

Pathological observations of porcine respiratory disease complex (PRDC) in the wild boar (*Sus scrofa*)

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Abstract Porcine respiratory disease complex (PRDC) is a multifactorial respiratory syndrome related to the infection with different pathogens. Although most of these pathogens have been detected in the wild boar, the PRDC pneumonic lesions in this species have not been characterized. The aims of this study were to assess the presence of the main swine respiratory pathogens in wild boar populations from mid-western Spain and to describe the pathological features present in the lung from animals infected with PRDC pathogens. A pathological assessment based on five histological parameters was carried out in lung sections from 210 hunted wild boar. The presence of *Mycoplasma hyopneumoniae*, *Haemophilus parasuis*, *Actinobacillus pleuropneumoniae*, *Pasteurella multocida*, Aujeszky's disease virus, and porcine

circovirus type 2 (PCV2) in lungs was assessed by the use of specific PCR assays. Additionally, immunohistochemical techniques were carried out to detect swine influenza virus (SIV) and porcine reproductive and respiratory syndrome virus (PRRSV) infection in the lungs. Furthermore, the distribution of infected cells with PCV2 and the presence of *M. hyopneumoniae* throughout the pulmonary parenchyma were evaluated using immunohistochemistry and in situ hybridization assays in a subset of animals. Wild boar infected with *M. hyopneumoniae*, *H. parasuis*, or *P. multocida* showed the most severe lesions. *M. hyopneumoniae*, SIV, PCV2, and PRRSV were detected in single or mixed infections. Animals suffering from mixed infections with *M. hyopneumoniae* together with different viruses showed severe bronchopneumonia associated with interstitial pneumonia, suggesting that interactions between pathogens might increase the severity of pathological outcomes.

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Introduction

Porcine respiratory disease complex (PRDC) is a multifactorial respiratory syndrome that produces important economic losses in porcine production throughout the world (Maes et al. 2000). This syndrome mainly affects finishing pigs with a morbidity rate ranging from 10 to 40 % and a mortality rate ranging from 2 to 10 % (Harms et al. 2002; Kim and Chae 2004; Thacker 2001).

In domestic swine, animals affected by PRDC usually show cough, dyspnea, fever, decreased feed intakes, and growth retardation (Opriessnig et al. 2011). Pulmonary lesions in affected animals are mainly located in the cranioventral parts of the lung, where consolidation, discoloration, and atelectasia may be observed, although these features can vary depending on the pathogens involved (Harms et al. 2002). Microscopically, these pulmonary lesions are represented by bronchopneumonia with the presence of exudates within airways and alveolar spaces, peribronchitis and peribronchiolitis, and lymphoid hyperplasia, occasionally in combination with the presence of inflammatory infiltration in alveolar septa and type II pneumocyte hyperplasia (Harms et al. 2002; Kim and Chae 2004). The severity of the clinical presentation of PRDC in pigs depends on the interactions between the respiratory pathogens involved, environmental factors including management systems, and individual factors such as age or immunological status (Opriessnig et al. 2011).

Different infectious pathogens may be involved in the development of PRDC. Respiratory pathogens are commonly divided into primary pathogens, which are able to induce severe lesions in the respiratory tract as a result of their own virulence, and secondary or opportunistic pathogens that usually induce lesions in the respiratory tract in combination with other pathogens or factors (Opriessnig et al. 2011). The main primary respiratory pathogens involved in PRDC are viruses such as porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza virus (SIV), Aujeszky's disease virus (ADV), and porcine circovirus type 2 (PCV2), or bacteria such as *Mycoplasma hyopneumoniae* or *Actinobacillus pleuropneumoniae* (Brockmeier et al. 2002). Furthermore, *Pasteurella multocida* or *Haemophilus parasuis* are among the most common opportunistic respiratory pathogens (Brockmeier et al. 2002).

The majority of the pathogens involved in the development of PRDC in domestic pigs, like PRRSV (Reiner et al. 2009), SIV (Closa-Sebastiá et al. 2011), PCV2 (Cságola et al. 2006), *M. hyopneumoniae* (Sibila et al. 2010), *A. pleuropneumoniae* (Reiner et al. 2010), *P. multocida* (Risco et al. 2013a), or *H. parasuis* (Cuesta et al. 2013), have been also detected in wild boar (*Sus scrofa*). However, the clinical and pathological outcomes produced by these respiratory pathogens in wild boar are unknown yet.

The aims of this study were to assess the presence of the main swine respiratory pathogens in wild boar from mid-western Spain and to describe the pathological features of the lungs of naturally infected animals with PRDC pathogens.

Material and methods

Sampling area and animals

This study was carried out on a total of 210 hunted wild boar from 20 game estates in mid-western Spain (Fig. 1). The study

area has particular features in terms of ecology and climate. Briefly, the average annual precipitation reaches 623 mm and is concentrated in the months of November to April. The mean annual temperature averages 17.7 °C, being January the coldest and July the warmest month of the year. The vegetation is typical of Mediterranean forest, characterized by abundant *Quercus ilex* and *Quercus suber* trees with understoreys dominated by *Quercus coccifera*, *Cistus ladanifer*, and *Erica arborea*. In all the game estates included in this work, wild boar share habitat with red deer (*Cervus elaphus*) and in some cases with fallow deer (*Dama dama*), roe deer (*Capreolus capreolus*), or cattle.

Wild boar included in this work were hunted between October 2011 and February 2013. The sex and age of these animals were determined on the basis of the observation of their sexual organs and the dentition eruption pattern respectively (Boitani and Mattei 1992). Animals were divided into four different groups according to their ages: piglets (less than 6 months), juveniles (6 months–1 year), yearlings (1 year–2 years), and adults (more than 2 years) as described previously (García-Jiménez et al. 2012).

Necropsy examination of all animals was performed in the field with a detailed macroscopic inspection of the lungs. A piece from the right cranial lung lobe was taken and immersed in 10 % buffered formalin immediately after the examination. An additional adjacent piece from the same lobe was collected in sterile storage bags, kept cold for transport, and, in less than 6 h, frozen at –20 °C for further laboratory analysis. Finally, blood samples were collected from the heart or the thoracic cavity. These samples were centrifuged in the laboratory at 3000 rpm for 10 min in order to separate the sera before being stored at –20 °C until their utilization.

Histopathological study

All the animals studied in this work were hunted wild boar. For this reason, animals showed frequent pulmonary hemorrhages that could lead to misinterpretation in a gross lesional scoring system of these lungs. To avoid this fact, the pathological study was based only on the study of specific histopathological parameters as previously described (Opriessnig et al. 2004), easily distinguishable from the artifacts caused by the type of death (shot).

