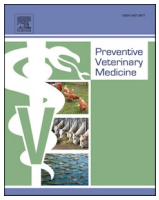
Preventive Veterinary Medicine 216 (2023) 105943

Contents lists available at ScienceDirect

Preventive Veterinary Medicine

journal homepage: www.elsevier.com/locate/prevetmed



Into the backyard: Multiple detections of PCV-2e in rural pig farms of Northern Italy. An unexpected ecological niche?

Giulia Faustini [a], Claudia Maria Tucciarone [a], Matteo Legnardi [a], Laura Grassi [a], Giacomo Berto [b], Michele Drigo [a], Mattia Cecchinato [a], Giovanni Franzo [a,*]

[a] Department of Animal Medicine, Production and Health (MAPS), University of Padua, Viale dell'Università 16, Legnaro 35020, PD, Italy
[b] Dipartimento di Prevenzione, AULSS 8 Berica, Via Giovanni Giuseppe Cappellari 6, Vicenza 36100, Italy

ARTICLE INFO

Keywords:
Italy
PCV-2e
Phylogenesis
Rural pigs
Vaccine

ABSTRACT

Porcine circovirus type 2 (PCV-2) is among the most burdensome viruses of the swine industry globally. Several genotypes have been periodically emerging, but just three of them (PCV-2a, PCV-2b, and PCV-2d) seem to circulate worldwide and be associated with the disease. Conversely, the spatial-temporal distribution of minor genotypes appears limited and their clinical relevance is still unclear. Recently PCV-2e was incidentally detected for the first time in Europe in a breeding farm in Northeastern Italy, while no connection could be established with countries where this genotype had been previously detected. To investigate circulating genotypes in the neglected rural context and provide a comparison with the most explored industrial context, a molecular survey was performed on samples collected in rural (n = 72) and industrial farms (n = 110) located in the same geographic area.

Phylogenetic analysis surprisingly evidenced PCV-2e circulation only in pigs reared in backyard farms (n = 5), while major genotypes (PCV-2a, −2b, −2d) circulate in both rearing contexts. However, the close genetic similarity between the herein detected PCV-2e strains and the previously reported one testify that, although unusual, such rural-to-industrial strains exchange affected also PCV-2e. The greater genetic and phenotypic diversity of PCV-2e genotype compared to other ones might threaten the protection granted by current vaccines. The present study suggests the rural context as an ecological niche for the circulation of PCV-2e, and even of other minor genotypes. PCV-2e detection in pigs with outdoor access further stresses the epidemiological role of backyard farms as interfaces for pathogen introduction, potentially ascribable to the different rearing approaches, lower managerial and biosecurity capabilities, and easier contacts with wildlife.

1. Introduction

Porcine circovirus type 2 (PCV-2) is one of the most relevant pathogens of the swine industry globally and it is responsible for severe economic losses and not-negligible control measures costs (Alarcon et al., 2013). PCV-2 is associated with several clinical and sub-clinical conditions, collectively named porcine circovirus diseases (PCVD), in which it may either act as the primary aetiological agent or, more commonly, in concert with other determinants of disease (Segalés et al., 2005).

PCV-2 belongs to the family of *Circoviridae* with a circular single-stranded DNA genome approximately 1.7 kb in length, which contains at least 6 ORFs (Li et al., 2018; Lv et al., 2014). The capsid protein (Cap) is the PCV-2 dominant immunogenic antigen, and it is encoded by ORF2, the gene with the highest genetic variability and thus the reference for molecular epidemiological studies and genotype characterization (Franzo et al., 2016a; Nawagitgul et al., 2000).

Different viral strains emerged in the last decades and, according to the classification proposed in 2018 by Franzo and Segalés, nine genotypes are currently recognized (from PCV-2a to −2 h) (Franzo and Segalés, 2018; Wang et al., 2020). PCV-2a, − 2b, and − 2d are the most persistently widespread genotypes, although some major changes have been observed in their prevalence over the years: after a first "genotype shift" from PCV-2a as the most prevalent to PCV-2b in mid-2000 s, a second shift has occurred from PCV-2b to PCV-2d (Franzo et al., 2016a; Xiao et al., 2015). This phenomenon might have been associated to the worldwide use of PCV-2 vaccines (Franzo et al., 2016b).

