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RAPID COMMUNICATION

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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

Giovanni Franzo | Claudia Maria Tucciarone | Michele Drigo | Mattia Cecchinato | Marco Martini | Alessandra Mondin | Maria Luisa Menandro

Department of Animal Medicine, Production and Health (MAPS), University of Padua, Legnaro, Italy

Correspodence
Giovanni Franzo and Maria Luisa Menandro, Department of Animal Medicine, Production and Health (MAPS), University of Padua, Legnaro, Italy.
Email: giovanni.franzo@unipd.it; marialuisa.menandro@unipd.it

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Summary

The genus Circovirus includes one of the most relevant infectious agents affecting domestic pigs, Porcine circovirus type 2 (PCV-2). The wild boar susceptibility to this pathogen has also been demonstrated although the actual epidemiological role of wild populations is still debated. In recent times, a new circovirus, Porcine circovirus type 3 (PCV-3), has been discovered and reported in the presence of several clinical conditions. However, no information is currently available about PCV-3 circulation and prevalence in wild boar. To fill this gap, 187 wild boar serum samples were collected in the Colli Euganei Regional Park (Northern Italy) and screened for PCV-3, demonstrating a high viral prevalence (approximately 30%). No gender differences were demonstrated while a lower infection prevalence was observed in animals younger than 12 months compared to older ones, differently from what described in commercial pigs. Almost all sampled animals were in good health conditions and no association was proven between PCV-3 status and clinical syndromes in wild animals. The genetic characterization of selected strains enlightened a relevant variability and the absence of closely related strains originating from domestic pigs. Therefore, the observed scenario is suggestive of multiple introductions from other wild or domestic swine populations followed by prolonged circulation and independent evolution. Worldwide, this study reports for the first time the high susceptibility of the wild boar to PCV-3 infection. The high prevalence and the absence of association with clinical signs support the marginal role of this virus in the wild boar population ecology. However, its epidemiological role as reservoir endangering commercial swine cannot be excluded and will require further investigations.

KEYWORDS
Epidemiology, Italy, PCV-3, Wild boar

1 | INTRODUCTION

Porcine cirvovirus type 3 (PCV-3), a member of the family Circoviridae, genus Circovirus, is a recently discovered virus (Palinski et al., 2017), which has been proven to infect commercial pigs in different regions of the world. At present, its presence has been reported in the USA (Palinski et al., 2017; Phan et al., 2016), Europe (Franzo, Legnardi, Hjulsager et al., 2018; Stadejek, Woźniak, Miłek, & Biernacka, 2017), Asia (Shen et al., 2018) and South America (Tochetto et al., 2018) with a variable but generally high prevalence (i.e.

approximately 10%–60%) (Franzo, Legnardi, Hjulsager et al., 2018; Stadejek et al., 2017; Zheng et al., 2017), which suggests a worldwide distribution and ancient origin. Since the first report, PCV-3 has been detected in the presence of several clinical syndromes, including porcine dermatitis and nephropathy syndrome (PDNS), reproductive disorders, respiratory signs (Ku et al., 2017; Palinski et al., 2017; Shen et al., 2018) and myocarditis (Phan et al., 2016). However, it has also been reported in asymptomatic animals (Zheng et al., 2017) and, although no extensive epidemiological studies have been published yet, few evidences are in favour of a PCV-3 true pathogenic role (Palinski et al., 2017) instead of a mere casual association (Franzo, Legnardi, Tucciarone et al., 2018). Similarly to Porcine circovirus type 2 (PCV-2) infection, several factors could contribute to trigger the overt clinical disease (Rose et al., 2009). In fact, PCV-3 was demonstrated in Spanish archive samples since 1996 (Klaumann et al., 2018) and the most recent common ancestor was dated back to the middle of the XX century (Fu et al., 2017), suggesting that some changes correlated with the modern farming systems could be involved in the rise of PCV-3 as a potential threat for swine industry.

