et al.* Lack of effect of piglet vaccination against Porcine circovirus type 2 (PCV2) on serum viral loads of Torque teno sus virus 2 (TTSuV2). *Vet Microbiol* 2012;157(1):8-12; doi: 10.1016/j.vetmic.2011.11.028

FIGURE LEGENDS

FIGURE 1 Percentage of PCV-3 frequency on tested farms distributed according to the analysed weeks of age and production periods for farms A, B, C and D.

FIGURE 2 Phylogenetic tree of PCV-3 based on the partial genomes obtained from pigs longitudinally sampled and the corresponding sequences from PCV-3 full genomes available at GenBank. The phylogenetic tree was constructed using the maximum-likelihood algorithm of MEGA 7 Software with 1,000 bootstraps replicates. The obtained sequences of the present study have been coloured in red.

TABLE 1 Production system, farm size and vaccination programs applied in piglets and sows in the farms under study.

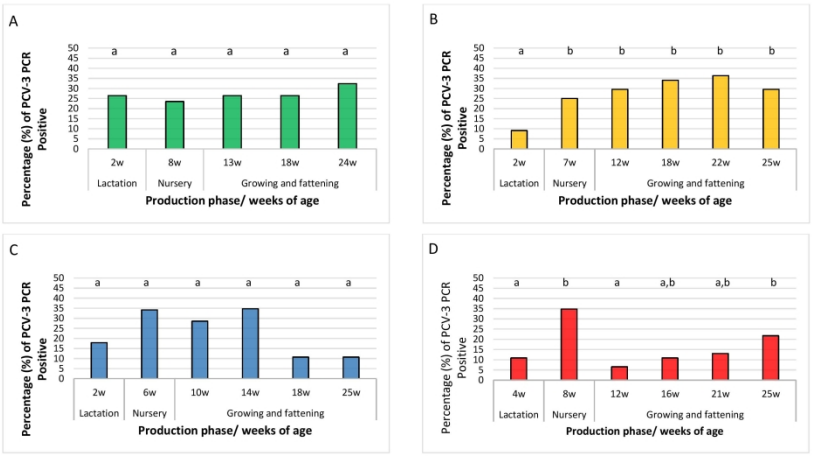
Farm ID	Production system	Herd size	Sow vaccination program*	Piglet vaccination program*
Farm A	Two-site, AI-AO	1,800 sows	ADV, PPV, Ery, EC, CP, PRRSV	PCV-2, Mhyo
Farm B	Multi-site, AI-AO	3,300 sows	ADV, PPV, Ery, EC, CP	PCV-2, Mhyo
Farm C	Two-site, AI-AO	800 sows	ADV, PPV, Ery, EC, CP, PRRSV, SIV	Mhyo
Farm D	Two-site, AI-AO	1,500 sows	ADV, PPV, Ery, EC, CP, PRRSV	Mhyo

AI-AO: all in-all out management practices

*ADV: *Aujeszky's disease virus*; PRRSV: *Porcine reproductive and respiratory syndrome virus*; PPV: *Porcine parvovirus*; PCV-2: *Porcine circovirus* 2; SIV: *Swine influenza virus*; Ery: *Erysipelothrix rhusiopathiae*; Mhyo: *Mycoplasma hyopneumoniae*; EC: *Escherichia coli*; CP: *Clostridium perfringens*

TABLE 2 Number and percentage of PCV-3 PCR positive and negative pigs during all the study period and number of PCV-3 PCR positive pigs during 1, 2, 3 and 4 or more sampling times.

