UNIVERSIDADE ESTADUAL PAULISTA – UNESP CÂMPUS DE JABOTICABAL

COINFECÇÕES POR *M. hyopneumoniae, M. hyorhynis e M. Flocculare* **EM LESÕES DE CONSOLIDAÇÃO PULMONAR DE SUÍNOS AO ABATE**

Marcela Manduca Ferreira

Médica Veterinária

2020

UNIVERSIDADE ESTADUAL PAULISTA – UNESP CÂMPUS DE JABOTICABAL

COINFECÇÕES POR *M. hyopneumoniae, M. hyorhynis* e *M. Flocculare* EM LESÕES DE CONSOLIDAÇÃO PULMONAR DE SUÍNOS AO ABATE

Marcela Manduca Ferreira

Orientador: Prof. Dr. Luis Guilherme de Oliveira

Dissertação apresentada à Faculdade de Ciências Agrárias e Veterinárias – Unesp, Câmpus de Jaboticabal, como parte das exigências para a obtenção do título de Mestre em Medicina Veterinária, área: Clínica Médica Veterinária

2020

F383c

Ferreira, Marcela Manduca
Coinfecções por Mycoplasma hyopneumoniae, Mycoplasma hyorhynis E Mycoplasma Flocculare em lesões de consolidação pulmonar de suínos ao abate / Marcela Manduca Ferreira. -- Jaboticabal, 2020
46 p.

Dissertação (mestrado) - Universidade Estadual Paulista (Unesp), Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal
Orientador: Luís Guilherme de Oliveira

1. Doenças respiratórias. 2. Pneumonia enzoótica suína. 3. Coinfecções por agentes respiratórios. 4. Doenças de suínos. I. Título.

Sistema de geração automática de fichas catalográficas da Unesp. Biblioteca da Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal. Dados fornecidos pelo autor(a).

Essa ficha não pode ser modificada.

unesp

UNIVERSIDADE ESTADUAL PAULISTA

Câmpus de Jaboticabal

CERTIFICADO DE APROVAÇÃO

TÍTULO DA DISSERTAÇÃO: COINFECÇÕES POR *M. hyopneumoniae*, *M. hyorhynis* e *M. Flocculare* EM LESÕES DE CONSOLIDAÇÃO PULMONAR DE SUÍNOS AO ABATE

AUTORA: MARCELA MANDUCA FERREIRA
ORIENTADOR: LUIS GUILHERME DE OLIVEIRA

Aprovada como parte das exigências para obtenção do Título de Mestra em MEDICINA VETERINÁRIA, área: Clínica Médica Veterinária pela Comissão Examinadora:

Prof. Dr. LUIS GUILHERME DE OLIVEIRA
Departamento de Clínica e Cirurgia Veterinária / FCAV / UNESP - Jaboticabal

Prof. Dr. MARCOS ROGÉRIO ANDRÉ VIDEOCONFERÊNCIA
Departamento de Patologia Veterinária / FCAV / UNESP - Jaboticabal

Prof. Dr. ABELARDO SILVA JÚNIOR VIDEOCONFERÊNCIA
Departamento de Veterinária-UFV / Viçosa/MG

Jaboticabal, 29 de maio de 2020

DADOS CURRICULARES DO AUTOR

MARCELA MANDUCA FERREIRA – Natural da cidade de Jaboticabal, São Paulo - Brasil, e nascida no dia 03 de setembro de 1992. É médica veterinária formada pela Universidade Federal de Uberlândia no ano de 2017. Foi bolsista de iniciação científica pelo programa de Medicina Veterinária Preventiva sob a orientação da Prof. Dr. Anna Monteiro Correia Lima durante o ano de 2016. No ano de 2017 realizou o estágio curricular obrigatório na empresa BRF, município de Uberlândia - MG. Em agosto de 2018, ingressou no programa de Pós-Graduação em Medicina Veterinária, área de Clínica Médica Veterinária, pela Faculdade de Ciências Agrárias e Veterinárias (FCAV) – Unesp/Jaboticabal, como bolsista CAPES, sob a orientação do Prof. Dr. Luis Guilherme de Oliveira.

Dedicatória

Aos meus pais, Rosana e Marcelo, por todo apoio, compreensão e amor incondicional.

AGRADECIMENTOS

A Deus por sempre conduzir e guiar meus passos na direção certa, me conferir discernimento, paciência e dedicação em toda a minha trajetória.

Aos meus Pais, Marcelo e Rosana, por tudo que fazem por mim, sem medir esforços. Sem vocês eu certamente não chegaria até aqui! Toda a minha gratidão e reconhecimento eu vos dedico. Amo vocês!

Ao meu namorado, Marco Antônio, por sempre se empenhar em me mostrar o lado bom das situações, por ser paciente, amigo, parceiro, e ainda me ajudar em algumas coletas no frigorífico. Obrigada pelo seu amor e companheirismo, amo você!

À minha psicóloga Roseli Isildinha Jovino, que faz parte da minha trajetória desde 2015, e acompanhou meu período de dois anos de mestrado. Obrigada pelo amor que você dedica ao que faz, sua contribuição foi de grande importância para esse título.

Ao meu orientador Prof. Dr. Luís Guilherme de Oliveira por ser exemplo de profissional e ser humano, por se fazer presente sempre que possível e dedicar empenho aos trabalhos que conduz.

Um agradecimento especial às colegas de laboratório Marina e Karina, que tiveram intensa participação no meu trabalho de mestrado. Minha jornada teria sido bastante complexa se eu não tivesse contado com tamanha ajuda de vocês. Obrigada pela amizade! E aos demais colegas do Laboratório de Medicina de Suínos: Henrique, Maria Eugênia, Gabriel, Fernando, Beatriz e Mariela por todo auxílio prestado, convívio e amizade.

À Faculdade de Ciências Agrárias e Veterinárias, Câmpus de Jaboticabal por toda estrutura fornecida para a realização deste trabalho.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001.

“O saber a gente aprende com os mestres e livros. A sabedoria se aprende é com a vida e com os humildes”

(Cora Coralina)

SUMÁRIO

COINFECÇÕES POR *Mycoplasma hyopneumoniae, Mycoplasma hyorhynis* **E** *Mycoplasma Flocculare* **EM LESÕES DE CONSOLIDAÇÃO PULMONAR DE SUÍNOS AO ABATE**

RESUMO – Poucos estudos sobre a ocorrência de coinfecção entre *Mycoplasma hyopneumoniae* (*Mhyo)* e outras espécies de micoplasmas – *Mycoplasma hyorhinis* (*Mhr)* e *Mycoplasma flocculare* (*Mfloc*) em lesões pulmonares de suínos foram registrados. A correlação entre a coinfecção por micoplasmas e o grau de lesões macroscópicas de consolidação pulmonar (LMCP) ainda não havia sido explorada no Brasil. Este trabalho teve como objetivo a detecção e quantificação de *Mhyo, Mhr* e *Mfloc* em LMCP de suínos ao abate*.* Foram selecionados 400 pulmões de suínos (idade média de 23-25 semanas), os quais foram divididos em cinco grupos (n=80), de acordo com o grau de lesões de consolidação pulmonar crânio-ventral, e coletados no momento do abate, em um frigorífico localizado na cidade de Guariba – SP. A detecção e quantificação dos micro-organismos foram realizadas por meio de qPCR multiplex com sonda de hidrólise. Diferenças estatísticas entre os grupos e a comparação entre os mesmos foram avaliadas, respectivamente, pelo teste de Kruskal-Wallis ($p<0,05$) e teste de Dunn ($p<0,05$), e a correlação entre os dados foi realizada pelo método de Spearman ($p<0,05$). Os resultados obtidos revelaram que LMCP estão diretamente correlacionadas à estimativa de *Mhyo* (rho=0,26), inversamente correlacionadas à estimativa de *Mfloc* (rho= - 0,15), e não apresentam correlação com a estimativa de *Mhr* (p=0,12). A extensão de LMCP exibiu correlação positiva com a coinfecção por *Mfloc* e *Mhr* (rho = 0,17), nenhuma correlação com *Mhyo e Mhr* (p=0,87)*,* e correlação negativa com *Mhyo e Mfloc* (rho= - 0,28). O estudo permitiu inferir que, no que diz respeito à extensão de LMCP, *Mhr* e *Mfloc* não apresentaram atividade oportunista da infecção primária por *Mhyo*, mas revelaram algum potencial agravante dessas lesões quando em conjunto. Além disso, *Mhyo* expressou comportamento inibitório em relação a *Mfloc*, sugerindo que um possa competir com a presença do outro.

Palavras - Chave: Pneumonia; doenças respiratórias; Mollicutes; pneumonia enzoótica; prevalência.

COINFECTION OF *Mycoplasma hyopneumoniae, Mycoplasma hyorhynis* **AND** *Mycoplasma flocculare* **IN LUNG CONSOLIDATION LESIONS OF SLAUGHTERED PIGS**

ABSTRACT – Few studies on the occurrence of co-infection between *Mycoplasma hyopneumoniae* (*Mhyo*) and other species of mycoplasmas - *Mycoplasma hyorhinis* (*Mhr*) and *Mycoplasma flocculare* (*Mfloc*) in swine lung injuries. The correlation between mycoplasma co-infection and the degree of macroscopic lung consolidation lesions (MLCL) has not yet been explored in Brazil. This work aimed to detect and quantify *Mhyo, Mhr* and *Mfloc* in MLCL of slaughter pigs. 400 pig lungs (mean age 23-25 weeks) were selected, which were divided into five groups (n = 80), according to the degree of lesions of the cranio-ventral lung consolidation, and collected at the time of slaughter, in a slaughterhouse located in the city of Guariba - SP. The detection and quantification of microorganisms were performed using a multiplex qPCR with hydrolysis probe. Statistical differences between the groups and the comparison between them were evaluated, respectively, by the Kruskal-Wallis test ($p < 0.05$) and Dunn's test ($p < 0.05$), and the correlation between the data was performed by Spearman's method ($p < 0.05$). The results obtained revealed that MLCL are directly correlated to the *Mhyo* estimate (rho = 0.26), inversely correlated to the *Mfloc* estimate (rho = - 0.15), and have no correlation with the *Mhr* estimate (p = 0, 12). The LMCP extension exhibited a positive correlation with *Mfloc* and *Mhr* co-infection (rho = 0.17), no correlation with *Mhyo* and *Mhr* (p = 0.87), and a negative correlation with *Mhyo* and *Mfloc* (rho = - 0.28). The study allowed to infer that, with regard to the extension of MLCL, *Mhr* and *Mfloc* did not present opportunistic activity of the primary infection by *Mhyo*, but revealed some potential aggravating of these injuries when together. In addition, *Mhyo* expressed inhibitory behavior towards *Mfloc*, suggesting that one can compete with the other's presence.