Wild boar populations are a relevant study subject in this sense. In fact, belonging to the same species (*Sus scrofa*), the wild boar has been proven to be susceptible to most of the pathogens infecting commercial pigs (Meng, Lindsay, & Sriranganathan, 2009). Nevertheless, the epidemiological role of wild populations as reservoir and menace to commercial farming is highly controversial, remarkably varying according to the particular disease. While wild populations are considered of pivotal relevance for Classic and African swine fever and hinder their eradication (Moennig, 2015), this linkage appears less evident for other viral infections, such as Porcine reproductive and respiratory syndrome virus (PRRSV) and PCV-2, and the direction of the viral flux is substantially unknown (Ruiz-Fons, Segales, & Gortazar, 2008). Other factors such as disease prevalence in domestic pigs, farming type, geographical and socio-economical features probably affect the interaction between wild and domestic pigs (Bengis, Kock, & Fischer, 2002). For instance, the increase in wild boar populations results in increased contact opportunities between the two groups, often mediated by rurally raised pigs (Massei et al., 2015). Additionally, the wild boar is increasingly managed by high-wire fencing, artificial feeding and selective culling (Vicente et al., 2004). As a result, some wild boar estates can mimic extensive pig breeding facilities, with high densities but limited or absent sanitary care, which represents a risk of both the nearby domestic pig and the wild population itself.

Based on these premises, a molecular epidemiological study was performed on the wild boar populations of the Colli Euganei Regional Park (Veneto, Italy) to investigate PCV-3 infection occurrence, prevalence and association with animal category and health status.

2 | MATERIAL AND METHODS

2.1 | Sampling sites, animal capture and sample management

The sampling area was part of the "Colli Euganei" park, which includes 15 Municipalities and covers an area of about 18,694 ha in Veneto region (North Eastern Italy).

Four hundred and ninety-three blood samples were collected from the resident boars during the routine culling campaign in 2014-2015, aimed to control the demographic growth.

Animals were captured through food traps by trained park staff who also collected blood samples. Capture date and place, animal gender, estimated age, weight and general health condition were recorded for each animal. Subjects were classified as Juveniles (<12 months of age), Subadults (>12 months and <24 months) and Adults (>24 months), based on tooth eruption patterns (Massei & Toso, 1993). Blood samples were centrifuged at 1,500 *g* for 10 min, and the obtained serum was stored at −20°C until processing.

Considering that each capture included mainly animals from the same litter, living in close contact and sharing the same environment, it may be assumed that they also shared common pathogens. On these bases, only one randomly selected sample for each capture was kept for further analysis. However, when animals of different age were found in the same trap, one sample for each age category was included. The minimum sample size was set according to a two-stage cluster sampling aiming to achieve a prevalence estimation with a precision of 0.05 and confidence level of 0.95. Expected prevalence, within and between cluster variance were set to 0.5, 0.2 and 0.1, respectively. Because of the absence of updated wild boar census in the considered region, an infinite size population was assumed to guarantee a conservative estimate. A total of 187 samples were therefore included in the study.

2.2 | PCV-3 diagnostic and sequence analysis

DNA was extracted from serum samples using the NucleoSpin®Blood kit (MACHEREY-NAGEL) and PCV-3 presence and viral titre were assessed using a previously validated real-time PCR diagnostic assay (Franzo, Legnardi, Centelleghe et al., 2018). Sequencing was attempted on all positive samples with a real-time PCR determined Cp lower than 33 to increase the likelihood of successful sequencing.

In particular, the whole Cap region was amplified (amplicon length: 707 bp) using the primers PCV3_1303F (5′-ACCGGAGGGG TCAGATTTAT-3′) and PCV3_8R (5′-TGCCGGGTAATACTAGCC3-3′). In brief, 2 μl of extracted DNA was added to a standard mix composed of 1× Phire Reaction.

