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Histopathologic patterns and etiologic diagnosis of porcine respiratory disease complex in Brazil

[*Padrões histopatológicos e diagnóstico etiológico do complexo de doenças respiratórias dos suínos no Brasil*]

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ABSTRACT

Porcine respiratory disease complex is a major health concern for the porcine industry, causing significant economic loss. In this study, a total of 156 samples from pigs referred to a diagnostic laboratory in Brazil for 15 months were analyzed by histopathology, bacterial isolation, PCR, and immunohistochemistry. Multiple infections were common, so 42.3% of the pigs had more than one pathogen detected in the lungs. Swine influenza virus was detected in 25.0% of the cases. Porcine circovirus type 2 was detected in 7.1% of the pigs, which was often associated with *Pasteurella multocida*. In addition, one case of porcine circovirus type 3 infection associated with granulomatous pneumonia was diagnosed. Bacteria were isolated in 125 cases, namely *Pasteurella multocida* (34.0%), *Glaesserella* (*Haemophilus*) *parasuis* (35.2%), *Streptococcus suis* (13.5%), and *Actinobacillus pleuropneumoniae* (7.7%). *Mycoplasma hyopneumoniae* was identified in 7.0% of the cases, and 18.6% of pigs carried *Salmonella* sp. The most common patterns of pulmonary inflammation were broncopneumonia, bronchointerstitial pneumonia, and pleuritis, in that order. This study demonstrated that histopathology is an efficient tool along with other laboratorial diagnostic tests for establishing an etiologic diagnosis in cases of porcine respiratory disease complex.

Keywords: *Pasteurella multocida*, influenza, *Streptococcus suis*, *Mycoplasma hyopneumoniae*, *Actinobacillus pleuropneumoniae*, *Glaesserella* (*Haemophilus*) *parasuis*, Porcine circovirus type 3

RESUMO

O complexo de doenças respiratórias de suínos é um dos principais problemas sanitários na suinocultura, causando perdas econômicas significativas. O presente estudo incluiu amostras de 156 suínos, que foram encaminhados a um laboratório de diagnóstico no Brasil, durante um período de 15 meses, sendo realizados histopatologia, isolamento bacteriano, PCR e imuno-histoquímica. Coinfecções por múltiplos patógenos foram comuns, correspondendo a 42,3% dos animais, que tiveram mais de um agente identificado nos pulmões. O vírus da influenza suína foi detectado em 25,0%. O circovírus suíno tipo 2 foi detectado em 7,1% dos animais, frequentemente associado à Pasteurella multocida. *Além disso, foi diagnosticado um caso de circovírus suíno tipo 3 associado à pneumonia granulomatosa. Foram isoladas bactérias em 125 casos, a saber:* Pasteurella multocida *(34,0%),* Glaesserella (Haemophilus) parasuis *(35,2%),* Streptococcus suis *(13,5%) e* Actinobacillus pleuropneumoniae *(7,7%).* Mycoplasma hyopneumoniae *foi identificado em 7,0%, e 18,6% dos animais tiveram isolamento de* Salmonella *sp. Os padrões mais frequentes de inflamação pulmonar foram: broncopneumonia, pneumonia broncointersticial e pleurite, nesta ordem. Este estudo demonstrou que a histopatologia é uma ferramenta eficiente, juntamente a outras técnicas laboratoriais de diagnóstico, para o estabelecimento de diagnóstico etiológico em casos do complexo de doenças respiratórias de suínos.*

Palavras-chave: Pasteurella multocida, *influenza,* Streptococcus suis, Mycoplasma hyopneumoniae, Actinobacillus pleuropneumoniae, Glaesserella (Haemophilus) parasuis, *circovírus suíno tipo 3*

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INTRODUCTION

Respiratory diseases in pigs are one of the most important health problems in highly technified porcine production systems, being a cause of economic losses due to deaths, treatments, low weight gain, and condemnation of carcasses at the abattoir (Sobestiansky *et al.*, 1987; Fraile *et al*., 2010; Opriessnig *et al*., 2011; Morés *et al*., 2015). Porcine Respiratory Disease Complex (PRDC) is a terminology that refers to swine mixed respiratory infections with multiple etiologic agents (Opriessnig *et al*., 2011). It is known that the environment, management practices, and the type of production system, might be relevant predisposing factors to PRDC supporting the notion of a multifactorial complex (Brockmeier *et al*., 2002; Fraile *et al*., 2010; Morés *et al.*, 2015).

Several different pathogens can colonize the respiratory tract of pigs. Although innate immunity may control most primary infections, immunocompromised pigs may develop lesions due to primary pathogens or when lesions are exacerbated by the pathogenicity of secondary infectious agents (Brockmeier *et al*., 2002). Indeed, some viruses and bacteria can induce important lesions because of a primary infection, but polymicrobial respiratory diseases are more common than single infections in porcine herds (Opriessnig *et al*., 2011). Conclusive etiologic diagnosis of respiratory diseases is challenging and usually requires multiple diagnostic tests. Although viruses are often considered primary agents that predispose to secondary bacterial infections, pathogenesis of multiple infections in the respiratory tract of pigs can be more complex so identification of primary or secondary infections may not be achievable (Opriessnig *et al*., 2011).

Viruses including swine influenza virus (SIV), porcine circovirus type 2 (PCV2) and porcine reproductive and respiratory syndrome virus (PRRS) are important primary agents in PRDC (Brockmeier *et al*., 2002). It is known that PCV2 infections may cause respiratory lesions including interstitial pneumonia, bronchial and bronchiolar fibroplasia and necrosis (Kim *et al*., 2003). However, PCV2 can be involved in subclinical infections with lymphoid depletion and, consequently, immunosuppression, predisposing to pulmonary infections.

