RESUMO.- [Agentes patogênicos de pneumonia em suínos abatidos no sul do Brasil.] Lesões sugestivas de pneumonia são frequentemente encontradas em altas prevalências em suínos abatidos no sul do Brasil. Para identificar quais microrganismos causam essas lesões, foram coletados 30 pulmões de suínos com lesão macroscópica sugestiva de pneumonia em cinco frigoríficos diferentes, totalizando 150 pulmões. Amostras para isolamento bacteriano, avaliação molecular, histopatológica e imuno-histoquímica (IHC) foram coletadas de cada pulmão. O escore de lesão pulmonar variou entre 1,53 e 2,83. O achado histopatológico mais observado foi a lesão sugestiva de infecção concomitante pelo vírus Influenza A e *Mycoplasma hyopneumoniae*, correspondendo a 55,3% (83/150), e em 54,2% (45/83) desses casos *Pasteurella multocida* tipo A foi isolado. Em 102 amostras (68%), houve lesão histopatológica sugestiva do envolvimento de mais de um agente infeccioso. *M. hyopneumoniae* foi o microrganismo mais frequente associado a lesões de pneumonia, estando presente em 92,1% (94/102) dos pulmões com coinfeções, seguido por IAV em 89,2% (91/102). Além das coinfeções, IAV lesões foram observadas também em seis amostras sem outro microrganismo patogênico detectado. A total de 46 amostras com lesão histopatológica sugestiva de IAV foram avaliadas por IHC e RT-PCR para IAV. A total de 35% (16/46) delas foram positivas por IHC e 13% (6/46) por RT-PCR. Conforme *M. hyopneumoniae*, 79,3% (119/150) das amostras foram positivas por qPCR e 84,9% (101/119) delas apresentaram lesão histopatológica sugestiva de pneumonia. Os resultados desse estudo sugerem a importância de IAV na patogênese de pneumonia em finais de fase, mesmo que este vírus é mais frequentemente relatado na fase de matadour. Além disso, nossos resultados enfatizam a importância de lesões coinfecciosas em suínos de finalização.

INDEX TERMS: Pneumonia, slaughter, pigs, Brazil, influenza A, *Mycoplasma hyopneumoniae*, *Pasteurella multocida*, coinfections, lungs, pneumatic lesions.
Respiratory diseases correspond to a multifactorial problem in pigs and it is one of the major concerns to swine production. The economic losses are caused by increased pig mortality and poor feed conversion rates (Haden et al. 2012). Pneumonic lesions are the most common finding in pig lungs at slaughter in many countries (Fraile et al. 2010, Fablet et al. 2012, Brewster et al. 2017, Karabasili et al. 2017). The relationship between pneumonic lesions and losses of pig carcasses quality is also known, measured as a reduction of the post-trimming carcass weight in 1.26kg (Brewster et al. 2017).

The Porcine Respiratory Disease Complex (PRDC) is characterized by the co-infection of two or more bacterial or viral agents that act in synergism exacerbating the clinical signs especially in growing and finishing pigs. The occurrence of pneumonia at the end of the finishing phase in Brazilian pig herds is frequent. Studies of the last two decades showed a prevalence of pneumonic lesions in pigs at slaughter up to 75.7% (Silva et al. 2002). In a recent study evaluating the etiology of pneumonia at the end of the finishing phase in Brazilian pig herds, as described by Markey et al. (2013). In the presence of pleuritis, necro-hemorrhagic areas of consolidation or nodules of necrosis in the lungs, microaerophilic incubation would be performed.

**Histopathological exam.** After 24h of formalin fixation, each lung was routinely processed and embedded in paraffin blocks. Histological sections of 3-5μm were stained by hematoxylin-eosin (HE), as described by Allen (1992), for further description of the histopathologic lesions. All samples that were suspected of Influenza A virus were classified in an acute (suppurative necrotizing bronchiolitis/bronchitis), subacute (proliferative bronchiolitis/bronchitis associated with bronchointerstitial pneumonia), or chronic (bronchiolitis obliterans and fibrous tissue) lesion (Watanabe et al. 2012). Lung samples with BAL (bronchus-associated lymphoid tissue) hyperplasia were considered indicative of *Mycoplasma hyopneumoniae* infection (Blanchard et al. 1992). Suppurative bronchopneumonia with fibrin deposition was considered indicative of bacterial involvement in the etiology of the lesion, especially *Pasteurella multocida*.

