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Molecular prevalence of *Mycoplasma parvum* in production cycle of technified swine herds

[*Prevalência molecular do* Mycoplasma parvum *em granjas de suínos tecnificados de ciclo completo*]

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ABSTRACT

Porcine hemoplasmosis is characterized as a geographically cosmopolitan disease caused by *Mycoplasma suis* and *Mycoplasma parvum.* Asymptomatic pigs are considered the focus of hemoplasmosis because they are carriers and reservoirs to new infections. This study aimed to determine the molecular occurrence of porcine hemoplasmas (PH) in the production cycle of technified farrow-to-finished swine herds. For this purpose, 20 swine herds were evaluated, where 501 whole blood samples were collected for qPCR and phylogenetic analyses for hemoplasmas. The epidemiological analysis was performed for the entire population and per the growth stage. The total prevalence for PH was 31.93% (161/501); 95% (19/20) of sampled herds were positive. The occurrence of PH by swine growth stages was nursery (30.47%), growing (31.29%), finishing (26.18%), and slaughter (40.25%). The quantification cycles (Cq) ranged from 3.18- 39.56 and the number of PH 16S rRNA copies per µL of DNA ranged from 5,57 $x10^{-2}$ to 2.23 $x10^{10}$. Sequencing and phylogenetic analysis of five selected samples showed 100% identity with *M. parvum* strain Indiana and two *M. parvum* sequences from Brazil/Goiás. This is the first report on PH in technified herds in Southeastern Brazil by growth stages.

Keywords: *Mycoplasma parvum*, bacteremia, carrier, growth stages

RESUMO

A hemoplasmose suína é uma doença geograficamente cosmopolita, causada por Mycoplasma suis *e* Mycoplasma parvum. *Suínos assintomáticos são considerados foco de hemoplasmose por serem portadores e reservatórios de novas infecções. Este estudo teve como objetivo determinar a ocorrência molecular de hemoplasmas suínos (HP) no ciclo de produção de rebanhos suínos tecnificados. Foram avaliados 20 rebanhos suínos e coletadas 501 amostras de sangue total para qPCR e análises filogenéticas para hemoplasmas. A análise epidemiológica foi realizada pela população e por estágio de crescimento. A prevalência total de HP foi de 31,93% (161/501); 95% (19/20) dos rebanhos amostrados foram positivos. A ocorrência de HP por fases de crescimento dos suínos foi: creche (30,47%), em crescimento (31,29%), acabamento (26,18%) e abate (40,25%). Os ciclos de quantificação (Cq) variaram de 3,18-39,56 e o número de cópias de rRNA PH 16S por µL de DNA variou de 5,57 $x10^{-2}$ a 2,23 $x10^{10}$. O sequenciamento e a análise filogenética de cinco amostras selecionadas mostraram 100% de identidade com a cepa indiana de* M. parvum *e duas sequências de* M. parvum *do Brasil / Goiás. Este é o primeiro relato de HP em rebanhos tecnificados, na região Sudeste do Brasil, por estágios de crescimento.*

Palavras-chaves: Mycoplasma parvum, bacteremia, portador, fases de criação

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INTRODUCTION

Porcine hemoplasmosis (PH) can be caused by *Mycoplasma suis* and *Mycoplasma parvum*. Both worldwide distributed microorganisms affect pigs at various developmental stages and are associated with severe or moderate chronic hemolytic anemia, dysgalactia, infertility, and immunosuppression, which can lead to economic losses (Hoelzle, 2008).

Mycoplasma parvum, the closest relative of *M. suis*, has a selective tropism for the plasmatic membrane of red blood cells, with more subclinical behavior. This agent presents bacillus to cocci morphology, and size varying from 0.2 to 0.5 μm. On the other hand, *M. suis* may have a ring-shape of up to 2.5μm in diameter (Nascimento *et al.*, 2014).

Guimarães *et al.* (2011) report a specific primer sequence of *M. suis* to qPCR. However, based on the phylogenetic analysis of the *M. suis* 16S rRNA gene (900 bp) these sequences are closely related to the *M. suis* and *M. parvum* cluster (Gatto *et al.*, 2019). At this moment, there is not a molecular method to distinguish these two species of hemotropic mycoplasmas.