Key words: Pneumonia; respiratory diseases; Mollicutes; enzootic pneumonia; prevalence.

LISTA DE ABREVIATURAS

%: Percentage

°C – degrees Celsius

Cq – Cycles quantity

DNA- Deoxyribonucleic acid

GBLOCK - Gene Fragments Available

LMCP – Lesões macroscópicas de consolidação pulmonar

mL – mililiter

MLCL – macroscopic lung consolidation lesions

Mhyo – Mycoplasma hyopneumoniae

Mhr – Mycoplasma hyorhinis

Mfloc – Mycoplasma flocculare

PEP – Porcine enzootic pneumonia

PES – Pneumonia Enzoótica Suína

pH – hydrogen potential

PRDC – Complexo de Doenças Respiratórias dos Suínos/ Porcine Respiratory diseases complex

qPCR – Reação em Cadeia pela polimerase quantitativa/ quantitative polymerase chain reaction

SQ - starting quantity

µL- microliter

USA – United States of America

LISTA DE GRÁFICOS E FIGURAS

Page

CAPÍTULO I – CONSIDERAÇÕES GERAIS

1. INTRODUÇÃO

Mycoplasma hyopneumoniae (Mhyo) é o principal micro-organismo do Complexo de Doenças Respiratórias de Suínos (PRDC), um distúrbio multifatorial envolvendo uma combinação de agentes infecciosos (Thacker, et al. 2006). E, nessas condições, a infecção primária por *Mhyo* prejudica a imunidade do hospedeiro, predispondo-o a infecções secundárias que agravam sua saúde (Kobisch and Friis, 1996). *Mhyo* é também o agente etiológico primário da pneumonia enzoótica suína (PES), uma enfermidade infecciosa frequentemente observada no mundo todo, em suínos de fase final de terminação (Pieters e Maes, 2019).

A PES caracteriza-se por levar a um quadro de broncopneumonia catarral, com lesões macroscópicas de consolidação pulmonar crânio-ventral (LMCP) (Thacker, 2006), as quais estão associadas à perdas econômicas significativas, principalmente devido à redução do ganho de peso médio diário e da eficiência alimentar dos animais (Pointon et al., 1985; Maes et al., 1996; Thacker e Minion, 2012) além da exigência de tratamento com antibióticos, afim de amenizar os prejuízos financeiros (Maes et al., 1996; Kyriakis et al., 2001).

A literatura traz poucos dados epidemiológicos sobre as diferentes espécies de micoplasma. Registros como estes foram reportados em alguns países como a França, a Espanha e a Tailândia, conforme descrito por Fourour et al. (2018), Assunção et al. (2005) e Makhanon et al. (2012), respectivamente. *Mycoplasma hyorhinis (Mhr)* e *Mycoplasma flocculare (Mfloc)* pertencem ao mesmo nicho ecológico que *Mhyo* (Siqueira et al. 2016). Uma vez que múltiplas infecções podem ser

particularmente graves (Fourour, et al. 2018), é importante entender de que maneira a presença concomitante destes micro-organismos podem impactar na extensão das LMCP dos suínos. Na suinocultura brasileira, ainda temos a mistura de lotes nas fases de creche e terminação, um dos fatores mais simples e de grande contribuição para a desestabilidade da saúde destes animais (Kummer, 2009).

Devido ao exposto sobre a importância das LMCP para o desempenho produtivo de suínos criados em sistemas intensivos, e à falta de informações literárias sobre a coinfecção por micoplasmas nessas lesões, este estudo teve como objetivo correlacionar as ocorrências de *Mhyo, Mhr* e *Mfloc,* com a extensão de LMCP em suínos ao abate.

2. REVISÃO DE LITERATURA

Os micoplasmas são as menores bactérias conhecidas com capacidade de auto-replicação. Eles pertencem à classe *Mollicutes* e acometem diversas espécies de animais, incluindo os suínos (ROTTEM et al., 2003). São estritamente dependentes do hospedeiro, têm um genoma pequeno e dificilmente crescem em meio de cultivo (BORDIN, 2012).

A PES, que possui como agente primário *Mhyo*, desempenha um papel primordial no PRDC, uma das principais causas de prejuízo para os produtores da espécie suína. Os prejuízos causados pela PES estão mais relacionados ao aumento dos custos com o tratamento, à queda no ganho de peso diário e, consequentemente, ao menor preço de mercado das carcaças (SIBILA et al., 2007). Gillespie (2013) apontou para um prejuízo, decorrente da entrada da enfermidade em um rebanho livre, de U$ 7,92 por animal, que pode chegar a U$ 10,12 quando associado a outros patógenos respiratórios como o vírus da influenza suína (SIV) (HADEN et al., 2012). Por outro lado, a erradicação do agente em um rebanho gera benefícios em torno de

U$837,375 por ano, e aproximadamente U$ 7,00 a mais por animal abatido (SILVA et al., 2019).

Outros micoplasmas de relevância encontrados em suínos incluem – *Mhr* que causa principalmente polisserosite e artrite; *Mhs,* que também leva à artrite em animais de terminação e crescimento, e *Mycoplasma suis,* que leva à anemia. Outros micoplasmas, incluindo *Mfloc*, *Mycoplasma sualvi, Mycoplasma hyopharyngis* e várias espécies de *Acholeplasma* podem ser isolados de suínos, mas não demonstram ser patogênicos. (TACKER & MINION, 2019).

Estão etiologicamente associados os agentes *Mhyo, Mhr* e *Mfloc* (SIQUEIRA et al., 2016). *Mfloc* foi considerado um patógeno associado ao PRDC, potencialmente oportunista no caso de coinfecção com *Mhyo* (CALCUTT et al., 2015), e seu genoma está intimamente relacionado a *Mhyo*, sendo que ambos compartilham vários fatores de virulência; no entanto, *Mfloc* é considerado um agente comensal do trato respiratório de suínos (PAES, et al., 2017). *Mhr* pode frequentemente estar presente em coinfecções respiratórias com patógenos virais, como também pode ser encontrado de forma comensal na cavidade nasal e no trato respiratório dos suínos (BUMGARDNER, et al., 2018). Dados sobre a prevalência de *Mfloc* e *Mhr* em trabalhos brasileiros são escassos.

Na indústria suína dos EUA, *Mhr* possui grande relevância no que diz respeito a problemas com artrite. Os impactos econômicos causados por este patógeno no país crescem anualmente, destacando-se o aumento nos custos com médicos veterinários, a diminuição da produtividade associada à redução do ganho de peso diário, e elevação da mortalidade (BUMGARDNER, 2018). A alta taxa de morbidade da PES resulta na alta prevalência que a enfermidade apresenta a nível mundial (MAROIS et al., 2007).

A elevada ocorrência de lesões pulmonares sugestivas de *Mhyo* em suínos abatidos no Brasil é de grande relevância. Baraldi, et al. (2019) afirmaram terem encontrado 72,4% de 908 pulmões de suínos de granjas da região Sudeste do Brasil, com LMCP. Galdeano et al. (2019) revelaram que 665 de 900 (73,9%) pulmões avaliados em um frigorífico que abate suínos do estado de Goiás, apresentaram LMCP semelhantes às de *Mhyo*.

No estado de Santa Catarina (Brasil), um dos principais produtores de suínos, estudo de Carrijo et al., (2012) revelou uma prevalência de 64,4% de animais positivos para *Mhyo*, enquanto que Tamiozzo et al., (2011) detectaram em três granjas de uma grande empresa, localizada em região produtora de suínos, aproximadamente 76% de lesão pulmonar sugestivas de PES nos animais abatidos. Já em outra pesquisa, realizada no estado de São Paulo, foram diagnosticadas 52% de amostras positivas coletadas em um frigorífico da região centro-oeste do estado (VICENTE et al., 2013).

Para controlar as infecções por *Mhyo*, vacinas comerciais inativadas são comumente utilizadas no mundo todo. Contudo, apesar de a vacinação permitir a redução de lesões pulmonares e aumentar o desempenho zootécnico das granjas, ela não impede a disseminação e a colonização do trato respiratório de suínos pelo micro-organismo. Estima-se que aproximadamente 70% do rebanho industrial de suínos em todo o mundo seja vacinado contra *Mhyo*. Essa porcentagem está aumentando nos países em que a produção de suínos vem sendo substituída por rebanhos comerciais intensivos (MARTELLO et al., 2014).

Mhyo adere-se ao epitélio respiratório ciliado e causa inicialmente ciliostase, destruição dos cílios e possivelmente morte celular epitelial. O processo de aderência, que é um pré-requisito para o início da PES, é um processo multifatorial e complexo (MEYNS et al., 2004). Exceto em casos graves, a doença é caracterizada apenas pelo aparecimento de tosse seca e não produtiva, e redução no ganho diário de peso (RODRÍGUEZ et al., 2016). Os animais infectados por *Mhyo* permanecem excretando o agente por longos períodos, segundo a literatura: 119 dias (FANO et al., 2005) e 214 (PIETERS et al., 2009).