The factual clinical relevance and wide distribution observed for the

* Corresponding author.
E-mail address: giovanni.franzo@unipd.it (G. Franzo).

https://doi.org/10.1016/j.prevetmed.2023.105943
Received 4 January 2023; Received in revised form 6 May 2023; Accepted 14 May 2023
Available online 18 May 2023

main genotypes contrast with the unclear pathogenicity (Franzo and Segalés, 2020) and scattered detection of other minor genotypes. Indeed their distribution appears restricted to certain geographical areas and time periods, without evidence of clear spatial-temporal epidemiological flows.

Among the minor PCV-2 genotypes, PCV-2e is of particular interest since its genetic and phenotypic divergence (Franzo and Segalés, 2018) may potentially jeopardize the effectiveness of current diagnostic methods and commercial vaccines (Franzo and Segalés, 2020; Karuppannan and Opriessnig, 2017).

Previously identified in the USA, Mexico, Japan, China, and South Korea (Davies et al., 2016; Harmon et al., 2015; Liu et al., 2018; Park et al., 2020), PCV-2e has been recently detected in Europe, in a breeding farm in Northeastern Italy in absence of clinical signs (Franzo et al., 2022). This incidental finding raised questions about PCV-2e introduction and paved the research path toward possible niches where this genotype may circulate.

The often-unexplored rural context, with its management peculiarities, represents a reality where uncommon circulating strains could find favoring conditions. In Italy, one of the major pigs producing countries, the swine market is mainly ruled by large commercial holdings, while backyard farming accounts for a minor share of the pig population (Augere-Granier and Members' Research Service, 2020). Italian intensive pig farms are mainly located in Northern Italy (https://www.istat.it/), where the few small family backyard farms represent an exception, only supplying local markets and/or domestic consumption needs.

The backyard breeding system management is extremely heterogeneous, but some elements are recurrent: low number of animals, no age compartmentalization, presence of animals purchased as adults from different industrial farms with no further vaccinations, poor effective biosecurity measures and outdoor access. Despite their marginal economic role and because of these inherently peculiar features, rural farms are pivotal epidemiologic observatories and interfaces, providing a different outlook on the determinants of pathobiology, spread and diversity of already known circulating infectious agents, and a potential privileged site for the identification of emerging ones (Cadenas-Fernández et al., 2019; Wiethoelter et al., 2015).

In the present study, an epidemiological survey was conducted on pigs reared in both rural and intensive farms in Northeastern Italy, to assess potential differences in circulating genotypes and explore alternative viral introduction flows between the two breeding environments.

2. Materials and methods

2.1. Sampling

Samples were obtained from animals, backyard and intensively raised, that were located in the same areas, productively interconnected, where PCV-2e was initially described (Franzo et al., 2022).

Rural samples originated from pigs reared in small family backyard farms (hereafter referred as rural farms) belonging to different municipalities of Northeastern Italy and regularly slaughtered for home consumption or the local market at the end of 2021 and during 2022. The rural farms were randomly selected among those delivering animals to the involved slaughterhouses, which acted as sampling sites. Sampled animals were visited ante-mortem, and post-mortem inspection was conducted on their organs. Lungs and lymph nodes were collected from animals that were randomly selected at the slaughterhouses by the official veterinarians. Farm location and sampling date were recorded. Lungs and lymph nodes from rural pigs were processed homogenizing both tissue specimens, including lesions when present, adding 10 ML of PBS 1X (phosphate buffer saline) for gram of tissue.

PCV-2 positive archived samples collected by private companies in the same geographic area from pig reared in intensive farms (hereafter also called industrial farms) were included in the study. The samples (i.e., lymph nodes, lungs, oral fluid, blood, or serum) were routinely collected between 2019 and 2022 for monitoring activities or from pigs showing clinical signs ascribable to PCV-2 infection. PCV-2 identification was initially performed by the private companies' laboratories with a real-time PCR assay performed using PCRmax DNA Porcine Circovirus 2 kit (Cole-Parmer Srl, Cernusco sul Naviglio, Milan, Italy). A subset of positive samples was randomly selected from a broader collection and subsequently processed for molecular characterization.

All biological samples were stored at − 80 °C until DNA extraction.

2.2. PCV-2 extraction and detection

For both rural and industrial sample sets, DNA was extracted from 100 μL of sample homogenate using the Viral DNA/RNA kit (A&A Biotechnology, Gdansk, Poland) according to the manufacturer's instructions. Extracted DNA was stored at − 80 °C until further processing.