Although there are important primary pulmonary infections by bacteria, including, *Mycoplasma hyopneumoniae*, *Actinobacillus pleuropneumoniae*, and *Bordetella bronchyseptica*, numerous bacteria can cause secondary infections resulting in exacerbation of respiratory clinical signs and lesions (Brockmeier *et al*., 2002). Infections with *A. pleuropneumoniae* are usually associated with necrosis of pulmonary parenchyma and pleuritis. Necrotizing lesions in these cases are due to the exotoxins ApxI, II and III (Frey, 1995).

Important secondary bacterial infections in the respiratory tract of pigs are due to infections by *Glaesserella* (*Haemophillus*) *parasuis*, *Streptococcus suis*, and *Trueperella* (*Arcanobacterium*) *pyogene*s. *Salmonella enterica* serotype Choleraesuis may be associated to primary or secondary pulmonary infections, due to the systemic behavior of this bacterium (Brockmeier *et al*., 2002).

In Brazil, the most common pathogens associated with PRDC are: *Pasteurella multocida*, Swine Influenza Virus A, Porcine Circovirus type 2 (PCV2), and *Mycoplasma hyopneumoniae* (Morés *et al*., 2015; Paladino *et al*., 2017). Importantly, the porcine reproductive and respiratory syndrome virus (PRSS) has not been diagnosed in Brazil (Ciacci-Zanella *et al*., 2004; Gava *et al.*, 2021).

Considering the economic losses due to PRDC, an accurate diagnosis is critical for the swine industry. Once the frequency and importance of different respiratory pathogens may vary among countries or regions, studies describing correlating lesions and specific agents are important to support veterinary pathologists, clinicians, and farmers (Fraile *et al*., 2010; Hansen *et al*., 2010; Morés *et al*., 2015). Although there are some studies on the etiology of PRDC in various countries (Hansen *et al*., 2010; Jimenez *et al*., 2014; Haimi-Hakala *et al*., 2017; Cheong *et al*., 2017) only a few of them include histopathology (Hansen *et al*., 2010). In Brazil, reports describing etiology of PRDC in Brazilian porcine herds are scarce (Schimidt *et al*., 2016; Galdeano *et al*., 2019) and none of them describe the association of the etiologic and pathologic diagnosis. Therefore, the aim of this study was to evaluate cases of PRDC from

Brazilian swine herds and correlate the morphologic lesion with the etiologic agent.

MATERIAL AND METHODS

Swine tissue samples from 156 pigs with history of respiratory disease referred to a private diagnostic laboratory (*Instituto de Pesquisas Veterinárias Especializadas* - IPEVE, Belo Horizonte, Brazil) from April 2018 to July 2019 were included in this study. All tissue samples were properly collected for histopathology and, at least, one ancillary diagnostic method for pathogen identification. Animals were divided into three categories: suckling (1 - 27 days of age; n = 9), nursery (28 - 72 days of age; n = 82), and finishing pigs (72 - 150 days of age; n = 65).

Gross changes were described, and tissue samples were sent fresh, for bacteriology and PCR analyses, or fixed in 10% buffered formalin for histopathology and immunohistochemistry. Detailed information about the history, category and gross lesions is provided in the Supplementary Table S1. Diagnosis was performed based on histologic lesions associated to results of bacteriology, immunohistochemistry, and/or molecular analyses of samples selected based on clinical history and/or histopathologic findings. Supplementary Table S1 also indicates which laboratorial technique was employed in each case.

Samples of lungs and lymph nodes were fixed in 10% buffered formalin for 48 hours, processed for paraffin embedding, and 3 µm-thick sections were stained with hematoxylin and eosin (HE) for histologic evaluation.

Selected samples of lymph nodes were submitted to a private accredited diagnostic laboratory (CEDISA – http://www.cedisa.org.br/) for immunohistochemistry for *in situ* detection of antigens of PCV2, *Mycoplasma hyopneumoniae*, and SIV.

Fresh samples of lymph nodes, lungs, trachea, pleura, brain, heart, and pericardium were submitted to bacteriology for diagnosis of *Glaesserella parasuis***,** *Streptococcus suis*, *Pasteurella multocida*, *Actinobacillus pleuropneumoniae* and *Trueperella pyogenes*. Plates were incubated at 37^{o}C with 5% CO_2.

For isolation of *G. parasuis*, samples were plated on blood agar with *Staphylococcus aureus* nurse streak, to prevent satellite growth. Isolates were morphologically analyzed by Gram staining (Gram-negative cocobacillus), and biochemical analysis included catalase, CAMP, and urease.

Isolation of *S. suis* was performed by plating samples on blood agar to identify alpha or beta hemolytic strains. Gram-stained smears smears of isolates demonstrated Gram-positive cocci arranged in chains, and biochemical analysis included catalase, amylase and NaCl.

For isolation of *P. multocida* samples were cultivated on blood agar to identify colonies with gray mucoid pattern or with gray non-mucoid pattern. Gram-stained smears demonstrated small Gram-negative coccobacillus, and biochemical analysis included catalase, oxidases, hyaluronidase and acriflavine to differentiate serotypes type A and D.

For isolation of *A. pleuropneumoniae* samples were plated on blood agar with 5% sheep blood streaked with *Staphylococcus aureus*, as NAD donor, generating hemolysis. Selected colonies (morphologically compatible with *A. pleuropneumoniae*) were then plated on tryptose agar. Gram-stained smears demonstrated Gram-negative coccobacilli, and biochemical analysis included NAD requirement, hemolysis, catalase, CAMP test, urea, acid production from arabinose, lactose, maltose, mannitol, melibiose, sucrose, and trehalose.

For isolation of *T. pyogenes*, samples were cultivated on blood agar to identify flat small colonies with mild hemolysis. Gram-stained smears demonstrated pleomorphic Gram-positive coccobacillus, and biochemical analysis included catalase and NaCl.

Occasionally samples were processed for isolation of *Salmonella* spp. by incubating samples in selective culture growth medium followed by culture on green shine agar, to identify pink small and irregular colonies. Gram staining demonstrated Gram-negative bacilli, and biochemical analysis included indol, citrate, urease, and lysine as well as motility.

