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Original article

Epidemiological study on porcine circovirus type 2 (PCV2) infection in the European wild boar (*Sus scrofa*)

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Abstract – Porcine circovirus type 2 (PCV2) is considered as the causative agent of postweaning multisystemic wasting syndrome (PMWS) in domestic pigs, where the virus is ubiquitous as evidenced by serological surveys. We present the results of the first nationwide sero-survey on the presence of PCV2 antibodies in European wild boars, and report the first PMWS case in a wild boar from Spain. Sera from 656 hunter harvested wild boars from 45 different geographical sites and 22 additional imported animals were analysed by means of an immunoperoxidase monolayer assay (IPMA). We also examined the tissues from 55 healthy and one diseased wild boars for the presence of PCV2 nucleic acid and PMWS lesions by in situ hybridisation and histopathology, respectively. Additionally, abundance estimates of wild boars and field interviews were carried out on 30 sampling sites. The prevalence of medium to high PCV2 serological titres among the examined wild boars was 47.89 ± 1.9%. Seropositive wild boars appeared in all but one of the geographical regions analysed. Seroprevalence and titre of PCV2 antibodies were closely related to the management of the wild boar populations. Wild boars from intensively managed, farm-like populations had higher prevalence than wild boars living in more natural situations. The effect of wild boar abundance and management on PCV2 antibody prevalence was further evidenced by the high correlation existing between the relative abundance estimates of animals and the percentage of wild boars with medium to high levels of PCV2 antibodies. PCV2 nucleic acid was detected in the tissues of three wild boars. One of these was diagnosed as PMWS. The results, in addition to information on piglet mortalities, suggest a potential role of PMWS in piglet mortality in intensively managed wild boar populations.

European wild boar / porcine circovirus type 2 / epidemiology / postweaning multisystemic wasting syndrome

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1. INTRODUCTION

Porcine circovirus type 2 (PCV2) is nowadays considered as the etiological agent of postweaning multisystemic wasting syndrome (PMWS), a recently described disease of nursery and growing domestic pigs [15]. The disease was initially described in Canada [8] but, during the last seven years, PMWS cases have been described worldwide, except in Oceania [21]. This condition is clinically characterised by wasting, respiratory distress, pallor of the skin and, very occasionally, jaundice. Due to the relatively unspecific clinical signs, the establishment of a final diagnosis of PMWS is based on three different criteria [20, 24]: clinical signs (those cited above), the presence of very specific lesions in lymphoid tissues (consisting of lymphocyte depletion and granulomatous inflammation) and the presence of PCV2 in these tissues. The latter two criteria are very specific for PMWS and closely associated, since a marked correlation between the severity of the lesions and the amount of viral antigen or genome is observed systematically [3, 10].

It is known from serological surveys that PCV2 is ubiquitous since it is present in both PMWS affected and non-affected farms [14], with a serological prevalence close to 100% in finishing pigs worldwide [21]. Furthermore, it is known that the wild boar (*Sus scrofa*) is also susceptible to PCV2 infection [19]. This latter data also applies to Spain, where a 34.6% PCV2 seroprevalence was obtained using a limited number of sera of wild boars from the Catalonian region [22]. Moreover, the first case of PMWS in a free living European wild boar was recently described in Germany [23], which clearly indicates that both the domestic pig and the wild boar are susceptible not only to PCV2 infection but also to the development of PMWS. The European wild boar is the most widely distributed ungulate in the Spanish mainland, and its range and densities have largely increased in the last three decades [6, 18]. Due to its abundance, it is also one of the most popular game species. In order to increase hunting harvest, wild boar populations are increasingly managed by high-wire fencing, artificial feeding, and restocking with farm bred individuals. As a result, some wild boar hunting estates resemble extensive pig breeding facilities, with high densities but almost no sanitary care. These changes in wildlife management have already risen concerns regarding the control of infectious and parasitic diseases [4, 7], and may also affect PCV2 epidemiology.

