UNIVERSIDADE ESTADUAL PAULISTA – UNESP CÂMPUS DE JABOTICABAL

OCORRÊNCIA E DIVERSIDADE GENÉTICA ASSOCIADA À INFECÇÃO POR MICOPLASMAS HEMOTRÓPICOS EM SUÍNOS DOMÉSTICOS NO BRASIL

Karina Sonálio

Médica Veterinária

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Jaboticabal, 20 de fevereiro de 2020

DADOS CURRICULARES DO AUTOR

KARINA SONALIO – Nascida em 5 de janeiro de 1993, na cidade de Arroio Trinta, Santa Catarina - Brasil, é médica veterinária formada pela Universidade Federal de Uberlândia (UFU) em 2018. Foi bolsista do Programa Ciência sem Fronteiras, tendo como instituição acolhedora a Iowa State University (EUA) no ano de 2014. Ainda, foi bolsista do Programa de Educação Tutorial Medicina Veterinária - UFU de 2015 a 2018, sob a orientação do Prof. Dr. Marcus Vinícius Coutinho Cossi. No ano de 2017 participou do Programa Supervisor Trainee na empresa BRF, na cidade de Uberlândia. Em 2018, ingressou no programa de Pós-Graduação em Medicina Veterinária, área de Clínica Médica Veterinária, pela UNESP - Câmpus de Jaboticabal, como bolsista CAPES, sob a orientação do Prof. Dr. Luis Guilherme de Oliveira e coorientação do Prof. Dr. Marcos Rogério André.

"Compreender constantemente. Trabalhar sempre. Descansar, quando se mostre necessária a pausa de refazimento. Parar nunca."

Francisco Cândido Xavier

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SUMÁRIO

	Página
CERTIFICADO DA COMISSÃO DE ÉTICA NO USO DE ANIMAIS	xii
RESUMO	xiii
ABSTRACT	xiv
LISTA DE TABELAS	xv
LISTA DE GRÁFICOS E FIGURAS	xvi
CAPÍTULO I – CONSIDERAÇÕES GERAIS	1
1 INTRODUÇÃO	1
2 REVISÃO DE LITERATURA	3
2.1 Etiologia da hemoplasmose em suínos	3
2.2. Ocorrência e aspectos epidemiológicos associados à doença	4
3 REFERÊNCIAS	8
CAPÍTULO II – MOLECULAR CARACTERIZATION AND GENETIC DIVERSITY ASSOCIATED WITH SWINE HEMOTROPHIC MYCOPLASMAS IN DOMESTIC PIGS IN BRAZIL ……..	11
ABSTRACT.....	11
1 INTRODUCTION	13
2 MATERIAL E METHODS	15
2.1 Study design.....	15
2.2 Epidemiologic data collection.....	16
2.3 Sampling and DNA extraction.....	16
2.4 Conventional PCR for the mammals-*gapdh* gene……..…….….	16
2.5 Porcine hemoplasmas-qPCR based on the 16S rRNA gene…….....	17
2.6 cPCR for mycoplasmas based on the 16S rRNA gene……..	18
2.7 cPCR for mycoplasmas based on the 23S rRNA gene……..…..	19
2.8 Cloning of 16S and 23S rRNA amplicons……	19
2.9 Amplification reaction of plasmid DNA fragments	20
2.10 Sequencing.....	21
2.11 Sequence analyses …….	21

2.12 Phylogenetic analysis..... 21
2.13 Statistical analyses..... 22
3 RESULTS 23
3.1 High occurrence of porcine hemoplasmas on qPCR ……..……. 23
3.2 Genetic diversity and coinfection of *M. parvum* genotypes in pigs …….. 26
3.3 Characterization of *M. parvum* ……..…….. 28
3.4 Impact of *M. parvum* on pig production……..……. 31
4 DISCUSSION..... 32
5 CONCLUSION..... 36
6 CONFLIT OF INTEREST..... 36
7 ACKNOWLEDGEMENTS..... 36
8 REFERENCES..... 36

CERTIFICADO COMISSÃO DE ÉTICA PARA EXPERIMENTAÇÃO ANIMAL

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de Jaboticabal

CERTIFICADO

Certificamos que o projeto de pesquisa intitulado **"Prevalência e fatores de risco associados à infecção por *Mycoplasma suis* em suínos de terminação"**, protocolo nº 005173/18, sob a responsabilidade do Prof. Dr. Luís Guilherme de Oliveira, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 08 de outubro de 2008, no decreto 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), da FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS, UNESP - CÂMPUS DE JABOTICABAL-SP, em reunião ordinária de 19 de abril de 2018.

Vigência do Projeto	03/05/2018 a 31/01/2020
Espécie / Linhagem	*Sus scrofa domesticus*
Nº de animais	450
Peso / Idade	130Kg / 150 dias
Sexo	Ambos os sexos
Origem	Granjas tecnificadas no estado de Goias

Jaboticabal, 19 de abril de 2018.

Fabiana Pilarski

Profª Drª Fabiana Pilarski
Coordenadora – CEUA

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OCORRÊNCIA DE MICOPLASMAS HEMOTRÓPICOS EM SUÍNOS DOMÉSTICOS NO BRASIL

RESUMO – *Mycoplasma suis* e *Mycoplasma parvum* se ligam fortemente aos eritrócitos e causam hemoplasmose em suínos. Os animais infectados podem ser portadores assintomáticos ou apresentar sinais inespecíficos, acometendo todas as faixas etárias. No Brasil, os estudos sobre a ocorrência e a diversidade genética associada aos hemoplasmas suínos (HS) são escassos. Portanto, este trabalho teve como objetivo detectar, quantificar e caracterizar a diversidade genética da HS em animais terminados de granjas tecnificadas no estado de Goiás, Brasil. Amostras de sangue total de 450 de 30 granjas diferentes localizadas no estado de Goiás, foram coletadas em tubos contendo ácido etilenodiaminotetracético (EDTA) e armazenadas a -80 °C. A extração de DNA seguiu o método “in house” descrito previamente. A PCR convencional (cPCR) direcionada ao gene endógeno *gapdh* foi realizada para evitar resultados falso-negativos nos testes seguintes. Os ensaios de PCR em tempo real quantitativa (qPCR) foram realizados a fim de detectar e quantificar DNA de Mycoplasma baseado no gene 16S rRNA para HS. Para avaliar a diversidade genética da HS, realizou-se a clonagem e sequenciamento dos genes 16S rRNA e 23S rRNA para hemoplasmas. Os resultados da qPCR mostraram uma ocorrência de HS de 68,89%, onde todas as fazendas tinham pelo menos dois animais positivos. A quantificação na qPCR variou de $8.43x10^{-1}$ a $4.69x10^{6}$ µL, e 52.71% das amostras apresentaram $1x10^{3}$ copies/µL. Além disso, o teste do coeficiente de Spearman revelou que a ocorrência de HS está inversamente associada ao número de partos por semana, leitões desmamados por semana e peso de abate. Já a análise filogenética utilizando os métodos de Máxima Verossimilhança e Bayesiana, demonstrou que os 22 clones obtidos a partir do fragmento 16S rRNA formaram um único cluster intimamente relacionado a *M. parvum*. Ainda, sete genótipos foram encontrados nas análises genéticas dessas 22 sequências, mesmo se tratando de um gene conservado. Análises filogenéticas por Máxima Verossimilhança e Bayesiana realizadas para os sete clones do fragmento 23S rRNA, resultaram em um clado associado a *M. parvum* (NR121958). Portanto, os resultados demonstram pela primeira vez a presença de *M. parvum* em suínos amostrados no estado de Goiás, e confirmam a existência de diferentes genótipos de *M. parvum* circulando entre granjas de suínos no Brasil.

Palavras – Chave: hemoplasmas suínos, *Mycoplasma parvum*, granjas tecnificadas, genótipos, suínos domésticos

OCCURRENCE OF HEMOTROPIC MICOPLASMAS IN DOMESTIC PIGS IN BRAZIL

ABSTRACT – *Mycoplasma suis* and *Mycoplasma parvum* are strongly bound to erythrocytes and cause hemoplasmosis in pigs. Infected animals can be asymptomatic carriers or have nonspecific signs, affecting all age groups. In Brazil, studies on the occurrence and genetic diversity associated with porcine hemoplasmas (PH) are scarce. Therefore, this work aimed to detect, quantify and characterize the genetic diversity of PH in finished animals from technified farms in the state of Goiás, Brazil. Whole blood samples from 450 animals from 30 different farms located in the state of Goiás, were collected in tubes containing ethylenediaminetetraacetic acid (EDTA) and stored at -80°C. DNA extraction followed the "in house" method previously described. Conventional PCR (cPCR) targeting the *gapdh* endogenous gene was performed to avoid false negative results in the following tests. Quantitative real-time PCR (qPCR) assays were performed in order to detect and quantify *Mycoplasma* DNA based on the 16S rRNA gene for PH. To assess the genetic diversity of PH, cloning and sequencing of the 16S rRNA and 23S rRNA genes for hemoplasmas was performed. The results of the qPCR showed a PH occurrence of 68.89%, where all farms had at least two positive animals. The quantification in the qPCR varied from $8.43x10^{-1}$ to $4.69x10^{6}$ µL, and 52.71% of the samples presented $1x10^{3}$ copies / µL. Furthermore, the Spearman coefficient test revealed that the occurrence of PH is inversely associated with the number of births per week, piglets weaned per week and slaughter weight. Phylogenetic analysis using the Maximum Likelihood and Bayesian methods, demonstrated that the 22 clones obtained from the 16S rRNA fragment formed a single cluster closely related to *M. parvum*. In addition, seven genotypes were found in the genetic analysis of these 22 sequences, even though it is a conserved gene. Phylogenetic analyzes by Maximum Likelihood and Bayesian performed for the seven clones of the 23S rRNA fragment, resulted in a clade associated with *M. parvum* (NR121958). Furthermore, the results demonstrate for the first time the presence of *M. parvum* in pigs sampled in the state of Goiás and confirm the existence of different genotypes of *M. parvum* circulating among pig farms in Brazil.

Keywords: porcine hemoplasmas, *Mycoplasma parvum*, technified farms, genotypes, domestic swine

LISTA DE TABELAS

Página

Tabela 1. Parameters for PH-qPCR assays 23

Tabela 2. Herd-level prevalence and range of qPCR results …… 25

Tabela 3. Genotype identification of *Mycoplasma parvum* 16S and 23S rRNA sequences from five different samples 27

LISTA DE GRÁFICOS E FIGURAS

Página

Figura 1. Location of the farms according to its geographical coordinates... 15

Figura 2. Box plot showing qPCR quantification cycle (Cq) results and distribution of starting quantity (SQ) 24

Figura 3. Genotypic network of previously deposited 16S rRNA PH sequences and the sequences obtained on this study, generated with TCS v.1.21 software. GEN #1 to GEN #7 represent the seven genotypes obtained from the 22 16S rRNA sequences ……... 28

Figura 4. Phylogenetic tree based on an alignment of ~800 bp fragment of *Mycoplasma* spp. 16S rRNA sequences, using ML method and the TIM1+I+G evolutionary model. Numbers at the nodes correspond to bootstrap. Accession numbers are indicated in the sequences. *Mycoplasma* spp. Sequences detected in the present study are highlighted in bold and colored in purple……. 29

Figure 5. Phylogenetic tree based on *Mycoplasma* spp. 23S rRNA sequences, using the Bayesian method and the TIM1+G evolutionary model. Numbers at the nodes correspond to bootstrap……... 30

Figura 6. Network analysis of *Mycoplasma* 16S rRNA sequences obtained from pigs sampled in the present study, compared to mycoplasma sequences retrieved from GenBank. The analysis was performed with Splitstree software using the parameters “Neighbor-Net and” Uncorrected p-distance”. *Mycoplasma* spp. sequences detected in the present study are colored in purple. *M. suis* sequences are

colored in blue, while *M. parvum* sequences are colored in yellow……..……. 31

CAPÍTULO 1 – CONSIDERAÇÕES GERAIS

1. INTRODUÇÃO

Micoplasmas hemotrópicos são parasitas de hemácias que acometem diversas espécies, incluindo os suínos. Dentre os hemoplasmas suínos, encontram-se *Mycoplasma suis*, *Mycoplasma parvum* e *Mycoplasma haemosuis*, descrito recentemente. São micro-organismos pequenos, desprovidos de parede celular e que ainda não foram cultivados "in vitro*". M. suis* possui grande tropismo pelas hemácias ocasionando quadros de anemia infecciosa, enquanto *M. parvum* e *M. haemosuis* são menos estudados e parecem não ter ação patogênica para os suínos.