Selected samples of lungs and trachea were submitted to a private accredited laboratory

(CEDISA – http://www.cedisa.org.br/) for PCR aiming to detect genomic sequences of SIV, *Mycoplasma hyopneumoniae*, and porcine circovirus 3 (PCV3).

Lung lesions were morphologically categorized based on the pattern of inflammatory changes in the pulmonary parenchyma as follow: (i) septal inflammatory infiltrate characterizing interstitial pneumonia; (ii) intraluminal alveolar and/or bronchiolar inflammatory infiltrate, characterizing bronchopneumonia, (iii) intraluminal and septal inflammatory infiltrate, categorizing bronchointerstitial pneumonia; and (iv) pleural inflammation, characterizing pleuritis or pleuropneumonia. Pulmonary parenchyma necrosis, bronchial and/or bronchiolar epithelial damage, including intraepithelial microabscesses, inflammation and necrosis, and bronchial associated lymphoid tissue (BALT) hyperplasia were assessed separately from the predominant morphologic pattern of pulmonary inflammation.

Frequencies of agents were compared among different categories (age groups) using the Fisher's exact test (Sampaio, 2010).

RESULTS

Data on age, sex, histopathologic findings, diagnostic techniques and results of each pig included in this study are summarized in Supplementary Table S1.

Considering all 156 pigs included in this study, lung etiologic evaluation demonstrated that 42.3% (66/156 pigs) had more than one pathogen detected, 53.8% (84/156 pigs) had only one pathogen detect and 3.8% (6/156 pigs) had no pathogens detect in their lungs. From agents considered as primary infections, SIV was the most frequent, and it was detected in 25% of the cases (39/156 pigs), followed by *A. pleuropneumoniae* that was detected in 7.7% of the cases (12/156 pigs). *M. hyopneumoniae* was detected in 7.0% (11/156) of pigs included in this study, affecting only nursery and finishing pigs, with 6 and 5 cases in these categories, respectively.

Among those pathogens considered secondary agents, *G. parasuis* and *P. multocida* were the most frequently detected, corresponding to 35.2% (55/156) and 34.0% (53/156) of the cases, respectively. Interestingly, no other pathogen was detected in 52.7% (29/55 pigs) and 43.4% (23/53 pigs) of the pigs with *G. parasuis* and *P. multocida* infections, respectively. In contrast, considering all 156 pigs, *Salmonella* spp. was isolated from 18.6% (29/156 pigs) of the pigs. Among these cases, 89.7% (26/29 pigs) were associated to other pathogens. Figure 1 demonstrates distribution of single and coinfections among SIV and the two most common secondary bacteria.

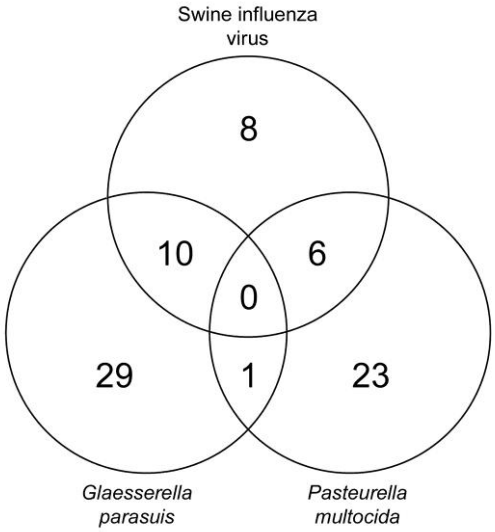

Figure 1. Venn diagram demonstrating cases of single or co-infections of swine influenza virus (SIV) and the two most common secondary bacterial agents, *Glaesserella parasuis* and *Pasteurella multocida*.

PCV2 was detected in lymph nodes of 7.1% (11/156 pigs) of pigs with any pattern of pulmonary lesions. Among these cases, 90.9% (10/11 pigs) had PCV2 associated to other pathogens. There was one finishing pig that was PCR positive to PCV3 with granulomatous interstitial pneumonia containing multiple multinucleated giant cells as well as granulomatous nephritis and lymphadenitis.

Frequencies of each pathogen isolated from these 156 pigs with pulmonary disease, including frequencies by age category (nursery, suckling and finishing) are demonstrated in Table 1 and Figure 2. Frequencies of association of different pathogens in mixed pulmonary infections among these 156 pigs with pulmonary disease are demonstrated in Table 2.

Table 1. Frequencies of pathogens detected by bacteriology, PCR or immunohistochemistry in 156 pigs with porcine respiratory disease syndrome, considering three age categories (suckling= 1-27 days, nursery= 28-72 days, and finishing= 72-150 days)