Buffer, 200 μM dNTPs, 0.6 μM of each primer and 0.4 μl of Phire Hot Start II DNA Polymerase. Sterile nanopure water was added to bring the final volume up to 20 μl. The following thermal protocol was selected as follows: 98° C for 30 s followed by 45 cycles of 98°C for 5 s, 60°C for 5 s and 72°C for 15 s. A final extension phase of 1 min at 72°C was also performed. Amplification and

specificity of bands were visualized using a SYBR safe stained 2% agarose gel. DNA sequencing was performed at Macrogen (Macrogen Europe, Madrid, Spain) using the same primers. All chromatograms were visually inspected with Finch TV programme 1.4.0 (2004–2006 Geospiza Inc) and consensus sequences were obtained using the ChromasPro (ChromasPro Version 1.5; Technelysium Pty Ltd, South Brisbane, Australia; http://technelysium.com.au/wp/chromaspro/).

A collection of PCV-3 *cap* sequences was downloaded from GenBank and nucleotide sequences were translated and aligned at amino acid level to those obtained in the preset study using the MAFFT method implemented in TranslatorX (Abascal, Zardoya, & Telford, 2010). The amino acid sequences were then back translated to nucleotide using the same programme. The alignment was trimmed to a region of 402 nucleotides to achieve a complete coverage still including the maximum number of sequences. The strength of phylogeny signal was assessed using TREE-PUZZLE (Schmidt, Strimmer, Vingron, & Haeseler, 2002). At last, a phylogenetic tree was reconstructed using the Maximum likelihood method implemented in PhyML (Guindon et al., 2010) selecting as substitution model the one with the lowest AIC calculated using JmodelTest (Darriba, Taboada, Doallo, & Posada, 2012). The robustness of the clade reliability was evaluated using the fast nonparametric version of the aLRT (Shimodaira–Hasegawa [SH]-aLRT), developed and implemented in PhyML 3.0 (Anisimova & Gascuel, 2006).

2.3 | Statistical analysis

PCV-3 prevalence and 95% CI, calculated using the asymptotic (Wald) method based on a normal approximation, were obtained using the *prevalence* (Devleesschauwer et al., 2014) package in R.

Association between PCV-3 infection, group age and health status was evaluated using a $\chi 2$ or Fisher's exact test values. The presence of statistical significant differences in the viral titre among groups was evaluated using the Mann–Whitney (two groups) or Kruskal–Wallis test (more than two groups), followed by post hoc Mann–Whitney tests with Bonferroni correction. The statistical significance level was set to $p < 0.05$.

3 | RESULTS AND DISCUSSION

The current complete lack of data about PCV-3 circulation in the wild boar prevents any tentative epidemiological analysis and discourages further, more focused, investigations.

As a result, the study of PCV-3 circulation in wild populations is interesting to evaluate host susceptibility, epidemiological role and clinical consequences and eventually to plan and implement adequate control measures. Moreover, it could be realistically assumed that most of the stressful factors associated with intensive farming do not act on these feral populations. Thus, it can be speculated that: (a) if PCV-3 was a primary pathogen, able to cause disease, infected wild animals should display a lower health condition; (b) whether other cofactors were required or PCV-3 represented an

