

Implementation of histopathological techniques and transmission electron microscopy for research of *Mycoplasma hyopneumoniae* in swine

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Abstract— *Mycoplasma hyopneumoniae* is a fastidious bacterium, an important member of swine respiratory disease complex, like Porcine Enzootic Pneumonia, affecting the non-specific defense mechanism of the respiratory tract, high mucociliary system, predisposing the pigs to secondary pathogens. The objective of this study is to implement precise diagnostic techniques for identification of *Mycoplasma hyopneumoniae*. 19 swine lungs fragments were collected from slaughterhouses and submitted by histopathological techniques. The presence of mucocellular exudate in 78.94% of the samples was observed in the bronchi and bronchioles, absence of eyelashes in 63.15% and lymphoid tissue hyperplasia associated with the bronchus in 42.10%. In pulmonary parenchyma, thickening of alveolar wall and interstitial bronchopneumonia were observed in 68.42%, hemorrhage in 47.36%, which 36.84% had hemosiderin and 15.78% lung consolidation. The presence of mycoplasma by the negative staining technique was identified in all samples, also the labeling of epitopes by colloidal gold immunostaining, using monoclonal antibody. In immunohistochemistry techniques and in situ hybridization, the labeled epitope and genome were observed confirming the presence of *Mycoplasma hyopneumoniae* in the State of São Paulo. Therefore, the bronchial epithelium is the best tissue to collect the sample for an accurate diagnosis and the best method of diagnosis is the negative staining technique for screening and colloidal gold immunocytochemistry techniques to identify *Mycoplasma* species.

Keywords—*Mycoplasma hyopneumoniae*, Porcine Enzootic Pneumonia, Immunohistochemistry and Hybridization.

I. INTRODUCTION

Mycoplasma hyopneumoniae is a small fastidious bacterium with a simple morphologically structure and absence of cell wall. (DEMINA et al., 2009; JUNQUEIRA and CARNEIRO, 2012). It is the etiologic agent of Swine Enzootic Pneumonia, causing the Porcine Respiratory Disease Complex (PRDC) (MARE and SWITZER, 1965; GOODWIN et al, 1965).

Is the most important disease that affects the respiratory system of pigs causing large economic losses for pigs (ROSS et al., 1999), due to the high morbidity and reduction of feed conversion that decreases average daily weight gain (CONCEICAO and DELLAGOSTIN, 2006).

Diagnoses for this bacterium by ELISA available on the market have low sensitivity and cross-reactions with other mycoplasmas present in the lungs of the swine (ROSS and STEMKE 1995; ERLANDSON et al., 2005).

The objective of this study is to implement precise diagnostic techniques for identification of *Mycoplasma hyopneumoniae*.

II. MATERIAL AND METHOD

2.1. Ethics Statement

This study was approved by Animal Experimentation Research Ethics Committe of the Instituto Biológico (protocol 138/14).

2.2. Experimental Design

Two lung fragments were collected from 19 male pigs weighing approximately 100 kg, at a slaughterhouse school of the University of São Paulo - Campos Pirassununga (São Paulo, Brazil).

The first fragment was submitted for analysis of electron microscopy, by negative staining techniques and electron immunocytochemistry. The second fragment was submitted to histopathological analyzes, H.E., immunohistochemistry and *in situ* hybridization.

2.3. Negative Contrasting

For this technique, 2 cm of pulmonary fragments were suspended in 0.1 M phosphate buffer - pH 7.0, placed in contact with metallic grids and previously covered in collodion and carbon films, negatively contrasted with 2% ammonium molybdate and pH 5.0 (BRENNER and HORNE, 1959; HAYAT and MILER, 1990).

2.4. Electronic Immunocytochemistry

In this paper, the immunostaining technique with negatively contrasted colloidal gold particles was used, following the protocol developed by Knutton (1995).

The copper grid was placed on 40 µl of pulmonary fragments suspension, then deposited on drops of the monoclonal antibody to *Mycoplasma hyopneumoniae* diluted 1:80. After this procedure the grids were incubated in drops of protein A conjugated with 10 nm colloidal gold (diluted 1:20 in 0.5% PBS) and contrasted with 2% ammonium molybdate, pH 5.0.