A PES crônica é a forma progressiva do PRDC e envolve outros agentes deste complexo, levando a sintomas clínicos mais graves (febre, dispnéia) e lesões pulmonares extensas. É frequentemente difícil determinar a prevalência exata de pneumonia por *Mhyo,* uma vez que a presença de coinfecções com outros patógenos respiratórios, incluindo *Pasteurella multocida, PRRSV, vírus da influenza suína (SIV) e PCV2* podem dificultar a obtenção de diagnóstico preciso (THACKER & MINION, 2012).

A infecção por *Mhyo* pode ocorrer logo após o nascimento, porém o quadro mais grave da doença se apresenta principalmente durante a fase de terminação,

momento em que as lesões pulmonares são mais evidentes (SIBILA et al., 2009). Essas lesões são caracterizadas por áreas de consolidação pulmonar, mais comumente em lobos cardíacos (sendo o direito mais frequentemente acometido), apicais, intermediário e partes mais craniais dos lobos diafragmáticos; em geral, essas lesões regridem em torno de 12 ou 14 semanas após infecção deixando apenas uma cicatriz (PIETERS & MAES et al., 2019).

As lesões típicas de pneumonia são as mais frequentemente observadas em abatedouros de suínos, estimando-se uma prevalência mundial entre 19% e 79% (FABLET et al., 2012). Segundo Vicca et al., (2002), altas taxas de prevalência e incidência da enfermidade em um rebanho não necessariamente implicam na presença e intensidade das lesões pulmonares. No entanto, Almeida et al. (2020), por meio de um ensaio experimental, afirmou que LMCP tendem a regredir à medida que a infecção por *Mhyo* evolui no tempo.

Um estudo realizado por Pereira et al., (2017) sugeriu que a colonização e o aparecimento de lesões causadas por *Mhr* seja mais precoce do que aquelas causadas por *Mhyo*; a média de idade de animais positivos para *Mhr* neste experimento foi de 57,32 dias, e para *Mhyo* de 116,31 dias; no entanto, o trabalho referido não incluiu suínos de terminação para diagnosticar possíveis infecções com os micoplasmas nesta fase. De acordo com Clavijo et al., (2017) a prevalência de colonização por *Mhr* pode variar com a idade dos suínos e entre as granjas avaliadas.

A nível microscópico, as principais lesões associadas à presença de *Mhyo* no pulmão são broncopneumonia catarral caracterizada por grande infiltração de neutrófilos, linfócitos e macrófagos no lúmen e hiperplasia de BALTs (HILLEN et al., 2014). Outras alterações como o aumento da densidade de células produtoras de muco e espessamento do epitélio pulmonar também estão associadas à presença de *Mhyo* no sistema respiratório de suínos (RODRÍGUEZ et al., 2016).

Mfloc é descrito como um micro-organismo comensal da mucosa do trato respiratório do suíno (MARTELLO et al., 2014), e não possui capacidade patogênica aparente (FRIIS, 1974; STRASSER et al., 1992). Pode ser encontrado em pulmões ou nas cavidades nasais (THACKER & MINION, 2012). No entanto, *Mfloc* parece aderir ao epitélio celular ciliado sem danificá-lo, ao contrário de *Mhyo* (PAES et al., 2017).

Foi demonstrado que o *Mfloc* é capaz de induzir infiltrações linfocíticas nas cavidades nasais e no tecido peribrônquico (FRIIS, 1974). Esses achados foram posteriormente confirmados por Armstrong et al., (1987). Esta espécie é de importância notória para a indústria suína devido às semelhanças antigênicas com *Mhyo*, que podem complicar sua diferenciação tanto antigenicamente, após cultura e isolamento, quanto sorologicamente (BEREITER et al., 1990).

Mhr também pode ser frequentemente encontrado no trato respiratório de suínos saudáveis (BUMGARDNER, 2018), sendo que animais do período pós-desmame são mais suscetíveis (LIN, 2006). A maioria das infecções por *Mhr* são geralmente assintomáticas (GOMES NETO, et al., 2012), sendo que casos de pneumonia, artrite, polisserosite, e conjuntivite já foram descritos em conjunto com a infecção por este micro-organismo (TACKER & MINION, 2012).

3. REFERÊNCIAS

ALMEIDA, Henrique MS et al. Cytokine expression and *Mycoplasma hyopneumoniae* burden in the development of lung lesions in experimentally inoculated pigs. **Veterinary Microbiology**, p. 108647, 2020.

ARMSTRONG, C. H..; FREEMAN, M. J.; SANDS-FREEMAN, L. Cross-reactions between *Mycoplasma hyopneumoniae* and *Mycoplasma flocculare*--practical implications for the serodiagnosis of mycoplasmal pneumonia of swine. **Israel journal of medical sciences**, v. 23, n. 6, p. 654-656, 1987.

BARALDI, Thaís G. et al. Antibodies against *Actinobacillus pleuropneumoniae*, *Mycoplasma hyopneumoniae* and influenza virus and their relationships with risk factors, clinical signs and lung lesions in pig farms with one-site production systems in Brazil. **Preventive veterinary medicine**, v. 171, p. 104748, 2019.

BEREITER, M. et al. Evaluation of the ELISA, and comparison to the complement fixation test and radial immunodiffusion assay for detection of *Mycoplasma hyopneumoniae.* In: **Proceedings, International Pig Veterinary Society, 11th Congress, July 1-5, 1990, Lausanne, Switzerland.** Swiss Association of Swine Medicine, 1990.

BORDIN, L.C. **Detecção do *Mycoplasma suis* em granjas com transtornos reprodutivos**. Dissertação apresentada à Faculdade de Medicina Veterinária da Universidade do Estado de Santa Catarina – UDESC, como requisito para obtenção do título de Mestre em Ciência Animal. Lages, Santa Catarina, 2012.

ASSUNÇÃO, P. et al. The occurrence of mycoplasmas in the lungs of swine in Gran Canaria (Spain). **Veterinary research communications**, v. 29, n. 6, p. 453-462, 2005.

BUMGARDNER, E. A.; BEY, R. F.; LAWRENCE, P. K. A p37-based ELISA used to monitor anti–Mycoplasma hyorhinis IgG in serum from pigs immunized with inactivated M. hyorhinis vaccines. **Journal of Veterinary Diagnostic Investigation**, v. 30, n. 5, p. 755-759, 2018.

CALCUTT, M. J. et al. Complete genome sequence of *Mycoplasma flocculare* strain Ms42T (ATCC 27399T). **Genome announcements**, v. 3, n. 2, p. e00124-15, 2015.

CARRIJO, K. D. F. diagnóstico de *Mycoplasma hyopneumoniae* e circovírus suíno tipo 2 em tecidos pulmonar, renal e linfóide e de *leptospira spp*. em suínos abatidos sob inspeção sanitária. 2012.

CLAVIJO, M. J. et al. Infection dynamics of *Mycoplasma hyorhinis* in three commercial pig populations. **Veterinary Record**, p. vetrec-2016-104064, 2017.

FANO, E.; PIJOAN, Carlos; DEE, Scott. Dynamics and persistence of *Mycoplasma hyopneumoniae* infection in pigs. **Canadian Journal of Veterinary Research**, v. 69, n. 3, p. 223, 2005.

FABLET, C. et al. Infectious agents associated with respiratory diseases in 125 farrow-to-finish pig herds: a cross-sectional study. **Veterinary microbiology**, v. 157, n. 1-2, p. 152-163, 2012.

FOUROUR, S. et al. A new multiplex real-time TaqMan® PCR for quantification of *Mycoplasma hyopneumoniae, M. hyorhinis* and *M. flocculare*: exploratory epidemiological investigations to research mycoplasmal association in enzootic pneumonia-like lesions in slaughtered pigs. **Journal of applied microbiology**, 2018.

FRIIS, N. F. *Mycoplasma suipneumoniae* and *Mycoplasma flocculare* in comparative pathogenicity studies. **Acta Veterinaria Scandinavica (Denmark)**, 1974.

GALDEANO, José Vanderlei Burim et al. Cross-sectional study of seropositivity, lung lesions and associated risk factors of the main pathogens of Porcine Respiratory Diseases Complex (PRDC) in Goiás, Brazil. **Porcine health management**, v. 5, n. 1, p. 23, 2019.

GILLESPIE, Tom. Mycoplasma infection costs in a naïve population. In: **Allen D. Leman Swine Conference**. 2013. p. 51.

GOMES NETO, J.C.G. et al. Mycoplasma-associated arthritis: critical points for diagnosis. **Journal of Swine Health and Production**, v. 20, n. 2, p. 82-86, 2012.

HADEN, C. et al. Assessing production parameters and economic impact of swine influenza, PRRS and Mycoplasma hyopneumoniae on finishing pigs in a large

production system. **Denver: American Association of Swine Veterinarians**, p. 75-76, 2012.

HILLEN, Sonja et al. Occurrence and severity of lung lesions in slaughter pigs vaccinated against Mycoplasma hyopneumoniae with different strategies. **Preventive veterinary medicine**, v. 113, n. 4, p. 580-588, 2014.

KURAMAE-IZIOKA, E. E. et al. Morphological and molecular characterization of Colletotrichum spp. from citrus orchards affected by postbloom fruit drop in Brazil. **European Journal of Plant Pathology**, v. 103, n. 4, p. 323-329, 1997.

KOBISCH, M.; FRIIS, N. F. Swine mycoplasmoses. **Revue Scientifique et Technique-Office International des Epizooties**, v. 15, n. 4, p. 1569-1614, 1996.

LAURITSEN, K. T. et al. Absence of strictly age-related resistance to *Mycoplasma hyosynoviae* infection in 6-week-old pigs. **Veterinary microbiology**, v. 130, n. 3-4, p. 385-390, 2008.