Pathogens Number of pigs tested (n)		Age category Suckling (n = 9)	 Nursery (n = 82)	 Finishing (n = 65)	Total (n = 156)
Glaesserella parasuis (tested, n = 156)	Alone	1.8% (1/55)	41.8% (23/55)	9.1% (5/55)	52.7% (29/55)
	Mixed	3.6% (2/55)	38.2% (21/55)	5.4% (3/55)	47.3% (26/55)
	Total	33.3% (3/9)	53.7% (44/82)	12.3% (8/65)	35.2% (55/156)
Pasteurella multocida (tested, n = 156)	Alone	1.9% (1/53)	11.3% (6/53)	30.2% (16/53)	43.4% (23/53)
	Mixed	1.9% (1/53)	18.9% (10/53)	35.8% (19/53)	56.6% (30/53)
	Total	22.2% (2/9)	19.5% (16/82)	53.8% (35/65)	34.0% (53/156)
Swine influenza vírus (tested, n = 82)	Alone	5.1% (2/39)	12.8% (5/39)	2.6% (1/39)	20.5% (8/39)
	Mixed	7.7% (3/39)	53.8% (21/39)	17.9% (7/39)	79.5% (31/39)
	Total	55.6% (5/9)	31.7% (26/82)	12.3% (8/65)	25.0% (39/156)
	Tested	100% (5/5)	52% (26/50)	29.6% (8/27)	47.6%(39/82)
Salmonella spp. (tested, n = 156)	Alone	3.4% (1/29)	6.9% (2/29)	0	10.3% (3/29)
	Mixed	3.4% (1/29)	48.3% (14/29)	37.9% (11/29)	89.6% (26/29)
	Total	22.2% (2/9)	19.5% (16/82)	16.9% (11/65)	18.6% (29/156)
	Alone	0	33.3% (7/21)	19.0% (4/21)	52.4% (11/21)
	Mixed	0	33.3% (7/21)	14.3% (3/21)	47.6% (10/21)
	Total	0	17.1% (14/82)	10.8% (7/65)	13.5% (21/156)
Actinobacillus pleuropneumoniae (tested, n = 156)	Alone	0	0	25% (3/12)	25% (3/12)
	Mixed	8.3% (1/12)	8.3% (1/12)	58.3% (7/12)	75% (9/12)
	Total	11.1% (1/9)	1.2% (1/82)	15.4% (10/65)	7.7% (12/156)
Porcine circovirus type 2 (tested, n = 31)	Alone	0	9.1% (1/11)	0	9.1% (1/11)
	Mixed	0	27.3% (3/11)	63.6% (7/11)	90.9% (10/11)
	Total	0	4.9% (4/82)	10.8% (7/65)	7.1% (11/156)
	Tested	0% (0/2)	23.5% (4/17)	58.3% (7/12)	35.5% (11/31)
Mycoplasma hyopneumoniae (tested, n = 26)	Alone	0	0	0	0
	Mixed	0	54.5% (6/11)	45.4% (5/11)	100.0% (11/11)
	Total	0	7.3% (6/82)	7.7% (5/65)	7.0% (11/156)
	Tested	0% (0/1)	40% (6/15)	45.4% (5/11)	42.3% (11/26)
Trueperella pyogenes (tested, n = 156)	Alone	0	0	50.0% (2/4)	50% (2/4)
	Mixed	0	0	50.0% (2/4)	50% (2/4)
	Total	0	0	6.2% (4/65)	2.6% (4/156)
Porcine circovirus type 3 (tested, n = 1)	Alone	0	0	100.0% (1/1)	100.0% (1/1)
	Mixed	0	0	0	0
	Total	0	0	1.6% (1/65)	0.6% (1/156)
	Tested	0	0	100.0% (1/1)	100.0% (1/1)

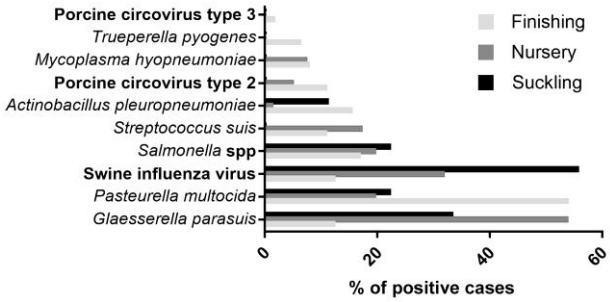

Figure 2. Percentage of positive cases for various pathogens associated with porcine respiratory disease syndrome, considering three age categories (suckling= 1-27 days, nursery= 28-72 days, and finishing= 72-150 days).

Table 2. Number of pigs with association of infectious agents diagnosed by bacteriology, PCR, or immunohistochemistry among 156 pigs with porcine respiratory disease syndrome, considering three age categories (suckling= 1-27 days, nursery= 28-72 days, and finishing= 72-150 days)

Pathogens (mixed infection)	Suckling (n = 9)	Nursery (n = 82)	Finishing (n = 65)	Total (n = 156)
Gp + Pm	0	1	0	0.6% (1/156)
Gp + Sal	1	3	1	3.2% (5/156)
Gp + SIV	1	8	1	6.4% (10/156)
Gp + My	0	1	0	0.6% (1/156)
Gp + PCV2	0	0	1	0.6% (1/156)
Gp + SIV + My	0	2	0	1.3% (2/156)
Gp + SIV + PCV2	0	1	0	0.6% (1/156)
Gp + Sal + SIV	0	3	0	1.9% (3/156)
Gp + SIV + PCV2 + My	0	1	0	0.6% (1/156)
Gp + Pm + My	0	1	0	0.6% (1/156)
Ss + Sal	0	2	1	1.9% (3/156)
Ss+ Sal + Pm	0	1	0	0.6% (1/156)
Ss + Pm + My	0	0	1	0.6% (1/156)
Ss + APP	0	0	1	0.6% (1/156)
Ss + PCV2	0	1	0	0.6% (1/156)
Ss + SIV	0	1	0	0.6% (1/156)
Pm + Sal	0	2	3	3.2% (5/156)
Pm + Sal + My	0	0	1	0.6% (1/156)
Pm + APP	0	0	3	1.9% (3/156)
Pm + APP + SIV	0	0	1	0.6% (1/156)
Pm + Tp	0	0	2	1.3% (2/156)
Pm + SIV	1	3	2	3.8% (6/156)
Pm + SIV + My	0	0	1	0.6% (1/156)
Pm + SIV + PCV2	0	0	1	0.6% (1/156)
Pm + My	0	1	1	1.3% (2/156)
Pm + PCV2	0	0	2	1.3% (2/156)
Pm + PCV2 + My	0	0	1	0.6% (1/156)
Sal + SIV	0	1	1	1.3% (2/156)
Sal + APP	0	1	2	1.9% (3/156)
Sal + PCV2	0	0	2	1.3% (2/156)
APP + SIV	1	0	0	0.6% (1/156)
Ss + Sal + SIV	0	1	0	0.6% (1/156)
Ss + Pm	0	1	0	0.6% (1/156)
Total	44.4% (4/9)	43.9% (36/82)	44.6% (29/65)	44.2% (69/156)

Abbreviations: Gp: *Glaesserella parasuis*; Pm: *Pasteurella multocida*; Sal: *Salmonella* spp.; SIV: swine influenza virus; My: *Mycoplasma hyopneumoniae*; PCV2: porcine circovirus type 2; Ss: *Streptococcus suis*; Tp: *Trueperella pyogenes*; APP: *Actinobacillus pleuropneumoniae*.