2.5. Histopathology

The lung fragments were fixed in paraformaldehyde 10% diluted in 0.1M PBS buffer, pH 7.0 for 72 hours, dehydrated in increasing series of ethyl alcohol concentrations, diaphanized in xylol and paraffin embedded in subsequent sections of 4µm and stained by the H.E. technique.

2.6. Immunohistochemistry

This technique was processed according to information from Kit LSAB + System-HRP K0690 (DAKO) and the antigen-antibody reaction was revealed by the DAB + Substrate K3468 chromogen.

2.7. In Situ Hybridization

The pairs of primers used for this technique were purchased by Life Technologies and performed according to the Dako K0620 - GenPoint commercial *in situ* Hybridization Kit protocol.

The sequences of the primers used for this reaction were: forward 5'-Biotin-GTC TAT CAA AAT TGC CAA TC-3' and the reverse 5'-Biotin-TCC CAT AAC CTT GTC TTC AG-3', from nucleotides 851-870 And 1351-1370 respectively. The primers were labeled with biotin for visualization with the chromogen DAB + Substrate K3468 (KWON et al., 2002).

2.8. Observation and Images Registration

For the techniques of electron microscopy, the observation and recording of the image was performed in the transmission electron microscope Philips EM 208. In the histopathological techniques, the Carl Zeiss Axio ScopeA ® light microscope was used and the images were captured using the ZEN ® software.

III. RESULTS

3.1. Negative Contrasting

In this technique, pleomorphic particles with smooth and clear edges, gray central parts and less homogeneous, could be visualized in all 19 samples, being characteristic of *Mycoplasma* (Fig 1).



FIG 1 –PHOTOMICROGRAPHY OF MYCOPLASMA BY NEGATIVE STAINING TECHNIQUE IN ELECTRON MICROSCOPE TRANSMISSION. BAR 340 NM (SUCK ARROWS). SOURCE: ELECTRON MICROSCOPY LABORATORY STREAMING BIOLOGICAL INSTITUTE SP.

3.2. Electronic Immunocytochemistry

The specific epitope labeling for *Mycoplasma hyopneumoniae* with colloidal gold was observed in the 19 lung fragments used for this technique (Fig 2).

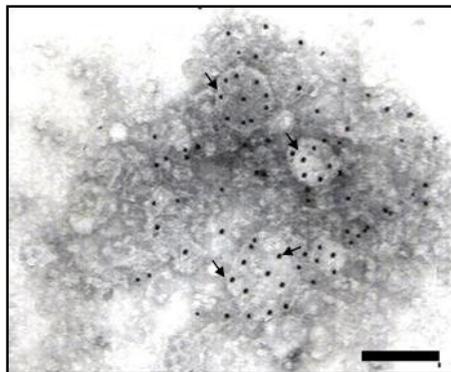


FIG 2 – PHOTOMICROGRAPHY MARKING OF MYCOPLASMA BY IMMUNOCYTOCHEMISTRY TECHNIQUE WITH COLLOIDAL GOLD IN NEGATIVE STAINING ELECTRON MICROSCOPE IN TRANSMISSION. 140 nm BAR (SUCK ARROWS). SOURCE: ELECTRON MICROSCOPY LABORATORY STREAMING BIOLOGICAL INSTITUTE SP.

3.3. Histopathology

The presence of mucocellular exudate in the bronchi and bronchioles was found in 78.94% (Fig 3), lack of eyelashes in 63.15%, bronchial associated lymphoid tissue hyperplasia (BALT) in 42.10% (Figure 4). In the pulmonary parenchyma, it was possible to visualize the thickening of the alveolar wall and interstitial bronchopneumonia in 68.42% (Fig 5), hemorrhage in 47.36%, among which 36.84% had hemosiderin and 15.78% consolidation pulmonary.