LIN, J. H. et al. *Mycoplasma hyorhinis* in Taiwan: diagnosis and isolation of swine pneumonia pathogen. **Veterinary microbiology**, v. 115, n. 1-3, p. 111-116, 2006.

MAKHANON, M. et al. Comparison of detection procedures of *Mycoplasma hyopneumoniae, Mycoplasma hyosynoviae,* and *Mycoplasma hyorhinis* in lungs, tonsils, and synovial fluid of slaughtered pigs and their distributions in Thailand. **Tropical animal health and production**, v. 44, n. 2, p. 313-318, 2012.

MAROIS, Corinne et al. Isolation of Mycoplasma hyopneumoniae from different sampling sites in experimentally infected and contact SPF piglets. **Veterinary microbiology**, v. 120, n. 1-2, p. 96-104, 2007.

MARTELLO, C. et al. Orthologous surface proteins from *Mycoplasma hyopneumoniae* and *Mycoplasma flocculare*: in silico comparison and heterologous expression of differential extracellular domains. In: **BMC proceedings**. BioMed Central, p. P157, 2014.

MEYNS, T. et al. Quantification of the spread of *Mycoplasma hyopneumoniae* in nursery pigs using transmission experiments. **Preventive veterinary medicine**, v. 66, n. 1-4, p. 265-275, 2004.

PAES, J. A. et al. Secretomes of *Mycoplasma hyopneumoniae* and *Mycoplasma flocculare* reveal differences associated to pathogenesis. **Journal of proteomics**, v. 154, p. 69-77, 2017.

PEREIRA, C.E.R et al. *Mycoplasma hyorhinis* infection in early cases of mycoplasmal pneumonia in swine and evaluation of diagnostic assays. **Pesquisa Veterinária Brasileira**, v. 37, n. 10, p. 1057-1063, 2017.

PIETERS, M. et al. An assessment of the duration of *Mycoplasma hyopneumoniae* infection in an experimentally infected population of pigs. **Veterinary microbiology**, v. 134, n. 3-4, p. 261-266, 2009.

PIETERS, M. G.; MAES, D. Mycoplasmosis. **Diseases of Swine**, p. 863-883, 2019.

RODRÍGUEZ, F. et al. Relationship between expression of interleukin-5 and interleukin-13 by epithelial cells and bronchiolar changes in pigs infected with *Mycoplasma hyopneumoniae*. **Journal of comparative pathology**, v. 154, n. 2-3, p. 165-168, 2016.

ROTTEM, S. Interaction of mycoplasmas with host cells. **Physiological reviews**, v. 83, n. 2, p. 417-432, 2003.

SIBILA, M. et al. Exploratory field study on *Mycoplasma hyopneumoniae* infection in suckling pigs. **Veterinary microbiology**, v. 121, n. 3-4, p. 352-356, 2007.

SIBILA, M. et al. Current perspectives on the diagnosis and epidemiology of *Mycoplasma hyopneumoniae* infection. **The Veterinary Journal**, v. 181, n. 3, p. 221-231, 2009.

SILVA, Gustavo S. et al. Benefit-cost analysis to estimate the payback time and the economic value of two Mycoplasma hyopneumoniae elimination methods in breeding herds. **Preventive veterinary medicine**, v. 168, p. 95-102, 2019.

SIQUEIRA, F. M. et al. New insights on the biology of swine respiratory tract mycoplasmas from a comparative genome analysis. **BMC genomics**, v. 14, n. 1, p. 175, 2013.

SIQUEIRA, F. M. et al. Mycoplasma non-coding RNA: identification of small RNAs and targets. **BMC genomics**, v. 17, n. 8, p. 743, 2016.

STRASSER, M. et al. Immunological and pathological reactions in piglets experimentally infected with *Mycoplasma hyopneumoniae* and/or *Mycoplasma flocculare*. **Veterinary immunology and immunopathology**, v. 31, n. 1-2, p. 141-153, 1992.

TAMIOZZO, Pablo Jesús et al. Monitoramento da presença de *Mycoplasma hyopneumoniae* em granjas de suínos durante a implementação de programas de erradicação. **Ciência Rural**, v. 41, n. 4, p. 699-705, 2011.

THACKER, Eileen L. Mycoplasmal diseases. **Diseases of swine**, 2006.

THACKER, E. L., MINION, F.C. Mycoplasmosis. In: Zimmerman JJ, Karriker LA, Ramirez A, Schwartz KJ, Stevenson GW (eds). **Diseases of Swine**. 10th ed. pp. 779-797. Wiley-Blackwell, Ames, 2012.

WOOLLEY, Lauren K. et al. Evaluation of clinical, histological and immunological changes and qPCR detection of *Mycoplasma hyopneumoniae* in tissues during the early stages of mycoplasmal pneumonia in pigs after experimental challenge with two field isolates. **Veterinary microbiology**, v. 161, n. 1-2, p. 186-195, 2012.

VICCA, Jo et al. Patterns of *Mycoplasma hyopneumoniae* infections in Belgian farrow-to-finish pig herds with diverging disease-course. **Journal of Veterinary Medicine, Series B**, v. 49, n. 7, p. 349-353, 2002.

VICENTE, A. F. et al. Seropositivity for *Mycoplasma hyopneumoniae* in pigs at a slaughterhouse in the central region of São Paulo. **Arquivo Brasileiro de Medicina Veterinária e Zootecnia**, v. 65, n. 6, p. 1899-1903, 2013.

CAPÍTULO II – Co-infections by *Mycoplasma hyopneumoniae, Mycoplasma hyorhinis* and *Mycoplasma flocculare* in macroscopic lesions of lung consolidation of pigs at slaughter

Marcela Manduca Ferreira[1], Marina Lopes Mechler Dreibi[1], Karina Sonálio[1], Henrique Meiroz de Souza Almeida[1], Maria Eugênia Silveira Ferraz[1], Ana Paula Prudente Jacintho[1]

Dominiek Maes[2], Luís Guilherme de Oliveira[1]*

[1]São Paulo State University, School of Agricultural and Veterinarian Sciences, Jaboticabal, São Paulo, Brasil.

[2]Faculty of Veterinary Medicine – Ghent University- Ghent, Belgium

* Corresponding author: luis.guilherme@unesp.br

ABSTRACT

Infections with *Mycoplasma hyopneumoniae* (*Mhyo*) *Mycoplasma hyorhinis* (*Mhr*) and *Mycoplasma flocculare* (*Mfloc*) are common in swine. However, the degree of co-infections and the correlations between these mycoplasma co-infection and the severity of macroscopic lung consolidation lesions (MLCL) have not yet been explored in Brazil. This study aimed to quantify *Mhyo, Mhr,* and *Mfloc* in MLCL of slaughter pigs, and to assess correlations with the

degree of MLCL in slaughter pigs. To this end, five groups of lungs were made based on severity of lung lesions, and 80 lungs were collected for each group (400 lungs in total). The Mycoplasmas were quantified using a multiplex qPCR. Statistical differences and comparison between the groups were evaluated, respectively, by the Kruskal-Wallis test ($p<0.05$) and Dunn's test ($p<0.05$), and the correlation between the data was performed by Spearman's method ($p<0.05$). The results revealed that the extent of MLCL showed a positive correlation with the *Mhyo* estimate (rho=0.26; $p<0.05$), a negative correlation with the *Mfloc* estimate (rho= -0.15; $p<0.05$), and no significant correlation with the *Mhr* estimate (p=0, 12). The extension of MLCL showed a positive correlation with the co-infection by *Mfloc* and *Mhr* (rho=0.17; $p<0.05$), and no significant correlation with *Mhyo* and *Mhr* (p=0.87), and a negative correlation with *Mhyo* and *Mfloc* (rho= -0.28; $p<0.05$). This study allowed to infer that, regarding the extension of MLCL, *Mhr* and *Mfloc* did not present opportunistic activity in relation to primary infection by *Mhyo*, but revealed some potential aggravation of these lesions. In addition, *Mhyo* expressed inhibitory behavior towards *Mfloc*, suggesting that one can compete with the other's presence.

Keywords: porcine enzootic pneumonia, pigs, Mollicutes, respiratory diseases.

1. INTRODUCTION

Mycoplasma hyopneumoniae (*Mhyo*) is the main pathogen of the Porcine Respiratory Disease Complex (PRDC), a multifactorial disorder involving a combination of infectious agents (Thacker, et al. 2006). Primary *Mhyo* infection impairs the host's immunity, predisposing it to secondary infections that aggravate its health (Kobisch and Friis, 1996). *Mhyo* is also the primary etiologic agent of porcine enzootic pneumonia (PEP), an infectious disease frequently observed worldwide in end-stage pigs (Pieters and Maes, 2019).

PEP is characterized by catarrhal bronchopneumonia with macroscopic lesions of cranio-ventral lung consolidation (MLCL) (Thacker, 2006), associated with significant economic losses due to the reduction in average daily weight gain, feed efficiency (Pointon et al., 1985; Maes et al., 1996; Thacker and Minion, 2012), and treatment with antibiotics (Maes et al., 1996; Kyriakis et al., 2001).

The literature provides little epidemiological data on the occurrence of different species of mycoplasma in pigs. There are records reported in some countries. In France (Fourour, 2018), prevalence rates of 59.5%, 3.4% and 34.7% were detected for *Mhyo, Mycoplasma hyorhinis (Mhr)* and *Mycoplasma flocculare* (*Mfloc),* respectively. In Thailand, Makhanon et al. (2012) revealed 40.3%, 12.3% 64.6% prevalence for *Mhyo, M. hyosynoviae (Mhs)* and *Mhr,* respectively, and in Spain, Assunção et al. (2005) detected 33%, 37%, 24% and 15% prevalence for *Mhyo, Mhr, Mfloc* and *Mhs*, respectively.

