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Short Communication

Molecular detection of *Mycoplasma suis* in extensive pig production systems in the State of Maranhão, northeast Brazil

Detecção molecular de *Mycoplasma suis* em suínos de criações extensivas no estado do Maranhão, nordeste do Brasil

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Abstract

Mycoplasma suis is a bacterium that causes hemoplasmosis in pigs. This agent is capable of adhering to the surface of porcine erythrocytes, inducing structural changes on these cells. In Brazil, there are few reports about the disease, its causal agent, and the economic impact of this pathogen on pig production systems and farm sanitation. The present study aimed to investigate the occurrence of *M. suis* in extensive swine farms located in the counties of Itapecuru Mirim, Santa Rita and Rosario, State of Maranhão, northeast Brazil. For such purpose, 64 blood samples of pigs from these facilities were tested for *M. suis* using a 16S rRNA gene-based quantitative real-time PCR (qPCR); 82.3%, 65.2% and 25% of blood samples of swine from farms in the cities of Itapecuru Mirim, Santa Rita and Rosario were positive for *M. suis* by qPCR, respectively. This study shows, for the first time, that *M. suis* circulates in pig populations from the state of Maranhão, Northeast Brazil.

Keywords: Swine, *Mycoplasma suis*, qPCR, hemoplasmosis.

Resumo

Mycoplasma suis é uma bactéria que causa a hemoplasmose em suínos. Este agente é capaz de se aderir à superfície dos eritrócitos de suínos, ocasionando deformações estruturais nestas células. No Brasil, poucos são os relatos acerca do parasita, da infecção e de seus impactos econômicos nas esferas produtiva e sanitária. O objetivo deste estudo foi investigar, por meio da PCR em tempo real quantitativa (qPCR) baseada no gene 16S rRNA, a ocorrência de *M. suis* em 64 amostras de sangue de suínos de criações extensivas dos municípios de Itapecuru Mirim, Santa Rita e Rosário, localizados no estado do Maranhão. Foram obtidos um percentual de 82,3%, 65,2% e 25% de amostras positivas na qPCR para *M. suis* nos municípios de Itapecuru Mirim, Santa Rita e Rosário, respectivamente. Este estudo mostra que *M. suis* circula entre os suínos de criações extensivas no estado do Maranhão.

Palavras-chave: Suínos, *Mycoplasma suis*, qPCR, hemoplasmose.

Introduction

Mycoplasma suis, the etiologic agent of porcine hemoplasmosis, is a pathogen showing a worldwide distribution (MESSICK, 2004) and causing significant economic losses in the pig industry (GUIMARÃES et al., 2007). Considering that Brazil occupies the fourth place in the pork export in the world (BRASIL, 2016), knowledge about the occurrence of this pathogen in the pig production systems in our country and its impacts in the sanitary and economic sphere are of paramount importance. This agent belongs to the group of hemotrophic mycoplasmas, or hemoplasmas, that comprises non-cultivable bacteria that lack a cell wall. They are capable of binding to the surface of erythrocytes from a number of mammals causing structural changes on these cells (HOELZLE, 2008).

Regarding the epidemiology of swine hemoplasmosis, mechanical transmission of this bacterial organism occurs through syringes,

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needles or contaminated surgical instruments (HENRY, 1979). Additionally, other routes of transmission have been speculated, since the agent has been detected in urine samples, water used for drinking, vaginal and nasal secretions, and environmental dust samples (DIETZ et al., 2014). Moreover, congenital infection has been reported in newborn piglets (vertical transplacental transmission) (HENDERSON et al., 1997).

Under natural conditions, the incubation period of the disease may vary greatly, and is dependent on individual susceptibility, severity of infection, and physiological stress response of each animal. Infected animals may be asymptomatic for months until the disease becomes clinically apparent. Hemolytic anemia is one of the clinical signs in affected pigs, and may ultimately result in death. Suckling piglets and finishing swine are preferentially affected, but the disease can also occur in growing pigs and sows, even though many infected animals often do not show any clinical signs (HOELZLE, 2008).

Direct blood smear examination under the light microscope is routinely used for the diagnosis of *M. suis* infection. Due to the low sensitivity and specificity of this method associated with the fact that blood samples are infrequently submitted to diagnostic laboratories for disease surveillance and monitoring, porcine hemoplasmosis is frequently underdiagnosed in many swine populations (STRAIT et al., 2012). On the other hand, molecular techniques, such as quantitative real time PCR assays (qPCR), show high sensitivity and specificity, allowing accurate detection of hemotrophic mycoplasmas in blood samples. Considering the low risk of contamination, high levels of standardization, specificity, sensitivity, reproducibility, and reliability presented by qPCR, this technique should be used in the diagnosis of *M. suis* infection (GUIMARÃES et al., 2011).

Considering that *M. suis* has only been detected in pigs from the states of Santa Catarina and Rio Grande do Norte so far, additional studies assessing the prevalence of this agent in swine herds in Brazil are needed in order to estimate how spread is this pathogen in swine production systems in the country. Therefore, the present study aimed to investigate the occurrence of *M. suis* in extensive swine farms located in the State of Maranhão, northeast Brazil, using molecular techniques.

