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Real-time PCR quantification and histopathological findings of *Mycoplasma hyopneumoniae* infection in the lungs of pigs slaughtered in São Luís, Maranhão, Brazil[1]

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ABSTRACT.- Rodrigues O.A.F., Dias E.F., Freitas F.M., Moreira W.F.F., Bezerra N.P.C., Ribeiro D.L.S., Carvalho Neta A.V., Abreu-Silva A.L., Machado R.Z. & Ribeiro L.S.S. 2023. **Real-time PCR quantification and histopathological findings of *Mycoplasma hyopneumoniae* infection in the lungs of pigs slaughtered in São Luís, Maranhão, Brazil.** *Pesquisa Veterinária Brasileira 43:e07233, 2023.* Programa de Pós-graduação Profissional em Defesa Sanitária Animal, Universidade Estadual do Maranhão, São Luís, MA 65365-000, Brazil. E-mail: larissa.sarmento@uema.br

Porcine enzootic pneumonia (PES), mainly caused by the bacteria *Mycoplasma hyopneumoniae*, is the main cause of respiratory problems in pigs. Infection by *M. hyopneumoniae* leads to production losses and the predisposition of affected animals to secondary infections, which may result in the condemnation of carcasses and organs due to lung lesions at the time of slaughter. The objective of the research was to evaluate the infection by *M. hyopneumoniae* in pigs submitted to slaughter in São Luís Island/MA, using molecular and histopathological diagnostic methods. One hundred fifty lung samples were collected from inspected (n=65) and non-inspected (n=85) slaughter pigs on São Luís Island, Maranhão, from July 2019 to August 2021. Of the 150 DNA samples collected, 121 showed an amplified product for Cyt B in the PCR assay. Thus, 121 samples were submitted to qPCR of *M. hyopneumoniae*, of which 44 (36.36%) showed positive results. The mean amount of bacterial load ranged from 1.20×10^1 to 7.20×10^4, with a mean of 1.73×10^4 copies. Of the reagent samples, 81.81% (36 samples) were obtained from non-inspected slaughter, while 18.18% (8 samples) were obtained from slaughterhouses. In the histopathological analysis, 44 positive qPCR samples were evaluated, of which 28 (63.63%) presented results compatible with the main inflammatory process associated with the presence of *M. hyopneumoniae*, that is, bronchial-associated lymphoid tissue hyperplasia (BALT). Three samples that showed the highest bacterial load (qPCR: 5.63×10^3, 2.19×10^4 and 7.23×10^4) showed more evident lesions in this study. The microscopic findings associated with the quantifications indicated a relationship between the amount of bacterial load and the presence of microscopic lesions; higher bacterial load in lung tissue is associated with increased histopathologic staining for BALT hyperplasia. In conclusion, the results point to the circulation of the etiological agent in the sampled animals and the need for preventive measures on pig farms in Maranhão with the involvement of producers, sanitary defense and inspection agencies.

INDEX TERMS: *Mycoplasma hyopneumoniae*, swine enzootic pneumonia, pig farming.