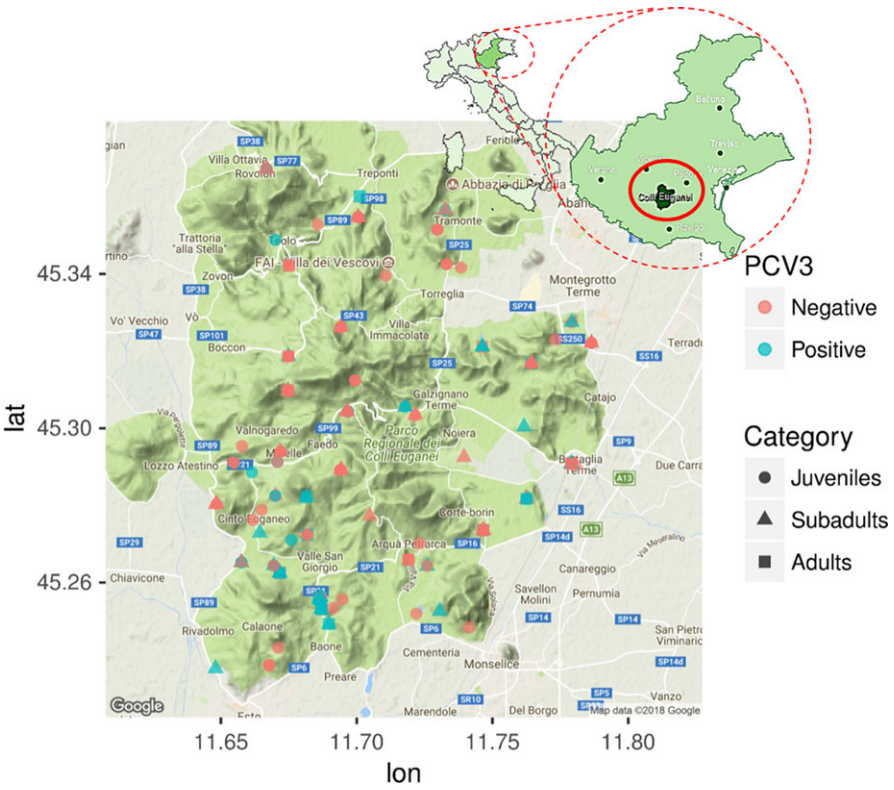

FIGURE 1 Maps of the Colli Euganei Regional Park. Capture sites have been geolocalized and the distribution of positive and negative samples is colour-coded. The animal age is also reported using different point shapes. The position of Colli Euganei Regional Park in Italy is reported in the top-right insert

incidental finding in diseased animals, no association should be identified between infection and sanitary status. Therefore, the study of PCV-3 infection in wild animals could contribute to enlighten its pathogenic role in the commercial swine.

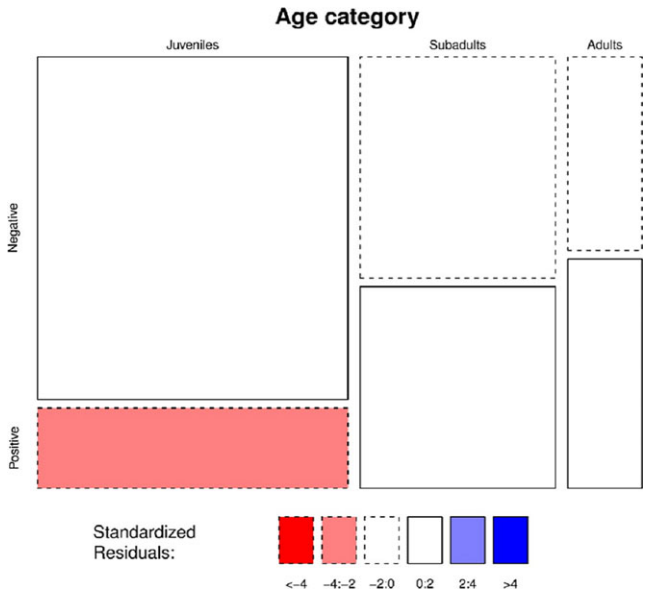

FIGURE 2 Mosaic plot depicting the relationship between PCV-3 status and animal age category. The area of each cell is proportional to the count size. Cells have been colour-coded and lines dotted based on standardized residuals (a standardized residual greater than 2 or lower than -2 is indicative of statistical significance)

Samples were successfully obtained from different areas of the Colli Euganei Regional Park (Figure 1). As expected, most of the samples originated from juvenile animals (n = 100) and to a lesser extent from subadults (n = 63) and adults (n = 24). Males (n = 91) and females (n = 96) were sampled with comparable frequency. Adults males were significantly underrepresented in the study (p = 0.021).

As an adult female to male ratio of approximately 2 has been reported by other authors (Merta, Bobek, Albrycht, & Furtek, 2015), our data could be representative of the actual population structure. However, considering the unavoidable convenience nature of this kind of sampling, other factors such as a lower likelihood of capturing adult males because of behavioural differences could have affected the sex representativeness.