From an epidemiological point of view, asymptomatic animals are considered hemoplasma reservoirs, since they carry the aforementioned agents but have no clinical signs or show clinical improvement without eliminating the parasite, thus contributing to the agent maintenance in the herds (Wu *et al.*, 2006), causing silent and persistent economic losses.

The prevalence of hemoplasmas in Brazilian pig herds is still unknown since most studies are intended for reports of molecular occurrence in matrices (Guimarães *et al.*, 2011; Gatto *et al.*, 2019; Sonalio *et al.*, 2020), intensive breeding (Petri *et al.*, 2020), extensive low-tech breeding (Toledo *et al.*, 2016), wild boars (Dias *et al.*, 2019).

Few studies have investigated the occurrence of porcine hemoplasmas (PH) in all growth stages in pig herds (e.g., farrow-to-finishing, nursery/weaning, weaning-to-finishing, and slaughter) and the parasite distribution in different Brazilian regions.

This study aimed to determine the molecular occurrence of PH in the production cycle of technified farrow-to-finished swine herds.

MATERIAL AND METHODS

The study was approved by the Ethics Committee on Animal Use (CEUA) of the School of Agricultural and Veterinary Sciences of the São Paulo State University, UNESP, Campus Jaboticabal (FCAV – UNESP), protocol n° 11.317/16.

Based on member registrations and data provided by the Paulista Association of Pig Breeders (Associação Paulista de Criadores de Suínos, APCS) there are 41 full cycle swine farms with approximately 80,000 animals, located in various regions of São Paulo state.

Of the 41 suggested farms, only 20 agreed to participate. Considering that the present study and the involved researchers were not sanitary authorities, producer participation was voluntary following the explanatory presentation on the project and design.

Some producers did not join the project allegedly due to biosecurity guarantee or suspended rearing activities during the sampling period. In the participating 20 pig farms in the state of São Paulo, the target population was 37,670 animals, to which a probabilistic selection methodology was applied.

To obtain the sample, the target population was classified into four growth stages as follows, nursery (21 - 65 days), growing (66 - 110 days), finishing (111 - 160 days), and slaughtering (170 - 190 days). To standardize the data collection and animal age, data were collected on the midpoint for each interval: nursery (45 days), growing (90 days), finishing (140 days), and slaughter (180 days).

The sample size required to determine prevalence was calculated using the formula below, already corrected for the estimated population size. The calculation was performed using Epi Info software.

$$n = \frac{N * p * q * Z^2}{(N-1) * E^2 + p * q * Z^2}$$

n = sample size to be examined
N = target population size
p = expected prevalence = 0.35 (35%)
q = 1 – expected prevalence (0.65)
Z ≅ 1.96 (confidence level = 95%)
E = error (0.05 or 5%)

According to the formula, the required sample size was 348 animals. Since laboratory resources were available, 501 samples were examined.

To maintain the animal ratio of each stage in the sample similar to the ratio in the phase in the target population, 128 nursery, 121 growing, 64 finishing, and 188 slaughtering samples were examined.

The data analysis was performed (a) for the entire population, (b) per growth stage, and (c) per state region. Thus, specific weights were calculated for the general analysis, per growth stage, and per state region.

The pig farm was considered as the primary sampling unit, except for the analysis per region in situations where all herds in the region were examined, or where only one herd was examined. In this case, the animal was considered as the primary sampling unit. In the general and regional analyses, stratification according to the growth stage was adopted.

The weight of each sampled animal in the population was obtained to calculate the prevalence rate of infected animals. For this, the probability of selecting each animal in the sample was initially determined according to the following equation (Dohoo *et al.*, 2009).

$$\frac{n}{N} * \frac{m}{M}$$

Where:
n = number of sampled herds
N = number of herds in the population
m = number of animals sampled on the farm
M = number of animals on the farm

Animal weight was given by the inverse of the selection probability, i.e., 1/[(n/N)*(m/M)]. Thus, weight is the number of animals represented by the sampled animal.

The blood samples were collected by venipuncture of the right jugular vein, packed in sterile vacuum tubes, and treated with ethylenediaminetetraacetic acid anticoagulant (EDTA-K2) for further molecular detection of PH.

Whole blood samples were transported in refrigerated isothermal boxes to the Veterinary Clinical Pathology Laboratory of the School of Agricultural and Veterinary Sciences of the São Paulo State University (FCAV-UNESP). Then, sample aliquots were separated in free RNA/DNA cryotubes and stored at - 20 °C until molecular techniques were performed at the Immunoparasitology Laboratory (FCAV-UNESP).