Considering the age category, SIV was diagnosed in all categories, totaling 39 cases, but it was more frequently detected in nursery (31.7%, 26/82) and suckling pigs (55.6%, 5/9) when compared to finishing pigs (12.3%, 8/65; $p = 0.0025$). In contrast, *A. pleuropneumoniae*, which was diagnosed in 12 pigs belonging to all categories, was significantly ($p = 0.004$) more frequent in finishing pigs (15.4%, 10/65) than in nursery (1.2%, 1/82) or suckling (11.1%, 1/9) combined. *G. parasuis* and *P. multocida* were diagnosed in all categories, but with higher frequencies in nursery (53.7%, 44/82) and finishing pigs (53.8%, 35/65). *Streptococcus suis* was diagnosed in nursery and finishing pigs, with higher frequency at the nursery category (17.1%, 14/82). *Salmonella* spp. was diagnosed at all categories with similar frequencies: 22.2% in suckling (2/9), 19.5% in nursery (16/82), and 16.9% in finishing (11/65) pigs. PCV2 was diagnosed only in nursery (4.9%, 4/82) and finishing pigs (10.8%, 7/65), with frequencies that were statistically similar in these two categories ($p > 0.05$).

Pigs carrying SIV represented 25.0% (39/156 pigs) of the cases, and had predominantly bronchointerstitial pneumonia (46.15% - 18/39)

or bronchopneumonia (46.15% - 18/39) patterns, compared to interstitial pneumonia (7.7% - 3/39) (Table 3). Among these cases, 43.6% (17/39 pigs) had bronchiolar epithelial lesions, including epithelial hyperplasia (8/39 pigs), epithelial hyperplasia with intraepithelial microabscesses (5/39 pigs; Figure 3), necrotizing bronchitis/bronchiolitis (3/39 pigs; Figure 4), and bronchiolitis (1/39). No bronchiolar lesions were observed by histopathology in 56.4% (22/39) of the pigs with SIV (Table 4). Bronchiolar lesions were also seen in 10 pigs with negative diagnosis for SIV, including epithelial hyperplasia (30% - 3/10 pigs) and necrotizing bronchiolitis (70% - 7/10 pigs). There were other 13 pigs with bronchiolar microscopic lesions, but PCR or IHQ for SIV were not performed in these cases. The frequency of bronchiolar epithelial hyperplasia associated with intraepithelial microabscesses was significantly higher in SIV-positive animals, indicating that this is a reliable microscopic indication of SIV infection (Table 4).

Pigs with *A. pleuropneumoniae* infection had bronchopneumonia as the predominant morphologic pattern (91.7% - 11/12 pigs) (Table 4). Among these pigs, 50% (6/12 pigs) also had fibrinous pleuritis and 50% (6/12 pigs) had multifocal or focally extensive areas of necrosis associated with inflammation (Table 3).

Pigs infected with *M. hyopneumoniae* had bronchopneumonia in 63.6% (7/11) of the cases, and bronchointerstitial pneumonia in 36.4% (4/11) of the cases. Importantly, only two pigs that tested positive for *M. hyopneumoniae* 18.2% (2/11) had bronchus-associated lymphoid tissue (BALT) hyperplasia (Figure 5).

Table 3. Number of cases of a given pathogen detected in the lungs of 156 pigs with porcine respiratory disease syndrome according to the morphologic patterns identified by histopathology

Histopathologic pattern	Pathogens										Total
	SIV	APP	Gp	Pm	Ss	Tp	Sal	PCV2	My	PCV3	
Bronchointerstitial pneumonia	18	1	32	14	7	2	11	4	4	0	93
Bronchopneumonia	18	11	16	39	11	2	13	6	7	1	124
Interstitial pneumonia	3	0	7	0	3	0	5	1	0	0	19
Fibrinous pleuritis	11	6	20	12	9	1	8	2	2	0	71
Pulmonary parenchymal necrosis	4	6	0	8	0	0	1	1	1	0	21
Bronchiolar hyperplasia	8	0	8	3	3	0	4	1	1	0	28
Bronchiolitis	1	0	2	1	1	0	1	0	1	0	7
Necrotizing bronchiolitis	3	0	3	4	3	0	4	0	2	0	19
Bronchiolar hyperplasia with microabcesses	5	0	5	2	2	0	1	0	1	0	16
BALT hyperplasia	5	2	11	9	5	0	5	2	2	0	41

Abbreviations*: Gp:* Glaesserella parasuis*; Pm:* Pasteurella multocida*; Sal:* Salmonella *spp.; SIV: swine influenza virus; PCV2: porcine circovirus type 2; Ss:* Streptococcus suis*; Tp:* Trueperella pyogenes*; APP:* Actinobacillus pleuropneumoniae*; My:* Mycoplasma hyopneumoniae; *PCV3: porcine circovirus type 3.*

Table 4. Frequency of bronquiolar damage characterized as bronquiolar hyperplasia, necrotic bronchiolitis and bronchiolar hyperplasia admixed to intraepithelial microabscesses in pigs tested for swine influenza virus (SIV)

Bronquiolar lesions	SIV positive	SIV negative	P value
Bronchiolar hyperplasia	20.5% (8/39)	14.0% (6/43)	0.5594
Necrotizing bronchiolitis	7.7% (3/39)	16.3% (7/43)	0.3183
Bronchiolar hyperplasia with microabcesses	12.8% (5/39)	0% (0/43)	0.0211

Pigs infected with SIV, *A. pleuropneumoniae*, *Salmonella* spp., and PCV2 represented 80.1% (125/156 pigs) of the animals with respiratory disease. Among these cases, pigs had bronchopneumonia (52% - 65/125 pigs) or bronchointerstitial pneumonia (40% - 50/125 pigs) as the predominant morphologic patterns (Table 3), and less frequent interstitial

pneumonia (8% - 10/125 pigs). Among these cases with opportunistic pathogens, 31.2% of the pigs (39/125) had fibrinous pleuritis associated with any of those morphologic patterns of pneumonia. Among the 39 pigs with fibrinous pleuritis 51.3% (20/39) had *G. parasuis* infection (Figure 6).