Thus, considering the ubiquitous distribution of PCV2 among domestic pig populations, and the known risk factors for PMWS in domestic pigs such as poor hygiene and crowding [21], we hypothesised (1) that the seropositivity to PCV2 is higher in intensively managed wild boar populations than in more natural ones and (2) that cases of PMWS may eventually occur in farm-like situations, affecting the hunting harvest. Hence, we undertook a nation-wide sero-survey for PCV2 antibodies in wild boars, tested a sub-sample of them for PMWS by in situ hybridisation combined with histopathology, estimated wild boar abundance using a faecal pellet-group survey, and examined game management techniques through direct interviews.

2. MATERIALS AND METHODS

2.1. Sampling sites

Between 2000 and 2003, samples were collected from 656 hunter-harvested wild boars in 45 Spanish localities (sampling sites) (Fig. 1). Sampling took place during the regular hunting season, from September to March. Sampled hunting areas range in size from 408 to 52 000 hectares (ha), means 9 212 ± 18 008 ha for open areas, 4 036 ± 3 157 ha for fenced areas, and 1 366 ± 1 069 ha for intensively managed ones. The number of boars shot on one hunting day ranged from 1 to 120 (means

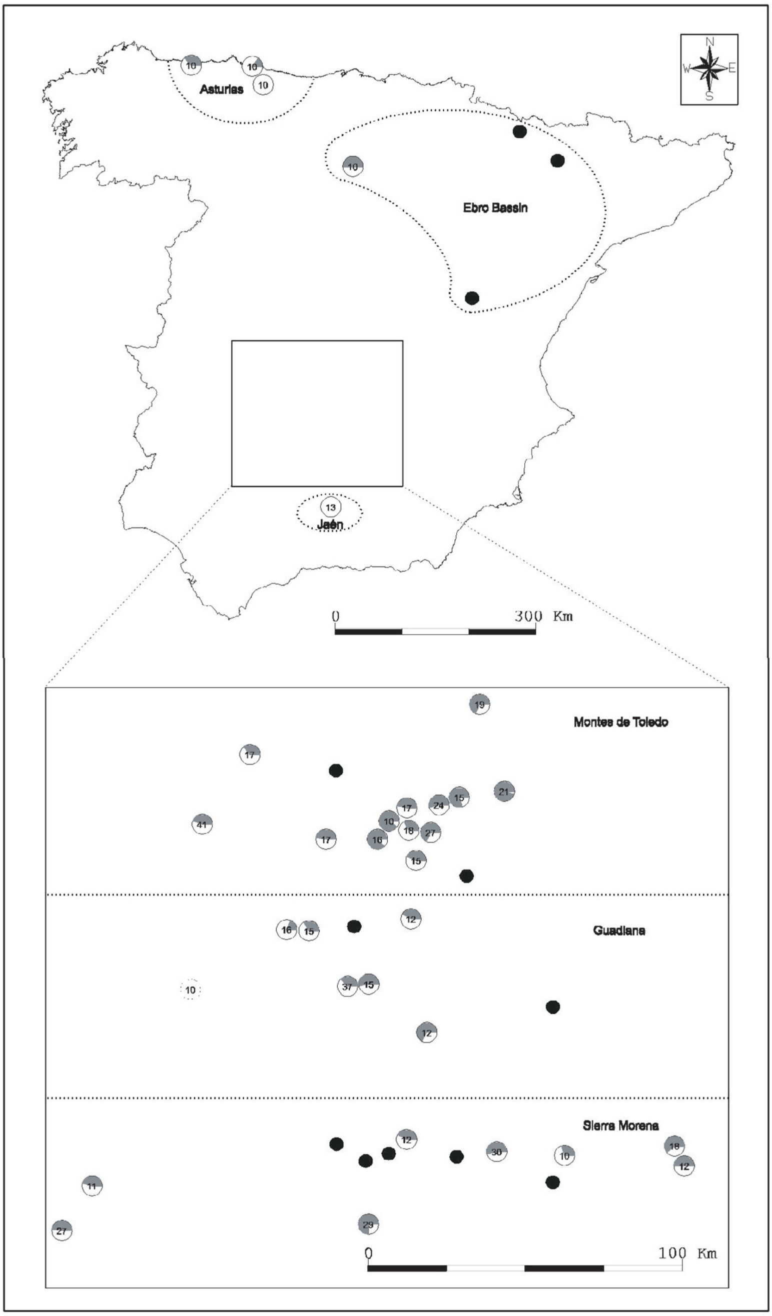

Figure 1. Map of peninsular Spain showing the sampling sites, the number of sera analysed per site and the prevalence of wild boars with medium to high titres of antibodies against PCV2 (percentage of grey colour in relation to the whole circle). Dots represent sampling sites where less than 10 samples were obtained.