Overall, 62 boars tested PCV-3 positive, corresponding to an infection prevalence of 33.15% (95CI 26.41%–39.90%). These results are fully comparable with the PCV-3 frequency occurring in domestic pigs in Italy (Franzo, Legnardi, Tucciarone et al., 2018), and within the range reported in other countries (Franzo, Legnardi, Hjulsager et al., 2018; Stadejek et al., 2017; Zheng et al., 2017). However, the prevalence in juvenile animals (i.e. younger than 12 months) appeared lower than in older animals, differently from what reported in commercial pigs (Figure 2) (Franzo, Legnardi, Tucciarone et al., 2018). This could be due to a lower animal density causing a delayed

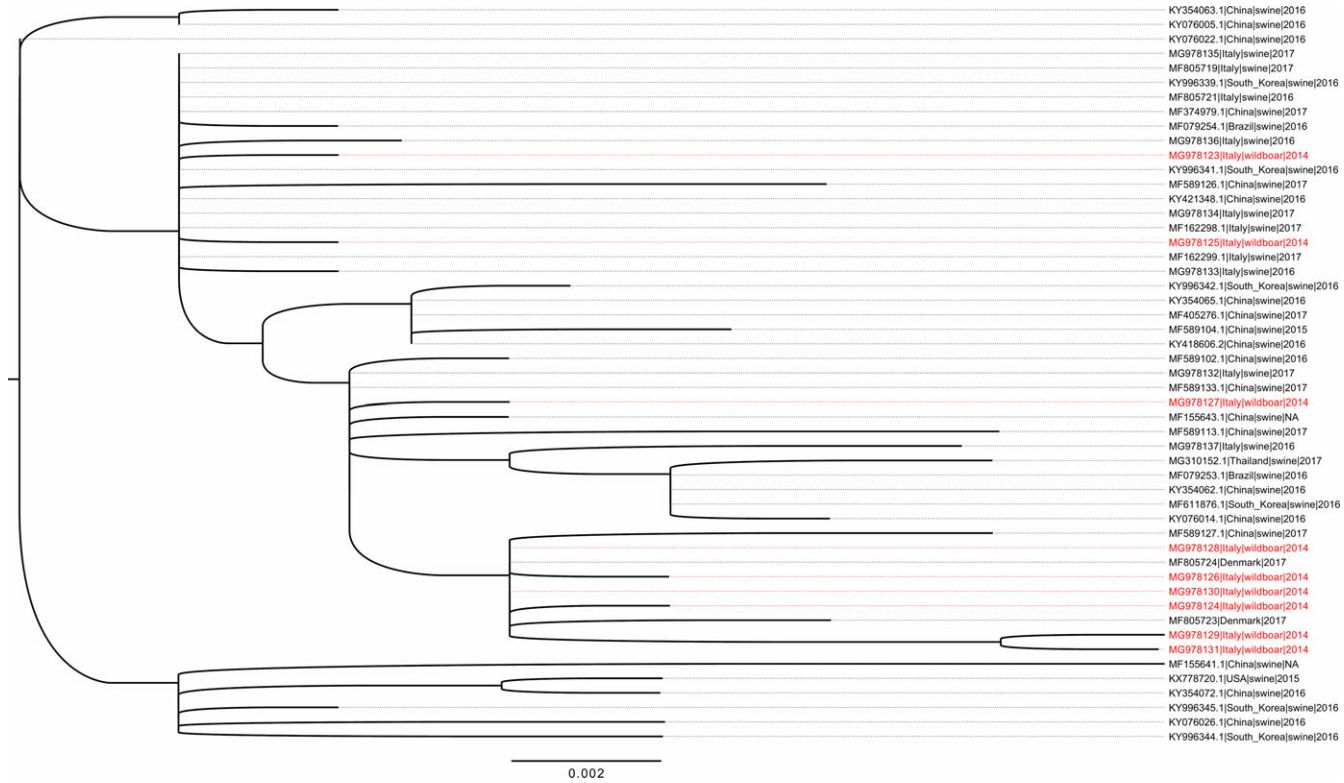

FIGURE 3 Maximum likelihood phylogenetic tree based on partial Cap gene sequence. Strains obtained from boars have been highlighted in red. A phylogenetic tree reconstructed based on a broader sequence data set has been provided as Supplementary Figure S1

infection and lower transmission rate. Nevertheless, further and more extensive studies will be necessary to confirm this hypothesis.