Mfloc and *Mhr* are etiologically associated with *Mhyo* (Siqueira et al. 2016). *Mfloc* was considered a pathogen associated with PRDC, potentially opportunistic in the case of co-infection with *Mhyo* (Calcutt, et al. 2015). However, the others authors related that *Mfloc* is a commensal microorganism of the pig respiratory tract (Friis, 1974; Strasser, et al. 1992;

Martello, et al. 2014). *Mhr* infections are generally asymptomatic (Gomes Neto, et al. 2012), with cases of pneumonia, arthritis, polyserosite and conjunctivitis having already been collected together with infection by this microorganism (Tacker and Minion, 2012), its role in lung lesions is not clear, however, the current study may clarify this role. According to Bumgardner et al. (2018), *Mhr,* can often be present in respiratory co-infections with viral pathogens.

Since multiple infections can be particularly serious (Fourour, et al. 2018), it is essential to understand how the concomitant presence of these microorganisms can influence the extent of swine's MLCL. In Brazilian pig farming, mixingbatches of animals from different origins in the nursery and finishing phases are still performed, which is a risk factor for transmission of pathogens and destabilization of the health status of these animals (Kummer, 2009).

Given the importance of MLCL for the performance of swine in intensive pig production systems and the lack of literature information on the co-infection by mycoplasmas in these lesions, this study aimed to correlate the quantification of *Mhyo, Mhr,* and *Mfloc* with the extent of MLCL in pigs at slaughter.

2. MATERIAL AND METHODS

2.1 Experimental design and sample selection

The study has been submitted and approved approved by the Ethics Committee on Animal Experimentation of Agricultural and Veterinary Sciences (CEUA) under protocol #017285/18. The collection of the samples from slaughter pigs was carried out in a slaughterhouse located in the city of Guariba - SP, Brazil, where 23-25 week-old pigs were slaughtered from the Southeast and Midwest regions of Brazil.

The number of samples was determined using the EpiInfo® software (CDC, USA), in which an error of 5% was stipulated, an expected prevalence of 50% and a number of stratum equal to five, a sample N of 400 was obtained, aiming to estimate the prevalence of the three agents. To increase the representativeness of the study, collections were carried out at ten different moments, seeking a greater variety of origins.

Four hundred lungs were selected for the collection, to obtain five groups of 80 animals each. The group allocation was based on the methodology described by Piffer and Brito (1991), in which each of the lung lobes were individually classified, with scores 0 to 4 (Table 1; Fig. 1). Considering that the right cardiac lobe (RC) is the most frequently affected by MLCL, according to Pieters and Maes (2019), the division of the groups considered only the scores of the RC, which were the collected lobes for further PCR analyses. However, all lung lobes (apical, cardiac, diaphragmatic, and intermediate) were scored for calculating the percentage of total MLCL area by multiplying the lobe score by its relative weight, according to Piffer and Brito (1991).

The samples were stored in sterile plastic bags (Whirl Pack), immediately placed in a polystyrene box with ice and transported to the Swine Medicine Laboratory (Unesp/Jaboticabal-SP). In the laboratory, fragments of the interior of the lobes (around the bronchi) were collected in duplicate with sterile scalpel blades and forceps. Between each collection, the forceps were flamed and kept in boiling water, and the edges exchanged. These fragments were deposited in DNAses and RNAses-free plastic microtubes (Axygen, USA) and stored in a freezer at -20ºC until qPCR analysis. A third fragment, approximately 5 mm thick, was collected from the transition areas between unaffected lung tissue and tissue with MLCL, stored in a properly identified cassette. These samples were submerged in a 10% buffered

formalin solution (pH 7.0) in the approximate proportion of 10:1 tissue formalin, further transferred by alcohol 70°, for routine histopathological analysis.

Table 1. Lung lobes classification in different groups according to the macroscopic lung consolidation lesion score of 400 lung samples collected in a commercial swine slaughterhouse.

Groups (scores) n = 80	Extension of the lung consolidation lesion by lobe (% of the lung area) (Piffer e Brito. 1991)
0	0 (no lung consolidation)
1	1 - 25
2	26 - 50
3	51 - 75
4	76 - 100

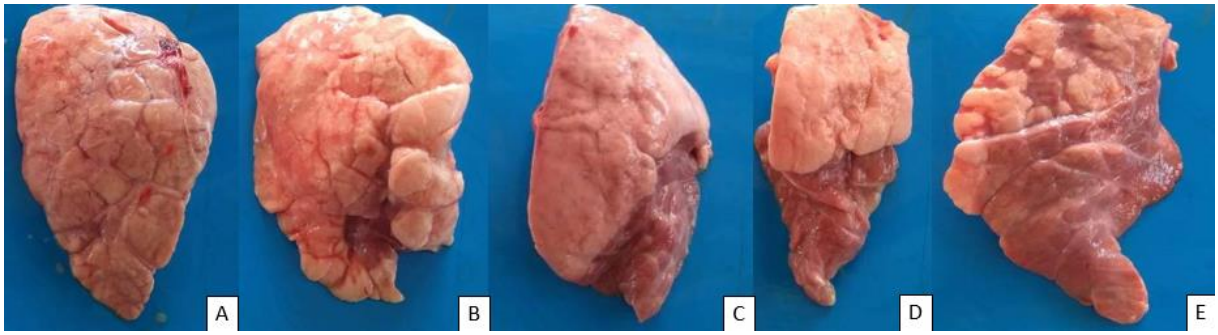

Fig 1. RC lobes collected in a commercial swine slaughterhouse with different degrees of MLCL. A - G0 (lobe with score 0 of lesion - without lesion); B - G1 (lobe with score 1 of lesion - 1 to 25% of lesion area); C - G2 (lobe with score 2 of lesion - 26 to 50% of lesion area); D - G3 (lobe with score 3 of lesion - 51 to 75% of lesion area); E - G4 (lobe with score 4 of lesion - 76 to 100% of lesion area).

2.2 Microscopic lesions score

After 24 hours in formalin solution, the fragments for histopathology were transferred to alcohol 70° and routinely processed for Hematoxylin and Eosin (HE) staining. The microscopic cuts were read under a light microscope, and microscopic tissue lesions were

classified into five different degrees according to the methodology adopted by Casalmiglia et al. (2000). Briefly, the score ranged from 0 to 4 with: 0 = no lesion; 1 = lesions of interstitial pneumonia and/or catarrhal bronchopneumonia; 2 = mild or moderate infiltration of neutrophils, macrophages and lymphocytes in the airways and alveoli; 3 = Perivascular or peribronchiolar lymphoplasmacytic hyperplasia, type II pneumocyte hyperplasia and the presence of edema in the alveoli and 4 = the same lesions of 3 plus the presence of perivascular and peribronchiolar lymphoid nodes. Degrees 1 and 2 are nonspecific, while 3 and 4 are already considered specific for PEP.

2.3 Multiplex qPCR

An in-house extraction of genetic material (DNA) was performed following a protocol previously described by Kuramae-Izioka, (1997). The presence of inhibitors and the concentration of DNA were measured by spectrophotometry in a NanoDrop 2000 device (Thermo Fisher Scientific®, Wilmington, Delaware, USA). The analysis of absolute quantification by qPCR was used to quantify the genetic material of *Mhyo, Mhr,* and *Mfloc* in the extracted lung fragments collected.

The multiplex qPCR targets were the *p102* gene (adhesin), the *p37* gene (membrane lipoprotein), and the *fruA* gene (a component of a fructose transporter), from *Mhyo, Mhr* and *Mfloc*, respectively. Primers and hydrolysis probes used were previously described by Fourour et al. (2018) (Table 2).

The amplification reaction used was based on the published protocol by Fourour et al. (2018) with modifications. The qPCR reaction was composed of 2 µL of the DNA-template, 0.3 µL of each hydrolysis probe, 0.05 µL of each initiator oligonucleotide, 5 µL of Master Mix Go taq® (Promega, Madison, USA) and 1.8 µL of sterile ultrapure water (Nuclease-Free

Water, Promega®, Madison, Wisconsin, USA) q.s.p, totalizing 10 µL. Amplifications were performed using a CFX96™ Real-Time PCR Detection System thermocycler (Bio-Rad®, Marnes-la-Coquette, France) under the following conditions: an initial 3-minute denaturation cycle at 95°C, followed by 39 cycles at 95°C for 15 seconds and annealing / extension at 55.7°C for one minute. All samples were tested in duplicate, and the results were only accepted for those with a standard deviation lower than or equal to 0.5 cycle (Bustin et al., 2009). Samples with a deviation greater than 0.5 were retested in triplicates. The curves of the fluorophores FAM (target p102), TXR (target p37), and CY5 (target *fruA*) were analyzed, and the results were visualized with Bio-rad CFX Manager Version 3.0 (Bio-rad, France).

Table 2. Sequences of the primers and hydrolysis probes used for each target region of the multiplex qPCR - *p102* gene (*Mhyo*). *P37* gene (*Mhr*). and *FruA* gene (*Mfloc*). and the respective amplifier sizes. adapted from Fourour et al. 2018.