Lung tissue samples previously immersed in buffered formalin were processed following standard procedures and were routinely stained with hematoxylin and eosin for the histopathological study. Lung sections were subjected to blind examination (without knowing the microbiological study results). Five histopathological parameters were scored on a scale from 0 to 6 (0, normal; 1, mild multifocal; 2, mild diffuse; 3, moderate multifocal; 4, moderate diffuse; 5, severe multifocal; 6, severe diffuse) being the severity a subjective score but always determined by the same pathologists. In fact,

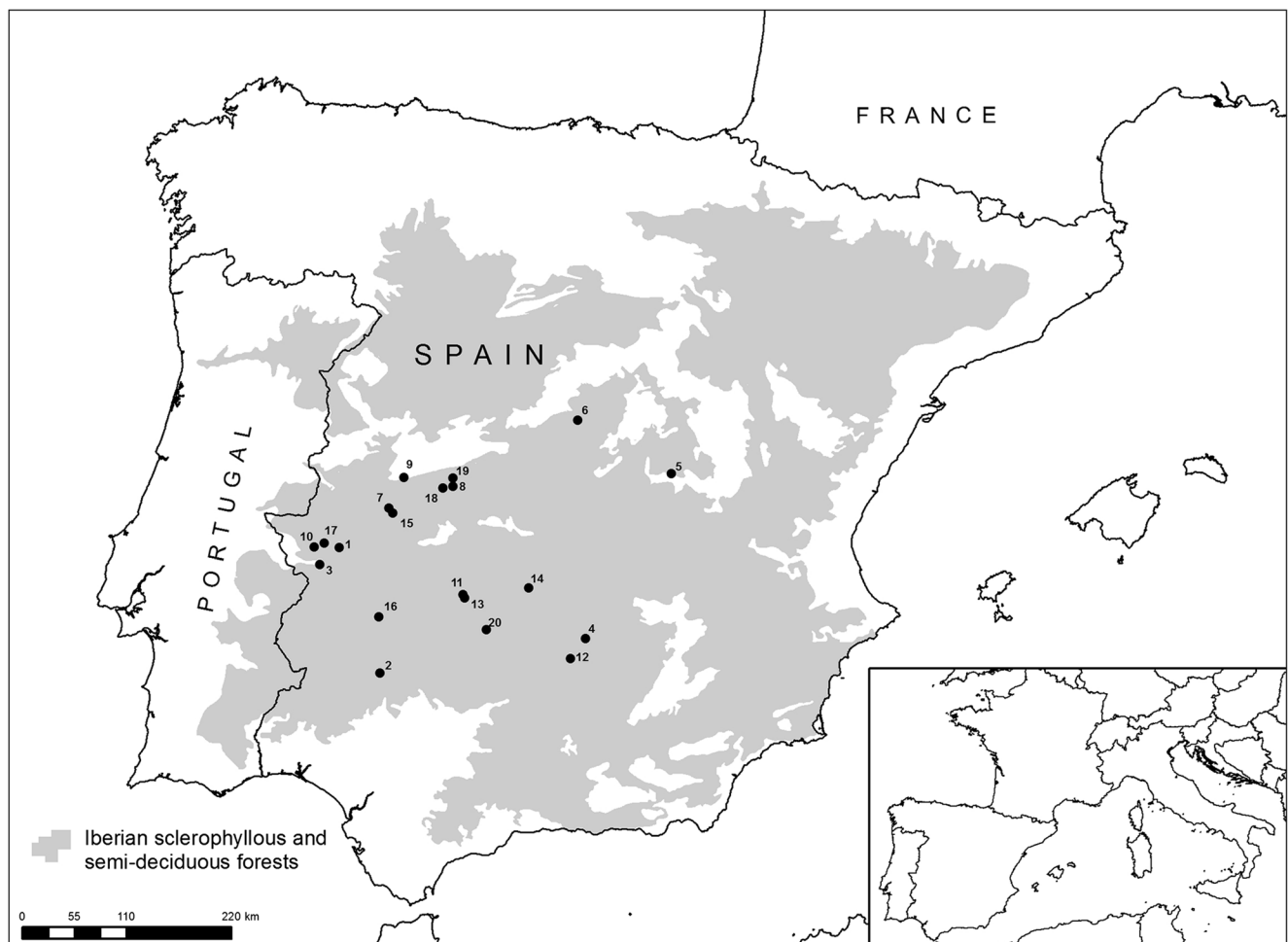


Fig. 1 Localization of the 20 game estates from southwestern Spain included in this study

the lesions were scored independently by two veterinary pathologists. In case of any discrepancy, the veterinary pathologists reviewed the slides and agreed the final score. These parameters were as follows: presence of alveolar septal infiltration with inflammatory cells (Fig. 2a), amount of exudates in alveoli and airways (Fig. 2b), peribronchial lymphoid hyperplasia (Fig. 2c), amount of inflammatory cells in the lamina propria of bronchi and bronchioles (Fig. 2d), and presence of necrosis in epithelial cells of bronchi and bronchioles (Fig. 2e) (Landolt et al. 2003; Opriessnig et al. 2004). Images of the different histological parameters showing different degrees of severity can be seen in the supplemental online file. The presence of tuberculosis-like granulomatous lesions (Fig. 2f) and lung nematodes was also assessed in the lung sections studied, since they are frequent in animals from the studied area (García-González et al. 2013; Martín-Hernando et al. 2007).