Samples from rural pigs were screened for PCV-2 with an in-house designed real-time PCR using DyNAmo Flash Probe qPCR Kit (Thermo Fisher Scientific, Waltham, MA, USA) as described in Franzo et al. (2020a).

ORF2 amplification was attempted both on rural and intensive farm positive samples using different primer pairs previously designed (Franzo et al., 2015). Biometra TAdvanced® Thermal Cycler (Analytik Jena GmbH, Jena, Germany) and Invitrogen™ Platinum™ II Taq Hot-Start DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA) were used to perform all the PCRs. Each reaction was performed on a total volume of 25 μL of a standard reaction mix containing 5 μL of DNA, 1X Platinum™ II PCR buffer, 0.6 μM of each primer, 0.2 mM of each dNTP, and one unit of Platinum™ Taq Hot-Start DNA Polymerase, following the thermal protocol described by the kit manual. Amplification and specificity of bands were checked by SYBR safe stained agarose gel electrophoresis, and all positive amplicons were purified using Applied Biosystems® ExoSAP-IT PCR Product Cleanup Reagent (Thermo Fisher Scientific, Waltham, MA, USA). The same PCR primers were used for Sanger sequencing of the amplicons in both directions at Macrogen Europe (Milan, Italy).

2.3. Sequence analysis

Chromatogram quality was evaluated using 4Peaks (Nucleobytes B. V., Aalsmer, the Netherlands) and consensus sequences assembly was performed with ChromasPro Version 2.0.0 (Technelysium Pty Ltd, South Brisbane, Australia). Sequences with multiple double peaks, suggestive of different strains co-infection, were excluded from the alignment, whereas those with only one double peak were duplicated, obtaining one sequence variant per each of the two nucleotides called.

All the obtained complete ORF2 sequences were aligned using the MUSCLE method implemented in MEGA X (Edgar, 2004; Kumar et al., 2018), and trimmed according to the reference dataset as suggested by Franzo and Segalés (Franzo and Segalés, 2018) for genotype characterization. Complete ORF2 sequences were then checked for recombination using the Genetic Algorithm for Recombination Detection method (GARD) implemented in Datamonkey (Kosakovsky Pond et al., 2006; Weaver et al., 2018).

A maximum likelihood (ML) phylogenetic tree was reconstructed using MEGA X, selecting the substitution model with the lowest Bayesian Information Criterion (BIC). To assess the robustness of the inferred clades, 1000 bootstrap replicates were performed. To broaden the dataset, a dedicated analysis was performed using the same approach including strains for which only partial ORF2 sequences were obtained.

Additionally, two different sets of aligned complete sequences were created, one for each rearing system (i.e., rural and industrial), and related pairwise p-distances were calculated using MEGA X. The alignments including all the obtained complete sequences, only the industrial strains, and only the rural strains were also investigated for episodic diversifying selection using Mixed Effects Model of Evolution (MEME)

(Murrell et al., 2012), with a significance value set to $p < 0.1$. To compare the genetic diversity of PCV-2 strains circulating in the industrial and rural context, MEME analysis was also performed excluding other genotypes from respective alignments.

2.4. Sequence accession numbers

Complete ORF2 sequences obtained were submitted to NCBI GenBank under the accession numbers OP899444 - OP899547. Partial ORF2 sequences are available with the accession numbers OP899548 - OP899564. The pairs of sequences with accession numbers OP899448/OP899449, OP899461/OP899462, OP899467/OP899468, OP899484/OP899485, OP899497/OP899498, OP899553/OP899555 are the result of duplication of the original sequence obtained from the same respective animal, performed because of the presence of one single mismatch. Accession numbers of rural strains are reported in Table S1.

3. Results

A total of 182 samples were included in the study: 72 samples were collected from family backyard farms (n = 45) and 110 were selected among PCV-2 positive archived samples collected in intensive farms (n = 65). All the farms were located in Northern Italy. In particular, the industrial farms were located in 3 different regions: Veneto (n = 48), Lombardia (n = 13), Emilia-Romagna (n = 4). The rural farms were sampled from the same regions: Veneto (n = 39), Lombardia (n = 5), Emilia-Romagna (n = 1).