Genes	N° of access on GenBank	Primers and probes	Nucleotide sequence (5' → 3')	Amplicon size (bp)
p102	[gb|AE017332.1|]	F_Mhp_*p102*	TAAGGGTCAAAGTCAAAGTC	
		R_Mhp_*p102*	AAATTAAAAGCTGTTCAAATGC	
		P_Mhp_*p102*	**FAMa**-AACCAGTTTCCACTTCATCGCC-**BHQ2d**	150
p37	[gb|CP003914.1|]	F_Mhr_*p37*	TTCTATTTTCATCTATATTTTCGC	
		R_Mhr_*p37*	TCATTGACCTTGACTAACTG	
		P_Mhr_*p37*	**TXRb**-CATCCTCTTGCTTGACTACTCCTG- **IB®RQ**	101
fruA	[gb|CP007585.1|]	F_Mfloc_*fruA*	TTAGCAGTTCCAATTTTATCAG	
		R_Mfloc_*fruA*	AAACCATAGGTATCTTTAAGTTG	
		P_Mfloc_*fruA*	**CY5c**-CAATTCCGCCAACTACAAATCCAG- **IB®RQ**	119

2.4 Testing for the presence of inhibitors in the extracted DNA

To check the presence of inhibitors in the extracted DNA samples and the occurrence of false negatives in the qPCR for micoplasmas, all samples were subjected to a conventional PCR for detecting the endogenous gene of Glyceraldehyde-3-Phosphate Dehydrogenase (*gapdh*), according to the protocol published Birkenheuer et al. (2003). The reaction was composed of: 0.5 µM of each of the initiators (Forward: 5'-CCTTCATTGACCTCAACTACAT-3 'and Reverse 5'-CCAAAGTTGTCATGGATGACC-3 ') (Invitrogen, USA), 1 U of Taq polymerase enzyme (Invitrogen, USA), 1X buffer solution (Invitrogen, USA), 1.5 mM MgCl2 (Invitrogen, 20 USA), 1mM dNTP mix (Invitrogen, USA), ultrapure water q.s.p. for 19 µL and 1 µL of DNA template, totaling 20 µL. The amplification reaction was performed in the following configurations: an initial denaturation cycle at 95 ° C, followed by 39 cycles of 95 ° C for 30 seconds, annealing at 50 ° C for 30 seconds and extension of 72 ° C for 1 minute and plus a final 5 minute extension in a MyCycler thermal cycler (Bio Rad, USA). In then the samples were subjected to electrophoresis on a 1% agarose gel (SigmaAldrich, USA) stained with 0.1% Ethidium Bromide (Sigma-Aldrich, USA). The gel was read on ChemiDoc MP transilluminator (Bio Rad, USA). The expected size of the amplified fragment was 437 bp. Only samples were used for the qPCR there was amplification of that gene. PCR negative samples for the gapdh gene were again subjected to DNA extraction.

2.5 Absolute quantification of fragments of the Mhyo p102, Mhr p37 and Mfloc fruA genes

Absolute quantification of fragments of the *Mhyo p102*, *Mhr p37*, and *Mfloc fruA* genes was performed using a standard curve with serial dilutions of constant ratio equal to 10, starting from 10^7 to 10^1. For this, synthetic DNA was used as a positive control (GBlock®, IDT, USA) containing fragments of 150, 101 and 119 bp, respectively, separated by a sequence of five

thymines, to be amplified by the pairs of primers shown in table 2. The synthetic DNA was diluted according to the manufacturer's guidelines and maintained in a stock concentration of 10^7 molecules / µL. Then, serial dilutions were performed using 45 µL of Tris-EDTA buffer (TE 24: 1) and 5 µL of solution at 10^7 µg of synthetic DNA. Quantification data were used only if the efficiency obtained was between 90% and 105% (Bustin et al., 2009). As a negative control, in qPCR reactions, sterile ultrapure water (Nuclease-Free Water, Promega®, Madison, Wisconsin, USA) was used q.s.p.

2.6 Data analysis and statistics

The following analyses were performed:

(1) examination by lobes - the MLCL scores in the RC lobes were correlated with the quantifications of each mycoplasmas;

(2) analysis of the whole lung - the percentages of total lung area with MLCL were correlated with the quantifications of each mycoplasmas; and

(3) histopathological examination - microscopy scores were correlated with MLCL scores in the RC lobes, with the percentages of total lung area of MLCL, and with the quantifications of mycoplasmas in the samples. The number of mycoplasma species present per sample was also correlated with the extent of MLCL in the RC lobes and the whole lung. Finally, the degree of MLCL of the RC lobes was correlated with the percentage of total lung area with MLCL in the samples. The normality of data for continuous variables was verified by the Shapiro-Wilks test ($p < 0.05$).

If there was a normal distribution of data, the difference between data groups was performed using Student t-test ($p < 0.05$), multiple comparisons using the Tukey test ($p < 0.05$), and correlations using Pearson's coefficient ($p < 0.05$). For non-parametric variables, significant

differences between groups were detected by the Kruskal-Wallis test ($p < 0.05$), the multiple comparisons between the sample means of the groups was performed by the Dunn test ($p < 0.05$), and associations between continuous variables and calculation of Spearman's coefficient ($p < 0.05$). The R software version 3.5.1 (R Core Team, 2018) was used for data analysis.

3. RESULTS

3.1 Prevalence of mycoplasmas in the different groups

The number of samples that tested positive for one of the three agents (*Mhyo*, *Mhr* or *Mfloc*), for two of the three agents (*Mhyo* and *Mhr* (i), *Mhyo* and *Mfloc* (ii), *Mhr* and *Mfloc* (iii)), and for the three agents (iv), in the different groups, are shown in Table 3.

Table 3. The number of positive lobe samples for *Mhyo*, *Mhr* and *Mfloc* and for co-infections by *Mhyo* and *Mhr* (i). *Mhyo* and *Mfloc* (ii). *Mhr* and *Mfloc* (iii). and by the three agents (iv). in the groups of scores of macroscopic lung consolidation lesion.

Group	*Mhyo*	*Mhr*	*Mfloc*	*(i)*	*(ii)*	*(iii)*	*(iv)*
G0 (n=80)	74	21	19	19	21	9	9
G1 (n=80)	75	18	15	17	12	5	3
G2 (n=80)	79	33	16	31	13	8	7
G3 (n=80)	79	28	9	29	9	7	7

G4 (n=80)	80	29	5	29	6	3	3

G0: RC lobe with score 0 - without lesion; G1: RC lobe with score 1 - 1 to 25% of lesion area; G2: RC lobe with score 2 - 26 to 50% of lesion area; G3: RC lobe with score 3 - 51 to 75% of lesion area; G4: lobe RC with score 4 - 76 to 100% of lesion area).

3.2 Correlations between mycoplasma quantifications and macroscopic and microscopic lesion scores

The qPCR results did not show normal distribution for any of the three pathogens. The Kruskall-wallis test revealed a significant difference between the groups for *Mhyo* ($p = 7.26x10-9$) and *Mfloc* ($p = 0.04$), but not for *Mhr* ($p = 0.12$).There was statistical difference ($p < 0.05$) between groups 0 and 1; 0 and 2; 0 and 3; 0 and 4; and 1 and 2 for *Mhyo*'s quantifications, but there was no difference between any groups for *Mhr* and *Mfloc*'s quantifications (Table 4).

Spearman's correlation showed a significant positive correlations between the quantification of *Mhyo* in the RC lobe and the gross and microsciopic lesions in that lobe, and the LMCL in the entire lung. The rho-values ranged between 0.2 and 0.3. There were no significant correlations for *Mhr* with the same variables. Significant negative correlations were found between the quantification of *Mfloc* in the RC lobe and the gross and microsciopic lesions in that lobe, and the LMCL in the entire lung. The rho-values ranged between -0.1 and -0.2.(Table 5).

In the co-infection scenario, there was a negative correlation between the quantification occurrence of *Mhyo* and *Mfloc* (rho = - 0.28); a positive correlation between the quantification of *Mhr* and *Mfloc* (rho = 0.17), and no significant correlation between the quantification of *Mhyo* and *Mhr* ($p = 0.87$).

Finally, a strong correlation was detected between the extent of MLCL in the RC lobes and the extent of MLCL in the total lung area of the samples ($p < 0.001$; rho = 0.88).

Table 4. Mean DNA quantifications ($\overline{x}$) of *Mhyo. Mhr.* and *Mfloc* in the groups of scores of MLCL - G0 (RC lobe with score 0 - without lesion); G1 (RC lobe with score 1 - 1 to 25% of lesion area); G2 (RC lobe with score 2 - 26 at 50% of lesion area); G3 (RC lobe with score 3 - 51 at 75% of lesion area); G4 (RC lobe with score 4 - 76 at 100% of lesion area).

Group	$\overline{x}$ ***Mhyo*** **(sd)**	$\overline{x}$ ***Mhr*** **(sd)**	$\overline{x}$ ***Mfloc*** **(sd)**
0	$3.3x10^4$ b (90.356)	$7.2x10^1$(433.03)	$3.2x10^1$ (115.44)
1	$4.2x10^4$a (135.838)	$7.3x10^1$ (384.11)	$5.9x10^2$(4.587.15)
2	$6.0x10^4$ c (105.630)	$1.1x10^2$ (528.02)	$1.2x10^2$ (782.90)
3	$5.1x10^4$ac (150.6090)	$1.0x10^3$(6.535.45)	$1.7x10^4$ (104.689)
4	$3.0x10^4$ac (46.210)	$4.9x10^2$(2.962.34)	$7.9x10^2$ (5.593)

[a, b, c] Within a column, different superscripts indicate a statistical difference between the groups

Table 5. Spearman correlations data (p and rho values) for: (1) analysis by lobes – the macroscopic lung consolidation lesion (MLCL) scores in the right cardiac lobes were correlated with the quantifications of mycoplasmas; (2) analysis of the whole lung - the percentages of total lung area with MLCL were correlated with the quantifications of mycoplasmas; and (3) histopathological examination - histopathology data were correlated with the quantifications of mycoplasmas in the samples.

	Quantification of		
Analysis	*Mhyo*	*Mhr*	*Mfloc*
By lobes	rho=0.26 p=1.24x10^{-07}	p=0.12	rho=-0.15 p=0.002
Whole lung	rho=0.27 p=6.58x10^{-08}	p=0.08	rho=-0.17 p=0.0009
Histopathological	rho=0.23 p= 1.26x10^{-05}	p=0.34	rho = -0.11 p=0.025

4. DISCUSSION

The findings of study revealed that the *Mhyo* estimate is significantly correlated with the extent of gross and histopathological lesions of the RC lobe and the gross lesions of the entire lung. On the other hand, *Mfloc* is negatively correlated with the same variables. As for co-infections, only *Mfloc* and *Mhr* exhibited apparent pathogenic capacity together, worsening

the MLCL. On the other hand, associated *Mhyo* and *Mfloc* showed a negative correlation with the extension of the MLCL.