Na infecção por *M. suis,* o agente pode aderir à superfície das hemácias ou invadi-las, fazendo com que o sistema imune do animal desencadeie o processo de apoptose dos glóbulos vermelhos, resultando em quadros de anemia hemolítica severa, além de danos vasculares e imunossupressão. Ainda, a doença pode apresentar-se de forma aguda ou crônica, sendo a última a mais comum. A fase aguda ocorre quando há intensa bacteremia, de forma que os animais podem apresentar anemia severa, febre, icterícia, enterite, necrose gangrenosa de orelhas, hipoglicemia, e até morte. Já a fase crônica costuma estar associada a baixa bacteremia e à redução do desempenho produtivo e reprodutivo dos suínos. Além disso, *M. suis* pode predispor os animais a infecções secundárias, permitindo que outros agentes os infectem e causem doença, o que pode desencadear um grande impacto econômico na suinocultura. Por outro lado, a infecção por *M. parvum* foi descrita como não-patogênica para suínos, mesmo em quadros de intensa bacteremia. Apesar de ser geneticamente parecido com *M. suis*, *M. parvum* infecta apenas alguns eritrócitos, e, portanto, não resulta em sinais clínicos.

Dentre os possíveis fatores associados à cronicidade da hemoplasmose suína, pode-se citar o uso de antimicrobianos em subdoses, prática comum na produção suinícola brasileira, que pode mascarar os sinais clínicos e perpetuar a infecção no rebanho, uma vez que o agente não é destruído e pode evadir-se à ação do sistema imune por mecanismos de resistência. Além disso, crescem as preocupações relacionadas ao mercado consumidor de carne suína que busca uma criação livre do

uso de antimicrobianos, prática que tende a desafiar o atual sistema produtivo brasileiro.

Ainda, a ocorrência de hemoplasmas em suínos tem sido reportada nos principais países produtores, incluindo no Brasil, onde *M. suis* e *M. parvum* já foram descritos. Apesar dos trabalhos realizados, dados sobre a ocorrência de hemoplasmas em suínos domésticos no Brasil ainda são escassos, bem como informações sobre variações genéticas desse agente. Portanto, o presente trabalho é importante para elucidar a hipótese de que a ocorrência de hemoplasmas em suínos terminados é alta e que existem diversidades genéticas entre o agente. Sendo assim, objetivou-se realizar a detecção e a quantificação de hemoplasmas suínos em animais de terminação pela técnica de qPCR visando o gene 16S rRNA, além de avaliar a ocorrência de diversidade genética com base nos genes 16S e 23S rRNA.

2. REVISÃO DE LITERATURA

2.1. Etiologia da hemoplasmose em suínos

Os micoplasmas hemotróficos (filo: Firmicutes; classe: Mollicutes), também conhecidos como hemoplasmas, são pequenas bactérias sem parede celular que apresentam tropismo pelos glóbulos vermelhos de diversos hospedeiros (do Nascimento et al., 2014). Até o momento, três espécies de hemoplasmas foram descritos em suínos: *Mycoplasma suis*, *Mycoplasma parvum* e *Mycoplasma haemosuis* (Kinsley, 1932; Splitter, 1950; Fu et al., 2017). Estes micro-organismo contam com a aquisição dos nutrientes presentes no sangue para sobreviver, fato que desempenha papel fundamental no desenvolvimento de um cultivo “in vitro” (do Nascimento et al., 2014).

Mycoplasma suis é uma bactéria que se liga fortemente aos eritrócitos dos suínos, e está ligado à ocorrência de anemia hemolítica em suínos mundialmente, cuja doença é responsável por perdas econômicas consideráveis na suinocultura (Ritzmann et al, 2009). Inicialmente, o agente foi nomeado como *Eperythrozoon suis* (Moulder, 1974), mas na década de 90, após o sequenciamento genético e inferências filogenéticas do gene 16S rRNA, verificou-se a proximidade da referida espécie com aquelas pertencentes ao gênero *Mycoplasma* (Neimark et al., 2001; Messick et al., 2002). Desde então, o agente foi reclassificado como *Mycoplasma suis* (*M. suis*) (Neimark et al., 2001).

Mycoplasma parvum foi relatado pela primeira vez em 1950 como uma bactéria não patogênica do suíno (Splitter, 1950). Ao contrário de *M. suis*, o *M. parvum* também se adere à superfície dos eritrócitos, entretanto, apenas algumas hemácias serão infectadas e eliminadas (do Nascimento et al., 2014). Segundo do Nascimento et al. (2014), micro-organismo nas formas de haste e cocóide são comuns na infecção por *M. parvum*, enquanto as formas de anel são frequentes em infecções por *M. suis*. A infecção por *M. parvum* pode ser caracterizada pela ausência de sinais clínicos, mesmo em infecções experimentais utilizando suínos esplenectomizados (do Nascimento et al., 2014). Por outro lado, a infecção por *M. suis* é caracterizada por febre e anemia hemolítica, que podem evoluir para a morte.

Estudos filogenéticos de *M. parvum* e *M. suis*, baseados em análises de sequência dos genes 16S rRNA e RNase P, sugerem que essas bactérias estão intimamente relacionadas (Watanabe et al., 2011). Alguns autores relataram que *M. parvum* pode ser um estágio imaturo de *M.* suis, encontrado simultaneamente com o estágio maduro (*M. suis*) (Dent et al., 2013). Ainda, a nível genômico, esses micro-organismo também são semelhantes, pois compartilham todas as sequencias de codificação conhecidas, bem como os mesmos genes relacionados aos fatores de virulência (do Nascimento et al., 2014).

2.2. Ocorrência e aspectos epidemiológicos associados à doença

Os micoplasmas são considerados os menores micro-organismo de vida livre capazes de auto-replicação. São estritamente dependentes do hospedeiro, têm um genoma pequeno e dificilmente crescem em meio de cultivo (Bordin, 2012). Apesar do *Mycoplasma suis* ser considerado espécie-específico, alguns pesquisadores chineses relataram a infecção desta bactéria em humanos, sugerindo a característica zoonótica do agente (Yuan et al., 2009).

A grande quantidade de casos subclínicos e de animais cronicamente infectados dificulta a obtenção de dados epidemiológicos. Além disso, os métodos de diagnóstico tradicionais para hemoparasitas, como exame de esfregaços sanguíneos corados pela acridinalaranja ou Giemsa têm demonstrado baixa sensibilidade e especificidade (Ritzmann et al, 2009). Atualmente, o método de eleição para o diagnóstico de hemoplasmas, é a técnica de PCR em tempo real quantitativa (qPCR) (Hoelzle et al, 2007; Guimarães et al., 2011). A infecção por *M. suis* tem sido relatada em vários países produtores de suínos como: Brasil, Estados Unidos, França Alemanha, China e Argentina. Segundo Guimarães et al. (2007), a prevalência de *M. suis* em suínos de terminação no sul do Brasil foi de 18,2%.

A transmissão experimental da hemoplasmose suína causada por *M. suis,* foi confirmada pelas vias intravenosa, oral, subcutânea e intraperitoneal, enquanto a infecção natural foi demonstrada pelo uso de material contaminado, como instrumentos cirúrgicos e agulhas (Henry, 1979). Ainda, ectoparasitas, como o piolho *Haematopinus suis*, a mosca dos estábulos (*Stomoxys calcitrans*) e o ácaro da sarna suína (*Sarcoptes scabiei* var. *suis*) também podem transmitir o agente, sendo

observado experimentalmente (Messick, 2004; Matos et al, 2007). Além disso, a alta densidade populacional na criação de suínos pode favorecer o contato entre os animais, com consequente transmissão do agente por meio de sangue e feridas, bem como pelo contato direto entre matriz infectada-leitão, ou ainda por transmissão vertical (Berrier e Gouge, 1954; Hoelzle et al., 2010; Stadler et al., 2019). Uma vez infectado, o período de incubação do agente, em condições naturais, é variável. Segundo Korn e Mussgay (1968), o período de incubação variou de 3 a 7 dias em infecções experimentais por via intravenosa.

Os mecanismos de patogenicidade desses hemoplasmas permanecem pouco conhecidos, pois a dificuldade de isolamento é um fator limitante (Hoelzle et al, 2014). Sabe-se que *M. suis* liga-se fortemente à superfície das hemácias através de ligações fibrilares, provocando deformações nessas células e consequente lise (Zachary et al., 1985). Groebel et al (2009), observaram em análise de microscopia eletrônica, baixa contagem de *M. suis* nas superfícies de hemácias, e alta contagem na qPCR, que sugere a capacidade do *M. suis* de invadir as hemácias. Este mecanismo garante a evasão do sistema imune, reduzindo a eficácia de antimicrobianos (Groebel et al., 2009), que pode justificar os quadros crônicos (Messick, 2004). Acredita-se ainda, que a alta frequência de casos subclínicos e crônicos ocorrem em razão do uso indiscriminado de antimicrobianos na produção de suínos (Messick, 2004).

A interação direta de adesão, invasão e captação de nutrientes entre micro-organismo e eritrócitos, associados à resposta do sistema imune, induzem à lise celular, caracterizando a anemia hemolítica autoimune (Hoelzle et al, 2014). Em manifestações agudas, os leitões desmamados e animais de terminação podem apresentar inapetência, febre, palidez na pele, dispneia, enterite, cianose e necrose gangrenosa de orelhas (Heinritzi, 1990). Na forma crônica, os sinais clínicos mais comuns são caracterizados por anemia, icterícia leve, nascidos com baixo peso, baixa eficiência reprodutiva de fêmeas e baixo desenvolvimento de animais de terminação (Henry, 1979; Hoelzle, 2014).

O diagnóstico pode ser realizado através de métodos diretos e indiretos. A reação de inibição da aglutinação pode gerar muitos resultados falsos negativos (Baljer et al., 1989). Já a técnica de esfregaços sanguíneos é barata e de fácil execução, porém possui baixa sensibilidade e especificidade (Hoelzle et al., 2007).

Zhang (2008) afirma que a imunofluorescência é 40% mais sensível que o exame microscópico, e considera o método de ELISA sensível e específico. Embora a literatura cite as técnicas de fixação de complemento, ELISA (Schuller et al., 1990; Zhang et al., 2008) e hemaglutinação indireta (Smith e Tamra, 1975), estas baseiam-se na detecção de anticorpos, que neste caso não são eficientes, pois a hemoplasmose pode ocorrer na forma intracelular, gerando títulos de anticorpos ondulantes durante a infecção (Schuller et al., 1990). Diante dessas dificuldades, pode-se perceber que a técnica de qPCR é o método mais sensível e específico utilizado atualmente (Guimarães et al., 2011). Essa técnica possui como características a padronização nos resultados, reprodutibilidade, automação e baixo risco de contaminação, gerando maior confiabilidade na obtenção dos resultados, diagnosticando também os portadores crônicos e assintomáticos (Guimarães et al., 2011).

Mundialmente observa-se que a prevalência da hemoplasmose suína varia de acordo com a técnica utilizada e com a região amostrada. Song et al. (2014), registraram 31,9% de prevalência no teste de ELISA, a partir de 4004 amostras de sangue de suínos na China. Em 2010, Hoelzle et al., ao avaliarem 359 javalis selvagens pela PCR convencional encontraram 10% de animais positivos *para M. suis*, enquanto na Argentina, 65,1% das amostras foram positivas, quando utilizadas as técnicas de PCR convencional e confirmação por Southern Blot (Pereyra et al., 2011).

Na Alemanha, Ritzmann et al. (2008) utilizaram a técnica quantitativa de real-time LightCycler PCR, e detectaram 13,9% de positividade ao avaliarem 1176 leitões. No Brasil, Kuchiishi et al. (2001) não encontraram animais positivos ao avaliarem 1400 esfregaços sanguíneos corados por Giemsa em amostras de sangue de suínos em um frigorífico em Santa Catarina. Guimarães et al. (2007) estudaram a ocorrência do referido agente em quatro rebanhos comerciais na região Sul, e a prevalência média foi de 18,2 %, utilizando a técnica de PCR. Bordin (2012) avaliou 80 amostras de sangue de fêmeas em 27 granjas de Santa Catarina e Paraná, encontrando 14 amostras positivas (17,5 %) de 11 diferentes rebanhos, utilizando o teste de qPCR. Toledo et al. (2016), ao analisarem amostras de granjas não-tecnificadas situadas no estado do Rio Grande do Norte, encontraram uma prevalência de 76,19% (112/147)

para hemoplasmas. A diferença entre os resultados encontrados no Brasil pode ser explicada pelos diferentes métodos diagnósticos utilizados, pelas categorias de animais amostrados, época do ano das colheitas, características dos rebanhos selecionados entre os estudos, dentre outros.