In sampled rural animals, no clinical signs were observed at antemortem visit. Although no precise record was available for each sample since they were obtained at the slaughterhouse, all carcasses were considered adequate for human consumption, therefore according to the Italian legislation no acute or severe lesions were present, and chronic pneumonitis, pleuritis and pericarditis were the most commonly observed lesions according to the veterinarians. Out of the 72 rural samples, 28 (38.9%) tested positive to PCV-2 at real-time PCR screening (Ct values are reported in Table S1), of which 25 (89.3%) were confirmed as positive at PCR. After sequencing, 22 complete and 2 partial ORF2 sequences were obtained. The PCR positive samples originated from 19 different farms, while the sequences obtained belonged to 18 of them.

Out of the 110 PCV-2 positive archived samples from intensive farms, 99 (90.0%) samples belonging to 57 farms were confirmed as positive at PCR. After sequencing, 77 complete and 14 partial ORF2 sequences were obtained. Five complete sequences and one partial sequence had only one double peak. They were therefore duplicated, leading to a total number of 82 complete and 15 partial ORF2 sequences from a total of 54 industrial farms. All sequences (n = 6) having a single double peak belonged to the same genotype (Fig. S1 and S2).

Both in rural and industrial sample sets, sequences with multiple double peaks were excluded (n = 1 rural sequence; n = 3 industrial sequences).

ML phylogenetic tree reconstruction allowed genotype characterization of obtained complete sequences according to the classification proposed by Franzo and Segalés in 2018 (Fig. 1 and S1). An additional ML phylogenetic tree including also partial sequences was generated (Fig. S2).

Among the 24 obtained rural strains, 3 (12.5%) belonged to genotype PCV-2a, 1 (4.2%) to genotype PCV-2b, 13 (54.2%) to genotype PCV-2d, 7 (29.2%) to genotype PCV-2e. For the majority of the rural farms (n = 14) only one sequence was obtained, hence only one genotype was found. More than one sequence were obtained only from four farms: two and one farms were positive multiple times to genotype − 2d and − 2e at different dates, respectively. Only one farm resulted positive to two genotypes (−2d and −2e). All the PCV-2e strains originated from Veneto region, 3 were collected from the same farm, located in Verona province; while the remaining 4 originated from 4 different farms located in a neighboring province, Vicenza (Table S1).