Respiratory pathogen detection by PCR

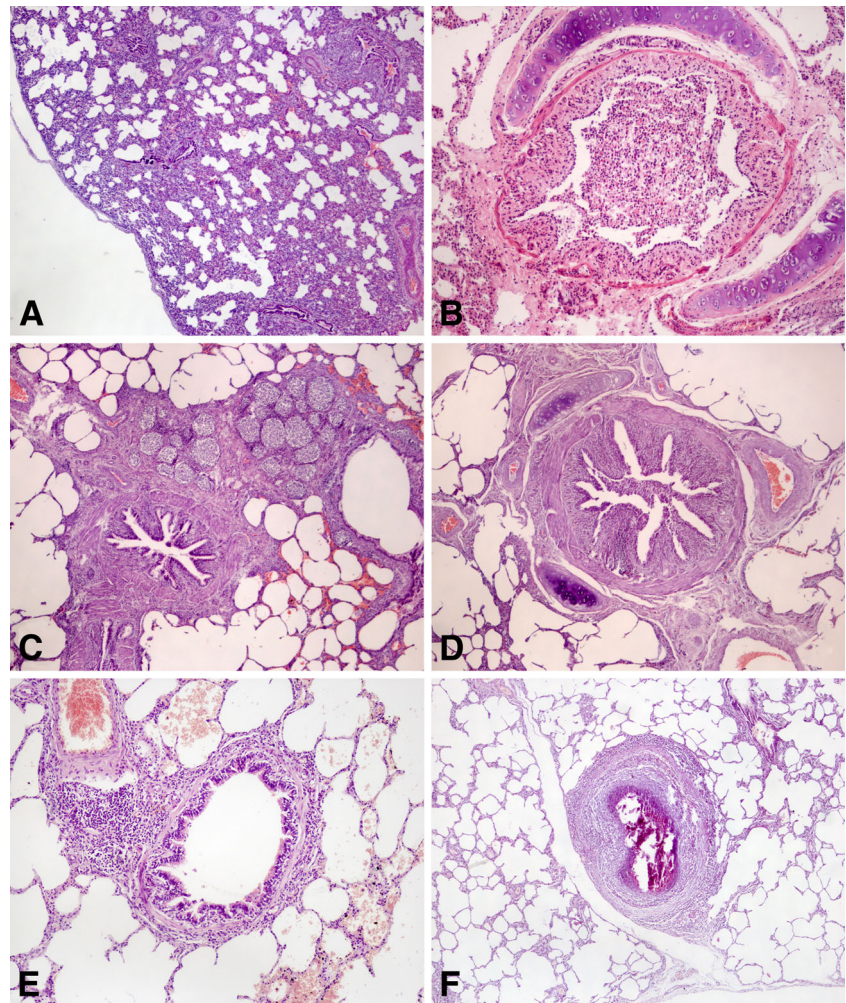
A set of specific PCR techniques was used to detect the presence of some of the most common respiratory pathogens in

wild boar lungs as it has been previously described in similar surveys (Reiner et al. 2010; Reiner et al. 2009; Sibila et al. 2010). DNA from the cranial lung lobe tissue stored at -20°C was extracted using a commercial QIAamp[®] DNA Mini kit (Qiagen Ltd., Crawley, West Sussex, RH10 9NQ, UK) following the manufacturer's recommendations. PCR assays were carried out following previously described protocols and using extracted DNA as template. Primers and protocols used to perform the PCR assays are summarized in Table 1.

Immunohistochemistry and in situ hybridization assays

The presence of PRRSV and SIV antigens and their distribution throughout the pulmonary parenchyma were assessed using immunohistochemistry (IHC) techniques. The avidin–biotin–peroxidase technique was used with the corresponding specific antibodies to detect PRRSV and SIV in lung sections following previously published procedures (Brookes et al. 2010; Gómez-Laguna et al. 2010). Due to logistic and funding reasons, IHC assays to detect SIV were carried out in 127 wild boar, whereas the presence of PRRSV was evaluated in 70 animals.

Fig. 2 Wild boar, lung. **a** Infiltration of inflammatory cells in alveolar septa. Hematoxylin and eosin (H-E) stain. **b** Airway exudates mainly formed by macrophages and neutrophils. H-E stain. **c** Severe lymphoid hyperplasia of bronchus-associated lymphoid tissues. H-E stain. **d** Inflammatory infiltrates within the bronchiolar lamina propria. H-E stain. **e** Nuclear pignosis associated to necrosis of bronchiolar epithelial cells. H-E. **f** Tuberculous granuloma with a mineralized core and an outer layer of macrophages, lymphocytes, and fibrous tissue. H-E stain



Furthermore, to assess the distribution of *M. hyopneumoniae* and PCV2 in infected lung tissues, different IHC and in situ hybridization (ISH) techniques were carried out in 26 wild boar. These animals were chosen among those that had been tested for the presence of SIV and PRRSV antigens. PCV2 antigens were detected using both IHC and ISH techniques, carried out as previously described (Drew et al. 2004; Rosell et al. 1999). The distribution of *M. hyopneumoniae* in pulmonary parenchyma was evaluated using a fluorescent ISH assay (Boye et al. 2001).

Statistical analyses

Mean scores obtained for each of the studied histopathological parameters were compared between animals infected and non-infected with each pathogen. A non-parametric test (Mann–Whitney *U* test) was used to compare means scored between groups. To avoid the possible influence of lung nematodes in obtained results, animals that showed the presence of parasites in the histopathological examination were not included in the statistical analysis

carried out to correlate the presence of CRPD pathogens and histopathological lesions.

In addition, mean scores obtained for each histological parameter were compared between age groups using Kruskal–Wallis test. All calculations were performed using the SPSS 15 software package (SPSS Inc., Chicago, IL, 60606, USA).

Results

Pathogen assessment results

The observed prevalences for respiratory pathogens studied in different groups of age are summarized in Table 2. Briefly, a moderate percentage of animals were infected by *M. hyopneumoniae* (24.80 %), PCV2 (19.5 %), and PRRSV (14.3 %), whereas the rest of the microorganisms studied were only found in a small percentage of animals (<10 %).

Prevalences obtained for *H. parasuis* and *P. multocida* were significantly higher in piglets than in the rest of the age groups ($p < 0.001$).

Table 1 Primers and protocols used to perform PCR assays to detect several respiratory pathogens in lungs from hunted wild boar

Pathogen	Primers	Final volume (μ l)	Sample volume (μ l)	Reagents	Protocol ^a	Cycles
<i>Mycoplasma hyopneumoniae</i> (1) ^b	649-f, 649-r (Sijjar et al. 1996)	25	5	Taq premix ^c = 1 \times Primers = 0.2 μ M	95° 30 s 59° 40 s 72° 30 s	30
<i>Mycoplasma hyopneumoniae</i> (2)	352f, 352r (Calsamiglia et al. 1999)	25	0.5 ^d	Taq premix = 1 \times Primers = 0.2 μ M	95° 30 s 59° 40 s 72° 30 s	30
<i>Haemophilus parasuis</i>	HPS-f, HPS-r (Oliveira et al. 2001)	25	1	Taq premix = 1 \times Primers = 0.3 μ M	72° 30 s 95° 30 s 59° 30 s 72° 2 min	30
<i>Actinobacillus pleuropneumoniae</i>	APXIV-f, APXIV-r (Cho and Chae 2003; Schaller et al. 1999)	20	2	Taq premix = 1 \times Primers = 0.4 μ M	95° 35 s 63° 90 s (1–5) ^e 59° 90 s (6–35) ^e	35
<i>Pasteurella multocida</i>	KMT1T7, KMT1SP6 (Townsend et al. 1998)	25	5	Taq premix = 1 \times Primers = 0.4 μ M	72° 1 min 95° 30 s 55° 40 s	30
Aujeszky's disease virus (1) ^b	gB1st05, gB1st03 (Yoon et al. 2006)	25	5	Taq premix = 1 \times Primers = 1 μ M	72° 30 s 95° 30 s 58° 30 s	30
Aujeszky's disease virus (2)	gB2nd05, gB2nd03 (Yoon et al. 2006)	25	0.5 ^d	Taq premix = 1 \times Primers = 1 μ M	72° 30 s 95° 30 s 58° 30 s	30
Porcine circovirus	CBB1, CBB2 (Cságola et al. 2006)	25	5	Taq premix = 1 \times Primers = 1 μ M	72° 30 s 95° 30 s 58° 30 s 72° 30 s	35