All analyses were performed following the standards established by the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (Bustin *et al.*, 2009).

DNA extraction from swine whole blood samples followed a protocol previously used by Toledo *et al.* (2016), Dias *et al.* (2019), Gatto *et al.* (2019), Petri *et al.* (2020) and Sonálio *et al.* (2020). After DNA extraction, duplicate sample aliquots were stored at -20°C for PCR.

DNA concentrations were measured by spectrophotometry using a Thermo Scientific NanoDrop 2000 Spectrophotometer (Thermo FisherScientific®, United States), at 260/280 nm absorbance, having as sample exclusion criteria those that did not reach 1.8 to 2.0 purity for the PCR technique.

To rule out the presence of inhibitors in DNA samples extracted from blood aliquots and the possibility of false negatives occurring in qPCR for PH (Gatto *et al.*, 2019), all DNA samples were submitted to a standard conventional PCR using *gapdh*-F (5'-CCTTCATTGACCTCAACTACAT-3') and *gapdh*-R (5'-CCAAAGTTGTCATGGATGACC-3') primer oligonucleotides, which flanked a 437 base pair (bp) fragment of the mammal endogenous glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) gene. The modified PCR technique adopted, and the amplification reaction used a final total volume reaction of 25 µL (5 µL sample DNA, 0.2 mM of each deoxynucleotide, 1 µM of each primer oligonucleotide, 0.75 mM

magnesium chloride, 0.25 µL Taq DNA Platinum Polymerase, PCR buffer and sterile ultrapure water q.s.p. 25 µL), with thermal sequence and amplification time as follows, initial denaturation at 94 °C for 3 min, 35 cycles consisting of denaturation at 94 °C for 1 min, annealing at 50 °C for 2 min and extension at 72 °C for 2 min, followed by final extension at 72°C for 7 min (Toledo *et al.*, 2016; Dias *et al.*, 2019; Gatto *et al.*, 2019; Petri *et al.*, 2020; Sonálio *et al.*, 2020).

Amplified products were subjected to horizontal electrophoresis on Ethidium Bromide stained 1.5% agarose gel (0.5 µL/mL) in pH 8.0 TEB running buffer at 90V/50mA for 90 min. The amplified products were compared to a 100 bp molecular weight marker, and the results were visualized and analyzed by an ultraviolet light transilluminator (Chemi-Doc, Bio-Rad®, United States) coupled to a computer with image analysis software (Image Lab, Bio-Rad®, United States).

The DNA samples extracted testing positive to the endogenous *gapdh* gene by conventional PCR were submitted to 16S rRNA gene-based qPCR to detect PH (*M. suis/M. parvum)* using oligonucleotides (Integrated DNA Technologies®, United States) F (5'-CCCTGATTGTACTAATTGAATAAG-3') and R (5'-GCGAACACTTGTTAAGCAAG-3') and the TaqMan hydrolysis probe (5'FAM-TGRATACACAYTTCAG-MGBNFQ3') as previously described by Guimarães *et al.* (2011). The qPCR assays were performed on the CFX 96 Thermal Cycler (BioRad®, United States) using 10 µL total final volume, containing a mixture of 1 µL sample DNA, 0.2 µM of each oligonucleotide and hydrolysis probe, PCR buffer (IQ Multiplex Power Mix, BioRad®, United States) and Sterile Ultrapure Water (Nuclease-FreeWater, Promega®, United States) q.s.p. 10 µl. The thermal sequence and amplification time adopted were as follows, 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s, 58.5 °C for 45 s, and 72 °C for 30 s.

To quantify the DNA copy number of the samples, the procedure consisted of serial dilutions with different concentrations of plasmid DNA containing the target sequence (2.0×10^7 to 2.0×10^0 copies/µL) to establish a standard curve. Such plasmid dilutions containing the target sequence were also used as positive reaction controls. In the qPCR assays, sterile ultrapure water was used as a negative control (Nuclease-Free Water, Promega®, United States). Plasmid copy number was determined by the following formula ((Xg/µL DNA/[plasmid size (bp) x 660]) x 6.022 x 10^{23} x plasmid copies/µL). Target copy number/µL was calculated based on 100% DNA extraction efficiency. Amplification efficiencies were calculated from the standard curve slope for each run based on the following equation (Bustin *et al.*, 2009): $E = \frac{10^{-1}}{slope}$. All positive samples or those whose replicate showed Cq (cycle of quantification) difference greater than 0.5 were repeated in triplicate according to Bustin *et al.* (2009).