Table I. Number of sampling sites, number of analysed sera (N), number of sera with medium to high antibody titres against PCV2 (No. positive), prevalence and 95% confidence intervals for 678 wild boar sera.

Area	No. sites	N	No. positive	Prevalence (%)	1.96 S. E. (95% C.I.)
Asturias	3	30	7	23.33	13.72
Ebro Bassin	4	24	12	50	20.384
Guadiana	9	124	43	36.67	8.46
Jaén	1	13	0	0	0
M. Toledo	15	277	156	58.42	5.905
S. Morena	13	188	96	51.06	7.056
Total	45	656	314	47.87	1.91

3.43 ± 2.04, 18.89 ± 28.69, 40.33 ± 37.16 for open, fenced and intensively managed estates, respectively). If more than 20 wild boars were available for sampling, we arbitrarily selected a random age and sex stratified subset of between 15 and 30 animals, depending on time availability and logistic constraints.

Sampling sites are representative of the biogeographically most relevant landscape units in peninsular Spain, with a bias towards the Mediterranean shrub-lands of the central and southern regions, where hunting activities are most important [5]. Thus, sampling sites were divided into six geographic regions, named Asturias, Ebro Basin, Guadiana, Jaén, Montes de Toledo, and Sierra Morena (Tab. I). Mediterranean shrub-lands are dominant in both mountainous central areas, Montes de Toledo and Sierra Morena. The Guadiana area consists of a fragmented Mediterranean habitat and is a transition between Montes de Toledo and Sierra Morena. These three areas are characterised by large densities of wild boars due to intensive big game management. Jaén is representative of the Betic mountain chains, which are located in south-western Spain and are characterised by extensive olive groves and a lack of intensive big game management. On the contrary, the Atlantic climate is represented by Asturias, a mountainous coastal region from the north of Spain, where no feed is supplemented due to the existing conflicts caused by the damage of wild boars to croplands. The last area is the Ebro Basin, with predominance of Mediterranean agro-ecosystems and diversity of management.

For each sampling site, we recorded the management conditions to which wild boars were subjected: open (164 samples from 16 sites), fenced (388 samples from 22 sites), or intensively managed areas with fencing, translocations and artificial feeding (104 samples from 7 sites).

2.2. Interviews and abundance estimates

We visited 30 of the sampling sites in September 2002, immediately before the hunting season, in order to carry out interviews regarding game management, and obtain field estimates of wild boar relative abundance based on dropping counts. No abundance estimates were carried out on sites sampled after September 2002. Through a personal interview, we obtained data from gamekeepers regarding the presence/absence of fencing, artificial feeding, and translocations of wild boars in the respective estate. We asked if any unusual disease outbreaks, especially in piglets, were recorded.

Artificial feeding was used in four out of seven open sites, in three of these sites it was only used previously to hunting days to attract boars. The other estate supplemented wild boars throughout the year. Seven out of 18 fenced estates fed boars previously to hunting and four out of 18 did it across the year. Finally, all but one farm-like managed estates indicated artificial feeding over the year, and the remainder one only supplemented in the summer (the limiting season from the point of view of food resources in Mediterranean habitats). Sanitary management was restricted to the oral administration of parasiticides (ivermectin) in food in one out of 18 fenced estates and three out of five intensive managed estates. No use of vaccines nor antibiotics was recorded through the interviews. In one occasion we were able to collect samples from 22 wild boars that had been illegally imported to a fenced hunting estate from two French farms. No other restocking of wild boars was reported in any study estate. Only in one fenced estate the possibility of hybridisation between wild boars and domestic pigs was reported in the interviews. The co-existence of wild boars with free roaming domestic pigs was not common in our study areas.