In addition, the coefficients of all the correlations evaluated were moderate to low, which means that other factors may be involved in the severity of the lesions. A strong correlation was detected between the extent of MLCL in the RC lobes and in the total lung area, suggesting that the greater the lesion in the RC lobes, the greater the lesion in the whole lung.

The high prevalence of pathogens associated with the swine respiratory system in Brazil is a fact that deserves to be highlighted. Baraldi, et al. (2019) detected high seroprevalence by ELISA of *Mhyo* and Swine Influenza virus (IAV-sw) in slaughter pigs (> 40%), in farms in the Southeast region of Brazil, and 72.4% of 908 lungs evaluated presented MLCL. Similarly, Galdeano et al. (2019) revealed that at least half of the assessed animals from 27/30 and 23/30 pig farms in the state of Goiás were seropositive for *Mhyo* and SIV, respectively, in the finishing and slaughter phases. Also, out of 900 lungs evaluated in the slaughterhouse, 665 (73.9%) presented MLCL similar to those characteristics of *Mhyo* infection.

In the current study, only 2% (8/400) of the pig lungs evaluated were negative for any species of mycoplasma studied. A total of 7.3% (27/400) of the samples, with different degrees of MLCL, presented co-infection by the three species of mycoplasmas studied. Interestingly, G0 (from 0 to 5% of the lesion area) and lungs classified as totally lesion-free lung area were positive for the three agents in, respectively, 11.2% (9/80) and 15.2% (7/46) of the samples. This indicates that even if there is no apparent lesions in the lungs, the lungs of healthy pigs may be colonized by these pathogens.

It was noted that, with the increase in the extent of MLCL in the RC lobes and lungs, the *Mhyo* estimate increased, the *Mfloc* estimate decreased, and there was no correlation with the *Mhr* estimate. These data suggest that only *Mhyo* can worsen MLCL, and that *Mhr* and

Mfloc are apparently not *Mhyo*'s opportunistic agents. Almeida et al. (2020), in an experimental study, stated that the MLCL showed a regressive trend as *Mhyo* infection evolved.

Regarding the samples positive for *Mhyo*, 84% were negative for *Mfloc*, while only two samples positive for *Mfloc* were negative for *Mhyo*. These data can generate different interpretations and questions. Firstly, these two pathogens may compete for the same site of action in the host cell, since their genomes are closely related to each other and that both share several virulence factors (Paes, et al., 2017). Furthermore, Vranckx et al. (2012) suggested that pathogenesis and virulence may be related to the rate of multiplication of the infectious agent, and, consequently, to the amount of microorganisms colonizing the respiratory tract of the pig. Therefore, *Mhyo* being more pathogenic than *Mfloc* (Thacker and Minion, 2012), the infection of this pathogen would be more evident.

In addition, the literature carry some data that may justify the negative correlation found between *Mhyo* and *Mfloc*. According to Paes, et al. (2018), the genomic differences observed between *Mhyo* and *Mfloc* so far do not clearly explain their differential virulence and pathogenicity phenotypes. Previous comparative phylogenetic and phylogenomic studies have provided evidence of a close relationship between *Mhyo* and *Mfloc* (Vasconcelos, et al., 2005; Stemke, et al., 1992; Siqueira, et al., 2013) which share most virulence-related genes known to these bacteria (Vasconcelos, et al., 2005). There are, however, differences between *Mhyo* and *Mfloc*, such as the absence, in *Mfloc*, of the glpO gene, associated with the production of hydrogen peroxide by *Mhyo* and cytotoxic activity (Ferrarini, et al., 2018; Ferrarini et al., 2016). Also, there is the presence of differential domains between orthologs of the P97 family of adhesins and other surface proteins (Dos Anjos Leal, et al., 2016).

Besides, 90% of the surface proteins predicted for *Mfloc* are shared with *Mhyo* (Siqueira, et al., 2013), and some antibodies against *Mhyo* might cross-react with *Mfloc* antigens (Bereiter

et al., 1990; Freeman et al., 1984). These data allow us to infer that *Mfloc* may be affected by antibodies resulting from *Mhyo* infection due to the lower capacity of evading the immune system, and consequently be eliminated from the lesion site, a fact demonstrated by the inverse correlation found in this study between the population of *Mhyo* and *Mfloc*, however further studies are needed.

Although Luehrs et al. (2017) suggested that *Mhr* alone could not cause lung lesions similar to *Mhyo* infection in fattening pigs, the current study revealed that *Mhr* and *Mfloc* might be able to intensify the MLCL when present simultaneously, which could be explained by the antigenic difference of both agents (Thacker and Minion, 2012). *Mhr* and *Mfloc* did not demonstrate an aggravating potential for infection caused by the primary PEP agent (*Mhyo*), which indicates that these microorganisms, considered secondary to PEP for decades (Falk et al., 1991; Kawashima et al., 1996; Kobayashi et al., 1996; Lin et al., 2006), may play pathogenic activity without the presence of *Mhyo*. However, this is an observational study at one point in time at the end of the fattening period, so it is not known how infections evolved during the fattening period, and further studies are needed.

No evidence was found concerning the opportunistic action of *Mhr* and *Mfloc* in animals infected by *Mhyo* concerning the manifestation of MLCL in swine. In a contradictory way, Fourour et al. (2019), suggested that co-infection by *Mhr* and *Mfloc* in pigs previously infected with *Mhyo* would induce an inflammatory response more specifically to increased concentrations of haptoglobin, which could be responsible for the decrease in the animals' average daily weight gain. When comparing the present study with Fourour et al. (2018), it can be inferred that the intensity of the MLCL is not necessarily associated with the worsening of the clinical signs of the acute phase of PEP.

It is worth mentioning that the dynamics of co-infections of pathogens in the swine respiratory tract is much more complicated since it involves several microorganisms from other classes. As a result of ciliostase caused by *Mhyo*, there is an increase in the number of secondary bacteria that colonize the lungs (Zielinski and Ross, 1993). Palzer et al. (2015) observed a high correlation between *Mhyo* and the influenza virus (SIV) ($p < 0.001$), and other agents such as *Mhr*, PRRSV of the EU type, *Pasteurella multocida* and *Bordetella bronchiseptica.*

This research revealed 87%, 32.3%, and 16% of positive samples for *Mhyo, Mhr,* and *Mfloc*, respectively. These data differ from those found by Fourour et al. (2018), in which, in the same order, 59.5% 3.4% and 34.7% of 671 lungs collected in the slaughterhouse were positive. On the other hand, this study reinforced one of the findings by Fourour et al. (2018), about the negative correlation between *Mhyo* and *Mfloc.*

The results obtained in this work were relevant to alert pig producers and veterinarians about the existence of other mycoplasmas, besides *Mhyo*, in the confined swine herds. It is important to note that the diagnosis of PEP should not be restricted to the necropsy exam to avoid false negatives, as mycoplasmas may occur even when there is no apparent MLCL in the animals. However, further studies are needed to complement the findings of the present one. It is pertinent to consider that the qPCR technique also detects dead cells, so the SQ values of mycoplasmas may be overestimated; in addition, other agents that can also cause MLCL, such as *Pasteurela multocida* and *IAV-sw*, were not investigated in the study.

5. CONCLUSIONS

According to the results the greater the extent of MLCL, the greater the concentration of *Mhyo* and, interestingly, the lower that of *Mfloc* in the lungs of pigs. *Mhr*'s estimate, in turn, showed no association with the degree of MLCL. The concomitant performance of *Mfloc* and

Mhr exhibited apparent pathogenic capacity, aggravating the MLCL, unlike what was observed between *Mhyo* and *Mhr*. Finally, *Mhyo* and *Mfloc* associates showed a negative correlation with the extent of MLCL, given that it induces several interpretations, and therefore requires more research.

6. CONFLICT OF INTEREST

The authors declared that there was no conflict of interest in this work.

7. ACKNOWLEDGMENTS

The authors would like to acknowledge the slaughterhouse Frimel - Guariba/SP. This study was financed with Master's scholarship by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001.

8. REFERENCES

Almeida, H. M., Mechler-Dreibi, M. L., Sonálio, K., Ferraz, M. E. S., Storino, G. Y., Rezende, F. B.,de Oliveira, L. G. 2020. Cytokine expression and Mycoplasma hyopneumoniae burden in the development of lung lesions in experimentally inoculated pigs. Veterinary Microbiology, 108647. https://doi.org/10.1016/j.vetmic.2020.108647

Assunção, P., De la Fe, C., Kokotovic, B., Gonzalez, O., Poveda, J. B. 2005. The occurrence of mycoplasmas in the lungs of swine in Gran Canaria (Spain). Veterinary research communications, 29(6), 453-462. doi: 10.1007 / s11259-005-1866-3

Baraldi, T. G., Cruz, N. D. R. N., Pereira, D. A., Galdeano, J. V. B., Gatto, I. R., Silva, A. F. D., de Oliveira, L. G. 2019. Antibodies against *Actinobacillus pleuropneumoniae*, *Mycoplasma hyopneumoniae* and influenza virus and their relationships with risk factors, clinical signs and lung lesions in pig farms with one-site production systems in Brazil. Preventive veterinary medicine, 171, 104748. https://doi.org/10.1016/j.prevetmed.2019.104748

Bumgardner, E. A., Bey, R. F., Lawrence, P. K. 2018. A p37-based ELISA used to monitor anti–Mycoplasma hyorhinis IgG in serum from pigs immunized with inactivated *M. hyorhinis* vaccines. Journal of Veterinary Diagnostic Investigation, 30(5), 755-759.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The

MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. doi:10.1373/clinchem.2008.112797

Calsamiglia, M., Collins, J. E., Pijoan, C. 2000. Correlation between the presence of enzootic pneumonia lesions and detection of *Mycoplasma hyopneumoniae* in bronchial swabs by PCR. Veterinary microbiology, *76*(3), 299-303. doi: 10.1016/s0378-1135(00)00245-5

Calcutt, M. J., Foecking, M. F., Heidari, M. B., McIntosh, M. A. 2015. Complete genome sequence of *Mycoplasma flocculare* strain Ms42T (ATCC 27399T). Genome Announc., *3*(2), e00124-15.