Samples that presented quantification above 10^3 copies of a fragment of 16S rRNA in qPCR assays were submitted to cPCR targeting a fragment of 800 bp from 23SrRNA *Mycoplasma* sp. gene. cPCR was performed using the following primers 23S_HAEMO_F (5′-TGAGGGAAAGAGCCCAGAC-3′) and 23S_HAEMO_R (5′-GGACAGAATTTACCTGACAAGG-3′). The amplification mixture contained 1× PCR buffer, 1.5 mM of MgCl2, 0.2 mM of each dNTP, 0.4 mM of each primer, 2.5 U of Taq Platinum DNA Polymerase (Life Technologies®, United States), 5µL of DNA template, and ultrapure water q.s.p 25µL. Cycling conditions consisted of three 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. Ultra-pure sterile water (Life Technologies®, United States) was used as a negative control in PCR assay. Results were visualized in 1% agarose gel stained with ethidium bromide (Life Technologies®, United States) in UV transilluminator (ChemiDoc MP Imaging System, Bio Rad®, United States). Only five amplicons showing high-intensity bands were sequenced. Amplified products were purified using the 'Exosap IT' kit (Applied Biosystems®, United States) according to the manufacturer's recommendations. The sequencing of amplified products was performed by the dideoxynucleotide chain termination method conducted in ABI PRISM 3130 DNA Analyzer sequencer (Applied Biosystems®, United States).

Electropherogram quality was checked by Phred-Phrap software version 23 good quality when scoring Phred > 20 (Ewing *et al.*, 1998). Using the same software, the consensus sequences were obtained by the alignment of the sense and antisense sequences (Ewing *et al.*, 1998). BLASTn tool (NCBI GenBank database) was used to evaluate the percentages of identity and query coverage of the obtained 23S rRNA PH sequences with those previously deposited in the GenBank database (Mongruel *et al*., 2020). The sequences obtained were aligned with other 23S rRNA sequences retrieved from GenBank using MAFFT software, version 7 (Katoh *et al.*, 2017). First, the model "best of fit" was selected by the program jModelTest2 (version 2.1.6) on 11 XSEDE19, under the Akaike Information Criterion (AIC) (Darriba *et al.*, 2012). Maximum likelihood tree inference was performed with IQ-TREE software (Trifinopoulos *et al.*, 2016). *Bacillus cereus* and *Bacillus subtilis* were used as an outgroup. The phylogenetic tree edition and rooting (outgroup) were performed using the Treegraph 2.0 beta software.

Data were tabulated in Microsoft Excel software (Microsoft Windows®, 2010) while the epidemiological analyses used the "Complex Sample Frequencies" feature of the EpiInfo™ 7.7.2.16 software (Centers for Disease Control and Prevention, CDC, 2018). The Wilson method was adopted to calculate the confidence interval of the ratio of herds with infected animals.

RESULTS

A total of 501 whole blood samples were collected, After DNA extraction, PH positive determination and quantification were based on 100% extraction efficiency, since all samples were positive for mammal endogenous mammal glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) gene.

The samples were processed in 21 different plates with ranging with reaction efficiency (E) (93.1 to 105%), correlation coefficient (R^2) (0.931 to 0.999), slope (-3.95 to -3,009) and y-intercept (y-int) (32.601 to 40.252). After screening for suspected positive samples, they were run in triplicates. Twelve plates were used to determine the number of PH copies with reaction efficiency ranging from 93.6 to 105% and the R^2 between 0.931 and 0.955 (Table 1).