^a Temperature and time used in denaturation, annealing, and extension steps are defined in this column. All the PCR protocols started with a first denaturation step (95° 3 min) and ended with a final extension step (72° 10 min)

^b Nested PCR with two steps

^c DreamTaq Green PCR Master Mix®, Thermo Scientific, Waltham, MA, USA

^d Amplified product from the first PCR of the nested PCR was used as template

^e Extension step with two different temperatures (63° cycles 1–5 and 59° cycles 6–35)

Table 2 Prevalence for the respiratory pathogens included in this study in piglets (0–6 months), juveniles (6–12 months), yearlings (13–24 months), and adult (over 24 months) male and female wild boar hunted harvested in southwestern Spain

Pathogen	Diagnosis technique	Total prevalence	Age-specific prevalence ^a
Viruses			
Porcine circovirus type 2	PCR	19.5 % (<i>n</i> =210)	Piglets 14.3 % (<i>n</i> =21) Juveniles 47.4 % (<i>n</i> =19) Yearlings 31.3 % (<i>n</i> =48) Adults 12.5 % (<i>n</i> =112)
Swine influenza virus	Immunohistochemistry assay	8.7 % (<i>n</i> =127)	Piglets 0 % (<i>n</i> =3) Juveniles 23.1 % (<i>n</i> =13) Yearlings 10 % (<i>n</i> =30) Adults 6.8 % (<i>n</i> =74)
PRRS virus	Immunohistochemistry assay	14.3 % (<i>n</i> =70)	Piglets 100 % (<i>n</i> =1) Juveniles 25 % (<i>n</i> =8) Yearlings 6.3 % (<i>n</i> =16) Adults 9.7 % (<i>n</i> =31)
Aujeszky's disease virus	PCR	11.3 % (<i>n</i> =160)	Piglets 0 % (<i>n</i> =11) Juveniles 0 % (<i>n</i> =17) Yearlings 17.1 % (<i>n</i> =35) Adults 12.5 % (<i>n</i> =88)
Bacteria			
<i>Haemophilus parasuis</i>	PCR	3.8 % (<i>n</i> =210)	Piglets 33.3 % (<i>n</i> =21) Juveniles 0 % (<i>n</i> =19) Yearlings 0 % (<i>n</i> =48) Adults 0.9 % (<i>n</i> =112)
<i>Actinobacillus pleuropneumoniae</i>	PCR	6.2 % (<i>n</i> =210)	Piglets 4.8 % (<i>n</i> =21) Juveniles 0 % (<i>n</i> =19) Yearlings 6.3 % (<i>n</i> =48) Adults 5.4 % (<i>n</i> =112)
<i>Mycoplasma hyopneumoniae</i>	Nested PCR	24.8 % (<i>n</i> =210)	Piglets 33.3 % (<i>n</i> =21) Juveniles 36.8 % (<i>n</i> =19) Yearlings 27.1 % (<i>n</i> =48) Adults 21.4 % (<i>n</i> =112)
<i>Pasteurella multocida</i>	PCR	1.4 % (<i>n</i> =209)	Piglets 14.3 % (<i>n</i> =21) Juveniles 0 % (<i>n</i> =19) Yearlings 2.1 % (<i>n</i> =48) Adults 0 % (<i>n</i> =112)

^a Ten out of the studied animals were not included in the estimation of prevalences by age since their ages were unknown

Histopathology

Histological lung lesions were found in 83.34 % of the studied animals. Each histopathological parameter studied was found in a different percentage of animals and showed different severity. For example, whereas the presence of peribronchial lymphoid hyperplasia was observed in 64.29 % of the animals with a mean score of 1.75, the presence of necrosis in epithelial cells of bronchi and bronchioles was only detected in 2.38 % of the animals. All the histopathological scores obtained are summarized in Table 3.

Histopathological lesions observed were influenced by the age of the animals (Fig. 3). Piglets showed a significant higher

score in the amount of exudates in airways and alveoli ($p<0.001$), compared to juveniles and yearlings, which presented a higher score of peribronchial lymphoid hyperplasia ($p=0.01$). Granulomatous lesions were detected in 42 animals, whereas lung nematodes were observed in 17 animals (Fig. 4a).

Correlation between respiratory pathogens and histological lesions

The mean scores obtained for each histopathological parameter assessed in both animals infected and non-infected with each pathogen are summarized in Table 4. Briefly, some of the lesional parameters assessed were more severe in

Table 3 Histopathological lesions found in lung sections from 210 wild boar evaluated. Mean score represents the severity found by each parameter in the wild boar population

Parameter	Mean score	Animals showing different degrees of lesions						
		No lesion	Mild multifocal	Mild diffuse	Moderate multifocal	Moderate diffuse	Severe multifocal	Severe diffuse
Alveolar septal infiltration	1.02	113 (53.8 %)	40 (19 %)	21 (10 %)	13 (6.2 %)	21 (10 %)	2 (1 %)	0 (0 %)
Bronchoalveolar exudates	0.43	178 (84.8 %)	10 (4.8 %)	3 (1.4 %)	8 (3.8 %)	6 (2.9 %)	3 (1.4 %)	2 (1 %)
Peribronchial lymphoid hyperplasia	1.75	75 (35.7 %)	42 (20 %)	28 (13.3 %)	19 (9 %)	29 (13.8 %)	5 (2.4 %)	12 (5.7 %)
Lamina propria inflammation	0.55	163 (77.6 %)	14 (6.7 %)	12 (5.7 %)	11 (5.2 %)	7 (3.3 %)	2 (1 %)	1 (0.5 %)
Necrosis of epithelial cells	0.07	205 (97.6 %)	1 (0.5 %)	0 (0 %)	3 (1.4 %)	0 (0 %)	1 (0.5 %)	0 (0 %)