Since ungulate droppings tend to be aggregated [16], we developed a simple method using dropping frequency (instead of their number) to estimate the relative abundance of European wild boars. Briefly, each count consisted of $n = 40$ transects of 100 m, divided into 10 sectors of 10 m in length. Dropping frequency was defined as the average of the number of 10 m sectors with wild boar droppings in each transect of 100 m ($DF = \Sigma Di/n$; where "D" is the number of dropping-positive sectors and ranges from zero to ten, and "n" is the number of 100 m transects, usually 40).

There were statistical differences in wild boar abundance indexes between sampling sites depending on the management type. Open hunting areas ($n = 7$) had a DF of 0.05 ± 0.05, fenced hunting areas ($n = 18$) had 0.44 ± 0.2, and intensively managed ones ($n = 5$) had 1.48 ± 0.8 ($F_{1, 29} = 16.76$, $P < 0.001$, $n = 30$).

2.3. Field necropsies

Each hunter-harvested wild boar (n = 656) was measured, weighed, and its sex determined. Thus, 348 females and 277 males were identified, but no sex was recorded for 31 of the sampled animals. Based on tooth eruption patterns, boars less than 7 months old were classified as piglets, boars between 7 and 12 months were classified as juveniles, those between 12 and 24 months as sub-adults, and those over 2 years as adults [17]. We estimated the fat condition using the kidney fat index (KFI) [12]. Samples of selected organs were collected and fixed by immersion in 10% neutral-buffered formalin. One wild boar piglet was found dead in an intensively managed hunting estate and processed as the hunter-harvested ones. Blood samples were collected from the heart during necropsy. Serum was obtained after centrifugation and stored at –20 °C until use.

2.4. Immunoperoxidase monolayer assay (IPMA)

A previously published protocol to detect PCV2 antibodies by means of an IPMA technique [13] was applied on 656 sera from the necropsied Spanish wild boars and the 22 imported ones. Serum dilutions were from 1:20 to 1:20 480, making serial four-fold dilutions. For statistical analyses, serologic results were grouped as negative or low (titre below 1:320) or medium to high (titre equal to or higher than 1:320) titres.

2.5. Histopathology and in situ hybridisation

Formalin-fixed samples of lung, lymph nodes, liver, spleen and/or kidney of a subsample of eight piglets, 13 juvenile, 23 subadult and 12 adult wild boars were dehydrated, embedded in paraffin wax, sectioned at 4 µm, and stained with haematoxylin and eosin (HE). Correlative tissue sections containing the same tissues were placed on Probe On Plus glass microscope slides (Fisher Scientific, Pittsburgh, USA) in order to perform an in situ hybridisation technique to detect PCV2 nucleic acid following previously published protocols [15].

2.6. Statistical analyses

Confidence intervals for standard errors of prevalence were estimated with the expression $S.E._{95\%C.I.} = 1.96[p(1-p)]/n^{1/2}$ [11]. To test for significant differences in PCV2 seroprevalence between sex, age-classes, and degree of management and interactions, log-linear analysis of contingency tables were employed, reporting the partial χ^2 values from a saturated model [2]. We treated both age and management degrees as categorical variables with three classes as described above. An additional number of analysed piglets were not included due to the low sample size ($n = 20$). We also used Spearman non-parametric correlations to study the relationship between density and the probability of having medium to high antibody titres (as continuous variables) and between the PCV2 antibody titres and KFI. ANOVA was employed to study the effect of management type on wild boar density. The level of significance was established at the 5% level.

3. RESULTS

Medium to high titres of antibodies to PCV2 were found in 332 of the 678 analysed sera (49 ± 1.88% of the total sample). Regarding only Spanish wild boars, medium to high titres of antibodies to PCV2 were found in 314 of the 656 sera (47.9 ± 1.91% of the total sample). Prevalence differed among geographical regions as shown in Table I. Figure 1 shows the percentage of sampled wild boars with medium to high PCV2 antibody titres. As shown in Figure 1, only three out of 35 sites with at least 10 sampled animals had no wild boars with medium or high antibody titres. A high prevalence of medium to high titres was found in the imported farmed wild boars from France (81.8 ± 16%).