Table 1. qPCR parameters for Porcine Hemoplasma (*Mycoplasma* spp) based on the 16S rRNA gene of the *M. suis/M. parvum* and copy number quantification from swine blood DNA samples collected in herds in São Paulo State

Reaction	Efficiency	Coefficient of correlation (R^2)	Slope	y-intercept	Mean Cq	Quantification mean
1-130918	104	0,984	-3,23	39,292	NA	0
1-180419	104.5	0,931	-3,576	32,601	28.89	6,81 x10^3
1-240419	101	0,994	-3,297	39,603	32.96	7,74 x10^3
1-260419	101.8	0,992	-3,279	39,691	37.76	7,22
1-290419	99.5	0,994	-3,335	39,179	30.06	1,93 x10^4
2-130819	93.6	0,964	-3,486	39,23	35.29	1,36 x10^1
2-240419	99.9	0,989	-3,324	40,252	31.61	3,92 x10^3
2-250119	101.1	0,978	-3,296	36,885	8.89	7,5 x10^9
2-260419	104.8	0,991	-3,211	38,513	29.83	7,81 x10^5
3-080319	105	0,989	-3,009	36,151	32.52	6,27 x10^3
3-260419	104.9	0,995	-3,211	38,627	30.59	3,82 x 10^3
4-260419	101.6	0,987	-3,284	37,909	32.19	4,04 x 10^3

NA: positive sample that was not quantified due to the Monte-Carlo effect.

The Monte-Carlo effect prevented some samples from being quantified due to a technique limitation observed when the sample has a low DNA copy number (Bustin *et al.*, 2009). Among the other plates, the quantification cycles (Cq) ranged from 3.18- 39.56 and the number of PH copies ranged from 5,57 x10^{-2} to 2.23 x10^{10}. The highest quantification was 2.23 x10^{10} copies of PH per µL of DNA in one of the samples.

To the best of our knowledge, this is the first report on PH in technified farrow-to-finish herds in the state of Sao Paulo. Tab. 2 shows the prevalence in relation to the PH positive animals,

first considering all sampled animals (general) and divided by the growth stage. The results indicate a PH prevalence rate of 31.93% (161/501, CI: 25.67 - 37.12%), 161 of 501 pigs from the studied 20 farrow-to-finish technified herds were infected.

Table 2. Number of animals Porcine Hemoplasma (Mycoplasma spp) positive in farrow-to-finish technified swine herds in São Paulo State, per growth stage: nursery (45 days), growing (90 days), finishing (140 days), and slaughtering (180 days). 1. positive percentage (% pos); 2. Prevalence rate considering the sampling design (PV); 3. Standard error (SE); 4. Confidence interval (CI) – 95%; 5. Effect of sampling design (EF)

Groups	Examined	Positive	% pos[1]	PV. (%)[2]	SE (%)[3]	CI (%)[4]	EF[5]
General	501	161	32.14	31.39	2.87	25.67 – 37.12	1.918
Nursery	128	34	26.56	30.47	1.75	19.15 – 41.80	5.411
Growing	121	36	29.75	31.29	7.49	15.61 – 46.97	3.133
Finishing	64	17	26.56	26.18	6.21	13.19 – 39.17	1.256
Slaughtering	188	74	39.36	40.25	5.29	29.18 – 51.31	2.172

Considering the growth phases, the results show a higher number of positive animals in the slaughtering phase 40.25% ± 5.29 (CI: 29.18 - 51.31), followed by the growing phase 31.29% ± 7.49 (CI: 15.61 - 46.97) and nursery 30.47% ± 1.75 (CI: 19.15 - 41.8), with similar rates. Still, the lowest positivity rate was observed for 140-day-old animals, with 26.18% ± 6.21 (CI: 13.19 - 39.17) (Fig. 1). These values are not significantly different, as shown by the confidence interval (CI).

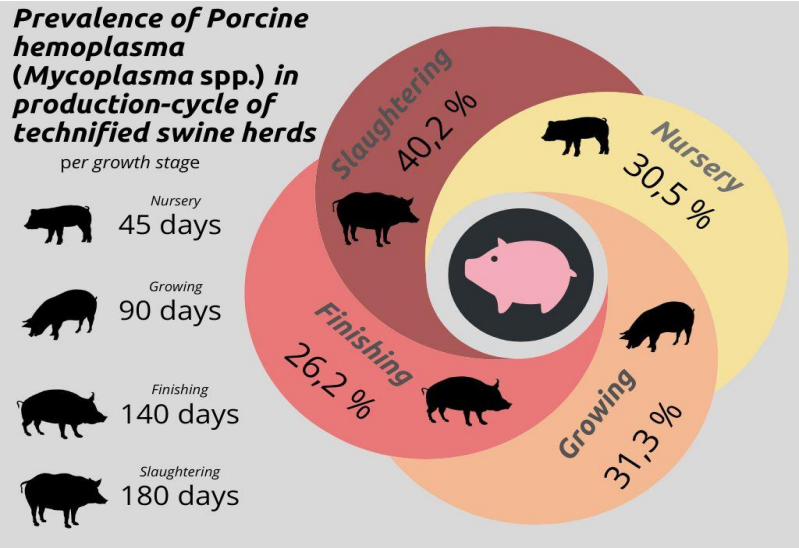

Figure 1. Graphic abstract of molecular prevalence of Porcine Hemoplasma (*Mycoplasma* spp) in production-cycle of technified swine herds.