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RESUMO.- [Quantificação por PCR em tempo real e achados histopatológicos da infecção por *Mycoplasma hyopneumoniae* em pulmão de suínos abatidos na cidade de São Luís, Maranhão, Brasil.] A pneumonia enzoótica suína (PES), causada principalmente pela bactéria *Mycoplasma hyopneumoniae*, é a principal causa de problemas respiratórios em suínos. A infecção por *M. hyopneumoniae* leva a perdas produtivas e a predisposição dos animais acometidos a infecções secundárias, o que pode resultar em condenação de carcaças e órgãos por lesões pulmonares no momento do abate. O objetivo da pesquisa foi avaliar a infecção por *M. hyopneumoniae* em suínos submetidos ao abate na Ilha de São Luís, por meio de métodos diagnósticos moleculares e histopatológicos. Para isso, foram coletadas 150 amostras de pulmão de suínos de abate inspecionado (n=65) e não inspecionado (n=85) na Ilha de São Luís/Maranhão, no período de julho de 2019 a agosto de 2021. Das 150 amostras de DNA coletadas, 121 apresentaram produto amplificado para Cyt B no ensaio de PCR. Assim, 121 amostras foram submetidas à qPCR de *M. hyopneumoniae*, das quais 44 (36,36%) apresentaram resultados positivos. A quantidade média de carga bacteriana variou de $1,20 \times 10^1$ a $7,20 \times 10^4$, com média de $1,73 \times 10^4$ cópias. Das amostras reagentes, 81,81% (36 amostras) foram obtidas de abate não inspecionado, enquanto 18,18% (8 amostras) foram obtidas em abatedouro. Na análise histopatológica, foram avaliadas 44 amostras positivas para qPCR, das quais 28 (63,63%) apresentaram resultados compatíveis com o principal processo inflamatório associado à presença de *M. hyopneumoniae*, ou seja, hiperplasia do tecido linfóide associado ao brônquio (BALT). Três amostras que apresentaram maior carga bacteriana (qPCR: $5,63 \times 10^3$, $2,19 \times 10^4$ e $7,23 \times 10^4$) foram mais evidentes neste estudo. Os achados microscópicos associados às quantificações indicaram uma relação entre a quantidade de carga bacteriana e a presença de lesão microscópica; a maior carga bacteriana no tecido pulmonar está associada a maior alteração histopatológica para hiperplasia BALT. Em conclusão, os resultados obtidos sinalizam para a circulação do agente etiológico nos animais amostrados e a necessidade de medidas preventivas nas criações de suínos do estado do Maranhão com envolvimento dos produtores, órgãos de defesa sanitária e inspeção.

TERMOS DE INDEXAÇÃO: *Mycoplasma hyopneumoniae*, Pneumonia Enzoótica Suína, Suinocultura.

INTRODUCTION

Brazil is the fourth largest producer and exporter of pork in the world after China, the European Union, and the United States of America (USA), with more than 80% of its products sold in the domestic consumer market (ABRA 2018). However, health challenges like respiratory and enteric diseases present obstacles to optimizing zootechnical results, reducing the country's competitiveness in the global market (Ciacci-Zanella et al. 2009).

In intensive swine farming, the onset of respiratory diseases has become common, mainly due to the confinement conditions in slaughters, which favor the accumulation of gases and the circulation of pathogens. It causes economic losses due to low animal performance, treatments, and/or disease prevention and control programs (Straw et al. 1990, Došen et al. 2007, Maes et al. 2008).

Porcine enzootic pneumonia (PEP) is the main cause of respiratory problems in swine, mainly caused by the bacterium *Mycoplasma hyopneumoniae* (Pieters et al. 2009). Its occurrence makes the host susceptible to bacterial or viral secondary infections, such as porcine circovirus type 2 (PCV-2) infection, both involved in the Porcine Respiratory Disease Complex, responsible for large losses in the national and international swine farming (Marois et al. 2007, He et al. 2011).

PEP is a highly contagious disease with a cosmopolitan distribution, characterized by high morbidity, low mortality, chronic cough, and growth retardation (Oboegbulem 1981, Tamiozzo et al. 2011). PEP needs attention due to its high morbidity, resulting in a decrease in feed conversion and average daily weight gain of swine (Conceição & Dellagostin 2006).

Considering the productive losses caused by PEP, the predisposition of animals to secondary infections, lung injuries at the time of slaughter, and the absence of studies on this disease in Maranhão, we investigated the infection by *M. hyopneumoniae* in swine submitted for slaughtering in São Luís, Maranhão.

MATERIALS AND METHODS

Animal ethics. The present study was approved by the Animal Experimentation Ethics Committee (CEUA) from the "Universidade Estadual do Maranhão" (UEMA) under protocol No. 020/2019.

Animals and collection of samples. Lung samples were collected from 65 pigs from the only technified farm of São Luís, which were submitted for slaughtering in a slaughterhouse with an inspection service (official Government service), and from 85 pigs from small producers in São Luís, which were slaughtered without inspection service. The collections were done from July 2019 to August 2021.