Frente a falta de informações relacionadas à infecção por hemoplasmas no Brasil, faz-se necessário conhecer outros dados relacionados à infecção, de forma que possamos atuar nestas lacunas para reduzir ou controlar a ocorrência da doença. De acordo com estudos realizados por Song et al. (2014), a prevalência de *M. suis* em fêmeas multíparas foi significativamente maior durante as estações de verão (65,3%) e outono (65%), resultado que pode estar correlacionado com a maior incidência de moscas e mosquitos, que por sua vez facilitam a transmissão. Da mesma forma, um trabalho realizado por Pereyra et al. (2010) na Argentina, demonstrou que idade, categoria produtiva, região geográfica e a procedência dos animais são fatores que favorecem a infecção por *M. suis*.

A infecção por *M. parvum* permanece pouco conhecida. Entretanto, o agente foi detectado recentemente na China, Brasil e Coréia. Fu et al. (2017), encontrou 42.6% de positividade para hemoplasmas (*M. suis* e *M. parvum*) em suínos de terminação. Estes autores descreveram ainda a ocorrência de um terceiro hemoplasma de suínos, o *M. haemosuis*. Além disso, Gatto et al. (2019) relatou 79.72% de prevalência de hemoplasmas em matrizes de descarte, e em análises filogenéticas, reportou pela primeira vez a ocorrência de *M. parvum* no Brasil. Da mesma forma, Seo et al. (2019) relatam a ocorrência desses três agentes em amostras de sangue de suínos na Coréia. Com base nessas pesquisas recentes, faz-se necessário conhecer a diversidade genética desses hemoplasmas, de forma que possamos atuar nas possíveis lacunas para reduzir ou controlar a ocorrência dos agentes em questão.

3. REFERÊNCIAS

Baljer G, Heinritzi K, Wieler L (1989) Indirect hemagglutination for *Eperythrozoon suis* detection in experimentally and spontaneously infected swine. **Journal of veterinary medicine** 36: 417–423

Berrier Jr HH, Gouge RE (1954) "Eperythrozoonosis transmitted in utero from carrier sows to their pigs." **Journal of the American Veterinary Medical Association** 98-100

Bordin LC (2012) **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.

Bustin S, Benes V, et al. (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. **Clinical. Chemistry** 55:611–622

Coetzee JF, Apley MD, Kocan KM (2006) Comparison of the efficacy of enrofloxacin, imidocarb, and oxytetracycline for clearance of persistent *Anaplasma marginale* infections in cattle. **Veterinary Therapeutics** 7:347–360

Groebel K, Hoelzle K, Wittenbrink MM, Ziegler U, Hoelzle LE (2009) *Mycoplasma suis* invades porcine erythrocytes. **Infection and immunity** 77(2):576-584

Guimarães AMS, Biondo AW, Lara AC, Messick JB (2007) Exploratory study of *Mycoplasma suis* (Eperythrozoon suis) on four commercial pig farms in southern Brazil. **The Veterinary Record** 160(2):50-53

Guimarães AMS, Vieira RFC, Poletto R, Vemulapalli R, Santos AP, De Moraes W, Cubas ZS, Santos LC, Marchant-Forde JN, Timenetsky J, Biondo AW, Messick JB (2011) A quantitative TaqMan PCR assay for the detection of *Mycoplasma suis*. **Journal of Applied Microbiology** 111:417-425

Henry SC (1979) Clinical observations on Eperythrozoonosis. **Journal of the American Veterinary Medical Association** 174:601-603

Heinritzi K, Plank G, Peteranderl W, Sandner N (1990) "Acid-base balance and carbohydrate metabolism in *Eperythrozoon suis* infected pigs." **Journal of Veterinary Medicine** 37:412-417

Hoelzle K, Engels M, Kramer MM, Wittenbrink MM, Dieckmann SM, Hoelzle LE (2010) Occurrence of *Mycoplasma suis* in wild boars (Sus scrofa L.). **Veterinary Microbiology** 43:405-409

Hoelzle K, Grimm J, Ritzmann M, Heinritzi K, Torgerson AH, Wittenbrink MM, Hoelzle LE (2007) Use of Recombinant Antigens to Detect Antibodies against *Mycoplasma suis*, with Correlation of Serological Results to Hematological Findings. **Clinical and Vaccine Immunology** 14:1616-1622

Hoelzle LE (2008) Review: Haemotrophic mycoplasmas: Recent advances in *Mycoplasma suis*. **Veterinary Microbiology** 130:215-226

Hoelzle LE, Helbling M, Hoelzle K, Ritzmann M, Heinritzi K, Wittenbrink MM (2007) First Light Cycler real-time PCR assay for the quantitative detection of *Mycoplasma suis* in clinical samples. **Journal of Microbiological Methods** 70:346-354

Hoelzle LE, Hoelzle K, Felder KM, Helbling M, Aupperle H, Schoon HA, Ritzmann M, Heinritzi K, Wittenbrink MM (2007b) MSG1, a surface localized protein of *Mycoplasma suis* is involved in the adhesion to erythrocites. **Microbes and infection** 9:466-474

Hoelzle LE, Zeder M, Felder KM, Hoelzle K (2014) Pathobiology of *Mycoplasma suis*. **The Veterinary Journal** 202:20-25

Matos MPC, Sobestiansky J, Ristow LE (2007) **Eperitrozoonose Suína**. In: Sobestiansky, J.; Barcellos, D. Doenças Dos Suínos. Goiânia: Cânone

Messick JB (2004) Hemotrophic mycoplasmas (hemoplasmas): a review and new insights into pathogenic potencial. **Veterinary Clinical Pathology** 33:2-13

Messick JB, Walker PG, Raphael W, Berent L, Shi X (2002) *'Candidatus mycoplasma haemodidelphidis*' sp. nov., *'Candidatus Mycoplasma haemolamae*' sp. nov. and *Mycoplasma haemocanis* comb. nov., haemotrophic parasites from a naturally infected opossum (*Didelphis virginiana*), alpaca *(Lama pacos*) and dog *(Canis familiaris*): phylogenetic and secondary structural relatedness of their 16S rRNA genes to other mycoplasmas. **International Journal of Systematic and Evolutionary Microbiology** 52:693-698

Neimark H, Johansson KE, Rikihisa Y, Tully JG (2001) Proposal to transfer some members of the genera Haemobartonella and Eperythrozoon to the genus Mycoplasma with descriptions of *'Candidatus Mycoplasma haemofelis'*, *'Candidatus Mycoplasma haemomuris*', *'Candidatus Mycoplasma haemosuis*' and *'Candidatus Mycoplasma wenyonii'*. **International Journal of Systematic and Evolutionary Microbiology** 51:891-899

Pereyra NB, Perez AM, Messick JB, Cane FD, Guglielmone AA (2011) "Estimación de la sensibilidad y especificidad de dos pruebas diagnósticas para la detección de *Mycoplasma suis* en Argentina utilizando un modelo bayesiano." **Archivos de medicina veterinaria** 43:117-125.

Ritzmann M, Grimm J, Heinritzi K, Hoelzle K, Hoelzle LE (2009) Prevalence of *Mycoplasma suis* in slaughter pigs, with correlation of PCR results to hematological findings. **Veterinary Microbiology** 133:84-91

Smith A, Tamra R (1975) An Indirect Hemagglutination Test for the Diagnosis of *Eperythrozoon suis* Infection in Swine. **American Journal of Veterinary Research**. 36:19-21

Song Q, Zhang W, Song W, et al (2014) Seroprevalence and risk factors of *Mycoplasma suis* infection in pig farms in central China. **Preventive veterinary medicine**. 117:215–221.

Toledo MA, Leite AI, Gonçalves LR, Sousa KC, Amaral RB, Silva GC, Machado RZ, André MR (2016) High occurrence of *Mycoplasma suis* infection in swine herds from non-technified farms in Mossoró, state of Rio Grande do Norte, Northeastern Brazil. **Revista Brasileira de Parasitologia Veterinária** 25:414-417

You Xiao-Xing, Yan-Hua Zeng, And Yi-Mou Wu (2006) "Interactions between mycoplasma lipid-associated membrane proteins and the host cells." **Journal of Zhejiang University-Science** 7:342-350

Yuan CL, Liang AiB, Yao CB, Yang ZB, Zhu JG, Cui Li, Yu Fei, Zhu NY, Yang XW,0 Hua XG (2009) Prevalence of *Mycoplasma suis* (*Eperythrozoon suis)* infection in swine and swine workers in Shangai, China. **American Journal of Veterinary Research** 70:890-894

Zachary JF, Smith AR (1985) "Experimental porcine eperythrozoonosis: T-lymphocyte suppression and misdirected immune responses." **American journal of veterinary research** 821- 830

[1]Capítulo II – MOLECULAR CARACTERIZATION AND GENETIC DIVERSITY ASSOCIATED WITH SWINE HEMOTROPHIC MYCOPLASMAS IN DOMESTIC PIGS IN BRAZIL

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Abstract

Mycoplasma suis and *Mycoplasma parvum* bind strongly to erythrocytes and can cause hemoplasmosis in swine, affecting several age groups. These animals may be asymptomatic carriers or show nonspecific signs. In Brazil, information on genetic diversity associated to porcine hemoplasmas (PH) has not been described yet. Therefore, this work aimed to detect, quantify and characterize the genetic diversity of porcine hemoplasmas in finishing pigs from technified farms in the state of Goiás, Brazil. Blood samples from 450 swine belonging to 30 different farms from Goiás, Brazil, were collected at the slaughterhouse. Quantitative real-time PCR (qPCR) assays were performed, for molecular detection and quantification of a 16S rRNA PH fragment. Cloning and sequencing of 16S and 23S rRNA amplicons were performed in order to evaluate the genetic diversity. Moreover, a questionnaire was applied to each farm manager to obtain epidemiological information about the herd. The results on qPCR showed herd prevalence of 68.89% for PH. Quantification values ranged from $8.43x10^{-1}$ to $4.69x10^{6}$ copies/µL, and 52.71% of the samples presented $1x10^{3}$ copies/µL. No risk factors were

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evaluated, once all farms had at least one positive animal. However, Spearman's coefficient test revealed that the occurrence of PH was inversely associated with number of farrows per week, weaned piglets per week and slaughter weight. Phylogenetic analysis based on Maximum likelihood and Bayesian methods, showed that the 16S rRNA and 23S rRNA sequences obtained from five samples formed a single cluster closely related to *M. parvum*. Genotype analysis using DNAsp software confirmed seven different genotypes from the 16S rRNA, and other four different were observed for the 23S cloned sequences, indicating that there are several genotypes of *M. parvum* circulating among pig farms in Brazil.

Keywords: porcine hemoplasmas, *Mycoplasma parvum*, molecular characterization, genotypes, intensive pig farming

1. Introduction

Hemotrophic mycoplasmas (HMs) are known for infecting several mammal's species, including wild and domestic pigs. Three hemoplasmas have been described in swine, namely *Mycoplasma suis* (Kinsley et al., 1932), *Mycoplasma parvum* (Splitter et al., 1950) and *M. haemosuis* (Fu et al., 2017). While *M. parvum* infects only a few red blood cells and, as a consequence, it is not associated with anemia in pigs (do Nascimento et al., 2014), *M. suis* is the main causative agent of Infection Anemia in Pigs (IAP), a re-emerging disease associated with economic losses worldwide (Hoelzle et al., 2008; Ritzmann et al, 2009). Even though HMs can infect pigs from different ages, they seem to be more prevalent in older animals, such as sows, boars and finishing pigs (Stadler et al., 2014; Gatto et al., 2019). Clinical signs vary according to the acute or chronic phases. Acute IAP caused by *M. suis* infection, is characterized by high bacteremia, fever, hypoglycemia and hemolytic anemia, while chronic disease varies from asymptomatic to decrease of feed intake and growth retardation (Hoelzle et al., 2008; Groebel et al., 2009). Therefore, chronic infected animals remain carriers and may become a source of infection for other pigs, making it harder to eliminate HMs from the herd (Hoelzle 2008).