animals infected with *M. hyopneumoniae*, *P. multocida*, or *H. parasuis*. A higher amount of airway and alveolar exudates, predominantly composed of macrophages and neutrophils, was observed in animals infected by *M. hyopneumoniae* (U Mann–Whitney=2919, $p=0.001$), *P. multocida* (U Mann–Whitney=133, $p=0.006$), and *H. parasuis* (U Mann–Whitney=255.5, $p<0.001$). In addition, a significant higher infiltration of inflammatory cells in alveolar septa was detected in animals infected by *H. parasuis* (U Mann–Whitney=441.5, $p=0.017$). Furthermore, lung sections showing nematodes had a significant higher amount of alveolar and airway exudates (U Mann–Whitney=884.5, $p=0.018$), lymphoid hyperplasia (U Mann–Whitney=712.5, $p=0.016$), and marked inflammation in the lamina propria of bronchi and bronchioles (U Mann–Whitney=327, $p<0.001$).

Immunohistochemistry and in situ hybridization

Specific antigens of PCV2, SIV, PRRSV, and *M. hyopneumoniae* were detected in lung sections evaluated by IHC and ISH techniques. PCV2 antigens were detected in 14 animals using IHC and were mainly found in macrophages located in bronchus-associated lymphoid tissues (BALT), although some alveolar macrophages also showed presence of PCV2 antigens. PCV2-infected cells were also detected using ISH assay in eight out of 14 animals that resulted positive to IHC methods showing a similar distribution (Fig. 4b). Immunopositive cells against SIV antigens detected in infected animals (11) were scarce, and only few epithelial cells of bronchi and bronchioles and alveolar macrophages showed a positive stain (Fig. 4c). Animals infected by PRRSV (10) showed few immunopositive macrophages located in BALT and alveolar septa, occasionally forming clusters of cells (Fig. 4d). *M. hyopneumoniae* DNA was detected in four animals using ISH assay, mainly as labeled bacteria attached to the apical border of bronchial and bronchiolar epithelial cells.

Five out of the 26 animals that were tested for all the pathogens using in situ detection techniques showed positive cells for more than one of these pathogens. Pathogen combinations obtained and the observed histological lesions are shown in Table 5. In general, animals infected only with viruses (in single or mixed infection) or single-infected with *M. hyopneumoniae* showed mild infiltration of inflammatory cells in alveolar septa and mild lymphoid hyperplasia. However, the two animals that presented mixed infections with viruses and *M. hyopneumoniae* showed a severe bronchopneumonia.

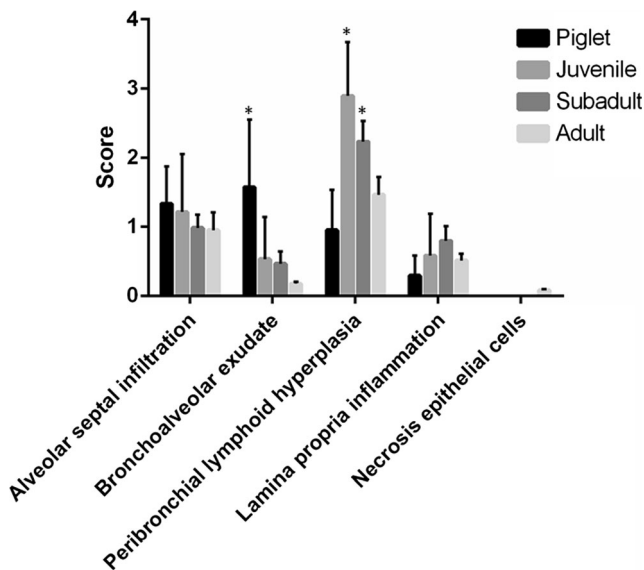
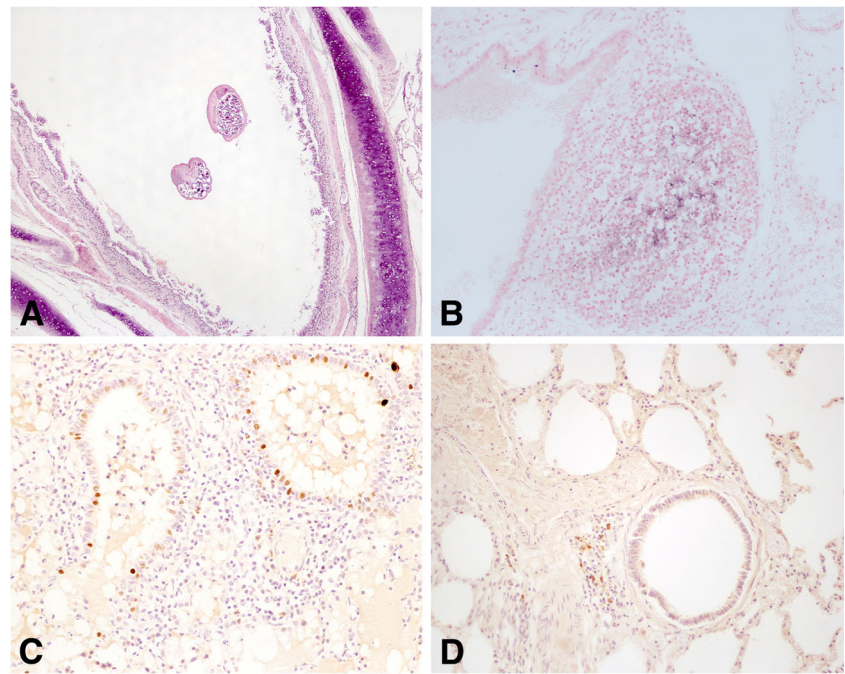


Fig. 3 Mean score values obtained for each histopathological parameter assessed in each age group. Asterisks show statistically significant differences ($p<0.05$) in mean lesional parameters between age groups

Discussion

Results obtained in this work show that wild boar can be infected by the main respiratory pathogens involved in PRDC. The presence of histopathological pulmonary lesions

Fig. 4 Wild boar, lung. **a** Nematodes within the airway. Hematoxylin and eosin stain. **b** Numerous macrophages positive against PCV2 detected by in situ hybridization assay. ISH, red counterstain. **c** Epithelial cells and macrophages immunomarked against SIV. IHC, Mayer's hematoxylin counterstain. **d** A cluster of macrophages immunomarked against PRRSV. IHC, Mayer's hematoxylin counterstain



was widely spread, finding lesions in 83.34 % of the studied wild boar. However, mean scores obtained for each histological parameter showed that the severity of lesions was often mild, although individual cases with severe lung lesions were also recorded.