Most of the herds were positive for PH (19/20 – 95%, IC: 76.39 – 99.11). Regarding the growth stage, the finishing and growing phases had the lowest positivity rates, 55% (CI: 34.24 - 74.18%) and 65% (CI: 43.29 - 81.88%), respectively. The nursery stage had 80% positivity (CI: 58.4 - 91.93%) while the slaughtering phase had the highest positivity (90% - CI: 69.90 - 97.21%).

Most positive samples could not have the PH 16S rRNA fragment copy number quantified due to low bacteremia (Tab. 3). The samples with low bacteremia and non-quantifiable due to the Monte-Carlo effect represented 45.3% (73/161) in general, and 58.8% (20/34) in the nursery, 57.1% (20/35) in the growing, 37.5% (6/16) in the finishing, and 35.53% (27/76) in the slaughtering phases.

Table 3. Porcine Hemoplasma (*Mycoplasma* spp) positivity in farrow-to-finish technified swine herds in the state of São Paulo determined by qPCR according to the copy number of a Porcine hemoplasma (PH) 16S rRNA *M. suis/M. parvum* gene fragment /µL

PH 16S rRNA nº of copies/µL DNA	General		Growth stage							
			Nursery 45 days		Growing 90 days		Finishing 140 days		Slaughtering 180 days	
Bacteremia	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
NA	73	45.3	20	58.8	20	57.1	6	37.5	27	35.53
10^{-1}	1	0.6	0	0.0	1	2.9	0	0.0	0	0.0
10^{-2}	7	4.3	2	2.9	0	0.0	0	0.0	6	7.9
10^{0}	23	14.3	5	14.7	2	5.7	0	0.0	16	21.1
10^{1}	17	10.6	2	5.9	3	8.6	4	25.0	8	10.5
10^{2}	6	3.7	1	2.9	1	2.9	1	6.2	3	3.9
10^{3}	21	13.0	3	8.8	2	5.7	5	31.2	11	14.5
10^{4}	7	4.3	1	2.9	3	8.6	0	0.0	3	3.9
10^{5}	2	1.2	0	0.0	1	2.9	0	0.0	1	1.3
10^{6}	1	0.6	1	2.9	0	0.0	0	0.0	0	0.0
10^{7}	1	0.6	0	0.0	1	2.9	0	0.0	0	0.0
10^{8}	1	0.6	0	0.0	1	2.9	0	0.0	1	1.3
10^{9}	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
10^{10}	1	0.6	0	0.0	0	0.0	0	0.0	0	0.0
TOTAL	161	100	34	100	35	100	16	100	76	100

NA: positive sample not quantifiable due to the Monte-Carlo effect.

Absolute quantification of the PH 16S rRNA gene fragment varied between the phases as follows, nursery (5.61×10^{-1} – 6.08×10^{6}), growing (5.57×10^{-2} – 2.23×10^{10}), finishing (1.56×10^{1} – 6.13×10^{3}) and slaughtering (1.32×10^{-1} – 1.11×10^{8}).

Five sequences obtained at cPCR for 23S rRNA showed high-intensity bands at agarose gel and were sequenced. All samples showed 100% of identity with three different sequences of *M. parvum*, one sequence from Indiana strain (NR121958) and two *M. parvum* sequences from Brazil/Goiás (MT232813/MT232820). Phylogenetic analysis clustered the sequences detected in the present study in a large clade with other sequences of *M. parvum* detected in Brazil and the USA, apart from the clade of *M. suis* (Fig. 2).