The proportion of animals with medium to high titres of antibodies differed significantly between management condition types (prevalence: partial $\chi^2 = 19.87$, $P < 0.001$). No significant differences were found between the sex or age classes (Fig. 2). Thus, independently of the sex and age structure, intensively managed wild boars had the highest values, while open populations had the lowest ones. Juveniles from intensively managed hunting estates showed an extremely high seropositivity as compared to juveniles from fenced or open populations (Fig. 3). No relationship was found between the kidney fat index and PCV2 antibody titres.

The effect of wild boar abundance and management on PCV2 antibody prevalence was further evidenced by the high correlation existing between the relative abundance estimates based on dropping counts, and the percentage of wild boars with medium to high levels of PCV2 antibodies (Spearman correlation, $r_s = 0.55$, $P < 0.001$, $n = 30$; Fig. 4).

Only 20 piglet sera were analysed: 12 from open populations (all with no or low PCV2 antibody titres), 5 from fenced estates (one of them with a medium to high titre), and only one from an intensively managed estate, also with a medium to high titre. Even though the probability of sampling piglets was similar in the three management types, the actual number of sampled piglets was lower than the expected number in the intensively managed estates (Chi square test, $\chi^2 = 19.33$, 2 d.f., $P < 0.001$). In the interviews, three out of five intensively managed estates reported increased piglet mortality. Dead or sick piglets in these estates were found mainly at feeding or watering places. In two of these estates the rangers reported that almost no piglets were seen at the feeding sites or during spotlighting surveys. In contrast, no observations regarding increased piglet mortality (nor lack of piglet sightings) were recorded in the 25 interviewed open or fenced hunting estates. For a subset of piglets from which KFI was

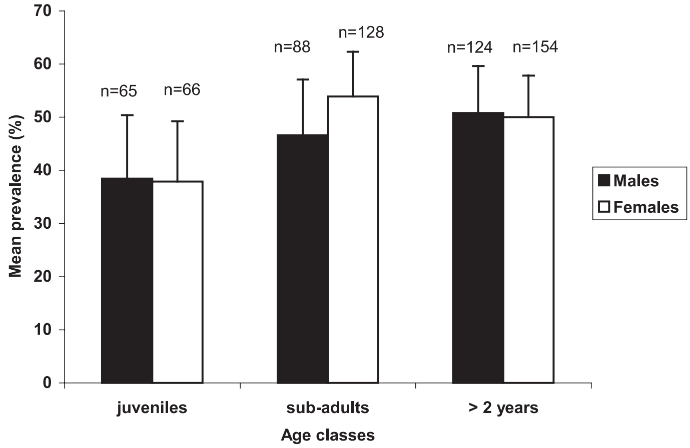

Figure 2. Percentage (and 95% C.I.) of wild boars with medium to high antibody titres against PCV2, depending on their age and sex. Piglets are not included.

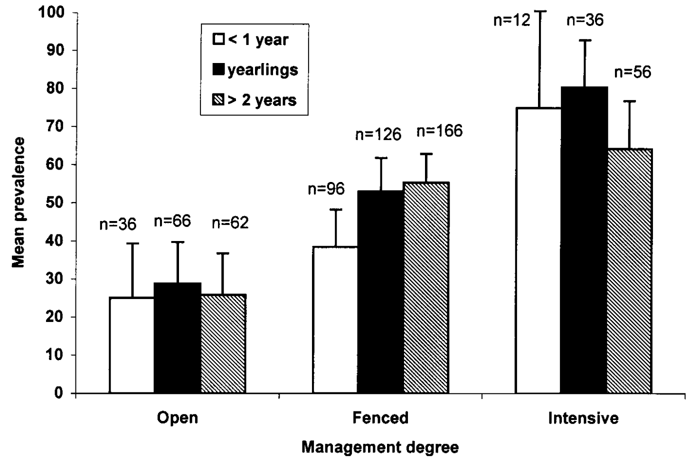

Figure 3. Percentage (and 95% C.I.) of wild boars with medium to high antibody titres against PCV2, depending on their age and on the management type of the sampling site.

available (n = 17), no correlation was evidenced between the PCV2 titre and KFI.