The classification of the 97 industrial strains identified only 3

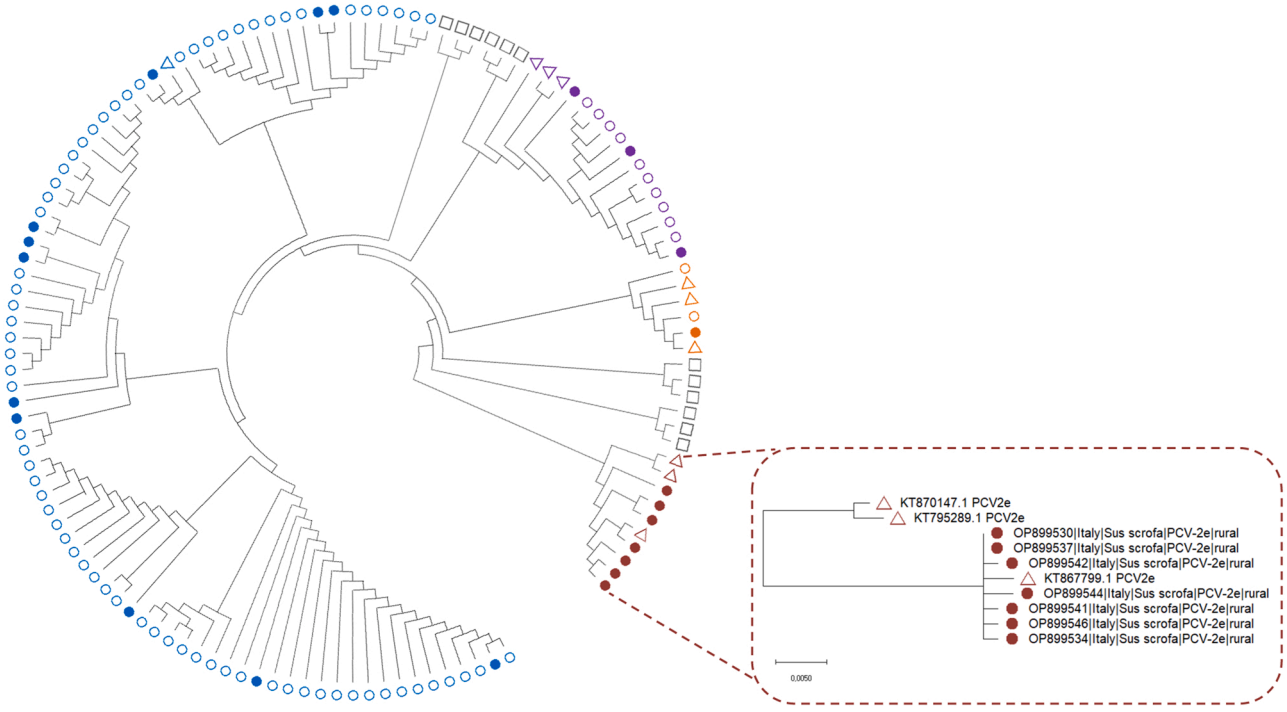


Fig. 1. Maximum likelihood phylogenetic tree estimated on the complete ORF2 alignment of PCV-2 strains obtained in the present study, marked with a circle. Rural sequences are color-filled. Reference sequences whose genotype have been detected in the present study have been marked with a triangle, while those not identified (−2c, −2 f, −2 g, −2 h) with a rectangle. Genotypes have been color coded: PCV-2a in purple, − 2b in orange, − 2d in blue, and − 2e in burgundy. PCV-2e strains are magnified in the right insert with the branch scale. Accession number, country, collection host, genotype and rearing system are reported for these sequences. Detailed information is reported for all obtained sequences in Fig. S1, in addition to bootstrap support.

genotypes, i.e. 11 (11.3%) PCV-2a, 3 (3.1%) PCV-2b and 83 (85.6%) PCV-2d. Out of the 54 farms from which sequences could be obtained, more than one sequence was obtained from 18: 12 farms resulted positive only to − 2d genotype, and 6 farms to two genotypes. In particular, 4 farms were positive to genotype − 2a and − 2d, one to − 2b and − 2d, and one to − 2a and − 2b. In the farms where genotype − 2d was identified with others, and sequence were more than 2 (n = 4), genotype − 2d was the predominant one.

No recombination events were identified.

The genetic distance of the PCV-2e strains herein reported compared to the ones detected by Franzo et al., (2022) was lower than 1% (i.e., 0.1–0.8%, depending on the specific comparison).

The mean pairwise genetic distance between all obtained complete sequences was 4.84% [interval: 0.00–19.60%]. Considering strains from industrial and rural farms independently, the mean p-distances were 2.96% [interval: 0.00–11.30%] and 9.58% [interval: 0.00–19.60%], respectively. Considering the high variability in p-distance values of rural strains, probably skewed by PCV-2e presence, rural strains pairwise p-distance was also calculated by excluding it. The rural scenario was more comparable to the industrial one after excluding PCV-2e genotype: the mean pairwise genetic distance was 4.53% [interval: 0.00–11.16%], while considering only PCV-2d genotype in the industrial and rural strain sets, the mean pairwise genetic distances were respectively 0.92% [interval: 0.00–15.43%] and 0.69% [interval: 0.00–1.42%].

The evaluation of episodic diversifying selection on all the obtained strains identified 5 sites under positive selection (at 68, 88, 133, 134, 169 amino acid residues). The same analysis performed separately on industrial and on rural strains detected 3 (133, 169, 185) and 6 (4, 68, 88, 134, 169, 208) sites under positive selection, respectively. Excluding PCV-2e genotypes from the rural strains set, MEME downsized to 3 (i.e., 4, 68, 169) the number of sites under diversifying selection.

Considering only PCV-2d strains, only one site (i.e., 133) under episodic positive selection was detected, whereas no evidence of positive selection was identified in rural PCV-2d strains.

4. Discussion

The incidental identification of PCV-2e genotype in 2021 in a breeding farm in Northeastern Italy (Franzo et al., 2022) renewed the interest in PCV-2 genotypes epidemiology in Italy, and broadly in Europe. In particular, questions arose concerning PCV-2e introduction and its actual circulation, that our study aimed to solve. In previous studies, the role of rural pigs was suggested as a potential source of viral circulation, evolution and introduction in intensive farms (Franzo et al., 2022, 2021). Therefore, the rationale of the study was to achieve a collection of intensive and backyard populations living in the same period and area of Northern Italy, where most pigs are raised, to describe the epidemiological scenario and perform a reasonable comparison.