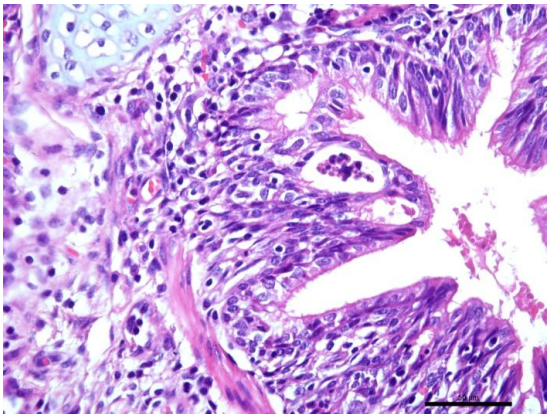

Figure 3. Nursery pig positive for swine influenza virus. Lung, bronchiolar epithelium. Intraepithelial microabscess containing neutrophils, with mild epithelial hyperplasia. Hematoxylin and eosin; bar = 50 µm.

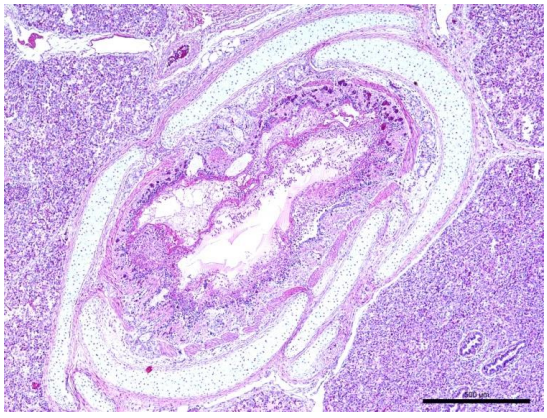

Figure 4. Finishing pig positive for swine influenza virus. Lung, bronchus. Severe and diffuse bronchial epithelial necrosis with abundant fibrinous exudate and cellular debris. Hematoxylin and eosin; bar = 500 µm.

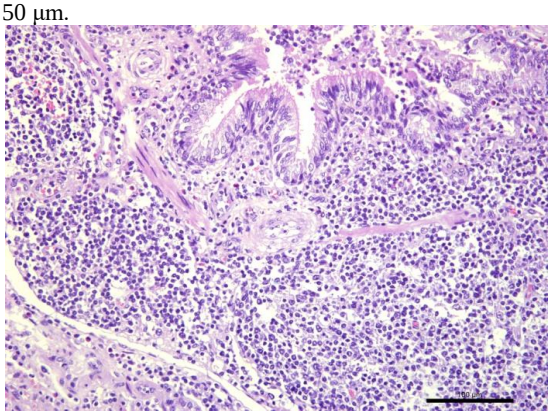

Figure 5. Finishing pig positive for *Mycoplasma hyopneumoniae*. Lung, bronchiolus. Moderate hyperplasia of bronchial-associated lymphoid tissue (BALT). Hematoxylin and eosin; bar = 100 µm.

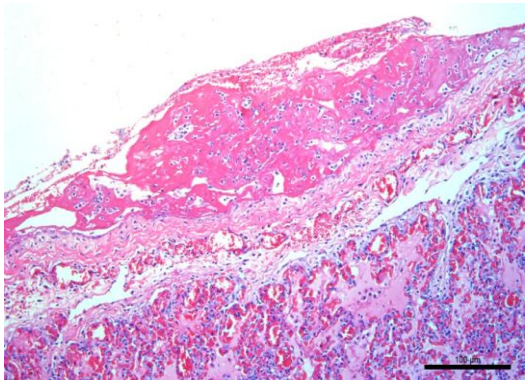

Figure 6. Finishing pig with coinfection by *Pasteurella multocida* and *Actinobacillus pleuropneumoniae*. Pleural surface. Diffuse and moderate acute fibrinous pleuritis, characterized by accumulation of fibrinous exudate on the pleural surface. Hematoxylin and eosin; bar = 100 µm.

Septicemic salmonellosis was observed in 18.6% (29/156) of the pigs, mainly in association with other pathogens (89.7% - 26/29 pigs; Figure 7), including *G. parasuis* (5/26 pigs; Figure 8), SIV (2/26 pigs), *Streptococcus* spp. (3/26 pigs; Figure 9), *P. multocida* (6/26 pigs; Figure 10), PCV2 (2/26 pigs) and *A. pleuropneumoniae* (3/26 pigs), or with more than one pathogen associated (5/26 pigs). Among these cases of septicemic salmonellosis, all morphologic patterns of pulmonary inflammation were observed including bronchointerstitial pneumonia in 37.9% (11/29 pigs), interstitial pneumonia in 17.2% (5/29 pigs; Figure 7) and bronchopneumonia in 27.6% (13/29 pigs). Among these cases 27.6% (8/29 pigs) had fibrinous pleuritis associated with inflammation of the pulmonary parenchyma. PCV2 was diagnosed in 7.1% of the pigs (11/156), mainly in association with other pathogens (90.9% - 10/11), including *G. parasuis* (1/11), *Streptococcus* spp. (1/11), *P. multocida* (3/11), *Salmonella* spp. (2/11), or with more than one pathogen associated (3/11). Among these cases,

bronchointerstitial pneumonia (36.4% - 4/11) and bronchopneumonia (54.5% - 6/11) were the predominantly morphologic patterns of pulmonary inflammation, whereas one pig had interstitial pneumonia and fibrinous pleuritis associated with inflammation of the pulmonary parenchyma were observed in 18.2% (2/11) of these cases.