Porcine hemoplasmas (PH) are very specialized agents and, so far, have never been successfully cultivated in vitro (Groebel et al., 2009; Schreiner et al., 2012). *M. suis* occurence was first recorded using microscopic examination of blood smears, which showed low sensitivity and specificity (Stadler et al., 2019). Nowadays, molecular techniques are commonly used to investigate the occurrence of hemothropic mycoplasmas worldwide, especially quantitative real-time PCR (qPCR), a very accurate test (Hoelzle et al., 2007; Guimarães et al., 2011). When using qPCR, *M. suis* prevalence in growing pigs ranged from 5% to 42.4% (Watanabe et al., 2012; Fu et al., 2017), while in sows it ranged from 19% to 80% (Hoelzle et

al., 2007; Guimarães et al., 2011), which means that this agent was more prevalent in older animals. Likewise, Toledo et al. (2016) found a 76.2% prevalence in growing pigs from non-technified farms, which raises the question about the situation of HMs occurrence in technified farms in Brazil, the fourth biggest pork producer in the world.

Mycoplasma suis can be transmitted experimentally by subcutaneous, intravenous, intraperitoneal, and oral inoculation (Henderson et al., 1997; Dietz et al., 2014). On the other hand, natural infections are mostly associated with mechanical transmission by contaminated needles and surgical instruments (Henry, 1979). Similarly, a research conducted by Stadler et al. (2019) indicates that *M. suis* could be vertically transmitted. It has also been proved that *M. suis* DNA was detected in the louse *Haematopinus suis*, which could play a role as a mechanical vector HMs (Acosta et al., 2019). Therefore, these findings increase the knowledge on HMs transmission and its maintenance on the herd.

At the genomic level, there are no reports on the diversity of hemoplasmas affecting pigs in Brazil. Even though *M. suis* and *M. parvum* have been detected in pigs from Brazil (Gatto et al., 2019), China (Fu et al., 2017) and Korea (Seo et al., 2019), little is known about *M. parvum* occurrence and its genetic diversity. Therefore, cloning and sequencing are important tools used to access the genetic diversity of fastidious agents, using DNA from field samples (Charlebois et al., 2014). The present study aimed to investigate the occurrence and genetic diversity of hemothropic mycoplasmas in domestic pigs from technified farms in Goiás, Brazil.

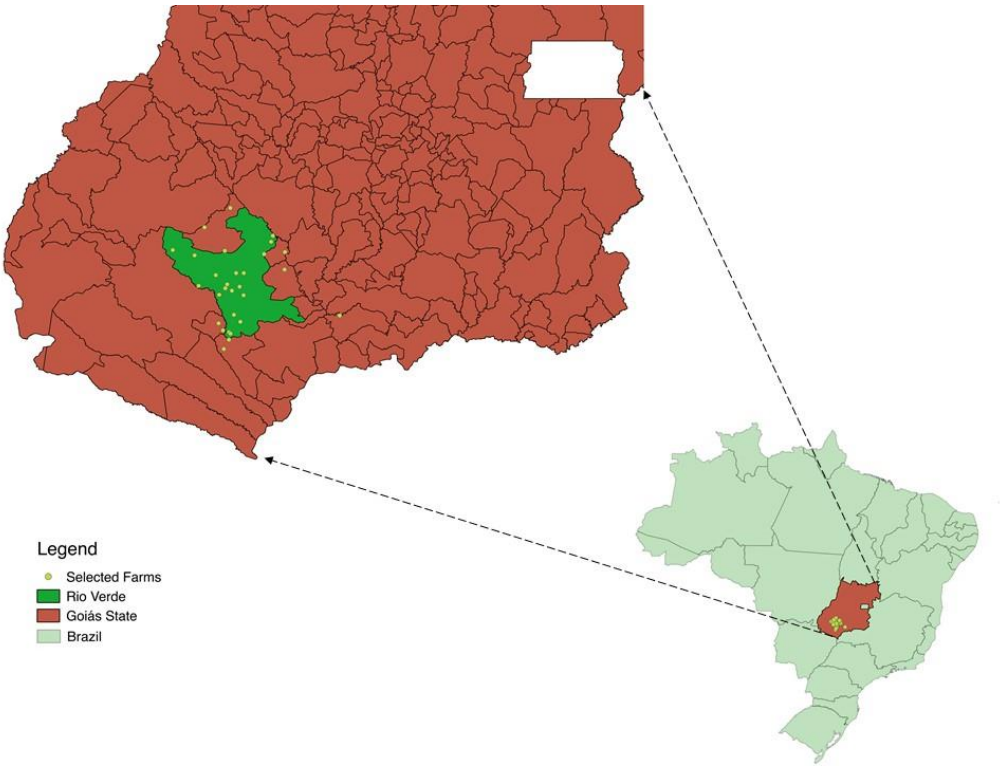
2. Materials e methods

2.1 Study design

This study was submitted and approved by the School of Agricultural and Veterinarian Sciences'Ethics Commission for Animal Experimentation (CEUA), under the protocol number 005173/18.

Sampling was conducted in 2017, when a total of 450 blood samples were collected from fattening pigs at the time of slaughter. The animals originated from 30 different technified farms in Goiás State, central-western Brazil (Figure 1), where 15 animals from each farm were sampled. The selected farms are multisite farrow-to-finish and have a herd size range from 400 to 4000 sows. Farms were selected by convenience according to the registry on Agência Goiana de Defesa Agropecuária – AGRODEFESA. The collection of blood was also approved by the slaughterhouse and the pig company.

Figure 1. Location of the farms according to its geographical coordinate.



2.2 Epidemiologic data collection

Prior to the slaughter, a visit was performed to the selected farms in order to fill out a structured questionnaire during an interview with farm owners. The questionnaire contained 38 questions regarding risk factors potentially related to the presence of porcine hemoplasma infection, such as: management regarding the production flow (continuous or all in-all out system), biosecurity, zootechnical indices, health status, and it was developed by our research group.

2.3 Sampling and DNA extraction

For all 450 pigs included in the trial, whole blood samples were taken from the jugular vein during slaughter and placed in suitable tubes containing ethylenediaminetetraacetic acid (EDTA). After collection, the samples were transferred to cryogenic microtubes, stored in liquid nitrogen and transported to the Immunoparasitology Laboratory at the Department of Veterinary Pathology of the School of Agricultural and Veterinarian Sciences at São Paulo State University (FCAV– UNESP – Jaboticabal) where they were stored at -80 °C until processing.

DNA extraction was performed from whole blood samples using the protocol previously described by Kuramae-Izioka (1997), with some modifications. Then, DNA concentration and quality of extraction (260/280 nm ratio) were measured using a Thermo Scientific NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific®, Wilmington, Delaware, USA).

2.4 Conventional (c)PCR for the mammals-gapdh gene

To check for the presence of inhibitors in the extracted DNA samples, and avoid the occurrence of false-negatives in the qPCR for PH, all DNA samples were subjected to a conventional PCR (cPCR) using the *gapdh*-F primers oligonucleotides

(5'CCTTCATTGACCTCAACTACAT-3') and *gapdh*-R (5'CCAAAGTTGTCATGGATGACC3'), which flanked a fragment of ~400 base pairs (bp) of the endogenous mammals *gapdh* gene. The cPCR technique followed the methodology proposed by Birkenheuer et al. (2003), with some modifications (Toledo et al., 2016).

2.5 Porcine hemoplasmas-qPCR based on the 16S rRNA *gene*

All 450 DNA samples showing positive results in the cPCR for the *gapdh* gene were subjected to qPCR assays for PH based on the 16S rRNA gene using the primers F (5'-CCCTGATTGTACTAATTGAATAAG-3') and R (5'- GCGAACACTTGTTAAGCAAG-3') and the TaqMan probe (5'FAM-TGRATACACAYTTCAGMGBNFQ3') (Thermo Fisher Scientific, Waltham, MA, USA) (Guimarães et al., 2011). All DNA samples were tested in triplicate. Porcine hemoplasmas-qPCR (PH-qPCR) assays were performed in a Thermal Cycler Model 96 CFX (BioRad®, Hercules, California, USA). The assay followed the protocol described by Guimarães et al. (2011) with some adaptations as described by Toledo et al. (2016). Amplification reaction was performed using a final volume of 10µL containing 1µL of DNA sample, 0.2 µM of each primer and probe, 5µL PCR buffer (GoTaq® Probe qPCR Master Mix, Madison, WI, USA) and sterilized ultrapure water (Nuclease-Free Water, Promega®, Madison, Wisconsin, USA) q.s.p 10 µL. Thermal conditions were 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds, 58.5°C for 45 seconds and 72°C for 30 seconds. Sensitivity of the assays were determined using 10-fold serial dilutions of pIDT SMART plasmids (Integrated DNA Technologies, Coralville, Iowa, USA) encoding the 16S rRNA *M. suis* sequence (insert containing 156 bp). In order to determine the assay limit of detection and quantification, standard curves were prepared by serial dilution, containing from 10^7 to 10^1 copy numbers of the plasmids. Furthermore, plasmids were used as positive controls and

ultrapure water were used as negative controls. All qPCR assays followed the standards on Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) (Bustin et al., 2009).

2.6 cPCR for mycoplasmas based on the 16S rRNA gene

In order to amplify the *Mycoplasma* spp. 16S rRNA gene fragments, qPCR-positive samples showing quantification values between 10^5 and 10^6 copies/µL were selected, as recommended by Gatto et al. (2019). The cPCR assays based on the 16S rRNA gene were performed using the primers HemMycop16S-41s (5′-GYATGCMTAAYACATGCAAGTCGARCG-3′) and HemMyco16S-938as (5′ – CTCCACCACTTGTTCAGGTCCCCGTC – 3′) (fragment I of ~800 bp) (Maggi et al., 2013). Amplification reaction was carried out using a final volume of 25 µL containing 10× PCR buffer, 1.0 mM MgCl2, 0.8 mM deoxynucleotide triphosphate (dNTP) mixture, 1.5 U Taq Platinum DNA Polymerase (Life TechnologiesTM, Carlsbad, California, USA), and 0.3 µM of forward and reverse primers. Thermal reaction was performed using 94 °C for 2 minutes followed by 55 repetitive cycles of 94 °C for 15 seconds, 68 °C for 15 seconds, and 72 °C for 18 seconds, and a final extension at 72 °C for 1 minute (Maggi et al., 2013). DNA from naturally infected pigs and ultra-pure water were used as positive and negative controls, respectively. All amplified samples were visualized in 1% agarose gel stained with ethidium bromide (Life Technologies™, Carlsbad, California, USA), and only amplicons showing high-intensity bands were cloned.

2.7 *cPCR for mycoplasmas based on the 23S rRNA gene*

To amplify *Mycoplasma* spp. 23S rRNA gene fragments, we selected the same samples used for the 16S rRNA cPCR. The assays were performed using the primers 23S_HAEMO_F (5'- TGAGGGAAAGAGCCCAGAC-3') and 23S_HAEMO_R (5-'-GGACAGAATTTACCTGACAAGG-3'), described by Vieira et al. (unpublished data). The amplification mixture contained 1x PCR buffer, 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.4 mM of each primer, 2.5 U of Taq Platinum DNA Polymerase (Life Technologies™, Carlsbad, California, USA), 5 µL of DNA template, and ultra-pure water q.s.p 25µL. Cycling conditions consisted of 3 min denaturation at 94 °C followed by 35 cycles of 94°C for 30 s, 54°C for 30 s and 72°C for 60 s, with a final extension of 72°C for 10 min (SimpliAmp® Thermal Cycler, Thermo Fisher Scientific, Waltham, USA). Positive and negative controls were used. Amplified samples were visualized in 1% agarose gel as described above.

2.8 Cloning of 16S and 23S rRNA amplicons

Cloning of 16S and 23S rRNA amplified products was performed with the pGEM-T Easy vector (Promega®, Madison, USA), following the manufacturer's recommendations. DNA and vector concentrations used in the binding reaction were determined in order to obtain an insert: vector ratio of 3:1. The DNA-binding reaction consisted of adding 40 ng of insert (DNA sample), 5 µL buffer, 1 µL of pGEM-T Easy vector, 1 µL of T4 Ligase enzyme and water sterile q.s.p. Solutions were kindly mixed and incubated at 16°C for 16 hours. Then, 50 µL of competent *E. coli* DH5α cells (10^9 - 10^{10} cfu/ng DNA) were added on the 10 µL binding reaction. The mix was kept in ice for 30 minutes, followed by thermal shock, where microtubes were placed into a water bath at 42ºC for 2 minutes. Subsequently, 250 µL of LB liquid medium (Tryptone; Yeast Extract; NaCl; distilled water q.s.p. (Invitrogen®, 9th Carlsbad, California,

USA) were added, and cells incubated at 37 °C for one and half hour, with agitation of 175 rpm. Then, 250 µL of this medium was added to Petri plates containing LB solid medium prepared with 100 µg/mL ampicillin, 40 µL X-gal (5-bromo-4-chloro-3-indolyl-β-Dgalactoside) (0.026%) and 20 µL IPTG (isopropylthio-β-galactoside) (0.82mM). Plates were incubated at 37°C for approximately 24 hours. Colonies of bacteria containing the clones (white colonies) were transferred to tubes containing 5 mL of LB liquid medium and 100 µg/mL ampicillin and incubated at 37ºC for 20 hours, followed by plasmid extraction, using the alkaline lysis method (Sambrook et al., 2006). Plasmid DNA samples were stored at -20°C.