Random sampling was performed using the EPI-Info 6 program with the following parameters: number of animals raised in the state (600,000 animals); estimated prevalence of 40%; confidence interval of 95%; and absolute precision of 10%. Thus, 150 pigs were evaluated by simple random sampling.

The samples were collected in duplicate, where a fragment was subjected to molecular analysis and stored in sterile microtubes at -20°C for further processing. The second fragment was subjected to histopathological analysis and then stored in flasks containing 10% buffered formalin. The analyses were performed in the Laboratory of Molecular Pathology and Veterinary Pathology of the Veterinary Medicine Course at UEMA. At the time of collection, an observation guide was used to note the places where the animals were raised and slaughtered.

DNA extraction. DNA extraction from lung samples was performed using the Wizard Genomic DNA Purification Kit (Promega®), according to the manufacturer's instructions. The concentration (ng/µL) and quality based on the 260/280 and 260/230 ratios of the extracted DNA were determined using a spectrophotometer and adjusted with ultrapure water to approximately 500ng/µL.

Molecular analysis of DNA extracted from swine lung. Conventional PCR for the endogenous Cytochrome B (*Cyt B*) gene

A PCR assay was performed using Cyt B of vertebrate mitochondrial DNA (mtDNA) to determine the quality of DNA extraction, the integrity of the extracted DNA, and/or the presence of reaction inhibitors, as described by Steuber et al. (2005), which produces a 359-base pair (bp) fragment.

Quantitative real-time PCR (qPCR). Positive samples for *Cyt B* were subjected to a qPCR assay specific for *Mycoplasma hyopneumoniae* based on the p183 gene, following the protocol described by Strait et al. (2008).

Amplification reactions were performed using a 7500® thermocycler (Applied Biosystems, Foster City, CA, USA). All samples were tested in duplicates. Quantification of DNA copies/µL was performed using gBlocks (Integrated DNA Technologies, Coralville, Iowa, USA) containing the target sequence for DNA amplification of *M. hyopneumoniae*. Serial dilutions were performed to construct standards with different concentrations of DNA containing the target sequence (2.0×10^7 copies/µL to 2.0×10^2 copies/µL). Ultrapure water (Invitrogen, Carlsbad, California, USA) was a negative control.

Histopathology. The lung fragments were subjected to histopathological analysis to evaluate the lesions previously identified in molecular diagnosis, being initially fixed in 10% buffered formalin for 24 h and then processed by routine histological techniques, which include the following steps: dehydration in alcohol, diaphonization in xylene, and embedding in paraffin. The embedded lungs were sectioned into sections of 5-µm thickness with a Microm HM 360 Rotary Microtome and stained with the hematoxylin and eosin (HE) technique (Allen 1992).

The histological sections were photographed using an image capture system composed of a digital camera coupled with an optical microscope (Leica ICC50 HD). The microscopic lesions were analyzed according to the findings of main lesions associated with the presence of *M. hyopneumoniae*, such as catarrhal bronchopneumonia, characterized by large infiltration of neutrophils, lymphocytes, and macrophages in the lumen and BALT hyperplasia (Hillen et al. 2014).

RESULTS

Of the 150 samples evaluated, 121 showed *Cyt B* amplified product in the PCR assay. The samples had an average DNA concentration of 500ng/µL. The 260/280 and 260/230 parameters exhibited means of 1.83 and 1.85, respectively. The *Cyt B* gene amplification product resulted in a single band on agarose gel, congruent to a 359-bp fragment (Fig.1), according to the primers described by Steuber et al. (2005).

Of the 121 samples subjected to qPCR for *Mycoplasma hyopneumoniae* detection, 44 (36.36%) were positive, with amounts of copies of the p183 gene fragment ranging from 1.20×10^1 to 7.20×10^4 and an average of 1.73×10^4 copies. The qPCR efficiency ranged from 100.09% to 104.08%; the coefficient of determination ranged from 0.998 to 0.999; the slope ranged from -3.32 to -3.228; and the y-intercept ranged from 38.82 to 40.18.

Of the positive samples, 81.81% (n=36/44) were obtained from small rural producers where slaughtering is performed without inspection service, whereas 18.18% (n=8/44) were obtained from a slaughterhouse inspected with animals by a technified farm. These results demonstrate wide pathogen dissemination among swine herds in São Luís.