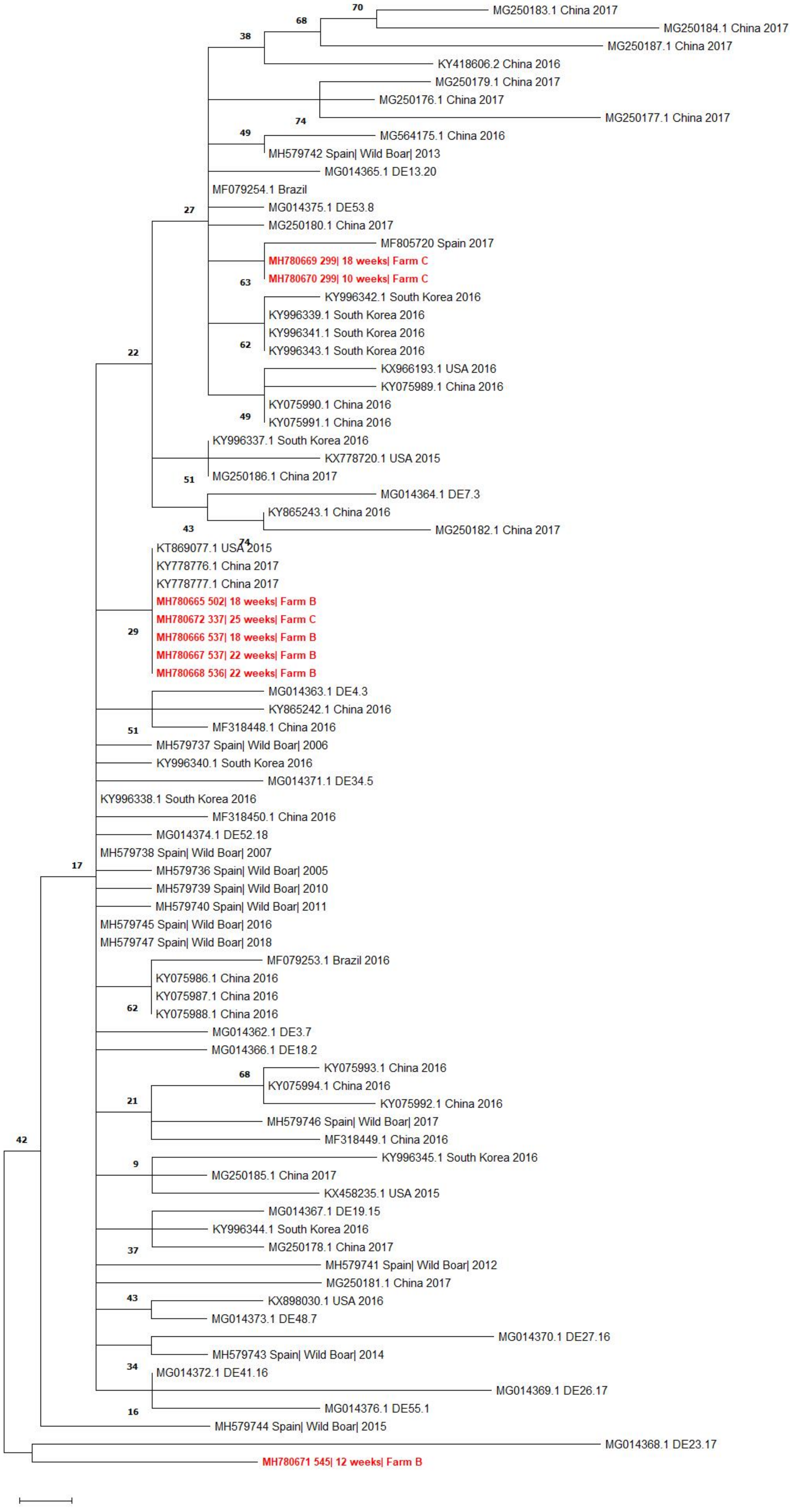
	PCV-3 PCR positive pigs along the study period (%)	PCV-3 PCR positive pigs at 1 sampling (%)	PCV-3 PCR positive pigs at 2 samplings (%)	PCV-3 PCR positive pigs at 3 samplings (%)	PCV-3 PCR positive pigs at ≥4 samplings (%)	Pigs PCV-3 PCR negative at all samplings (%)
Farm A	28/34 (82.35%)	15/34 (44.12%)	10/34 (29.41%)	2/34 (5.88%)	1/34 (2.94%)	6/34 (17.65%)
Farm B	32/44 (72.73%)	14/44 (31.82%)	7/44 (15.91%)	3/44 (6.82%)	8/44 (18.18%)	12/44 (27.27%)
Farm C	22/28 (78.57%)	12/28 (42.86%)	6/28 (21.43%)	3/28 (10.71%)	1/28 (3.57%)	6/28 (21.43%)
Farm D	34/46 (73.91%)	26/46 (56.52%)	5/46 (10.87%)	3/46 (6.52%)	0/46 (0%)	12/46 (26.09%)