2.9 Amplification reaction of plasmid DNA fragments

Plasmid DNA extracted from the clones was subjected to cPCR assay flanking the multiple cloning site (M13) of the pGEM T-easy plasmid, which includes the target gene inserts. For this purpose, the M13 forward (5'-GTAAAACGACGGCCAG-3 ') and M13 R (5'-CAGGAAACAGCTATGAC-3') primers described by Lau et al. (2010) were used. Amplification was performed using a total volume of 25 µL, containing 5 µL of the DNA sample and 20 µL reaction mixture composed of Taq buffer (PCR Buffer 10X-100nM Tris-HCl, pH 9.0, 500 mM KCl), 1.5 mM MgCl2 (Life Technologies®), 0.8 mM of dNTP mixture, 0.4 µM of each primer, 1.5 U Taq Platinum DNA Polymerase (Invitrogen®, Carlsbad, California, United States), and sterile ultrapure water. The reaction conditions were: initial denaturation at 95°C for 4 minutes, followed by 35 cycles of denaturation at 94°C for 40 seconds, annealing at 54°C for 40 seconds and extension at 72°C for 1.5 minutes, followed by a final extension step at 72°C for 10 minutes. Amplified samples were visualized in 1% agarose gel.

2.10 Sequencing

Amplified products were purified using the "Exosap IT" kit (Applied Biosystems, Cleveland, Ohio, USA) according to the manufacturer's recommendations. Quantification of total genomic material from the purified product was performed on NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific®, Wilmington, Delaware, USA). Sequencing of amplified products was performed by the dideoxynucleotide chain termination method (Sanger et al., 1977), conducted in ABI PRISM 3130 DNA Analyzer sequencer (Applied Biosystems).

2.11 Sequence analyses

The sequences obtained were first submitted to a screening test using Phred-Phrap software version 23 (Ewing et al., 1998; Ewing & Green, 1998). Then, BLAST program (Altschul et al., 1990) was used to analyze the sequences of the nucleotides (BLASTn) as well as to search for sequences with identity greater than 99%, previously deposited in GenBank (Benson et al., 2013). All BLASTn analysis were based on the program default settings.

2.12 Phylogenetic analyses

The DNA sequences originated from this study and those retrieved from GenBank were aligned using MAFFT software (Katoh et al., 2017). The Bayesian inference (BI) analysis was performed with MrBayes 3·1·2 (Ronquist & Huelsenbeck 2003) via CIPRES Science Gateway (Miller et al., 2010). The best evolutionary model was selected by the program jModelTest2 (version 2.1.6) on XSEDE (Darriba et al., 2012), under the Akaike Information Criterion (AIC) (Posada & Buckley, 2004). Maximum likelihood tree inference was performed with IQ-TREE software (http://iqtree.cibiv.univie.ac.at/) (Trifinopoulos et al., 2016). The tree was edited in TreeGraph 2.0 β (Stöver & Müller, 2010). Nucleotide sequence genealogies were analyzed

using Splitstree v4.11.3 software, based on the sequences from the present study and those previously deposited in GenBank. In order to infer the distance between genotypes and its occurrence in other areas, genotypic network using the software DNASp 5 and PopArt was completed (Clement et al., 2000).

2.13 Statistical analyses

To detect potential correlations between HMs prevalence and continuous variables, data normality was assessed using Sharpiro-Wilkins test ($p<0.05$). If the data presented normal distribution the Pearson correlation coefficient ($p<0.05$) was used to detect significant correlations, while for non-parametric data Spearman's rank coefficient test ($p<0.05$) was used. Therefore, mentioned analysis was performed using software R version 3.5.1 (R core team, 2018). Fisher's exact test ($p<0.05$) was used to detect association between positive herds and categorical variables investigated as risk factors. If a significant association was detected, the odds ratio (OR) and its 95% confidence interval were calculated using 2 x 2 contingency tables. Associations that presented $p<0.2$ were used in exploratory logistical regression models to detect significant associations. These analyses were performed using EpiInfo software version 7 (CDC, EUA).

3. Results

3.1 High occurrence of porcine hemoplasmas on qPCR

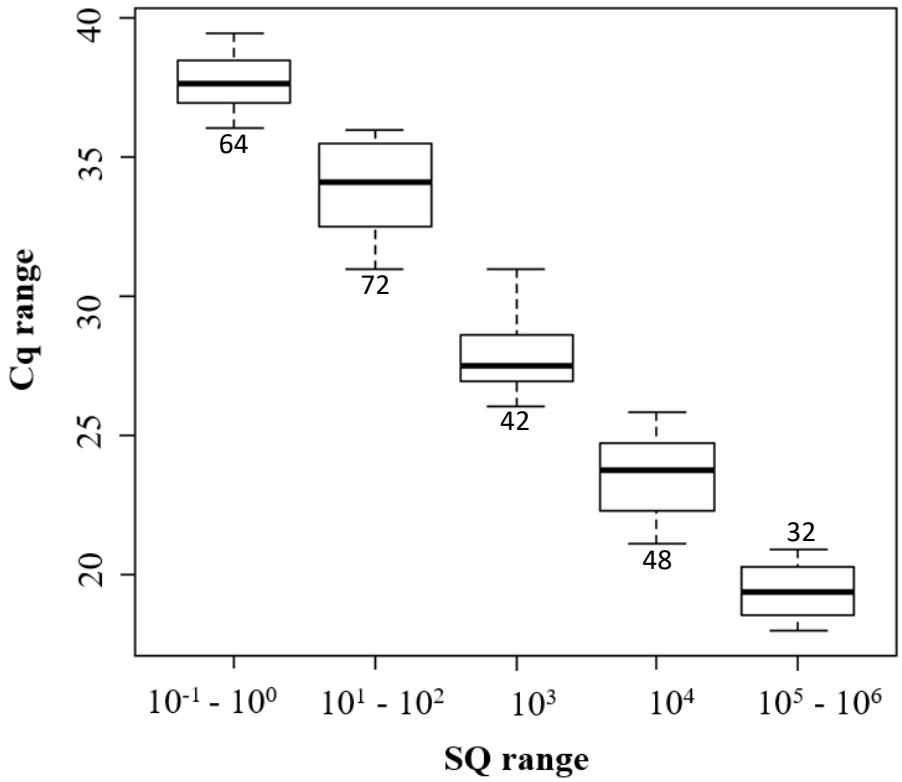
All samples tested positive for the mammals *gapdh* gene, indicating a successful DNA extraction. PH-qPCR parameters for each assay are presented on Table 1, according to the MIQE guidelines (Bustin et al., 2009). Samples were tested for PH-qPCR in triplicates, with reaction efficiency (E) ranging from 92.8 to 101.6% (average= 96.67), r^2, slope, and Y-intercept of qPCR assays ranged from 0.988 to 0.999 (average= 0.995), -3.506 to -3.285 (average= -3.381), and 39.061 to 40.920 (average= 40.436), respectively. Cycle quantification (Cq) ranged from 17.99 to 39.49, while starting quantification (SQ) ranged from $8.43x10^{-1}$ to $4.69x10^{6}$ copies/µL (Figure 2). Besides, 52.71% (136/258) of the quantified samples had SQ values lower than $1x10^{3}$ copies/µL.

Table 1. Parameters for PH-qPCR assays.

Plate number	Efficiency (E)	r^2	Slope	Y-intercept
1	100.4	0.992	-3.312	40.183
2	92.8	0.992	-3.506	41.235
3	94.7	0.991	-3.457	41.303
4	93	0.991	-3.502	41.524
5	99.5	0.996	-3.335	39.061
6	96.8	0.996	-3.400	40.07
7	100.5	0.997	-3.310	39.705
8	100.2	0.990	-3.316	40.172
9	100	0.998	-3.321	39.998
10	98.9	0.988	-3.348	39.826
11	96.1	0.993	-3.419	40.085
12	96.6	0.998	-3.405	40.17
13	100.9	0.997	-3.300	40.351
14	97	0.997	-3.397	40.563
15	95.4	0.998	-3.437	40.92
16	94.3	0.994	-3.467	40.865
17	101.6	0.999	-3.285	40.585
18	100.9	0.993	-3.301	40.466
19	96.3	0.999	-3.414	41.049

r^2: correlation coefficient.

Figure 2. Box plot showing qPCR quantification cycle (Cq) results and distribution of starting quantity (SQ).



Porcine hemoplasmas DNA was found in 68.89 % (310/450) of the tested samples. It was detected in 100% of the farms, with a within-herd prevalence ranging from 13.3% (2/15) to 100% (15/15). A total of 16 herds had a prevalence equal or higher than 80%, nine herds ranged from 40% to 74%, and five herds showed PH occurrence lower than 40% (Table 2). Detailed information regarding PH occurrence within-herd and its quantification data (number of copies/μL) can be found in Table S1. Farm numbers #16 and #23 had positive samples that could not be quantified due to its inconsistent quantification results (Cq values with a difference greater than 0.5) even when tested in triplicates. Likewise, other 48 samples presented the same inconsistency in the quantification results (Table S2). This fact is likely due to the Markov Chain Monte Carlo effect (Bustin et al., 2009), which represents an inherent limitation of the

qPCR technique, mainly in samples with a low number of DNA copies. Even though these samples could not be quantified, they were considered positive

Table 2. Herd-level prevalence and range of qPCR results.

Farm ID	Positive samples	Herd-level prevalence (%)	Cq range		SQ range	
1	14	93.33	23.18	38.81	6.02×10^{0}	1.74×10^{5}
2	14	93.33	24.55	39.43	1.72×10^{0}	7.06×10^{4}
3	15	100.00	35.28	38.91	1.12×10^{0}	2.56×10^{1}
4	14	93.33	35.81	39.33	8.43×10^{-1}	3.88×10^{1}
5	14	93.33	36.69	38.85	6.17×10^{0}	2.32×10^{1}
6	7	46.67	36.88	38.67	5.40×10^{0}	1.76×10^{1}
7	14	93.33	23.63	38.86	1.15×10^{0}	4.26×10^{4}
8	14	93.33	22.16	38.32	3.28×10^{0}	1.85×10^{5}
9	13	86.67	19.09	39.26	1.82×10^{0}	1.48×10^{6}
10	10	66.67	35.29	39.02	1.71×10^{0}	2.16×10^{1}
11	14	93.33	21.95	38.24	2.79×10^{0}	2.35×10^{5}
12	9	60.00	23.06	39.48	1.62×10^{0}	1.45×10^{5}
13	2	13.33	22.30	37.83	5.18×10^{0}	2.14×10^{5}
14	9	60.00	27.50	38.21	3.45×10^{0}	5.84×10^{3}
15	3	20.00	22.72	38.32	2.83×10^{0}	1.31×10^{5}
16*	2	13.33	-		-	
17	11	73.33	27.49	39.37	1.65×10^{0}	4.83×10^{3}
18	14	93.33	22.04	32.06	2.40×10^{2}	1.90×10^{5}
19	8	53.33	21.99	36.96	8.76×10^{0}	2.19×10^{5}
20	14	93.33	21.18	39.45	1.98×10^{0}	6.55×10^{5}
21	11	73.33	21.10	36.42	1.57×10^{1}	6.81×10^{5}
22	12	80.00	23.28	31.51	4.63×10^{2}	1.22×10^{5}
23*	3	20.00	-		-	
24	12	80.00	23.47	38.49	5.11×10^{0}	1.20×10^{5}
25	12	80.00	17.99	38.84	4.09×10^{0}	4.69×10^{6}
26	7	46.67	26.11	29.45	1.96×10^{3}	1.81×10^{4}
27	6	40.00	24.99	38.22	5.40×10^{0}	5.69×10^{4}
28	14	93.33	19.76	39.30	2.46×10^{0}	2.27×10^{6}
29	3	20.00	25.88	28.58	3.98×10^{3}	2.63×10^{4}
30	15	100.00	20.90	27.55	8.16×10^{3}	8.46×10^{5}

Cq = quantification cycle value; SQ = starting quantity value
*Cq and SQ ranges could not be defined due to the Monte Carlo Effect.