The pneumonic lesions induced by the respiratory pathogens assessed were variable. Wild boar infected with pathogens such as *M. hyopneumoniae*, *H. parasuis*, or *P. multocida* showed more severe pulmonary lesions than non-infected animals. Conversely, single infections with *A. pleuropneumoniae*, PCV2, or ADV were not related with pulmonary lesions.

Animals infected with *M. hyopneumoniae* showed a significant higher amount of airway and alveolar inflammatory

exudates. These results are in agreement with a recent work in which *M. hyopneumoniae* infections have been correlated with the presence of gross pneumonic lesions in free-ranging wild boar (Chiari et al. 2013), and confirm that the presence of this pathogen may also affect a variety of histopathological parameters.

Likewise, lesions found in animals infected with *H. parasuis* were similar to those recently described in a young wild boar infected with this pathogen, showing severe bronchopneumonia associated with interstitial pneumonia (Cuesta et al. 2013). The prevalence of *H. parasuis* infection found in this study was low (4.7 %), but interestingly, most of the infected animals were piglets (39.1 % of the piglets resulted infected). These

Table 4 Mean scores obtained for the five pulmonary pathological parameters studied in positive and negative animals against each respiratory pathogen

	SIV		PCV2		MHYO		HPS		APP		PM		Nematodes		PRRSV		ADV	
	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.
Alveolar septal infiltration	0.97	0.60	1.11	0.85	1.15	0.77	1.02 ^a	2 ^a	1.04	1.31	1.04	1.33	1.06	0.50	1.05	0.67	1.08	0.83
Bronchoalveolar exudates	0.29	0.40	0.40	0.48	0.23 ^a	1.00 ^a	0.29 ^a	3.25 ^a	0.42	0.38	0.37 ^a	2 ^a	0.41 ^a	0.75 ^a	0.23	0.67	0.28	0.00
Peribronchial lymphoid hyperplasia	1.72	1.80	1.61	1.95	1.60	1.92	1.71	0.93	1.67	1.77	1.66	1.33	1.68 ^a	3.00 ^a	1.61	2.33	1.64	1.56
Lamina propria inflammation	0.35	0.6	0.42	0.43	0.35	0.65	0.43	0.25	0.39	0.77	0.4	0	0.42 ^a	2.67 ^a	0.27	1	0.38	0.28
Necrosis of epithelial cells	0.11	0	0.09	0	0.08	0.06	0.07	0	0.08	0	0.07	0	0.07	0	0.14	0	0.09	0

SIV swine influenza virus, PCV2 porcine circovirus type 2, MHYO *Mycoplasma hyopneumoniae*, HPS *Haemophilus parasuis*, APP *Actinobacillus pleuropneumoniae*, PM *Pasteurella multocida*, PRRSV porcine reproductive and respiratory syndrome virus, ADV Aujeszky's disease virus

^a Statistically significant difference ($p < 0.05$) between mean scores obtained in positive and negative animals

Table 5 Pathogens and lesions found in immunohistochemical and in situ hybridization assays against *Mycoplasma hyopneumoniae* (MHYO), porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza virus (SIV), and porcine circovirus type 2 (PCV2)

Pathogen combination	Sample	Lesional score ^a	Histopathological lesion description
Single infections			
MHYO	2	3	Mild lymphoid hyperplasia
PRRSV	2	4.5	Mild infiltration of inflammatory cells in alveolar septa and mild lymphoid hyperplasia
PCV2	9	4.22	Mild foci of inflammatory infiltrate in alveolar septa and mild to severe lymphoid hyperplasia
Mixed infections			
PCV2 + SIV	1	1	Mild focus of inflammatory infiltrate in alveolar septa
PCV2 + SIV + PRRSV	2	4.5	Severe lymphoid hyperplasia
PCV2 + SIV + MHYO	1	20	Severe bronchopneumonia and interstitial pneumonia with presence of abundant exudates
PCV2 + SIV + PRRSV + MHYO	1	10	Severe bronchitis with inflammatory infiltrates in the lamina propria, lymphoid hyperplasia, and presence of alveolar exudates

^a Mean lesional score found in the group of animals infected with each pathogen or combination of pathogens (the lesional score was calculated as the sum of the scores for each histological parameter)

results suggest that *H. parasuis* infection may lead to pathological outcomes mainly in young animals that, in addition, are most susceptible to respiratory pathogens (Opriessnig et al. 2011).

Animals infected by *P. multocida* showed a higher amount of bronchoalveolar exudates. The presence of abundant inflammatory infiltrates and exudates in alveoli and airway lumen has been previously related with *P. multocida* infection in domestic swine (Pijoan 2006). However, this microorganism is considered a secondary respiratory pathogen and rarely produces severe lung lesions during single infections (Opriessnig et al. 2011). The three animals in which *P. multocida* was detected were co-infected with *M. hyopneumoniae* or *H. parasuis*, and, hence, *P. multocida* might have played a role as a secondary respiratory pathogen in these infections.

The detection of immunopositive cells against SIV and PRRSV in pulmonary parenchyma seemed not to influence the appearance of lung lesions in wild boar. In most of animals infected by these viruses, a positive reaction was detected in just a few cells, mainly macrophages but also epithelial cells from bronchi and bronchioles in the case of SIV. This fact could mean that infected animals received a low dose of infection or they were in the process of clearing the virus (Caswell and Williams 2007), which might be related with the lack of pulmonary lesions detected in infected animals. PCV2 and *M. hyopneumoniae* antigens were observed in typical target cells throughout the pulmonary parenchyma. Regarding PCV2 detection in situ, IHC assay (14 positive animals) resulted more sensitive than ISH assay (eight positive animals), as has been previously reported (Morandi et al. 2013).

Co-infections with some of the respiratory pathogens diagnosed in situ were detected in several lung samples. Respiratory co-infections may lead to more severe pathological outcomes (Opriessnig et al. 2011). In fact, it has been suggested that interactions between bacteria such as *M. hyopneumoniae* and respiratory viruses may enhance the development of lung lesions in domestic pigs (Brockmeier et al. 2002; Harms et al. 2002; Kim and Chae 2004; Thacker 2001). The results obtained in this work seem to be in agreement with this hypothesis. Wild boar infected with one or more viruses only showed a mild interstitial pneumonia and lymphoid hyperplasia, whereas two animals suffering concurrent infections of viruses and *M. hyopneumoniae* displayed more severe lesions.