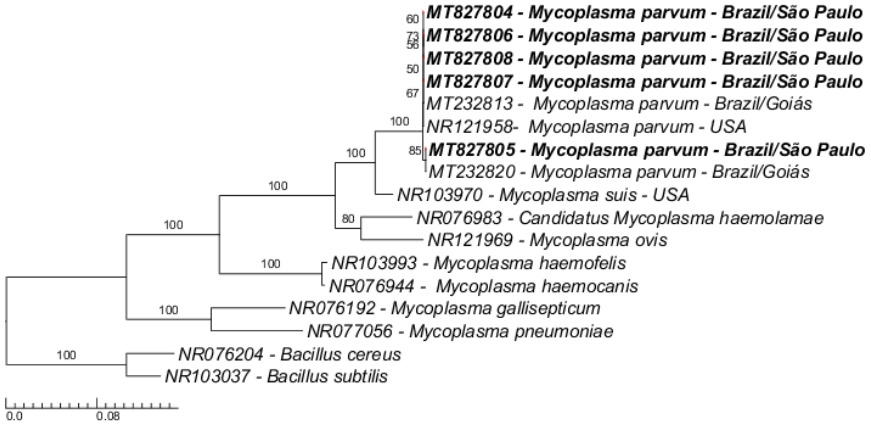

Figure 2. Phylogenetic tree based on Porcine Hemoplasma *(*M*ycoplasma* spp) 23S rRNA sequences. Phylogenetic analysis based on the Maximum likelihood, and the TrN + G evolutionary model. Accession numbers are indicated in the sequences. Porcine hemoplasmas sequences detected in the present study are highlighted in bold. The numbers at the nodes correspond to bootstrap values accessed with 1,000000 generations. *Bacillus cereus* and *Bacillus subtilis* were used as outgroup.

DISCUSSION

This molecular study is the first to detect Porcine Hemoplasma (*Mycoplasma* spp) rRNA in the entire production cycle and to report the molecular occurrence of hemoplasmas in pigs in the state of São Paulo, Southeastern Brazil. Although not all swine farmers adhered to the study, our investigation provided an important sampling towards the PH presence in the technified production system of São Paulo state.

The qPCR technique used in this study has high sensitivity and specificity for PH, was standardized by Guimarães *et al.* (2011) and widely reproduced by other authors (Toledo *et al.*, 2016; Dias *et al.*, 2019; Gatto *et al.*, 2019; Petri *et al.*, 2020; Sonálio *et al.*, 2020). The results indicate a PH prevalence rate of 31.93% (161/501, CI: 25.67 - 37.12%): 161 of 501 pigs from the studied 20 farrow-to-finish herds were infected. Similar to our results, the PH prevalence rate reported in many parts of the world ranges from 2.7% to 86% (Yuan *et al.*, 2009; Guimarães *et al.*, 2011; Song *et al.*, 2014; Toledo *et al.*, 2016; Fu *et al.*, 2017; Gatto *et al.*, 2019; Seo *et al.*, 2019; Petri *et al.*, 2020; Sonálio *et al.*, 2020).

Song *et al.* (2014) stated that prevalence rates vary based on geographical distribution, sampling time and design, farm-related characteristics (such as size, mechanization, sanitary conditions, etc.), and growth stage (Hoelzle, 2008).

Compared to non-technified swine herds, the prevalence of infected animals seen in our study (31.93%) was lower than the 76.19% (112/147) in Mossoró, Rio Grande do Norte, Brazil by Toledo *et al.* (2016), in the slaughtering phase. The São Paulo herds sampled are intensive and technified compared to the extensive northeastern herds with no access to nutritional programs, adequate facilities, breeding programs, and standardization of zootechnical management. Yuan *et al.* (2009) state that commercial pig cultures with poor zootechnical-sanitary conditions provide good conditions for the occurrence of *M. suis* vectors and other transmission and infection sources.

In the Zhejiang region of China, Fu *et al.* (2017) also found a similar prevalence rate in the growing phase, 42.4% (101/238), but considering that the age interval between 30 and 120 days overlaps the ranges determined by this study, the results possibly corroborate our findings. Seo *et al.* (2019) did not find a statistically significant difference between breeding and fattening pigs, even though a higher *Mycoplasma* prevalence was found in breeding pigs.

Brazilian laws allow some classes of antibiotics to be included in the feed as growth promoters (Gavioli *et al.*, 2013). However, to meet the grace period, the antibiotics are suspended between the finishing (140 days) and slaughtering (180 days) periods, thus, allowing recrudescence of the disease and an increase of the infection rate.