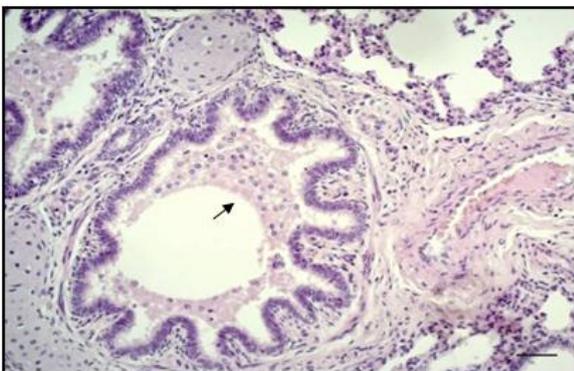


FIG 3 – PHOTOMICROGRAPH OF A HISTOLOGICAL CUT COLORED PIG LUNG BY HEMATOXYLIN AND EOSIN TECHNIQUE WITH MUOCOCELLULAR PRESENCE OF EXUDATE WITHIN THE BRONCHI (BLACK ARROW) (20μm bar).

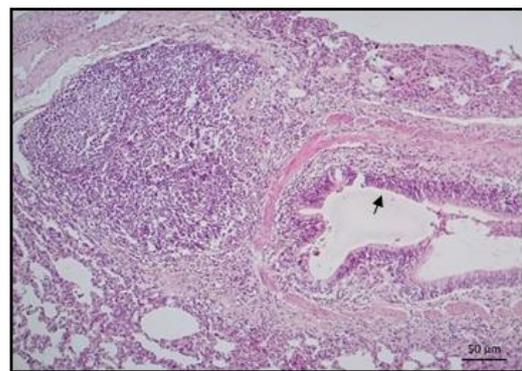


FIG 4 – PHOTOMICROGRAPH OF A HISTOLOGICAL CUT COLORED PIG LUNG BY HEMATOXYLIN AND EOSIN TECHNIQUE, WITH BALT HYPERPLASIA (ASTERISK).

3.4. Immunohistochemistry

The labeled epitope was observed by the antigen-specific antibody-*Mycoplasma hyopneumoniae* reaction in both the bronchial and bronchial epithelium and in the alveoli of the 19 histological sections of the lung fragments (Fig 6).

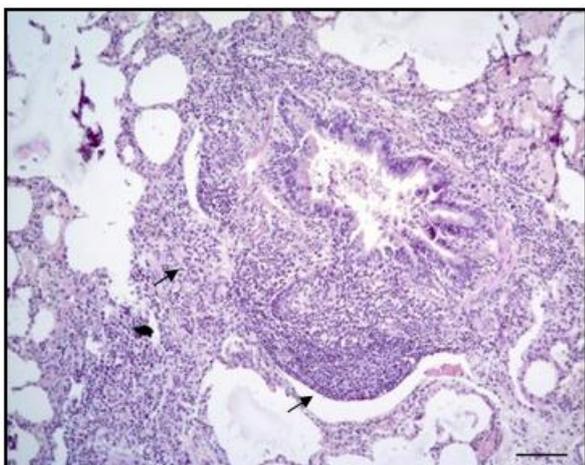


FIG 5 – PHOTOMICROGRAPH OF HISTOLOGICAL SECTIONS OF PORCINE LUNG STAINED WITH HEMATOXYLIN AND EOSIN TECHNIQUE, SHOWING AN INTERSTITIAL PNEUMONIA AND THICKENING OF THE ALVEOLAR WALLS (BLACK ARROWS) (100 µm bar).

3.5. *In Situ* Hybridization

The genome of *Mycoplasma hyopneumoniae* was observed in both bronchi and bronchiole epithelium and alveoli of the 19 histological sections lungs fragments (Fig 7).

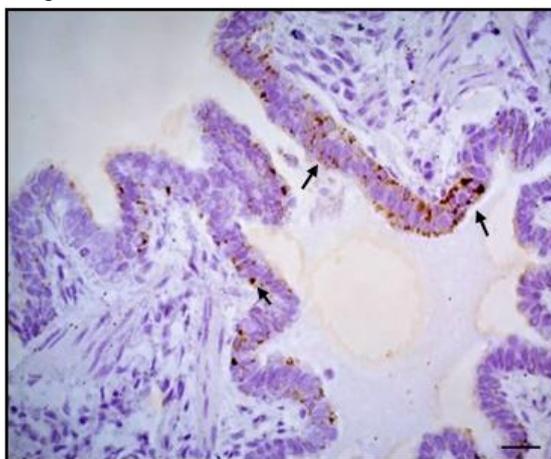


FIG 7 – PHOTOMICROGRAPH SHOWING POSITIVITY, THE MARKINGS BY MEANS OF IN SITU HYBRIDIZATION TECHNIQUE IN BRONCHIOLAR EPITHELIAL CELLS (BLACK ARROWS) (10 µm bar).