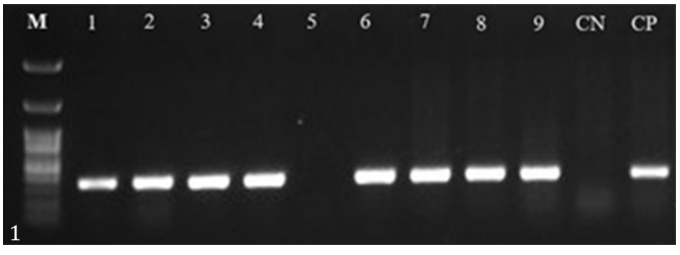

Fig.1. Electrophoresis on agarose gel (1.5%) and PCR of CytB from lung fragments of swine submitted for slaughtering in São Luís. M = molecular weight marker; NC = negative control; PC = positive control; 1, 2, 3, 4, 6, 7, 8, and 9 = positive samples; 5 = negative sample.

In the histopathological analysis, of the 44 qPCR-positive samples, 68.18% (n=30/44) were compatible with the inflammatory processes, such as the presence of BALT hyperplasia, interstitial pneumonia, alveolar edema, hemorrhage, congestion, and emphysema (Table 1). Of these samples, 43.33% (n=13/30) showed marked BALT hyperplasia, the main alteration associated with *M. hyopneumoniae* infection (Fig.2). On the other hand, 31.81% (n=14/44) of the analyzed fragments had discrete lesions or no lesions.

Regarding the association between the bacterial load obtained using qPCR and the histopathological lesions, the four samples with highlighted microscopic lesions showed higher bacterial load (qPCR: 1.52×10^4, 5.63×10^3, 2.19×10^4, and 7.23×10^5). Of the samples with little microscopic significance (14/44), one had a bacterial load similar to those with evident BALT hyperplasia (qPCR: 1.00×10^4). The inverse was also observed in a sample with a notable histopathological lesion, where bacterial quantification was among the lowest (qPCR: 1.98×10^0).

DISCUSSION

This study seems to be the first on *Mycoplasma hyopneumoniae* in swine in Maranhão, in which a positivity rate of 36.36% was found by qPCR in the analyzed fragments. Studies performed in other regions of Brazil revealed different positivity rates. Vicente et al. (2013) showed a positivity percentage of 52% in swine herds in the central-west region of the state of São Paulo. In contrast, Morés et al. (2015) showed a 74% of positivity rate in swine herds in the midwest and south regions of Brazil. However, no study has been conducted on the prevalence of *M. hyopneumoniae* in Maranhão. It may not restrict the sale of pork, even when contaminated, which favors the restricted information, as mentioned by Maes et al. (2018).

Most of the positive samples in qPCR (81.81%) were obtained from swine from small rural producers, which

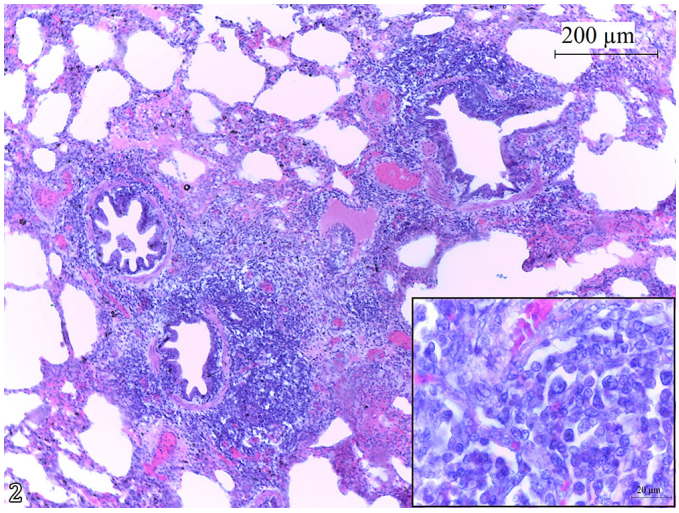

Fig.2. Histological section of swine lung. In the peribronchial region, marked multifocal lymphoid hyperplasia is observed. Alveoli sometimes filled with eosinophilic amorphous substance (edema), in addition to thickening of the alveolar region. Presence of discrete and multifocal areas of hemorrhage. HE, bar = 200µm. Inset: Lymphoid aggregate in greater detail at 1000X magnification. HE, bar = 20µm.