**Immunohistochemistry for Influenza A.** Samples with acute and subacute lesions suggestive of influenza virus infection in the histopathological evaluation were submitted to immunohistochemistry (IHC) for IAV. Briefly, the sample sections were placed on glass slides coated with poly-L-lysine and heated at 57°C until dry. The biotin-streptavidine-peroxidase Kit (Kit Lsab + System-HRP, Dako®, USA) was used to detect the presence of IAV. The endogenous activity of peroxidase was inhibited by immersion in the slides in methanol solution at 10% hydrogen peroxide for 10 minutes. Antigenic recovery was performed with enzymatic treatment using 0.05% protease XIV (Sigma®, USA) for 25 minutes at 37°C. All slides were incubated with primary antibodies Anti-I4A (Millsore, dilution 1:800) overnight at 4°C. The chromogen used was the 3.3-Diaminobenzidine (Dako®, USA) with Harris' Hematoxylin.
as counterstain (Watanabe et al. 2012). The IAV positivity immune staining was assessed according to the structure, being classified as marked in the epithelium, bronchi and bronchioles, and macrophages.

**Real-time PCR for Influenza A**. Samples with acute and sub acute lesions suggestive of influenza infection in the histopathological evaluation were also submitted to real-time RT-PCR for IAV. Due to the short time for detection of the virus in lung tissue (Schaefer et al. 2013), samples with characteristic chronic lesions were not submitted to this test. The extraction of RNA was performed with a commercial extraction kit based on a silica column (AllPrep DNA/RNA Mini Kit, Qiagen®, Germany) as recommended by the manufacturer. The PCR reaction for IAV was performed from the extracted RNA, as described by the CDC (Centers for Disease Control, Atlanta, United States of America; World Health Organization) (WHO 2010). Samples were evaluated in duplicate, and two positive and two negative controls were added to each analysis.

**Real-time PCR for Mycoplasma hyopneumoniae**. The DNA extraction was performed with a commercial extraction kit based on a silica column (Purelink Genomic DNA Mini kit, Thermo Fisher Scientific®, USA) following manufacturer’s instructions. The PCR was performed in all samples, according to Takeuti et al. (2017). The samples were evaluated in duplicate, and two additional positive and negative were added to each analysis.

**Statistical analysis**. Multiple pairwise comparison tests of proportion with Bonferroni correction were performed in R v3.2 (R Core Team 2013) to compare the prevalence of positive lungs for *P. multocida* by isolation and the prevalence of positive samples for IAV and *M. hyopneumoniae* by PCR among the companies. Chi-square analysis was performed to compare absence or presence of histopathological lesions, bacterial isolation and PCR results. Statistical significance was considered when *P*-values were less than 0.05.

**RESULTS**

**Pulmonary scoring at slaughter**. All selected lungs presented macroscopic pneumatic lesions. The only pattern of lesion observed was cranioventral consolidation (Fig. 1A).

The mean lung lesion score ranged from 1.53 to 2.83 among companies (Table 1). No lungs (0/150) had pleuritis, necro-hemorrhagic areas of consolidation or nodules of necrosis.

**Bacteriology**. *Pasteurella multocida* type A was the only bacterium isolated with relevance to pneumonic lesions. The bacterium was isolated in 43.3% (65/150) of the lungs, and company C did not have any lung sample with *P. multocida* type A isolation. However, companies D and E presented a higher number of lungs with *P. multocida* type A isolation when compared to companies A and C (Table 1).

**Histopathological evaluation**. There was a predominance of lung lesions suggestive of *Mycoplasma hyopneumoniae* and Influenza A virus (IAV) coinfection, representing 55.3% (83/150) of the lungs, followed by 28% (42/150) of samples suggestive of single *M. hyopneumoniae* infection (Fig. 1B), and 9.3% (14/150) of samples indicative of single IAV infection.

**Table 1. Lung lesion scoring and distribution of positive lungs for *Pasteurella multocida* type A isolation, and *Mycoplasma hyopneumoniae* and Influenza A virus suggestive lesions by histopathological evaluation in five companies assessed in the study**

| Company | A       | B       | C       | D       | E       |
|---------|---------|---------|---------|---------|---------|
| IAV     | 86.7%*  | 70.0%*  | 20.0%*  | 76.7%*  | 70.0%*  |
|          | (26/30) | (21/30) | (6/30)  | (23/30) | (21/30) |
| Mhyo    | 80.0%*m | 86.6%*m | 73.3%*  | 90.0%*  | 86.6%*m |
|          | (24/30) | (26/30) | (22/30) | (27/30) | (26/30) |
| PmA     | 33.3%*  | 50.0%*  | 0%      | 66.7%*a | 66.7%*a |
|          | (10/30) | (15/30) | (0/30)  | (20/30) | (20/30) |
| LLS     | 2.03    | 2.40    | 1.53    | 2.83    | 2.23    |

IAV = Influenza A virus, Mhyo = Mycoplasma hyopneumoniae, PmA = Pasteurella multocida type A, LLS = lung lesion score; Different superscript letters within a row indicate statistically significant difference (*P*<0.05) among the five companies (A, B, C, D, and E) based on multiple pairwise comparison tests of proportion analysis.