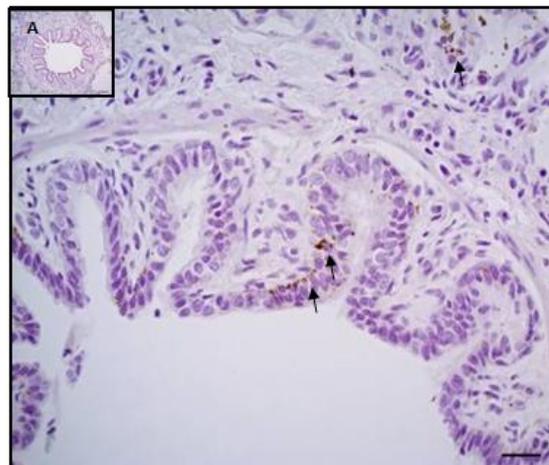


FIG 6 – PHOTOMICROGRAPH A IS SMALLER INCREASE IN PIG LUNG BRONCHIOLES (10x 10MICRON BAR). A PHOTOMICROGRAPH TO, IS THE EXPANSION OF A REGION OF THE PHOTOMICROGRAPH, DISPLAYING THE POSITIVE IMMUNOSTAINING OF EPITHELIAL CELLS OF THE BRONCHIOLES (BLACK ARROWS) (10 µm bar).

IV. DISCUSSION AND CONCLUSION

The identification of *Mycoplasma hyopneumoniae* was divided into 3 axes, according to their visualization, epitope marking and genome.

The agent was visualized through the negative staining presenting pleomorphic particles with the following characteristics: smooth and clear edges, gray in the center, less homogeneous and pleomorphic, corroborating with the characteristics described by Souza, et al. (2007). However, this technique does not allow identification at the species level, and therefore, the colloidal gold immunostaining technique was used to visualize and specify *Mycoplasma hyopneumoniae* species.

Due the small quantity of lung samples used in electron microscopy techniques, a correlation model was necessary between the identification of the agent and the histopathological lesions.

According to Sobestiansky et al., (1999) e Thacker (2006) the microscopic lesions vary with the disease progression, and the most observed lesion was bronchointerstitial pneumonia with BALT hyperplasia. In this study, it was observed that 68.42% presented interstitial bronchopneumonia and 42.10% presented BALT hyperplasia, corroborating with classic findings of the disease described above.

In addition, in 78.94% of the cases, a mucocellular exudate with neutrophils in the bronchi and bronchioles was observed, pulmonary consolidation of the parenchyma in 15.78% and thickening and hyperplasia of the alveolar walls with inflammatory infiltrate in 68.42%. Although these microscopic changes are not classic of *Mycoplasma hyopneumoniae* infection, Redondo et al. (2009) and Hillen et al. (2014) also described these findings in the discrimination of the disease. It was observed that in 63.15% there was a lack of eyelashes in the bronchi and bronchioles, an infectious characteristic also seen by Posá et al. (2013). These findings indicate that *M. hyopneumoniae* infection is extremely complex with classic and secondary characteristics that are relevant in clinical diagnosis.

Although markers are positive in histopathological lesions by immunohistochemical techniques and in situ hybridization, the fixation in 10% buffered formalin can alter the epitopes as a protein fixative as described by Souza (2007). Although the *in situ* hybridization technique is effective, Santos (2015) verified that *M. hyopneumoniae* genomics in Brazil is highly variable, identifying 39 different types of the species in 8 States, according to multiple locus variable number tandem repeat analysis (MLVA) which makes it difficult to specify probes.

Because of the possible epitope alteration and the high genotypic variability, the reliable method of histopathological findings was the positivity of both methods (immunohistochemistry and in situ hybridization) to confirm the pulmonary lesions caused by *Mycoplasma hyopneumoniae*, in the State of São Paulo, Brazil.

It was concluded that for a precise diagnosis of *M. hyopneumoniae* in São Paulo State, the best sample is the bronchial epithelium and the best diagnostic method is the electron microscopy technique (as screening) and immunocytochemistry with colloidal gold for identification level of agent species.

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