Dos Anjos Leal, F. M., Virginio, V. G., Martello, C. L., Paes, J. A., Borges, T. J., Jaeger, N., Ferreira, H. B. 2016. *Mycoplasma hyopneumoniae* and *Mycoplasma flocculare* differential domains from orthologous surface proteins induce distinct cellular immune responses in mice. Veterinary microbiology, doi:10.1016 / j.vetmic.2016.05.008

Dos Santos, L. F., Sreevatsan, S., Torremorell, M., Moreira, M. A., Sibila, M., Pieters, M. 2015. Genotype distribution of *Mycoplasma hyopneumoniae* in swine herds from different geographical regions. Veterinary microbiology, 175(2-4), 374-381. doi:10.1016/ j.vetmic.2014.11.018

Falk, K., Lium, B. M. 1991. An abattoir survey of pneumonia and pleuritis in Slaughter weight swine from 9 selected herds. Acta. Vet. Scand, 32, 79-88.

Ferrarini, M. G., Mucha, S. G., Parrot, D., Meiffrein, G., Ruggiero, B. J. F., Comte, G., Sagot, M. F. 2018. Hydrogen peroxide production and myo-inositol metabolism as important traits for virulence of *Mycoplasma hyopneumoniae*. Molecular microbiology, *108*(6), 683-696. doi:10.1111 / mmi.13957.

Ferrarini, M. G., Siqueira, F. M., Mucha, S. G., Palama, T. L., Jobard, É., Elena-Herrmann, B., Sagot, M. F. 2016. Insights on the virulence of swine respiratory tract mycoplasmas through genome-scale metabolic modeling. *BMC genomics*, *17*(1), 353. doi:10.1186 / s12864-016-2644-z.

Fourour, S., Fablet, C., Tocqueville, V., Dorenlor, V., Eono, F., Eveno, E. Marois, C. 2018. A new multiplex real-time TaqMan® PCR for quantification of *Mycoplasma hyopneumoniae, M. hyorhinis* and *M. flocculare*: exploratory epidemiological investigations to research mycoplasmal association in enzootic pneumonia-like lesions in slaughtered pigs. Journal of applied microbiology, 125(2), 345-355. doi: 10.1111 / jam.13770

Fourour, S., Tocqueville, V., Paboeuf, F., Lediguerher, G., Morin, N., Kempf, I., Marois-Créhan, C. 2019. Pathogenicity study of *Mycoplasma hyorhinis* and *M. flocculare* in specific-pathogen-free pigs pre-infected with *M. hyopneumoniae*. Veterinary microbiology, 232, 50-57. doi:10.1016 / j.vetmic.2019.04.010

Galdeano, J. V. B., Baraldi, T. G., Ferraz, M. E. S., de Souza Almeida, H. M., Mechler-Dreibi, M. L., Costa, W. M. T., de Oliveira, L. G. 2019. Cross-sectional study of seropositivity, lung lesions and associated risk factors of the main pathogens of Porcine Respiratory Diseases Complex (PRDC) in Goiás, Brazil. Porcine health management, 5(1), 23. https://doi.org/10.1186/s40813-019-0130-0

Kawashima, K., Yamada, S., Kobayashi, H., Narita, M. 1996. Detection of porcine reproductive and respiratory syndrome virus and *Mycoplasma hyorhinis* antigens in pulmonary lesions of pigs suffering from respiratory distress. Journal of comparative pathology, 114(3), 315-323. doi: 10.1016 / s0021-9975 (96) 80053-3

Kobayashi, H., Morozumi, T., Munthali, G., Mitani, K., Ito, N.,Yamamoto, K. 1996. Macrolide susceptibility of *Mycoplasma hyorhinis* isolated from piglets. Antimicrobial agents and Chemotherapy, 40(4), 1030-1032. doi: 10.1292 / jvms.58.11_1107

Kobisch, M., Friis, N. F. 1996. Swine mycoplasmoses. Revue Scientifique et Technique-Office International des Epizooties, *15*(4), 1569-1614.

Kummer, R., Gonçalves, M. A. D., Lippke, R. T., Marques, B. M. F., Mores, T. J. 2009. Fatores que influenciam o desempenho dos leitões na fase de creche. Acta Scientiae Veterinariae, 37(1), 195-209.

Kuramae-Izioka, 1997. A rapid, easy and high yield protocol for total genomic DNA isolation from Colletotrichum gloeosporioides and Fusarium oxysporum for RAPD. Rev. Unimar.

Lin, J. H., Chen, S. P., Yeh, K. S., Weng, C. N. 2006. *Mycoplasma hyorhinis* in Taiwan: diagnosis and isolation of swine pneumonia pathogen. Veterinary microbiology, 115(1-3), 111-116. doi:10.1016 / j.vetmic.2006.02.004

Luehrs, A., Siegenthaler, S., Grützner, N., Grosse Beilage, E., Kuhnert, P., & Nathues, H. 2017. Occurrence of *Mycoplasma hyorhinis* infections in fattening pigs and association with clinical signs and pathological lesions of Enzootic Pneumonia. Veterinary microbiology, doi: 10.1016 / j.vetmic.2017.02.001.

Makhanon, M., Tummaruk, P., Thongkamkoon, P., Thanawongnuwech, R., Prapasarakul, N. 2012. Comparison of detection procedures of Mycoplasma hyopneumoniae, Mycoplasma hyosynoviae, and Mycoplasma hyorhinis in lungs, tonsils, and synovial fluid of slaughtered pigs and their distributions in Thailand. Tropical animal health and production, 44(2), 313-318. doi: 10.1007 / s11250-011-0022-z

Paes, J. A., Machado, L. D. P. N., dos Anjos Leal, F. M., De Moraes, S. N., Moura, H., Barr, J. R., Ferreira, H. B. 2018. Comparative proteomics of *two Mycoplasma hyopneumoniae* strains and *Mycoplasma flocculare* identified potential porcine enzootic pneumonia determinants. Virulence, doi: 10.1080 / 21505594.2018.1499379.

Maes, D., Verdonck, M., Deluyker, H., de Kruif, A. 1996. Enzootic pneumonia in pigs. Veterinary Quarterly, 18(3), 104-109.

Pieters, M. G., Maes, D. 2019. Mycoplasmosis. Diseases of Swine, 863-883.

Piffer, I. A., Brito, J. R. F. 1991. Descricao de um modelo para avaliacao e quantificacao de lesoes pulmonares de suinos e formulacao de um indice para classificacao de rebanhos. EMBRAPA-CNPSA. Documentos.

Pointon, A. M., Byrt, D., Heap, P. 1985. Effect of enzootic pneumonia of pigs on growth performance. Australian veterinary journal, 62(1), 13-18. doi: 10.1111/j.17510813.1985.tb06032.x

R Core Team, 2018. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.URL https://www.R-project.org/.

Siqueira, F. M., Thompson, C. E., Virginio, V. G., Gonchoroski, T., Reolon, L., Almeida, L. G., Ferreira, H. B. 2013. New insights on the biology of swine respiratory tract mycoplasmas from a comparative genome analysis. BMC genomics, 14(1), 175. doi:10.1186 / 1471-2164-14-175

Stemke, G.W., Laigret, F., Grau, O., & Bove, J.M. 1992. Phylogenetic relationships of three porcine mycoplasmas, *Mycoplasma hyopneumoniae*, *Mycoplasma flocculare*, and *Mycoplasma hyorhinis*, and complete 16S rRNA sequence of M. flocculare. International Journal of Systematic and Evolutionary Microbiology, 42(2), 220-225. doi:10.1099 / 00207713-42-2-220

Thacker, E.L., 2006. Mycoplasmal disease. In: Straw, B.E., Zimmermann, J.J., D'Allaire, S.,Taylor, D.J. (Eds.), Disease of swine. Iowa State University Press, Ames, pp. 701–717.

Thacker, E.L., Minion, C., 2012. Mycoplasmosis. In: Zimmerman, J.J., Karriker, L.A., Ramirez, A., Schwartz, K.J., Stevenson, G. (Eds.), Diseases of swine, 10th ed. Wiley Blackwell, UK, pp. 779–808.

Thacker, E. L., Halbur, P. G., Ross, R. F., Thanawongnuwech, R., Thacker, B. J. 1999. *Mycoplasma hyopneumoniae* potentiation of porcine reproductive and respiratory syndrome virus-induced pneumonia. Journal of clinical microbiology, 37(3), 620-627.

Vasconcelos, A. T. R., Ferreira, H. B., Bizarro, C. V., Bonatto, S. L., Carvalho, M. O., Pinto, P. M., Assunção, E. N. 2005. Swine and poultry pathogens: the complete genome sequences of two strains of *Mycoplasma hyopneumoniae* and a strain of *Mycoplasma synoviae*. Journal of bacteriology, 187(16), 5568-5577. doi: 10.1128 / jb.187.16.5568-5577.2005

Vranckx, K., Maes, D., Marchioro, S.B., Villarreal, I., Chiers, K., Pasmans, F., & Haesebrouck, F. 2012. A vacinação reduz a infiltração de macrófagos no tecido linfóide associado ao brônquio em porcos infectados com uma cepa de *Mycoplasma hyopneumoniae*

altamente virulenta. BMC pesquisa veterinária , 8 (1), 24. doi:10.1186 / 1746-6148-8-24.

Zielinski, G. C., Ross, R. F. 1993. Adherence of *Mycoplasma hyopneumoniae* to porcine ciliated respiratory tract cells. American journal of veterinary research, 54(8), 1262-1269.