Fig. 1. **A** Lung of a pig selected for the study, dorsal view. Multifocal to coalescent, reddish and firm areas in the cranial and middle lobes are observed (consolidation). The cuts in the diaphragmatic lobe were performed by the slaughterhouse inspection. **B** Histological aspect of the lung of a pig selected for the study. There is bronchointerstitial pneumonia associated with BALT (bronchus-associated lymphoid tissue) hyperplasia, edema and mild inflammatory infiltrate of macrophages in alveolar spaces. These lesions were observed in cases of *Mycoplasma hyopneumoniae* pneumonia. HE, obj.20x. **C** Histological aspect of the lung of a pig selected for the study. An area of bronchiolitis obliterans is observed associated with moderate inflammatory infiltrate of neutrophils and macrophages in alveolar and bronchiolar lumen. There is also type II pneumocytes hyperplasia, and interstitial inflammatory infiltrate of lymphocytes and plasma cells. These lesions were considered compatible with chronic Influenza A pneumonia. HE, obj.40x.
Moreover, 7.4% (11/150) of lung samples did not show any suspected lesion in the histopathological examination. Taking all histopathological results into account, a total of 64.7% (97/150) of them resulted in microscopic lesions suggestive of IAV infection, isolated or in a coinfection with *M. hyopneumoniae*. The association of histological lesions and *P. multocida* type A isolation is summarized in Table 2.

**Immunohistochemistry and real-time RT-PCR for Influenza A**. A total of 46 samples with acute and subacute IAV suspected lesions in histopathological examination were selected for IHC and real time RT-PCR for IAV. A proportion of 35% (16/46) of them were positive by IHC with macropathographic marking, and 13% of the samples (6/46) were positive by real time RT-PCR.

**Detection of *M. hyopneumoniae* by real-time PCR (qPCR).** A total of 79.3% (119/150) of samples were positive for *M. hyopneumoniae* by qPCR. Companies A, B, D and E had similar results, ranging from 90% (A) to 100% (D) of positives samples, and the majority of samples also presented histopathological lesions suggestive of *M. hyopneumoniae* infection. On the other hand, company C presented only 16.6% (5/30) of positives samples by qPCR (Table 3).

**DISCUSSION**

All selected lungs of this study showed gross pneumonic lesions, as expected since it was a selection criterion in each slaughterhouse. The lungs of all companies showed high lesion scores, but company C presented the lowest scoring, which was in agreement with the laboratory results, since it was not possible to isolate *Pasteurella multocida* type A from any of the lungs. The presence of secondary bacteria in the lungs may result in more severe lesions (Loving et al. 2010), impacting in the performance of the pigs, leading to major economic losses (Pointon et al. 1985, Straw et al. 1990).

The only bacterium of importance in pig pneumonia isolated in this study was *P. multocida* type A. The isolation of this bacterium was expected since it is reported to be more prevalent than other *P. multocida* serotypes in lung confections. In a recent study, 90.9% of the *P. multocida* isolated in pigs lungs were type A and only 9.1% type D (Paladino et al. 2017). Morés et al. (2015) have detected 100% of *P. multocida* type A from pig lungs with *P. multocida* isolation. Although this serotype has been described as a primary role in the induction of lesions in the respiratory tract (Paladino et al. 2017, Oliveira Filho et al. 2018), in this study we did not observe lesions suggestive of *P. multocida* highly pathogenic strain. The frequent isolation of *P. multocida* type A (43.3%; 65/150) in our study is probably related to the correlation between *Mycoplasma hyopneumoniae* and *P. multocida* type A infections (Takeuti et al. 2013), as we found an average prevalence, in all lungs, of 79.3% of *M. hyopneumoniae* positive samples by qPCR, reaching 100% of the lungs from company D. The pathogenic mechanisms of this correlation are suggested to be due to the L-fucose composition increased by *M. hyopneumoniae* and thereby enhancing the adherence of *P. multocida* type A to the bronchial and bronchiolar epithelial cells (Park et al. 2016). In our study, 42.7% of the samples which presented *P. multocida* type A isolation also showed histopathological lesions suggestive of *M. hyopneumoniae* and/or IAV infection, which demonstrates the importance of *P. multocida* type A as a secondary causative agent of pig pneumonia (Takeuti et al. 2013, Morés et al. 2015). In this study, *P. multocida* was not considered as the primary agent of pneumonia in any case, while another study detected a low proportion of cases in which the bacterium was identified as the primary agent (Morés et al. 2015). As no lungs (0/150) showed pleuritis, necro-hemorrhagic areas of consolidation or nodules of necrosis, microaerophilic incubation was not performed. If the microscopic evaluation had presented suggestive lesions of *Actinobacillus pleuropneumoniae* or *Glaesserella parasuis* (formerly *Haemophilus parasuis*), the PCR testing would be performed in order to detect these agents.