3.2 Genetic diversity and coinfection of M. parvum genotypes in pigs

In order to investigate the presence of coinfections and genetic diversity of hemoplasmas infecting pigs, cloning and sequencing was performed. Of the 33 samples with SQ higher than 10^5 copies/μL, only five showed positive results on cPCR for *Mycoplasma* spp. targeting 16S rRNA, which resulted in 22 clones with ~800 bp length. The same five samples were used for the cPCR targeting 23S rRNA and resulted in seven clone sequences. BLASTn analysis for the 16S rRNA sequences revealed 94.16% to 94.98% identity with *Mycoplasma suis* strain Illinois 16S (CP002525.1), 99.07% to 99.87% identity with *Mycoplasma parvum* strain Morioka4 (AB610846.1) and 99.47% to 99.73% with *Mycoplasma parvum* strain Indiana (CP006771.1). Similarly, BLASTn analysis for the 23S rRNA sequences showed 99.12% to 99.90% identity with *Mycoplasma parvum* strain Indiana (NR121958.1), 91.07% to 93.42% identity with *Mycoplasma suis* strain Illinois (NR103970.1) (Table S3, Table S4).

Nucleotide diversity of the 16S and 23S rRNA sequences obtained from this study was performed. Sequences of the 16S rRNA fragment showed 7 genotypes with genotype diversity (Hd) value of 0.645 and 9 variable sites (S). Genotype #2 was the more prevalent, being detected in four of the five tested samples and present in 59.09% (13/22) of the clones. Moreover, samples 129, 292, 371 and 411 had at least two different genotypes. Genotypes #4, #5, #6 and #7 were represented by the sequences 411, 129, 292 and 371, respectively. Likewise, four genotypes were observed from the seven 23S rRNA sequences, with haplotype diversity of 0.810 and 5 variable sites, where genotype #1.1 occurred in three sequences. Genotype #3.1 was detected in two sequences and genotypes #2.1 and #4.1 were detected in the other two sequences (Table 3).

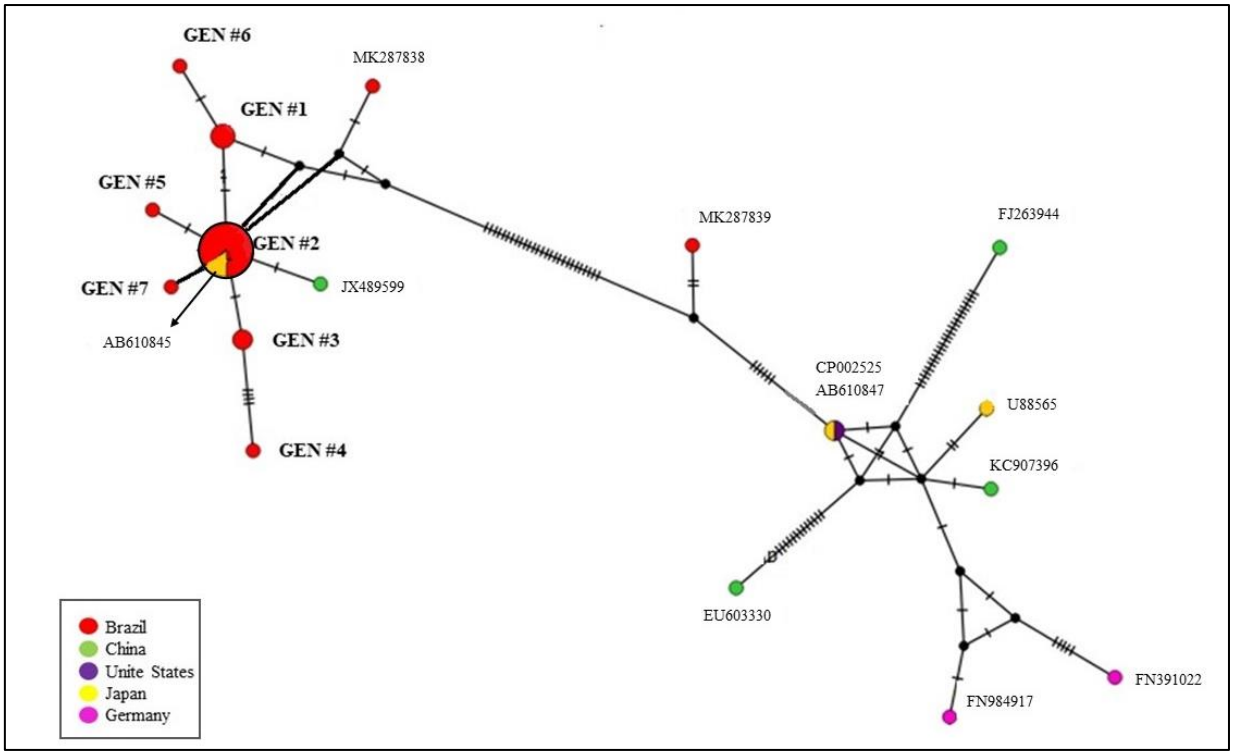
Table 3. Genotype identification of *Mycoplasma parvum* 16S and 23S rRNA sequences from five different samples.

		Samples				
Gene	**Genotype #**	**129**	**258**	**292**	**371**	**411**
16S	1	-	-	(3)	-	-
	2	(4)*	(3)	-	(2)	(4)
	3	-	-	-	(2)	-
	4	-	-	-	-	(1)
	5	(1)	-	-	-	-
	6	-	-	(1)	-	-
	7	-	-	-	(1)	-
23S	1.1	(1)	-	(2)	-	-
	2.1	-	(1)	-	-	-
	3.1	-	(1)	-	(1)	-
	4.1	-	-	-	-	(1)

*Numbers inside parentheses ( ) represent the total of sequences presenting the same genotype. For example: Sample 129 has four sequences with genotype #2, and one sequence with genotype #1.1

Additionally, genotype network analysis was performed using *Mycoplasma* spp. 16S rRNA sequences detected in pigs from studies performed in Brazil (Gatto et al., 2019), China (Fu et al., 2017), United States of America (Guimarães et al., 2011), Japan (Watanabe et al., 2011) and Germany (Felder et al., 2011). Thirty-five sequences were selected, and the analysis was performed with the TCS software v.1.2116 (Figure 3).

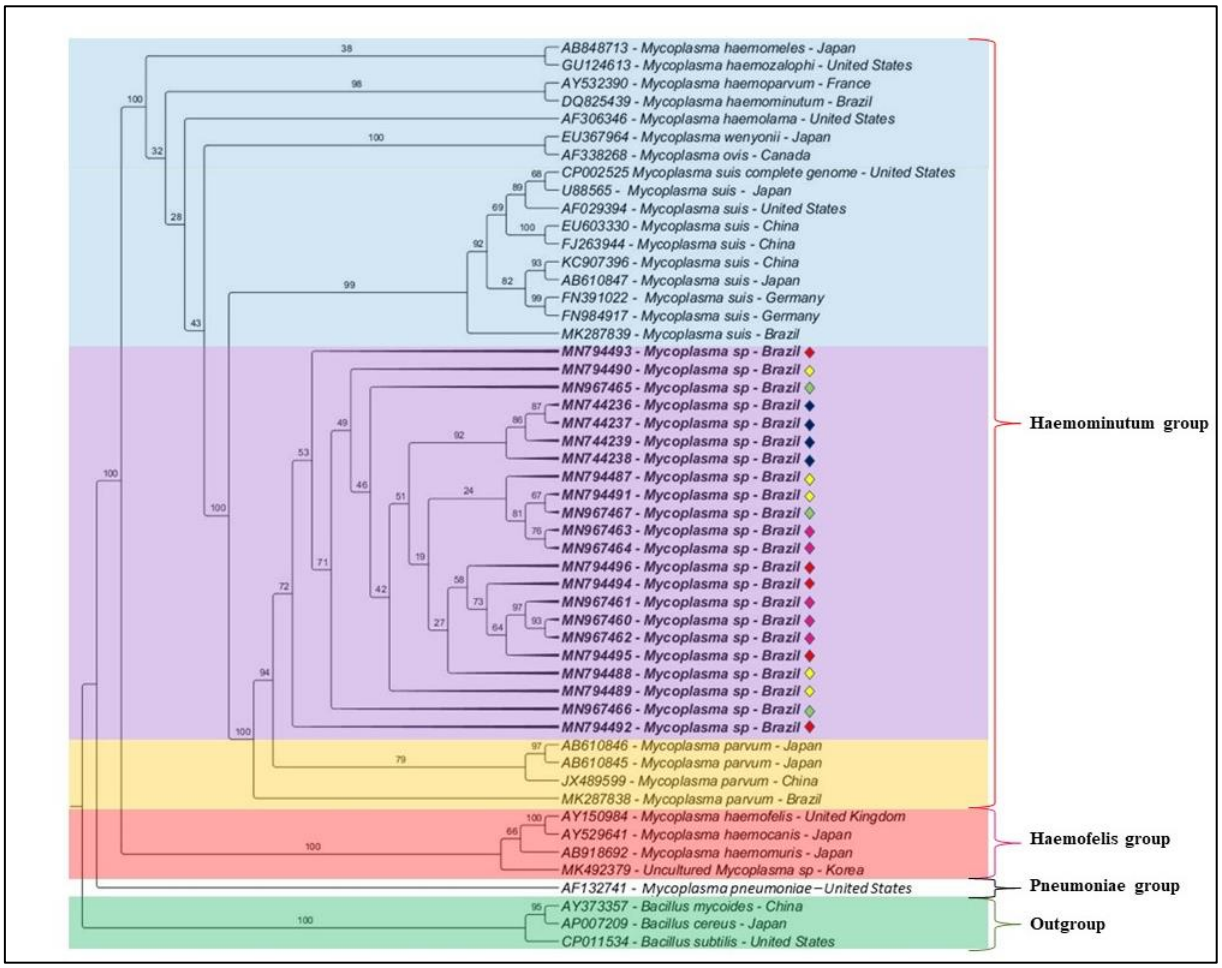
Figure 3. Genotypic network of previously deposited 16S rRNA PH sequences and the sequences obtained on this study, generated with TCS v.1.21 software. GEN #1 to GEN #7 represent the seven genotypes obtained from the 22 16S rRNA sequences.



3.3 Characterization of M. parvum

Phylogenetic inferences estimated by both Maximum Likelihood (ML) and Bayesian methods of *Mycoplasma* spp. 16S rRNA sequences presented similar results. Considering this, it is observed that the 22 sequences obtained from the 16S rRNA gene, formed a large clade, closely related to *M. parvum* detected in Japan. Sequences of *Bacillus* spp. that belong to the same Filo (Firmicutes) as *Mycoplasma* spp, were used as outgroup. Numbers at the nodes correspond to bootstrap values (Figure 4).

Figure 4. Phylogenetic tree based on an alignment of ~800 bp fragment of *Mycoplasma* spp. 16S rRNA sequences, using ML method and the TIM1+I+G evolutionary model. Numbers at the nodes correspond to bootstrap. Accession numbers are indicated in the sequences. *Mycoplasma* spp. Sequences detected in the present study are highlighted in bold and colored in purple.