Starch granules, characterized as foreign body, were observed in association to histiocytic inflammation, with epithelioid macrophages and multinucleated giant cells in six pigs (3.8% - 6/156; Figure 11). In these association to different pathogens was diagnosed including *G. parasuis*, *Streptococcus suis*, *Salmonella* spp., *P. multocida*, and SIV.

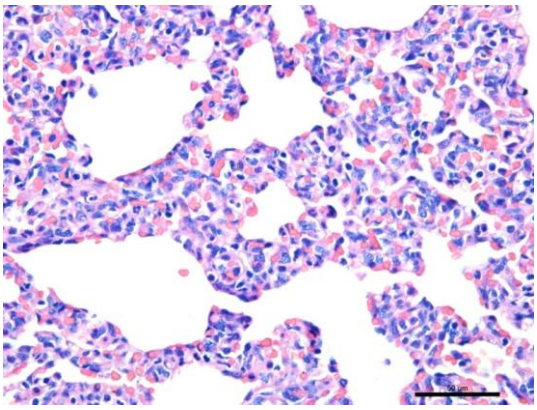

Figure 7. Suckling pig with *Salmonella* sp. infection. Lung. Acute interstitial pneumonia, characterized by diffuse thickening of the alveolar septa with moderate neutrophilic inflammatory infiltrate. Hematoxylin and eosin; bar = 50 μm.

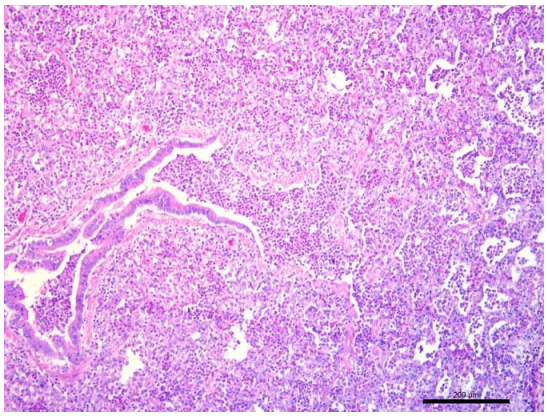

Figure 8. Nursery pig with *Glaesserella* (*Haemophillus*) *parasuis* infection. Lung. Bronchointerstitial pneumonia characterized by diffuse thickening of alveolar septa, and marked infiltration of neutrophils in alveolar and bronchiolar lumen. Hematoxylin and eosin; bar = 200 μm.

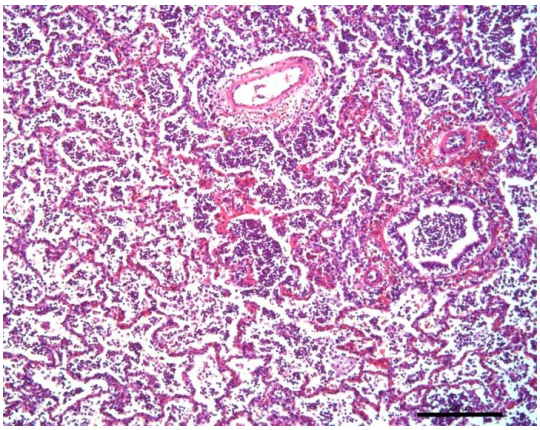

Figure 9. Finishing pig with *Streptococcus suis* infection. Lung. Bronchopneumonia, characterized by alveolar and bronchiolar lumen filled with neutrophils. Hematoxylin and eosin; bar = 200 μm.

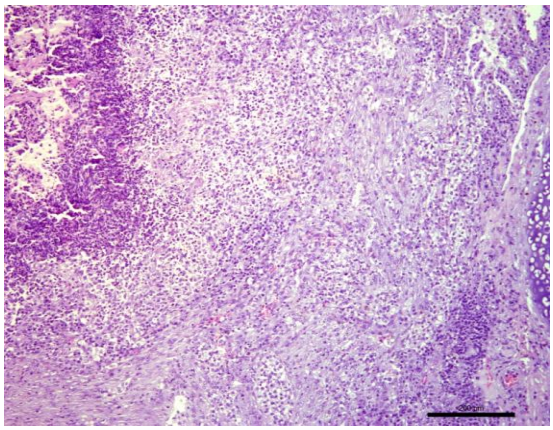

Figure 10. Finishing pig with *Pasteurella multocida* infection. Lung. Focal area of parenchyma necrosis (upper right) surrounded by abundant fibroblasts and collagen matrix (fibrosis). Hematoxylin and eosin; bar = 200 μm.

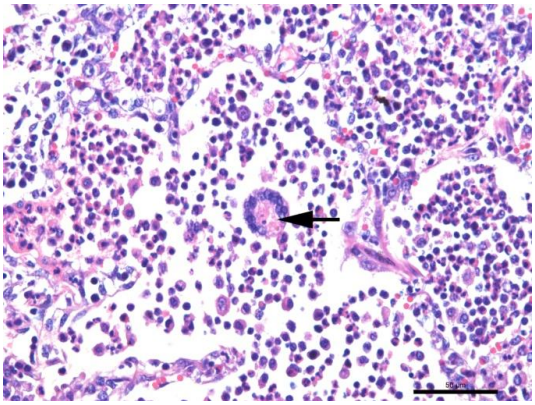

Figure 11. Finishing pig. Lung. Multinucleated giant cell (center) in alveolar space containing small amount of negatively stained particles (arrow), surrounded by moderate amount of neutrophils. Hematoxylin and eosin; bar = 50 µm.