In addition, other pathogens not frequently involved in PRDC development were also considered in the present study. Lesions associated to lung nematodes were recorded in the studied wild boar. Animals infected by lung nematodes showed severe lymphoid hyperplasia and a severe bronchitis and fibrosis, as it has been previously described in domestic pigs (Greve 2012). In addition, tuberculosis-like granulomatous lesions were found in 20 % of the population. Since tuberculosis was not included in the aims of this work, the presence of *Mycobacterium* spp. was not systematically assessed in wild boar showing granulomatous lesions. However, several studies have recently showed a high concordance between tuberculosis-like microscopic lesions and *Mycobacterium* spp. infections in wild boar from the studied area (Martín-Hernando et al. 2007; Risco et al. 2013b).

As a multifactorial disease, clinical signs presented in domestic pigs affected by PRDC pathogens may be enhanced by several factors such as age or management practices

(Opriessnig et al. 2011). In this work, more severe histological lesions were recorded in lungs belonging to piglets (bronchoalveolar exudates), juveniles, and wild boar yearlings (peribronchial lymphoid hyperplasia), suggesting that the pneumonic lesions may be more severe in young animals.

In addition, the severity of clinical outcomes produced by swine respiratory pathogens may be higher in farms with a high density of animals and more intensive management practices (Opriessnig et al. 2011). Currently, management practices such as fencing or supplying additional food are common in wild boar breeding, leading to artificial higher densities of animals (Gortázar et al. 2006). The number of wild boar farms in which these measures are applied is rising worldwide to meet the great demand of wild boar products in the meat industry (Hälli et al. 2012) and in the game business (Gortázar et al. 2006). The impact of PRDC pathogens could be higher in these “intensively managed” farms leading to more severe clinical outcomes and producing serious economic losses.

In conclusion, results obtained in this work show that infections with swine respiratory pathogens such as *M. hyopneumoniae*, *P. multocida*, or *H. parasuis* may produce lung lesions in wild boar. The presence of more severe lung lesions in the few animals co-infected with viruses and *M. hyopneumoniae* suggests that co-infections with respiratory pathogens might enhance the severity of respiratory pathology. However, more research including a higher number of animals and taking into account other factors that may influence the PRDC development, e.g., animal density, would be necessary to elucidate it.

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References

- Boitani L, Mattei L (1992) Aging wild boar (*Sus scrofa*) by tooth eruption. In: Spitz F, Janeau G, González G, Aulagnier S (eds) Ongules/ungulates 91. SFPEM-IRGM, Paris-Toulouse, pp 419–421
- Boye M, Jensen TK, Ahrens P, Hagedorn-Olsen T, Friis NF (2001) *In situ* hybridisation for identification and differentiation of *Mycoplasma hyopneumoniae*, *Mycoplasma hyosynoviae* and *Mycoplasma hyorhinis* in formalin-fixed porcine tissue sections. APMIS 109: 656–664
- Brockmeier SL, Halbur PG, Thacker EL (2002) Porcine respiratory disease complex. In: Brogden KA, Guthmiller JM (eds) NCBI bookshelf. ASM Press, Washington, pp 231–258
- Brookes SM, Nunes A, Choudhury B, Matrosovich M, Essen EC, Clifford D, Slomka MJ, Kuntz-Simon G, Garçon F, Nash B, Hanna A, Heegaard PMH, Quéguiner S, Chiapponi C, Bublöt M, Maldonado-García J, Gardner R, Foni E, Loeffen W, Larsen L, Van Reeth K, Banks J, Irvine RM, Brown H (2010) Replication, pathogenesis and transmission of pandemic (H1N1) 2009 virus in non immune pigs. PLoS ONE 5:e9068
- Calsamiglia M, Pijoan C, Trigo A (1999) Application of a nested polymerase chain reaction assay to detect *Mycoplasma hyopneumoniae* from nasal swabs. J Vet Diagn Investig 11:246–251
- Caswell JL, Williams KJ (2007) Respiratory system. In: Maxie MG (ed) Jubb, Kennedy and Palmer’s pathology of domestic animals. Saunders Elsevier, Philadelphia, pp 579–587
- Chiari M, Ferrari N, Zannoni M, Alborali L (2013) *Mycoplasma hyopneumoniae* temporal trends of infection and pathological effects in wild boar populations. Eur J Wildl Res 60:187–192
- Cho WS, Chae C (2003) PCR detection of *Actinobacillus pleuropneumoniae* apxIV gene in formalin-fixed, paraffin-embedded lung tissues and comparison with *in situ* hybridization. Lett Appl Microbiol 37:56–60
- Closa-Sebastiá F, Casas-Díaz E, Cuenca R, Lavín S, Mentaberre G, Marco I (2011) Antibodies to selected pathogens in wild boar (*Sus scrofa*) from Catalonia (NE Spain). Eur J Wildl Res 57:977–981
- Csárgola A, Kecskeméti S, Kardos G, Kiss I, Tuboly T (2006) Genetic characterization of type 2 porcine circoviruses detected in Hungarian wild boars. Arch Virol 151:495–507
- Cuesta JM, Risco D, Gonçalves P, García-Jiménez WL, Gil M, Fernández-Llario P, Hermoso de Mendoza J, Gómez L (2013) Fatal infection due to *Haemophilus parasuis* in a young wild boar (*Sus scrofa*). J Vet Diagn Investig 25:297–300
- Drew TW, Grierson SS, King DP, Hick D, Done S, Nester JA, Evans DPB, Grimbeek P, Banks M (2004) Genetic similarity between porcine circovirus type 2 isolated from the first reported case of PMWS in South Africa and North American isolates. Vet Rec 155:149–151
- García-González ÁM, Pérez-Martín JE, Gamito-Santos JA, Calero-Bernal R, Alcaide Alonso M, Frontera Carrión EM, Jaafar RF (2013) Epidemiologic study of lung parasites (*Metastrongylus* spp.) in wild boar (*Sus scrofa*) in southwestern Spain. J Wildl Dis 49:157–162
- García-Jiménez WL, Benítez-Medina JM, Fernández-Llario P, Abecia JA, García-Sánchez A, Martínez R, Risco D, Ortiz-Peláez A, Salguero FJ, Smith NH, Gómez L, Hermoso de Mendoza J (2012) Comparative pathology of the natural infections by *Mycobacterium bovis* and by *Mycobacterium caprae* in wild boar (*Sus scrofa*). Trans Emerg Dis 60:102–109
- Gómez-Laguna J, Salguero FJ, Barranco I, Pallarés FJ, Rodríguez-Gómez IM, Bernabé A, Carrasco L (2010) Cytokine expression by macrophages in the lung of pigs infected with the porcine reproductive and respiratory syndrome virus. J Comp Pathol 142:51–60
- Gortázar C, Acevedo P, Ruiz-Fons F, Vicente J (2006) Disease risks and overabundance of game species. Eur J Wildl Res 52:81–87
- Greve JH (2012) Internal parasites: helminths. In: Zimmerman J, Karriker L, Ramirez A, Schwartz K, Stevenson G (eds) Diseases of swine. Wiley, Ames, pp 908–920
- Hälli O, Ala-Kurikka E, Nokireki T, Skrzypczak T, Raunio-Saarnisto M, Peltoniemi OAT, Heinonen M (2012) Prevalence of and risk factors associated with viral and bacterial pathogens in farmed European wild boar. Vet J 194:98–101
- Harms PA, Halbur PG, Sorden SD (2002) Three cases of porcine respiratory disease complex associated with porcine circovirus type 2 infection. J Swine Health Product 10:27–30
- Kim J, Chae C (2004) Concurrent presence of porcine circovirus type 2 and porcine parvovirus in retrospective cases of exudative epidermitis in pigs. Vet J 167:104–106
- Landolt GA, Karasin AI, Phillips L, Olsen CW (2003) Comparison of the pathogenesis of two genetically different H3N2 influenza A viruses in pigs. J Clin Microbiol 41:1936–1941
- Maes D, Deluyker H, Verdonck M, Castryck F, Miry C, Vrijens B, De Kruif A (2000) Herd factors associated with the seroprevalences of four major respiratory pathogens in slaughter pigs from farrow-to-finish pig herds. Vet Res 31:313–327