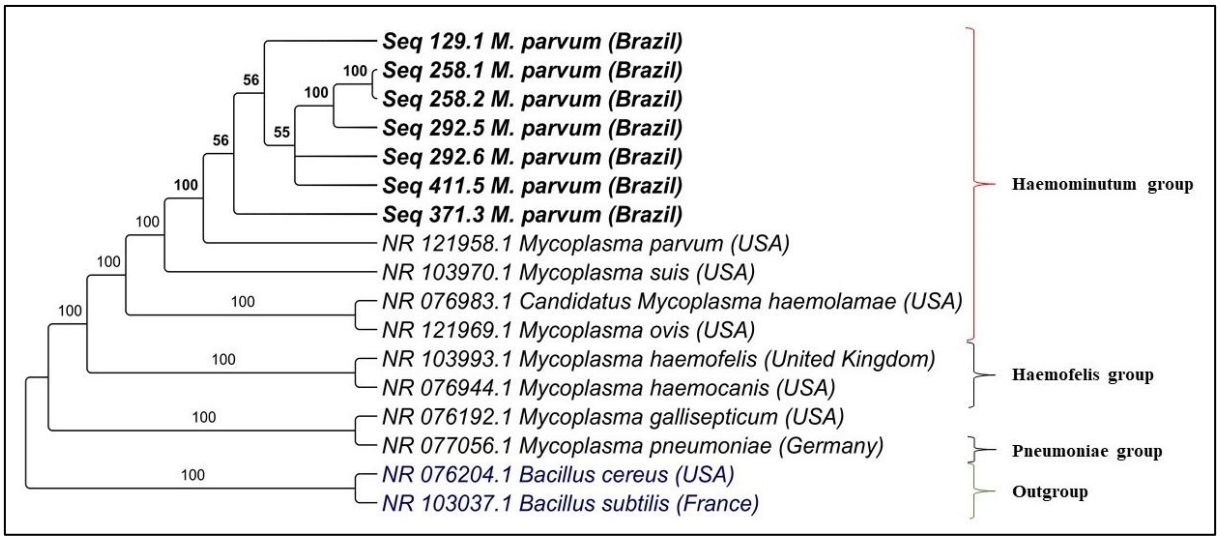


Sequences followed with ◆ correspond to sample 129. Samples 258, 292, 371 and 411 are followed by ◆, ◆, ◆ and ◆, respectively.

Similarly, phylogenetic analyses of *Mycoplasma* spp. 23S rRNA sequences estimated by the Bayesian and Maximum Likelihood methods, showed that all 23S rRNA clones are positioned in the *M. parvum* clade. The numbers at the nodes correspond to bootstrap values

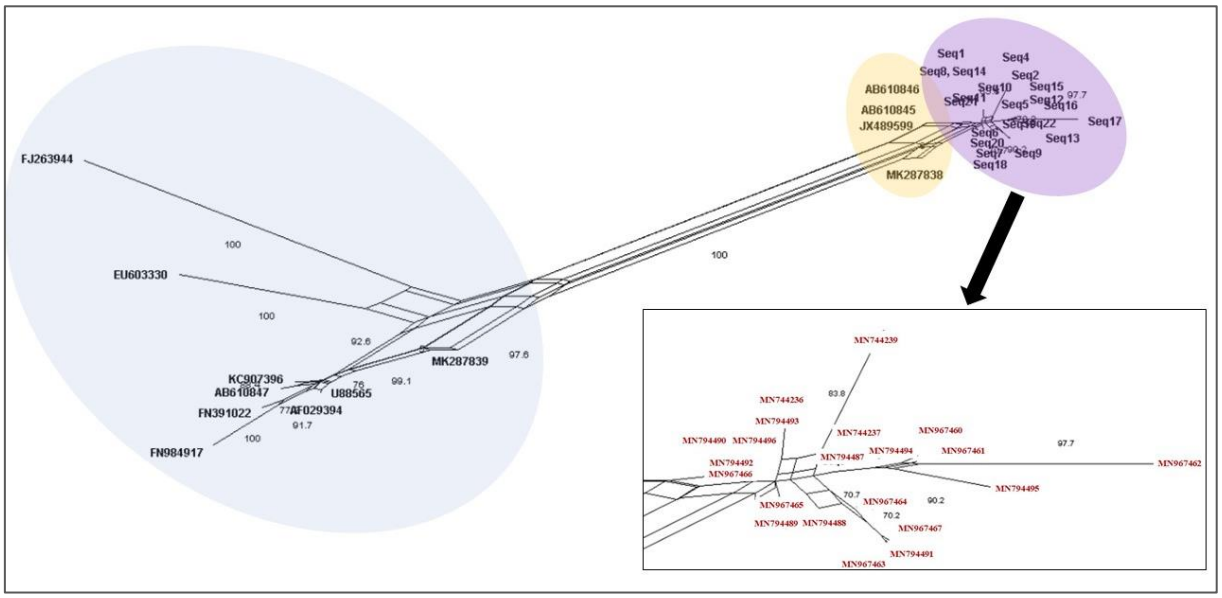
accessed with 1.000.000 generations. *Bacillus cereus* and *Bacillus subtilis* were used as outgroups (Figure 5).

Figure 5. Phylogenetic tree based on *Mycoplasma* spp. 23S rRNA sequences, using the Bayesian method and the TIM1+G evolutionary model. Numbers at the nodes correspond to bootstrap.



Additionally, Splitstree results corroborated with the phylogenetic analysis, and revealed one major cluster closely related to *M. parvum* (Figure 6).

Figure 6. Network analysis of *Mycoplasma* 16S rRNA sequences obtained from pigs sampled in the present study, compared to mycoplasma sequences retrieved from GenBank. The analysis was performed with Splitstree software using the parameters "Neighbor-Net and" Uncorrected p-distance". *Mycoplasma* spp. sequences detected in the present study are colored in purple. *M. suis* sequences are colored in blue, while *M. parvum* sequences are colored in yellow.



3.4 Impact of M. parvum on pig production

Risk factors could not be assessed because all farms had at least one positive sample. However, Spearman's coefficient test revealed that the occurrence of PH was inversely associated with number of farrows per week (p=0.001), weaned piglets/week (p=0.003) and slaughter weight (p=0.001). Coefficient values for the independent variables were -0.5556, -0.5179 and -0.5513, respectively. The information regarding the questionnaire is presented in Table S5.

4. Discussion

The present study aimed to investigate the occurrence and genetic diversity of hemoplasmas in domestic pigs from technified farms in Goiás, Brazil. For this purpose, 450 pigs from 30 farms were sampled at the time of slaughter. PH detection revealed a prevalence of 68.89%, corroborating the results found by Toledo et al. (2016) that were 76.19%. However, Toledo et al. (2016) analyzed samples from non-technified farms, without adequate nutritional programs, facilities, defined genetics, and standardized management techniques. Genetic diversity of 16S and 23S rRNA fragments was observed, resulting in 11 genotypes.

High occurrence of PH has been reported worldwide affecting all ages, but seems to be higher in older animals, like finishing pigs, boars and sows. In China, Fu et al. (2017) found 42.4% and 70.9% prevalence in growing pigs and sows, respectively. In Brazil, Gatto et al. (2019) detected a prevalence even higher (79.7%) in sows. On the other hand, our results showed a prevalence greater than that one detected in growing pigs from China (42%), Japan (5%) and Germany (14% and 10%) (Fu et al., 2017; Watanabe et al., 2011; Ritzmann et al., 2009). Moreover, the occurrence of PH in growing pigs worldwide ranges from 5% in Japan (Watanabe et al., 2012) to 86% in China (Yuan et al., 2009). Differences in the prevalence data may be explained by the chronic stage of infection, characterized by low bacteremia, which compromises DNA detection when using less sensitive tests, such as cPCR (Gatto et al., 2019),or by the age of the animals when samples were collected. According to a study developed in Brazil, the occurrence of PH in pigs with 65 days of life was 59%, however, when the same animals were sampled 105 days later, 95% of them tested positive on qPCR for PH (Sonalio et al.,unpublished data). It is also important to mention, that most pig productions in Brazil use antimicrobials as growth promoters until 15 days prior to slaughter, and this should

be considered, once occurrence can increase when taking out these drugs at the end of finishing fase.

Our results on qPCR showed that 57.42% (178/310) of the positive samples presented PH loads between 10^{-1} and 10^{3} copies/μL. Similarly, Gatto et al. (2019) described that 88% of the samples had DNA loads ranging from 10^{1} to 10^{4} copies/μL. However, level of bacteria might range from 10^{3} to 10^{9} copies/mL (Guimarães et al., 2011), which could be very variable from one animal to another. These findings also suggest that most of the animals on this study presented a chronic infection, characterized by low bacteremia and low quantification values (Gatto et al., 2019).

In order to access the genetic diversity of *Mycoplasma* spp., fragments of the16S and 23S rRNA gene were cloned. For this purpose, five positive samples showing bacterial loads between 10^{5} and 10^{6} copies/μL were used. Samples with lower bacterial loads did not show positive results on cPCR for PH, precluding the assessment of the genetic diversity on those samples. This fact could be related to the low sensitivity of cPCR assays (Hoelzle et al., 2003), when compared to qPCR assays, which have 4- to 40-fold higher sensitivity (Hoelzle et al., 2007).

The 22 sequences obtained from the five animals presented a high query cover and high identity to *M. suis* and *M. parvum* strains when tested on BLASTn. Phylogenetic analyses show that the 16S rRNA clone sequences formed a large clade, closely related to *M. parvum*. Similarly, Watanabe et al. (2011) reported the presence of two clusters in Japan: while the cluster A was closely related to *M. suis*, the cluster B comprised a new hemoplasma species closely related to *M. parvum,* suggesting a different taxon in the hemoplasma taxonomy. In addition, Seo et al. (2019) debates whether isolates previously identified as *M. suis,* but closer to *M. parvum* may need to be reclassified, plus, conclusions of earlier studies may need to be

reevaluated when appropriate molecular data becomes available (Seo et al., 2019). Even though 16S rRNA sequences seem to be a very effective tool in determining the phylogeny and taxonomy of Mollicutes, it hampers accurate differentiation of *M. suis* from *M. parvum* (Razin et al. 1998). As 16S rRNA gene sequences of *M. suis* and *M. parvum* have high sequence identity, it is difficult to design PCR primers distinguishing the two species (Watanabe et al., 2011).

Thus, the 23S rRNA PCR used in this study, seems effective to differentiate *M. suis* and *M. parvum.* BLASTn results for the seven 23S rRNA cloned sequences showed that these samples had, at least, 99.12% identity with *M. parvum* 23S rRNA gene previously deposited at GenBank under de accession number: NR121958. Conversely, the sequences identity *for M. suis* (NR103970) was lower than 93.42%, indicating that all cloned sequences originated from *M. parvum.* Interestingly, in the present study, phylogenetic analysis of 23S rRNA sequences revealed the occurrence of a Mycoplasma other than *M. suis*. All tested samples were closely related to *M. parvum* (NR121958). So, it is possible that *M. parvum* is the most common PH occurring in Brazil nowadays, which makes sense considering that *M. parvum* does not cause clinical signs on infected animals, what could keep the agent on the herd (do Nascimento, et al., 2014).

Genotypic diversity was observed on 16S and 23S rRNA sequences, totalizing 11genotypes, seven on 16S rRNA gene and four on 23S rRNA gene. Moreover, network analysis of the 16S rRNA samples, demonstrates proximity with *M. parvum* strains from Brazil, Japan and China. Therefore, nucleotide substitutions in base pairings correspond to evolutionary changes, which can generate new genotypes or species (Watanabe et al., 2011). Watanabe et al. (2011) also infers that point mutations occur continuously through the prokaryotic genome at every multiplication phase. So, analyses of more than one structure could

help the identification of closely related species, such *M. suis* and *M. parvum*. This would be very important, considering that *M. parvum* seems to be non-pathogenic, while *M. suis* is associated with economic losses worldwide.

Nonetheless, our results showed inverse correlations with number of farrows per week ($p=0.001$), weaned piglets/week ($p=0.003$) and slaughter weight ($p=0.001$), which means that when PH occurrence increases, number of farrows/week, weaned piglets/week and slaughter weight decreases, resulting in economic losses as described previously. Still, these losses might be connected to compromised metabolic functions resulted from *M. suis* infection, such as nutrient scavenging and competition, reduced energy production, oxidative stress and life span of RBCs (Hoelzle et al., 2014). Since the five sequenced samples were associated with *M. parvum*, it is likely that some of the non-sequenced samples are associated with *M. suis*, explaining the productive impact on pig production.

Even though the results represent only 18% of all pig farms in Goiás state, they rise questions regarding the sanitary conditions and biosecurity measures of the farms. Likewise, Yuan et al. (2009) demonstrated that poor sanitary conditions, ingestion of blood through contaminated feed and reuse of needles might contribute to high prevalence levels. Song et al. (2014) also suggested that elimination of mosquitoes and flies at farm level is an effective measure to control the spread of PH.

Thus, this is the first report of genotype diversity in *M. parvum* samples from domestic pigs. In addition, our results indicate the occurrence of coinfections by different genotypes in the same animal and inside a certain herd. Besides, PH occurrence was close to 70%, indicating that the disease may be neglected in commercial herds in Brazil. Finally, the real impact of PH coinfections in a single animal is still unknown.