Irrespective of these differences, the present study clearly demonstrates that the wild boar is susceptible to PCV-3 infection and that the viral circulation is widespread in this population. A similar scenario was previously described for the other major swine circovirus, PCV-2, whose prevalence in wild animals approximately overlaps the domestic pig one, even if relevant differences among-countries were observed (Ruiz-Fons et al., 2008; Vicente et al., 2004). However, differently from PCV-2, which was reported to be responsible for clinically relevant disease also in wild populations (Petrini et al., 2009; Ruiz-Fons et al., 2008; Vicente et al., 2004), no significant evidences of PCV-3 associated diseases were found in the present study ($p = 0.15$), being almost all considered animals healthy and in good conditions, irrespective of the PCV-3 infections. Although the only two animals in poor conditions were PCV-3 positive, the detected viral titres were low (i.e. approximately 10^4 genome copies/ml) and fully comparable to those of the other positive animals. As a result, the role of PCV-3 as a primary cause of animal wasting is unlikely. These evidences, together with the percentage of PCV-3-positive animal, further confirm that PCV-3 alone is unlikely to be the cause of overt clinical disease and suggest that other factors peculiar to intensive pig farming are likely required for the expression of a full virulence, if any.

Although PCV-3 relevance for wild boar appears to be negligible in a natural setting, a potential sanitary impact cannot be excluded on estates where boars are risen at higher densities for hunting, commercial or restocking purposes. On the other hand, because of the high prevalence, increasing wild population size and more frequent contact opportunities with the domestic species, it cannot be excluded that the wild boar might act as a virus reservoir for the domestic pig.

In the present study, nine partial cap sequences (Acc.Numbers MG978123-MG978131) were obtained and compared to others sampled in Italy and worldwide (Figure 3 and Figure S1). Surprisingly, despite the small considered region, PCV-3 strains identified in the present study exhibited a remarkable genetic diversity and sequences belonging to two major genetic groups were detected. Both clades included sequences sampled from commercial pigs in Italy, supporting some strain interchange between wild and domestic swine populations. Another nonconflicting hypothesis calls upon the wild boar origin in the park. In fact, the actual wild boar population has a very recent origin from subjects illegally introduced at the end of the 90′ (Scacco, Carnevali, & Riga, 2011). The tested animals were thus the descendent of relatively recent hybridization events possibly occurred among boars from other Italian regions, from other countries and domestic pigs. It can therefore be speculated that PCV-3 was introduced from different sources, as already suggested for PCV-2 in the Brazilian Pantanal (Franzo et al., 2015). The genetic heterogeneity of PCV-3 was proven to be relevant in the considered wild population (average 0.8%; range 0%–1.5%) and none of the strains from the boars appeared identical to the currently available from domestic pigs, suggesting that even if contacts were likely in the past, a prolonged and independent circulation among the wild animals of the park has occurred. Moreover, the presence of strains collected in other countries closely related to those reported in the present manuscript could suggest that frequent PCV-3 introductions in Italy affected both domestic and wild populations.

It is unfortunate that it must be stressed that the molecular epidemiology data about PCV-3 are still poor and sparse.

As a result, the currently available information cannot enlighten the origin of PCV-3 presence in the Italian wild boar nor solve the issue of viral flux directionality and, similarly to other swine pathogens, the role of the wild boar remains largely unknown (Ruiz-Fons et al., 2008). Further studies, comparing the genetic features of strains collected from the wild population and pig farms located in the park neighbourhood, could provide more conclusive evidences. In addition, the gathering of sequence data from other countries will surely contribute to the understanding of PCV-3 role and epidemiology in different animal populations.

In conclusion, the present study demonstrates for the first time the widespread circulation of PCV-3 in wild boar populations and its relevant genetic heterogeneity, suggestive of a prolonged circulation in the area. Based on our results, no association was demonstrated between animal infection and health status, posing in favour of a negligible PCV-3 pathogenic role, at least in the considered population.

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ORCID

Giovanni Franzo http://orcid.org/0000-0003-2991-217X
Marco Martini http://orcid.org/0000-0002-9252-6389

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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