- Martín-Hernando MP, Höfle U, Vicente J, Ruiz-Fons F, Vidal D, Barral M, Garrido JM, de la Fuente J, Gortazar C (2007) Lesions associated with *Mycobacterium tuberculosis* complex infection in the European wild boar. *Tuberculosis* 87:360–367
- Morandi F, Verin R, Sarli G, Canetti N, Scacco M, Panarese S, Poli A (2013) Antigen localisation of porcine circovirus type 2 (PCV2): distribution of the infection and PMWS diagnosis in Italian populations of free ranging wild boar. *LAR* 16:77–84
- Oliveira S, Galina L, Pijoan C (2001) Development of a PCR test to diagnose *Haemophilus parasuis* infections. *J Vet Diagn Investig* 13:495–501
- Opriessnig T, Thacker EL, Yu S, Fenaux M, Meng XJ, Halbur PG (2004) Experimental reproduction of postweaning multisystemic wasting syndrome in pigs by dual infection with *Mycoplasma hyopneumoniae* and porcine circovirus type 2. *Vet Pathol* 41:624–640
- Opriessnig T, Giménez-Lirola LG, Halbur PG (2011) Polymicrobial respiratory disease in pigs. *Anim Health Res Rev* 12:133–148
- Pijoan C (2006) Pneumonic pasteurellosis. In: Straw B, Zimmermann J, D’Allaire S, Taylor DJ (eds) *Diseases of swine*. Blackwell Publishing, Ames, pp 719–726
- Reiner G, Fresen C, Bronnert S, Willems H (2009) Porcine reproductive and respiratory syndrome virus (PRRSV) infection in wild boars. *Vet Microbiol* 136:250–258
- Reiner G, Fresen C, Bronnert S, Haack I, Willems H (2010) Prevalence of *Actinobacillus pleuropneumoniae* infection in hunted wild boars (*Sus scrofa*) in Germany. *J Wildl Dis* 46:551–555
- Risco D, Fernández-Llario P, Cuesta JM, García-Jiménez WL, Gil M, Gonçalves P, Martínez R, Gómez L, García A, Rey J, Hermoso de Mendoza M, Hermoso de Mendoza J (2013a) Fatal outbreak of systemic pasteurellosis in a wild boar (*Sus scrofa*) population from southwest Spain. *J Vet Diagn Investig* 25:791–794
- Risco D, Fernández-Llario P, García-Jiménez WL, Gonçalves P, Cuesta JM, Martínez R, Sanz C, Sequeda M, Gomez L, Carranza J, de Mendoza JH (2013b) Influence of porcine circovirus type 2 infections on bovine tuberculosis in wild boar populations. *Trans Emerg Dis* 60(Suppl 1):121–127
- Rosell C, Segalés J, Plana-Durán J, Balasch M, Rodríguez-Arriola GM, Kennedy S, Allan GM, McNeilly F, Latimer KS, Domingo M (1999) Pathological, immunohistochemical, and in-situ hybridization studies of natural cases of postweaning multisystemic wasting syndrome (PMWS) in pigs. *J Comp Pathol* 120:59–78
- Schaller A, Kuhn R, Kuhnert P, Nicolet J, Anderson TJ, MacInnes JI, Segers RPAM, Frey J (1999) Characterization of apxIVA, a new RTX determinant of *Actinobacillus pleuropneumoniae*. *Microbiology* 145:2105–2116
- Sibila M, Mentaberre G, Boadella M, Huerta E, Casas-Díaz E, Vicente J, Gortázar C, Marco I, Lavín S, Segalés J (2010) Serological, pathological and polymerase chain reaction studies on *Mycoplasma hyopneumoniae* infection in the wild boar. *Vet Microbiol* 144:214–218
- Sitjar M, Noyes EP, Simon X, Pijoan C (1996) Relationships among seroconversion to *Mycoplasma hyopneumoniae*, lung lesions, and production parameters in pigs. *J Swine Health Product* 4:273–277
- Thacker EL (2001) Immunology of the porcine respiratory disease complex. *Vet Clin North Am Food Anim Pract* 17:551–565
- Townsend KM, Frost AJ, Lee CW, Papadimitriou JM, Dawkins HJS (1998) Development of PCR assays for species- and type-specific identification of *Pasteurella multocida* isolates. *J Clin Microbiol* 36:1096–1100
- Yoon HA, Seong-Kug EO, Aleyas AG, Cha SY, Lee JH, Chae JS, Jang HK, Cho JG, Song HJ (2006) Investigation of pseudorabies virus latency in nervous tissues of seropositive pigs exposed to field strain. *J Vet Med Sci* 68:143–148