5. Conclusions

Our results indicate, for the first time, the occurrence of coinfections by different genotypes of *M. parvum* in the same animal and inside a technified pig herd. Molecular characterization of 16S and 23S partial gene of *M. parvum* was also described, along with porcine hemoplasmas occurrence (68.89%) in technified farms in Goiás State, Brazil.

6. Conflict of interest

The authors declare no conflict of interest.

7. Acknowledgements

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8. References

Acosta, D.B., Ruiz, M., Sanchez, J.P., 2019. First molecular detection of Mycoplasma suis in the pig louse Haematopinus suis (Phthiraptera: Anoplura) from Argentina. Acta Trop. doi:10.1016/j.actatropica.2019.04.007

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410. doi:10.1016/S0022-2836(05)80360-2

Benson, D.A., Cavanaugh, M., Clark, K., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J., Sayers, E.W., 2013. GeneBank. Nucleic Acids Res. 41, D36–D42.

Birkenheuer, A.J., Levy, M.G., Breitschwerdt, E.B., 2003. Development and evaluation of a seminested PCR for detection and differentiation of Babesia gibsoni (Asian genotype)

and B. canis DNA in canine blood samples. J. Clin. Microbiol. 41, 4172–4177. doi:10.1128/JCM.41.9.4172-4177.2003

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

Charlebois, A., Jacques, M., Archambault, M., 2014. Biofilm formation of Clostridium perfringens and its exposure to low-dose antimicrobials. Front. Microbiol. doi:10.3389/fmicb.2014.00183

Clement, M., Posada, D., Crandall, K.A., 2000. TCS: A computer program to estimate gene genealogies. Mol. Ecol. doi:10.1046/j.1365-294X.2000.01020.x

core Team, R., 2018. R: A Language and Environment for Statistical Computing. R Found. Stat. Comput. Vienna, Austria.

Darriba, D., Posada, D., 2012. jModelTest 2.0 Manual. Nat. Methods 9, 772. doi:10.1093/molbev/msn083

Do Nascimento, N.C., Dos Santos, A.P., Chu, Y., Guimaraes, A.M., Baird, A.N., Weil, A.B., Messick, J.B., 2014. Microscopy and genomic analysis of Mycoplasma parvum strain Indiana. Vet. Res. doi:10.1186/s13567-014-0086-7

do Nascimento, N.C., Guimaraes, A.M.S., Dos Santos, A.P., Chu, Y., Marques, L.M., Messick, J.B., 2018. RNA-Seq based transcriptome of whole blood from immunocompetent pigs (Sus scrofa) experimentally infected with Mycoplasma suis strain Illinois. Vet. Res. doi:10.1186/s13567-018-0546-6

Ewing, B., Green, P., 1998. Base-Calling of Automated Sequencer Traces Using *Phred.* II. Error Probabilities. Genome Res. 8, 186–194. doi:10.1101/gr.8.3.186

Ewing, B., Hillier, L.D., Wendl, M.C., Green, P., 1998. Base-calling of automated sequencer traces using phred. I. Accuracy assessment. Genome Res. 8, 175–185. doi:10.1101/gr.8.3.175

Felder, K.M., Hoelzle, K., Ritzmann, M., Kilchling, T., Schiele, D., Heinritzi, K., Groebel, K., Hoelzle, L.E., 2011. Hemotrophic mycoplasmas induce programmed cell death in red blood cells. Cell. Physiol. Biochem. 27, 557–564. doi:10.1159/000329957

Fu, Y., Shi, T., Xu, L., Wei, W., Lu, F., Zhang, X., Yuan, X., Li, J., Lv, J., Fang, W., 2017. Identification of a novel Hemoplasma species from pigs in Zhejiang province, China. J. Vet. Med. Sci. doi:10.1292/jvms.16-0545

Gatto, I.R.H., Sonálio, K., Amaral, R.B.D., Morés, N., Dalla Costa, O.A., André, M.R., de Oliveira, L.G., 2019. High frequency and molecular characterization of porcine

hemotrophic mycoplasmas in Brazil. Vet. Microbiol. 231. doi:10.1016/j.vetmic.2019.02.024

Groebel, K., Hoelzle, K., Wittenbrink, M.M., Ziegler, U., Hoelzle, L.E., 2009. Mycoplasma suis invades porcine erythrocytes. Infect. Immun. 77, 576–584. doi:10.1128/IAI.00773-08

Guimaraes, A.M.S., Biondo, A.W., Lara, A.C., Messick, J.B., 2007. Exploratory study of Mycoplasma suis (Eperythrozoon suis) on four commercial pig farms in southern Brazil. Vet. Rec. 160, 50–53. doi:10.1136/vr.160.2.50

Guimaraes, A.M.S., Vieira, R.F.C., Poletto, R., Vemulapalli, R., Santos, A.P., de Moraes, W., Cubas, Z.S., Santos, L.C., Marchant-Forde, J.N., Timenetsky, J., Biondo, A.W., Messick, J.B., 2011. A quantitative TaqMan PCR assay for the detection of Mycoplasma suis. J. Appl. Microbiol. doi:10.1111/j.1365-2672.2011.05053.x

Henry, S.C., 1979. Clinical observations on eperythrozoonosis. J. Am. Vet. Med. Assoc.

Hoelzle, K., Engels, M., Kramer, M.M., Wittenbrink, M.M., Dieckmann, S.M., Hoelzle, L.E., 2010. Occurrence of Mycoplasma suis in wild boars (Sus scrofa L.). Vet. Microbiol. 143, 405–409. doi:10.1016/j.vetmic.2009.11.015

Hoelzle, L.E., 2008. Haemotrophic mycoplasmas: Recent advances in Mycoplasma suis. Vet. Microbiol. doi:10.1016/j.vetmic.2007.12.023

Hoelzle, L.E., Adelt, D., Hoelzle, K., Heinritzi, K., Wittenbrink, M.M., 2003. Development of a diagnostic PCR assay based on novel DNA sequences for the detection of Mycoplasma suis (Eperythrozoon suis) in porcine blood. Vet. Microbiol. doi:10.1016/S0378-1135(03)00040-3

Hoelzle, L.E., Helbling, M., Hoelzle, K., Ritzmann, M., Heinritzi, K., Wittenbrink, M.M., 2007. First LightCycler real-time PCR assay for the quantitative detection of Mycoplasma suis in clinical samples. J. Microbiol. Methods 70, 346–354. doi:10.1016/j.mimet.2007.05.009

Hoelzle, L.E., Zeder, M., Felder, K.M., Hoelzle, K., 2014. Pathobiology of Mycoplasma suis. Vet. J. doi:10.1016/j.tvjl.2014.07.023

Katoh, K., Rozewicki, J., Yamada, K.D., 2018. MAFFT online service: Multiple sequence alignment, interactive sequence choice and visualization. Brief. Bioinform. doi:10.1093/bib/bbx108

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.

Lau, A.O.T., Cereceres, K., Palmer, G.H., Fretwell, D.L., Pedroni, M.J., Mosqueda, J., McElwain, T.F., 2010. Genotypic diversity of merozoite surface antigen 1 of Babesia

bovis within an endemic population. Mol. Biochem. Parasitol. doi:10.1016/j.molbiopara.2010.03.017

Maggi, R.G., Chitwood, M.C., Kennedy-Stoskopf, S., DePerno, C.S., 2013. Novel hemotropic Mycoplasma species in white-tailed deer (Odocoileus virginianus). Comp. Immunol. Microbiol. Infect. Dis. 36, 607–611. doi:10.1016/j.cimid.2013.08.001

Miller, M.A., Pfeiffer, W., Schwartz, T., 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees, in: 2010 Gateway Computing Environments Workshop, GCE 2010. doi:10.1109/GCE.2010.5676129

Posada D., B.T.R., 2004. Model Selection and Model Averaging in Phylogenetics : Advantages of Akaike. Syst.Biol. 53, 793–808. doi:10.1080/10635150490522304

Razin, S., Yogev, D., Naot, Y., 1998. Molecular Biology and Pathogenicity of Mycoplasmas. Microbiol. Mol. Biol. Rev. doi:10.1128/mmbr.62.4.1094-1156.1998

Ritzmann, M., Grimm, J., Heinritzi, K., Hoelzle, K., Hoelzle, L.E., 2009. Prevalence of Mycoplasma suis in slaughter pigs, with correlation of PCR results to hematological findings. Vet. Microbiol. 133, 84–91. doi:10.1016/j.vetmic.2008.06.015

Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574. doi:10.1093/bioinformatics/btg180

Sambrook, J., Russell, D.W., 2006. Preparation of Plasmid DNA by Alkaline Lysis with SDS: Minipreparation. Cold Spring Harb. Protoc. doi:10.1101/pdb.prot4084

Sanger, F., Nicklen, S., Coulson, A.R., 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U. S. A. doi:10.1073/pnas.74.12.5463

Schreiner, S.A., Hoelzle, K., Hofmann-Lehmann, R., Hamburger, A., Wittenbrink, M.M., Kramer, M.M., Sokoli, A., Felder, K.M., Groebel, K., Hoelzle, L.E., 2012. Nanotransformation of the haemotrophic Mycoplasma suis during in vitro cultivation attempts using modified cell free Mycoplasma media. Vet. Microbiol. doi:10.1016/j.vetmic.2012.05.022

Seo, M.G., Kwon, O.D., Kwak, D., 2019. Prevalence and phylogenetic analysis of hemoplasma species in domestic pigs in Korea. Parasites and Vectors. doi:10.1186/s13071-019-3638-x

Song, Q., Zhang, W., Song, W., Liu, Z., Khan, M.K., He, L., Fang, R., Li, P., Zhou, Y., Hu, M., Zhao, J., 2014. Seroprevalence and risk factors of Mycoplasma suis infection in pig farms in central China. Prev. Vet. Med. doi:10.1016/j.prevetmed.2014.07.006

Spliter, E.G., 1950. Eperytrozoon suis and Eperytrozoon parvum: two new blood parasites of swine. Science. 111:513-514

Stadler, J., Jannasch, C., Mack, S.L., Dietz, S., Zöls, S., Ritzmann, M., Hoelzle, K., Hoelzle, L.E., 2014. Clinical and haematological characterisation of Mycoplasma suis infections in splenectomised and non-splenectomised pigs. Vet. Microbiol. 172, 294–300. doi:10.1016/j.vetmic.2014.05.012

Stadler, J., Willi, S., Ritzmann, M., Eddicks, M., Ade, J., Hoelzle, K., Hoelzle, L.E., 2019. Detection of Mycoplasma suis in pre-suckling piglets indicates a vertical transmission. BMC Vet. Res. doi:10.1186/s12917-019-2001-y

Stöver, B.C., Müller, K.F., 2010. TreeGraph 2: Combining and visualizing evidence from different phylogenetic analyses. BMC Bioinformatics 11. doi:10.1186/1471-2105-11-7

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. doi:10.1093/nar/22.22.4673

Toledo, M.A., Leite, A.I., Gonçalves, L.R., Sousa, K.C.M. de, Amaral, R.B. do, Silva, G.C.P. da, Machado, R.Z., André, M.R., 2016. High occurrence of Mycoplasma suis infection in swine herds from non-technified farms in Mossoró, state of Rio Grande do Norte, Northeastern Brazil. Rev. Bras. Parasitol. Veterinária. doi:10.1590/s1984-29612016084

Trifinopoulos, J., Nguyen, L.T., von Haeseler, A., Minh, B.Q., 2016. W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis. Nucleic Acids Res. doi:10.1093/nar/gkw256

Watanabe, Y., Fujihara, M., Obara, H., Nagai, K., Harasawa, R., 2011. Two genetic clusters in swine hemoplasmas revealed by analyses of the 16s rRNA and RNase P RNA genes. J. Vet. Med. Sci. doi:10.1292/jvms.11-0293

Watanabe, Y., Fujihara, M., Suzuki, J., Sasaoka, F., Nagai, K., Harasawa, R., 2012. Prevalence of swine hemoplasmas revealed by real-time PCR using 16S rRNA gene primers. J. Vet. Med. Sci. 74, 1315–1318. doi:10.1292/jvms.12-0096

Yuan, C.L., Liang, A.B., Yao, C.B., Yang, Z.B., Zhu, J.G., Cui, L., Yu, F., Zhu, N.Y., Yang, X.W., Hua, X.G., 2009. Prevalence of Mycoplasma suis (Eperythrozoon suis) infection in swine and swine-farm workers in Shanghai, China. Am. J. Vet. Res. 70, 890–894. doi:10.2460/ajvr.70.7.890