One out of 56 wild boars had marked lymphocyte depletion with moderate histiocytic infiltration of lymphoid tissues, which are the hallmark lesions for PMWS. This animal was a 6-month-old piglet showing wasting that had been found dead in an intensively managed enclosure from Toledo province, central Spain. No serology was available for this animal. A marked amount of viral genome was detected in the tissues of this animal; the labelling was mainly located in the cytoplasm of macrophage-like cells in the lymph nodes, spleen, lung and liver, while a low amount of the PCV2 genome was also detected within the cytoplasm of epithelial tubular cells of the

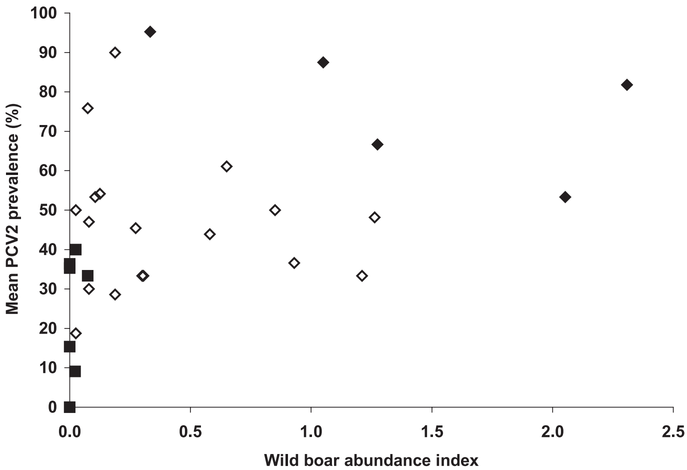

Figure 4. Mean seroprevalence of medium to high PCV2 antibody titres, plotted against abundance indexes (mean number of droppings every 100 m in 4 km transects) in 30 wild boar populations. ■ open, ◇ fenced, ◆ intensively managed.

kidney. Two more wild boars, juvenile individuals that had been shot, showed PCV2 nucleic acid in their tissues. One of them came from an intensive enclosure in Montes de Toledo and the other came from a fenced estate in Sierra Morena. They had a very low amount of viral nucleic acid within the cytoplasm of cells in the lymphoid follicles (dendritic-like cells) of lymph nodes; no labelling was observed in other tissues of these animals. It is remarkable that both individuals had high titres to PCV2 (1:20 480). The rest of the 53 wild boars had negative results by in situ hybridisation.

Other microscopic lesions observed in the studied group of animals were a variable degree of hyperplasia of the broncho-alveolar lymphoid tissue (n = 18; the presence of nematodes with morphological characteristics typical of *Metastrongylus* spp. were observed associated to these lesions), tuberculosis-like granulomatous lesions, consisting of a necrotic centre (mineralised or not) surrounded by epithelioid and scattered multinucleated giant cells in the lymph nodes, lung and/or liver (n = 14), catarrhal-purulent bronchopneumonia (n = 8) and fibrino-necrotising pleuropneumonia (n = 1).

4. DISCUSSION

This paper presents the first large-scale study of PCV2 infection in European wild boars. The high seroprevalence to PCV2, along with its widespread geographical distribution, suggest an endemic status of PCV2 in the wild boar in Spain. This is in agreement with a previous report that already showed the PCV2 antibody presence in north-eastern Spanish wild boars [22]. Moreover, we report the first confirmed PMWS case in a Spanish wild boar, further confirming that European wild boars can develop PMWS [23].

The three localities with no animals with medium to high titres were placed in the northern, southern, and south-western extremes of the study area, and corresponded to open, low density wild boar populations. In contrast, high titres were frequently observed in the Mediterranean woodlands of Montes de Toledo and Sierra Morena, where wild boars are abundant, populations are not fragmented, and management is often intensive. Indeed, the percentage of wild boars with medium to high antibody titres was maximal in intensively managed populations. This fact shows that management conditions have a clear effect on PCV2 prevalence in wild boars. This density dependence was expected since the seroprevalence in domestic swine reared under intensive conditions is also extremely high [14]. Moreover, since wild boar management conditions are becoming increasingly intensive in Spain, the risk of increasing PCV2 prevalence in wild boars is also growing; this situation also applies to many European countries [18], since natural wild boar populations are increasing.