slaughter without inspection service and sell in open markets. From the data obtained at the time of collection, it was found that the slaughtering was performed without any hygienic precautions, as the animals were obtained from small producers, which lacked veterinary medical assistance or technical guidance regarding the sanitary measures for swine farms. The conjuncture of such factors, according to Schwartz (2001) and Ferracini & Piassa (2021), corroborates the incidence of PEP, which is mainly caused by *M. hyopneumoniae*.

On the other hand, according to Constable et al. (2020), in slaughterhouses, approximately 40% to 80% of pig lungs raised intensively present macroscopic lesions characteristic of PEP. This is due to the rapid spread of the disease in farms with high animal density and a lack of sanitary management

Table 1. Mean quantification of *Mycoplasma hyopneumoniae* and histopathological findings in the lung of pigs slaughtered in São Luís

Samples	qPCR (quantity mean)	BALT hyperplasia*	Interstitial pneumonia**	Edema***
		Score	Score	Score
58	4.01×10^1	–	+++	–
59	1.12×10^2	–	–	–
60	5.83×10^2	–	–	–
61	8.14×10^2	++	+	–
62	5.71×10^2	–	–	–
63	1.01×10^3	+	++	+
64	5.57×10^2	–	–	–
65	4.93×10^3	+	–	–
78	1.20×10^0	–	–	–
85	7.50×10^1	+	–	–
86	1.99×10^1	+	+	–
88	5.87×10^0	+	+	–
93	6.47×10^1	+++	+++	+
94	3.16×10^0	++	+++	+
96	1.16×10^1	++	+++	+
97	7.95×10^0	–	–	–
99	8.83×10^1	++	++	–
101	1.98×10^0	+++	+	–
102	7.91×10^0	+++	++	+
104	2.49×10^0	+	++	+
105	1.81×10^1	–	–	–
106	2.14×10^2	+++	+++	+
107	2.20×10^0	+	–	–
108	4.60×10^0	+	+	–
111	2.73×10^0	+	+	–
122	3.56×10^1	–	–	–
129	3.72×10^1	–	–	–
130	1.18×10^2	++	+	+
131	4.56×10^1	++	+	–
132	4.90×10^0	–	+	–
133	1.00×10^3	–	–	–
134	2.19×10^4	+++	++	–
135	5.18×10^2	+	+	–
136	6.73×10^1	++	+++	–
137	1.52×10^3	++	++	+
138	5.01×10^1	–	–	–
139	2.44×10^2	–	–	–
140	3.97×10^1	+	+	–
143	5.63×10^3	++	+++	+
144	7.20×10^4	+++	+++	+
146	3.64×10^1	–	–	–
148	1.92×10^1	–	+	–
149	4.58×10^0	+	++	+
150	7.35×10^2	+	+++	+

* BALT hyperplasia (bronchus-associated lymphoid tissue): [–] absent, [+] mild, [++] moderate, [+++] marked; **Interstitial pneumonia: [–] absent, [+] mild, [++] moderate, [+++] marked; ***Alveolar edema: [–] absent, [+] present.

(Ferracini & Piassa 2021). In a study performed by Ferraz et al. (2020) in the State of Minas Gerais, with 333 pigs with macroscopic lesions in the lung raised in an intensive system, 100% positivity for *M. hyopneumoniae* was verified by qPCR.

In the present study, only swine from a slaughterhouse with inspection service was from a technified farm. Despite this rearing condition being favorable to the dissemination of bacteria, a lower percentage of positivity was observed in the samples (17.39%). This factor may be related to the biosecurity standards followed by the farm. According to Maes et al. (2008), the main management practices adopted to reduce the spread of *M. hyopneumoniae* include an "all in/all out" production system, proper acclimatization of gilts, balanced stocking, prevention of other respiratory diseases, and optimal stocking conditions.