DISCUSSION

In this study, the association of histopathology evaluation with molecular, immunohistochemical and/or bacteriologic analysis was a crucial tool for an accurate diagnosis of porcine respiratory disease complex. A relevant infectious agent was identified in more than 95% of the cases of pigs with clinical respiratory disease in this study, which indicates that appropriate laboratorial diagnostic approach, based on histopathology as a screening method, is efficient for establishing an etiologic diagnosis. According to Caswell and Williams (2016), increasing number of auxiliary tests available does not decrease the importance of morphological analysis even if the principal agent involved in death is had been already identified, mainly because underlying causes are also important and need to be investigated as well. Importantly, coinfections or superinfections are very common in cases respiratory diseases of pigs (Saade *et al.*, 2020). Therefore, this study represents a contribution since it is important to recognize morphological patterns associated with natural and often multiple infections, which contrasts with morphologic descriptions of experimentally induced lesions by a single pathogen under controlled conditions and specific time-points post infection (Müller *et al*., 2003; Takemae *et al*., 2018). In addition, under experimental conditions animals are challenged with one specific strain of the pathogens, which may not represent the variability among field strains (Oliveira Filho *et al*., 2018).

SIV infection is an important cause of respiratory disease in pigs. In this study, 29% (39/156) of the pigs were carrying SIV. However, among those pigs 59% (23/39 pigs) had no bronchiolar lesions, which possibly represent animals with early SIV infection, since necrosis and neutrophilic intraepithelial infiltrate in bronchioli is observed beginning only at 24 to 48 hours post infection with SIV (Janke, 2014). In these cases, molecular analysis is an important tool to detect the virus. Interestingly, ten animals with bronchiolar epithelial hyperplasia and necrotizing bronchiolitis were SIV-negative. Importantly, SIV-induced lesions may remain after viral clearance (Janke, 2014) so histopathology is very useful tool for a proper differential diagnosis in those cases.

The most frequently isolated bacterial pathogens in this study were *G. parasuis* and *P. multocida*. *G. parasuis* is a common pathogen in the technified swine industry in all pig ages (Oliveira and Pijoan, 2014) and bronchopneumonia with fibrinous pleuritis is the expected histopathological pattern in those cases (Caswell and Williams, 2016; Vahle *et al*., 1995). The large number of pigs with respiratory disease associated to *P. multocida* infection in this study is in good agreement with a previous study in Brazil (Paladino *et al*., 2017). In these cases of pulmonary damage by *P. multocida* bronchopneumonia was the most common histopathological pattern, which has also been previously described (Pors *et al*., 2011, 2013; Caswell and Williams, 2016).Although considered a secondary pathogen (Brockmeier *et al*., 2002; Opriessnig *et al*., 2011), *P. multocida* were isolated in eight cases of necrotizing pulmonary lesions in this study. One possibility is that those pigs were infected with a recently described highly pathogenic *P. multocida* (Oliveira Filho *et al*., 2018), which may cause necrotizing lesions as a single agent (Paladino *et al*., 2017), resulting in similar histopathological patterns of parenchyma necrosis as observed in this study. An important differential in these cases is *A. pleuropneumoniae* infection.

Mycoplasma hyopneumoniae is a pathogen associated with the condition known as enzootic pneumonia, although very often this organism is associated with other pathogens in the lung (Hillen *et al.*, 2014). Microscopically, BALT hyperplasia is an important morphologic marker

of *M. hyopneumoniae* infection (Hillen *et al*., 2014; Know *et al*., 2002). However, only two out of 11 pigs positive for *M. hyopneumoniae* in this study had BALT hyperplasia, which is in good agreement with previous reports indicating that the diagnosis and interpretation of *M. hyopneumoniae* infection may be quite challenging (Buddle and O'Hara, 2005). In some cases in this study, the infectious agent detected by different diagnostic tests was associated with lesions that did not quite are expected for those agents, as occurred in some cases of pigs infected with *A. pleuropneumoniae* and *M. hyopneumoniae*. Importantly, the expected patterns of lesions are largely based on studies based on experimental infections, which contrasts with naturally occurring disease that often is a result of coinfections as demonstrated in this study.

PCV2 is a common and very important cause of multisystemic disease in pigs, which is often associated with pulmonary lesions (Oppressing *et al.*, 2020). Importantly, PCV3 was detected in one case in this study, associated with granulomatous pneumonia, a presentation similar to PCV2-induced systemic disease. PCV3 is a recently described virus, associated with multiple lesions, particularly the condition known as porcine dermatitis and nephropathy syndrome (Arruda *et al*., 2019; Jiang *et al*., 2019). PCV3 has been previously diagnosed in Brazil (Tochetto *et al*., 2017).

T. pyogenes is not a specific agent of porcine respiratory disease complex, being considered an opportunistic bacterium, part of respiratory, skin and reproductive microbiota. However, *T. pyogenes* is an emerging pathogen with potential to cause economic losses for the swine industry (Jarosz *et al*., 2014).

Animals with pulmonary interstitial reaction and septicemic salmonellosis were frequently observed in this study, a situation which histopathology may also be useful to confirm systemic and pulmonary lesions of salmonellosis.

Finally, cases of lesions attributable to starch aspiration were also identified in this study. Similarly to previous reports, here we found a bronchopneumonia with epithelioid or multinucleated cells in alveolar lumen, containing negative cytoplasmic particles morphologically compatible with starch (Corner and Jericho, 1972). Starch-associated pneumonia was considered in this study in spite of not being associated with any specific infectious agent, but because it is a relevant differential diagnosis for infectious agents. As a matter of fact, it was considered an incidental finding in pigs affected by different pathogens in this study.

In conclusion, association of histopathology and traditional microbiologic methods, along with other ancillary diagnostic methods, particularly immunohistochemistry and PCR, are powerful tools for the differential and etiologic diagnosis of porcine respiratory disease complex, which is highly desirable since an accurate diagnosis in these cases is critical for implementing appropriate control and mitigation protocols.

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