In our opinion, while the wild boar is considered a potential reservoir of viruses for the domestic swine, such as classical swine fever virus, Aujeszky disease virus and bovine viral diarrhoea virus [1, 9, 25, 27], it is likely that this situation is the contrary for PCV2.

The PMWS affected animal was a 6-month-old wild boar within the age category of piglets. However, the presentation of PMWS in the domestic swine is around 2 to 4 months of age [21], while it is rare or very rare at slaughter age, which would be the equivalent age for this diseased wild boar piglet. The same situation applied to the already described case of PMWS in a German wild boar [23] which was a 10-month-old juvenile; histopathological lesions observed in both cases were identical to those described in domestic pigs suffering from PMWS. At present it is not possible to assess the reasons for the difference in age presentation. Living conditions, age of infection and infection load with PCV2 are potential factors that influence the progress of the disease resulting from this viral infection. Furthermore, it is known that a non-specific activation of the immune system and concurrent viral/bacterial infections can trigger or worsen PMWS presentation [21]. Taking into account the general histopathological results of the studied wild boars, where evidence of bacterial and parasitic infections occurred, it would not be surprising that these concomitant processes may influence the final outcome of PCV2 infection towards the development of PMWS in certain animals. Also, an inverse relationship may occur since PCV2 infection may trigger the development of other processes, such as tuberculosis. However, further studies on young wild boars, and especially in those with age comparable to the natural occurring PMWS in domestic swine, are needed.

The present study was based on hunter-harvested animals and not on clinical cases, except for the piglet with PMWS. Therefore, it was not surprising to detect only few individuals with PCV2 nucleic acid in their tissues, although in low amounts. This latter finding probably represents a subclinical infection and/or a recovery phase of the disease, as suggested for domestic swine with a similar amount of viral nucleic acid in lymphoid tissues [20]. A third possibility suggested for the domestic pigs, an initial phase of infection, is probably non-applicable since most of the cases were older than seven months of age.

Piglets are less interesting to hunters due to their small size and trophy, and because Spanish hunting regulations forbid shooting "reproducing animals". Thus, wild boar piglets are less frequently shot than elder age classes. Although the number of wild boars with definitive diagnosis of PMWS is minimal, three facts suggest that PMWS might play a role in the population dynamics of intensively managed populations: first, fewer piglets than expected were sampled from intensively managed areas; second, juveniles from these conditions had an extremely high seroprevalence (suggesting relatively recent past PCV2 infection), which contrasts with data from other management types; and third, a number of these farm-like areas reported abnormal piglet mortalities. However, if PMWS is of importance in wild boar piglets, the control of the disease would be very difficult in this population since the control of the disease in domestic pig farms is carried out through all in-all out management, strict hygiene and other zootechnical measures [21]. These options are very difficult to apply in intensively managed wild boar populations, and almost impossible in non-intensively managed ones.

The main weaknesses of this study are the lack of information from part of continental Spain, including the north-west and the whole Mediterranean coast, and the limited information on piglets less than seven months of age, which are most likely to develop PMWS. Moreover, retrospective studies on old wild boar samples would be needed to elucidate the temporal evolution of PCV2 presence in Spanish wild boar populations. The abundance index used in this study needs a validation regarding factors such as habitat, time of the year and age and sex related differences in defecation behaviours, that exceeds the frame of this survey.

Our study highlights the need to include all host classes and management situations in the sampling of wildlife species for epidemiological surveys [26]. In order to improve our knowledge on PCV2 epidemiology, and particularly in order to determine the importance of PMWS as a mortality factor in wild boar piglets, future studies should pay special attention to the piglet age-class and should consider radio-tagging in order to permit true mortality estimates and a prompt retrieval of the carcasses.

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