The most astonishing finding in this study was the high detection frequency of PCV-2e in rural farms, which contrasts with the lack of detections in the industrial farms, suggesting a prolonged circulation of this elusive genotype.

The lack of distinct clustering of rural strains (Fig. 1, S1 and S2), which appear to be interspersed among industrial ones is indicative of the likely viral flow between the two environments. The extremely low genetic distance between the strains detected in rural farms and the one previously reported indicates a common epidemiological cluster. Therefore, PCV-2e introduction in commercial herds is likely to have occurred also. Why this event appears rarer and the circulation of this genotype in industrial farms extremely limited remains to be clarified.

While the prevalent genotype in both rural and industrial pigs was PCV-2d, confirming the genotype shift currently observed worldwide (Franzo et al., 2016a), the greater diversity observed in rural viral communities both in terms of the number of circulating genotypes and genetic distances might be due to some peculiarities of rural farming. Pig-flow, all-in-all-out, high hygiene standards and compartmentalization are rarely performed in rural settings, which could favor a wider within-farm circulation. Furthermore, mixing of animals purchased from different farms (Correa-Fiz et al., 2018), as well as outdoor access allowing direct and indirect interactions with external reservoirs may indeed foster the introduction and circulation of different variants, compared to the isolation that strongly implemented biosecurity measures guarantee in intensive farms.

Management could not only facilitate viral circulation, but it might also increase the risk of long-term pathogen persistence. Pigs reared in rural farms often do not receive any vaccination or, in lately purchased animals, any booster. A different immune status, and diverse pathogen communities, in comparison to those normally observed in intensively raised animals, can thus be expected and might allow for PCV-2e persistence.

Of note, PCV-2 rural strains were collected from animals at slaughter, testifying a late and/or more persistent infection in absence of overt clinical signs. This scenario could be the result of different conditions in the two systems, involving both stressors and competition/synergism between PCV-2 strains and other pathogens. The intensive rearing system favors co-infections with other pathogens and other stressing conditions, whose presence often represents a key element for the clinical onset of a multifactorial disease such as the one caused by PCV-2. Additionally, it could be hypothesized that less virulent strains-less acute infections, like the one potentially caused by PCV-2e (Oh et al., 2022), may benefit from the conditions of rural farming, while in the industrial setting, less virulent strains may be outcompeted by more virulent ones (i.e., the major ones) (Segalés et al., 2013), which are effectively maintained by denser and high-turnover population. Accordingly, all backyard animals did not show any clinical sign, suggesting that lower virulence is plausible. However, the low sample size and heterogeneity of rural sampled animals prevent any definitive conclusion and further studies are then necessary to investigate a potential correlation between genotypes, persistent infections, clinical conditions, and farming system. The neglected role of this farming sector in securing pathogen persistence and spread over the Italian territory, representing a potential menace for intensive farming, was already proposed for other viruses, although based on mathematical modeling only (Franzo et al., 2021; Franzo et al., 2020b). Intensive farms typically apply strict biosecurity measures and a hierarchical pig flow. Contact with rural and wild animals should be prevented through rigorous external biosecurity measures, cleaning and disinfection of vehicles and fomites entering the farm, and adequate employees' education. Nevertheless, breakages and new virus introduction have been reported for PRRSV, testifying some gaps in the overall process that allowed strain introduction, including from backyard farms, as previously modeled (Franzo et al., 2021). On the other hand, although rarely, rural farms might acquire animals from intensive ones, especially subjects with poor performances and retarded growth, and workers in the intensive sector, although highly discouraged, might sometime raise pigs for personal consumption, creating risky situations. However, reconstructing the precise contacts among specific farms was not possible since no accurate monitoring and recording system of these potential connections has currently been applied, and the above-mentioned hypothesis, although plausible, must be considered anecdotical. The benefits of systematic data collection is therefore clear and should represent a field of further research and development in the near future. Moreover, the potential role of wildlife cannot be excluded as previously suggested for PCV-2 and PCV-3 (Franzo et al., 2019, 2020a).