*Mycoplasma hyopneumoniae* is one of the most relevant bacteria in swine production (Pieters & Maes 2019). It is the causative agent of Enzootic Pneumonia and has an important role in the PRDC, facilitating other bacteria or virus infections (Thacker et al. 1999, Park et al. 2016, Pieters & Maes 2019). A total of 79.3% of the lungs were positive for *M. hyopneumoniae* by qPCR and four out of five companies presented a high

### Table 2. Relationship between *Pasteurella multocida* type A isolation and histopathological lesions suggestive of IAV and *Mycoplasma hyopneumoniae* coinfections

| Histological aspect | Total | Bronchointerstitial pneumonia with BALT hyperplasia | No evidence of BALT hyperplasia |
|---------------------|-------|---------------------------------------------------|--------------------------------|
| Positive            |       |                                                   |                                |
| Negative            |       |                                                   |                                |
| IAV                 |       |                                                   |                                |
| Yes                 |       |                                                   |                                |
| No                  |       |                                                   |                                |

### Table 3. An overall view of correspondence of lungs with histopathologic *Mycoplasma hyopneumoniae* (Mhyo) suspected lesions and *M. hyopneumoniae* qPCR results regarding the five companies enrolled in the study

| Histological aspect | Companies | Mhyo qPCR |
|---------------------|-----------|-----------|
|                     | A         | B         | C         | D         | E         |
| Positive            | 70% (21/30) | 80% (24/30) | 13.3% (4/30) | 90% (27/30) | 83.3% (25/30) |
| Negative            | 30% (9/30)  | 20% (6/30)  | 86.7% (23/30) | 10% (3/30)  | 16.7% (4/30)  |

qPCR = quantitative polymerase chain reaction, BALT = bronchus-associated lymphoid tissue; Different letters within a row indicate a statistically significant difference (P<0.05) based on chi-square analysis.
Agents of pneumonia in slaughtered pigs in southern Brazil

The presence of more than one infectious agent within all lungs from five companies was detected in 68% of the samples regarding histopathological, bacteriological and molecular findings. This data demonstrated the importance of coinfections in pig lungs at slaughter age, being *M. hyopneumoniae*, IAV and *P. multocida* type A the three most frequent microorganisms identified in our study. Although *M. hyopneumoniae*, *P. multocida* and Influenza A virus had already been described as the most relevant aggravating of PRDC in Brazil within pigs from all ages (Rech et al. 2018), the high frequency of IAV in slaughtered pigs was an unexpected finding as previous work have observed only a 2.8% frequency in fattening pigs (Morés et al. 2015). These findings have an important economic impact, since the economic losses due to coinfections between IAV and *M. hyopneumoniae* were estimated to be 16 times greater than single infections caused only by *M. hyopneumoniae* (Haden et al. 2012). Furthermore, our results suggest that those microorganisms should be taken into account in order to control pig pneumonia at the end of the finishing phase in Brazilian pig farms.

**CONCLUSION**

The data of this work highlight the coinfection roll of *Mycoplasma hyopneumoniae*, Influenza A virus (IAV) and *Pasteurella multocida* type A as the main agents of the Porcine Respiratory Disease Complex (PRDC) in Brazilian swine production (Rajão et al. 2015). In addition, PRRSv and PRCv are important viruses in the world pig industry but they have never been reported in Brazil (Rech et al. 2018), so the absence of suggestive lesions for the agents was expected.
Respiratory Disease Complex in pigs from Brazil. Moreover, we highlight the high frequency of lesions caused by IAV and the identification of the virus by real time RT-PCR in pigs at slaughter age, even though IAV is not typically associated with infections and clinical cases in pigs in this phase.

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Conflict of interest statement.- The authors declare that they have no competing interest.

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