Different letters in superscript mean statistically significant differences ($p<0.05$) among different tested weeks of age.

FIGURE 1 Percentage of PCV-3 frequency on tested farms distributed according to the analysed weeks of age and production periods. A= Farm A; B= Farm B; C= Farm C; D= Farm D.

297x210mm (300 x 300 DPI)


70
MG250183.1 China 2017
68
MG250184.1 China 2017
38
MG250187.1 China 2017
KY418606.2 China 2016
MG250179.1 China 2017
MG250176.1 China 2017
MG250177.1 China 2017
74
49
MG564175.1 China 2016
MH579742 Spain| Wild Boar| 2013
MG014365.1 DE13.20
MF079254.1 Brazil
27
MG014375.1 DE53.8
MG250180.1 China 2017
MF805720 Spain 2017
MH780669 299| 18 weeks| Farm C
MH780670 299| 10 weeks| Farm C
63
KY996342.1 South Korea 2016
KY996339.1 South Korea 2016
KY996341.1 South Korea 2016
62
KY996343.1 South Korea 2016
22
KX966193.1 USA 2016
KY075989.1 China 2016
KY075990.1 China 2016
49
KY075991.1 China 2016
KY996337.1 South Korea 2016
KX778720.1 USA 2015
51
MG250186.1 China 2017
MG014364.1 DE7.3
KY865243.1 China 2016
43
MG250182.1 China 2017
74
KT869077.1 USA 2015
KY778776.1 China 2017
KY778777.1 China 2017
MH780665 502| 18 weeks| Farm B
MH780672 337| 25 weeks| Farm C
29
MH780666 537| 18 weeks| Farm B
MH780667 537| 22 weeks| Farm B
MH780668 536| 22 weeks| Farm B
MG014363.1 DE4.3
KY865242.1 China 2016
MF318448.1 China 2016
51
MH579737 Spain| Wild Boar| 2006
KY996340.1 South Korea 2016
MG014371.1 DE34.5
KY996338.1 South Korea 2016
MF318450.1 China 2016
MG014374.1 DE52.18
MH579738 Spain| Wild Boar| 2007
17
MH579736 Spain| Wild Boar| 2005
MH579739 Spain| Wild Boar| 2010
MH579740 Spain| Wild Boar| 2011
MH579745 Spain| Wild Boar| 2016
MH579747 Spain| Wild Boar| 2018
MF079253.1 Brazil 2016
KY075986.1 China 2016
KY075987.1 China 2016
62
KY075988.1 China 2016
MG014362.1 DE3.7
MG014366.1 DE18.2
68
KY075993.1 China 2016
KY075994.1 China 2016
21
KY075992.1 China 2016
MH579746 Spain| Wild Boar| 2017
MF318449.1 China 2016
42
KY996345.1 South Korea 2016
9
MG250185.1 China 2017
KX458235.1 USA 2015
MG014367.1 DE19.15
KY996344.1 South Korea 2016
MG250178.1 China 2017
37
MH579741 Spain| Wild Boar| 2012
MG250181.1 China 2017
43
KX898030.1 USA 2016
MG014373.1 DE48.7
MG014370.1 DE27.16
MH579743 Spain| Wild Boar| 2014
34
MG014372.1 DE41.16
MG014369.1 DE26.17
MG014376.1 DE55.1
16
MH579744 Spain| Wild Boar| 2015
MG014368.1 DE23.17
MH780671 545| 12 weeks| Farm B
0.001