The microscopic findings of the positive samples (68.18%; n=30/44) for *M. hyopneumoniae* indicated the presence of lesions caused by this bacterium, i.e., BALT hyperplasia. This is the main microscopic lesion resulting from catarrhal bronchopneumonia caused by this bacteria, as mentioned by Redondo et al. (2009), Thacker & Minion (2012), and Hillen et al. (2014). In a study on the lungs of swine with a previous history of respiratory disease, Almeida et al. (2012) reported that the most prominent microscopic finding was BALT hyperplasia, evident in 11 (73.33%) of the 15 samples evaluated. In the same study, qPCR was performed, finding amplified material in 100% of the bronchial swabs. Considering that *M. hyopneumoniae* is the main causative agent for PEP and that this disease has a chronic evolutionary characteristic (Thacker & Minion 2012), the variability of microscopic findings and bacterial quantification will depend on the age of the infected animal, stage of the infection, and presence of secondary infectious agents (Mattsson et al. 1995, Calsamiglia et al. 1999).

Although BALT hyperplasia is a histopathological lesion characteristic of *M. hyopneumoniae* infection used in diagnosis, the pathogen has already been detected in animals that did not show these microscopic lesions (Sørensen et al. 1997). The present study confirmed the presence of *M. hyopneumoniae* DNA in lung samples without characteristic lesions (31.81%), which showed a low bacterial load revealed by qPCR. A study performed by Andrade (2018) reported a similar result, showing the presence of the pathogenic agent even in the absence of a lesion in lung fragments. Thus, the dose, circulating strain, and management practices are the factors directly related to the occurrence of the disease (Vicca et al. 2003). Furthermore, Pieters et al. (2009) reported that the agent might not be definitively eliminated from the respiratory tract of the infected animal, even after the resolution of pulmonary lesions, rendering the animal an asymptomatic carrier capable of infecting other animals.

By comparing the results of bacterial quantification with those of histopathological lesions, it was found that the four samples with the highest bacterial load also had diffused histopathological lesions characteristic of *M. hyopneumoniae* infection. Almeida (2019) reported a positive relationship between agent quantification and the presence of microscopic lesions, demonstrating that the high bacterial load in lung tissue is associated with intense characteristic histopathological staining and BALT hyperplasia. This correlation occurs because the infection promotes strong activation of alveolar macrophages and lymphocytes, thereby facilitating the production of several pro-inflammatory cytokines and antibodies, as well as producing perivascular and peribronchial lymphoid hyperplasia (Blanchard et al. 1992, Kobisch & Friis 1996, Rodríguez et al. 2004, Choi et al. 2006). The progression of this inflammatory condition results in bronchoconstriction and obstruction of the airway, with consequent formation of atelectatic lesions in the lungs, which can be observed macroscopically during slaughtering or necropsy (Maes et al. 2008, Redondo et al. 2009, Thacker & Minion 2012, Deblanc et al. 2013).

CONCLUSION

This study detected the DNA of *Mycoplasma hyopneumoniae* by qPCR analysis associated with histopathological lesions and confirmed the presence of BALT hyperplasia in the lungs of pigs. The results indicated that *M. hyopneumoniae* might circulate in the animals. Thus, preventive measures are required in swine farms in Maranhão with the involvement of producers, sanitary defense, and inspection agencies.

Authors' contribuitions.- Odinéa A.F. Rodrigues contributed to the general study, mainly collection and execution, laboratory analysis and interpretation and manuscript preparation. Fernanda M. Freitas, Wendel F.F. Moreira, Ana Lúcia Abreu-Silva contributed to the execution, histopathology analysis, and interpretation. Elaine F. Dias, Diego Luiz S. Ribeiro, Alcina V. Carvalho Neta contributed to the study design, quantitative PCR analysis, and interpretation. Nancyleni P.C. Bezerra, Rosângela Z. Machado, and Larissa S.S. Ribeiro contributed to the study design, study execution, and interpretation and final approval of the manuscript.

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Conflict of interest statement.- The authors declare no conflicts of interest.

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