Nevertheless, our findings provide consistent proof of the reliability of the epidemiological role of these neglected pig populations and testify the need for further monitoring activities and more integrated control efforts involving both rural and industrial farms. If such pattern is peculiar to the Italian situation or can be generalized to other countries and regions should also be investigated.

Rural and industrial contexts were also compared from the

evolutionary pressure perspective, to evaluate the strength of selective pressure acting on their respective PCV-2 populations. When evaluated only on PCV-2d to remove the confounding effect of between-genotypes variability, a higher genetic distance was present between strains circulating in the industrial context, as expected. This evidence is indicative of an environment favoring a more heterogeneous viral population. Stronger natural selection and/or among-strain competition can thus be inferred, although an effect of the larger time span in which industrial samples were collected cannot be excluded. Accordingly, double peaks were displayed exclusively in the intensive sample set, confirming the presence of a more heterogeneous viral community, which could be favored by intensive farming system features (e.g., higher animal densities) or stronger selective pressures.

Comparable results were obtained assessing the episodic selective pressures acting on strains circulating in rural and industrial settings. As demonstrated by other studies, different sites under positive selection burst were detected in the Cap protein, which is the main constituent of the viral capsid and target of the host immune system. Accordingly, almost all the herein detected positively selected sites (8 out of 9) were located within previously described epitope regions (Lekcharoensuk et al., 2004; Mahe et al., 2000; Saha et al., 2012; Trible et al., 2012). When the most divergent genotype, PCV-2e, was excluded from the analysis, the same number of sites was detected for both rural and industrial strains. However, it is relevant to point out that all called sites were different between populations, except for site 169, strengthening the differential pressures hypothesis. Additionally, when the more balanced PCV-2d dataset was evaluated, only site 133 (an already recognized immunodominant epitope) was identified among the industrial strains as subjected to episodic positive pressure.

Therefore, the higher variability and pressure detected on the whole rural dataset are due to the differential genotype composition, likely caused by less constrained among-farm circulation, rather than stronger selective pressures. The viral population affecting rural animals may experience unconventional and less intense selective forces. In the industrial context, in addition to the stronger natural selection and abovementioned management-related stressors affecting some physiological paths (Martínez-Miró et al., 2016; Grau-Roma et al., 2011), the vaccination status is undoubtedly paramount in the evolutionary forces (Franzo et al., 2016b). The different immune status in pigs reared in rural farms mainly due to lacking, delayed or non-boosted vaccinations is more likely associated with a weaker positive pressure in comparison to what is normally expected in intensively raised animals. Moreover the lower host size and density might also prevent the development of big viral populations, necessary for selective pressures to act.

Nevertheless, the intrinsic diversity of PCV-2e should warn about the increased risk of immune-escaping circulating variants, if PCV-2e will massively spread in intensive farms (Franzo et al., 2016a). Current PCV-2 commercial vaccines are mainly based on the PCV-2a genotype, but still provide cross-protection against PCV-2b and PCV-2d (Franzo and Segalés, 2020). If such cross-protection extends to PCV-2e is still unproven.

5. Conclusions

The present study, besides updating on PCV-2 diversity in Northern Italy, confirms the circulation of the highly divergent genotype PCV-2e on the Italian territory, the first and only country in Europe where this genotype has so far been identified. PCV-2e identification exclusive to rural farms emphasizes their epidemiological role: the rural context appears as a privileged niche in which PCV-2e, and potentially other pathogens, replicate and circulate. Nevertheless, the actual source of PCV-2e introduction in these farms and hence in Italy, remains obscure. Similarly, the PCV-2e circulation in other neighboring countries and the generalizability of present results to other areas should be investigated. A more accurate system to monitor or at least estimate the animal exchanges and other contact points between rural and intensive farms, sometimes following underground pathways, should also be developed. Finally, the extension of the epidemiological survey to the wild population should be considered in order to deeply investigate plausible virus flows and more clearly draw the contact networks behind the introduction of PCV-2e genotype in Italy and Europe. Monitoring the circulation of such a divergent genotype like PCV-2e should be of primary interest since its introduction into intensive farms could lead to more severe clinical conditions and further prompt evolutionary phenomena.

Funding statement

This work was supported by the Department of Animal Medicine, Production and Health, University of Padua [grant number BIRD225455/22].

Animal ethics

All samples originated from routine diagnostic activity and no additional experimental procedures were performed.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.prevetmed.2023.105943.

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