The infection rates are high in breeders, ranging from 79.7 to 80% in samples from southern Brazil (Paraná, Santa Catarina, and Rio Grande do Sul states) (Guimarães *et al.*, 2011; Gatto *et al.*, 2019). In China, Song *et al.* (2014) reported a prevalence of 48.2%. Although our study did not sample breeders, it should be emphasized that PH can be transmitted by the direct contact between newborn piglets and infected sows, which act as reservoirs of the agent on the farm due to the long period in the productive cycle (Hoelzle, 2008). Thus, the disease spreads to the litter in the productive system through disputes, cannibalism, vectors, among others.

Based on seroprevalence studies per age group in the technified herds of the Hubei region (China), Song *et al.* (2014) found an increased prevalence at 21 days (13%) compared to pigs older than 71 days. Similarly, in the present study, a prevalence of 30.47% was observed for 45-day old animals and 40.25% for 180-day old pigs in the slaughtering phase.

Yuan *et al.* (2009) investigated the positive correlation of *M. suis* with age, sex, and location, in pigs from poorly-technified herds in Shanghai (China) and reported no significant difference in prevalence rates for the variables analyzed. However, despite the high prevalence of 86% (148/172), the above authors did not report on the prevalence rates per age group. Complementing the statement by Gatto *et al.* (2019), that the differences in prevalence among rearing stages can be explained by chronic infection behavior and low bacteremia.

Since Song *et al.* (2014) also found a correlation between age group and prevalence for positive animals, the age group is of fundamental importance for understanding porcine hemoplasmosis epidemiology. The age range can be considered as a measurement of the exposure time to the agent since PH presence and infection increase with the advancing developmental stage. Additionally, the stress-related challenges inherent to swine breeding favor the establishment of new infections and disease resurgence (Wu *et al.*, 2006; Hoelzle, 2008; Song *et al.*, 2014).

In the literature, Gatto *et al.* (2019) reported a 100% infection rate, therefore, all sows (53/53) sampled from swine herds in southern Brazil were positive. In China, Song *et al.* (2014) found 95.65% positivity for PH in 66/69 of the herds investigated. Still, in the Shanghai region in China, Yuan *et al.* (2009) reported that 49% (32/65) have infected animals, however, the results were not classified by growth stage or weight whereas the different prevalence rates were justified by the different sampling methodology, geographic distribution, and PH copy number (bacteremia). Among breeders from technified rearing herds in southern Brazil, absolute quantifications of the same PH gene fragment (bacteremia) were also variable (Gatto *et al.*, 2019).

Guimarães *et al.* (2011) stated that the PH is fundamental to indicate the presence of the agent in the farm, given that swine hemoplasmosis presents nonspecific clinical signs in the acute phase or resurgence of the chronic cases. Also, bacteremia levels may fluctuate throughout the month associated with several stress-causing factors in animals or indiscriminate inclusion of antibiotics in food (Wu *et al.*, 2006; Gatto *et al.*, 2019). It is noteworthy that despite low bacteremia, positive animals can be a significant source of infection since they act as chronic carriers, silent and present false-negative in blood smears and conventional PCR assays (Hoelzle, 2008).

The phylogenetic tree based on the 23S rRNA gene of hemoplasmas showed that the five sequences obtained corresponded to *M. parvum*. The sequences of this study were closely related to two *M. parvum* recently reported in pigs from Brazil/Goiás (Petri *et al.*, 2020; Sonalio *et al.*, 2020) and Indiana (USA) strain of *M. parvum* (Nascimento *et al.*, 2014).

CONCLUSIONS

To the best of our knowledge, this is the first report on Porcine Hemoplasma (*Mycoplasma* spp) in technified farrow-to-finish herds by growth stages. Also, it brings the first report of PH in pigs in the state of São Paulo, Brazil. The highest prevalence in the slaughtering stage may be associated with antibiotic suspension between the finishing (140 days) and slaughtering (180 days) periods, thus, allowing recrudescence of the bacteremia and an increase in infection rate. The low bacteremia, which was estimated by a qPCR assay targeting a fragment of 16S rRNA gene, observed in pigs evaluated reinforces the chronic characteristic of PH with healthy animals, positive for mycoplasma but without clinical signs. Sequencing and phylogenetic analysis of five selected samples showed the occurrence of *M. parvum* in southeast Brazil. Our observations about the production stage and the agent spatial distribution may call for future studies regarding the presence and behavior of this disease in other neighboring states.

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