Material and Methods

Study population

Between August 2016 and August 2017, blood samples were collected, by convenience, from 64 pigs during the dry season. The sampled animals (16 males and 48 females) age ranged from 1 to 3 years. The sampling was performed in extensive swine farms located in the counties of Itapecuru Mirim (17 samples) (Latitude: -3.39501, Longitude: -44.3601 3°23'42" South, 44°21'36" West), Santa Rita (23 samples) (Latitude: 3°23'42" South, Longitude: 44°21'36" W), and Rosario (25 samples) (Latitude: 2°56'24" South, Longitude: 44°14'27" West), state of Maranhão, northeast Brazil. Blood samples collected by venipuncture in vaccutainer tubes containing EDTA-anticoagulant were stored in sterile 1.5 mL microtubes and kept at -20 °C until molecular analysis.

The research proposal was approved by the Animal Experimentation Ethics Committee (AEEC) from the Maranhão State University (UEMA) program in Brazil, Protocol Nº 004/2015 AEEC/UEMA).

DNA extraction and conventional PCR assay (cPCR) for the endogenous glyceraldehyde-3-phosphate dehydrogenase gene (gapdh)

DNA extraction from whole blood was performed using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, California, USA), according to the manufacturer's recommendations. Extracted DNA samples were then stored at -20 °C until PCR analysis.

An endogenous (internal) control was used in order to rule out any false negative results due to the presence of PCR inhibitors. For such purpose, DNA samples were submitted to a previously described conventional PCR assay aiming to amplify the mammalian *gapdh* (glyceraldehyde-3-phosphate dehydrogenase) gene (endogenous control of the reaction) (BIRKENHEUER et al., 2003). The amplified products were subjected to horizontal electrophoresis in ethidium bromide-stained agarose gel and visualized under an ultraviolet light (UV) transilluminator. Images were analyzed using the software Chemi Doc Imaging System (BioRad®).

16S rRNA gene-based quantitative real-time PCR (qPCR) for the detection of M. suis

DNA samples showing positive results in *gapdh*-PCR assay were submitted to a previously described TaqMan quantitative real-time PCR (qPCR) based on 16S rRNA for the detection of *M. suis* (GUIMARÃES et al., 2011). Serial dilutions with different concentrations of plasmid DNA containing the 16S rRNA target sequence were performed in order to construct the standard curve (2.0×10^7 copies/µL at 2.0×10^0 copies/µL). The number of plasmid copies was calculated using the formula (Xg/µL DNA/[plasmid (pb) size x 660]) x 6.022×10^{23} x plasmid copies/µL. The amplification efficiency (E) was calculated from the slope of the standard curve in each run using the following formula: $E = 10^{-1/slope}$. To determine the limit of detection and quantification of the TaqMan assay, standard curves were generated by serial dilutions from 10^7 to 10^0 copy numbers of plasmids.

Results

All swine blood samples were positive in cPCR assays based on *gapdh* gene, ruling out the occurrence of false negative results. Out of 64 sampled animals, 35 (54.7%) were positive for *M. suis* by qPCR: 14 (82.3%) from the city of Itapecuru Mirim, 15 (65.2%) from Santa Rita, and 6 (24%) from the city of Rosario. The Table 1 shows the mean of quantification cycles (Cq) and number of 16SrRNA-*M. suis* copies/µL of DNA from blood samples of pigs raised in farms located in the aforementioned counties.

The samples were processed in two different plates with reaction efficiency (E), slope and R^2 of 90.4% and 91.1%; -3.577 and -3.555;

Table 1. Mean Cqs, number of copies/µL, and reaction parameters (efficiency, correction coefficient, R^2, and y-intercept) for DNA extracted from blood samples of swine raised in farms located in the counties of Itapecuru Mirim, Santa Rita, and Rosário, State of Maranhão, Brazil.

Sample	Mean Cqs	Quantification mean	Efficacy	R^2	Slope	Y-interceptor
	25.59	5.89×10^4				
	22.59	4.36×10^5				
	27.15	2.07×10^4				
	20.50	1.77×10^6				
	24.29	1.43×10^5				
	26.39	3.48×10^4				
	26.50	3.23×10^4				
Itapecuru Mirim	25.83	5.22×10^4	91.1%	0.992	-3.555	42.050
	32.54	6.07×10^2				
	26.77	2.67×10^4				
	28.52	8.31×10^3				
	26.34	3.72×10^4				
	22.17	5.78×10^5				
	28.86	6.65×10^3				
	26.08	1.66×10^4				
	28.32	7.15×10^3				
	27.95	9.05×10^3				
	25.82	1.71×10^4				
Santa Rita	27.44	1.27×10^4	91.1%	0.992	-3.555	42.016
	31.20	1.11×10^3				
	33.10	3.22×10^2				
	26.28	2.67×10^4				
	28.65	5.74×10^3				
	30.50	8.36×10^2				
	29.13	4.21×10^3				
	34.35	1.44×10^2				
	24.79	7.05×10^4				
	31.36	1.01×10^3				
	33.02	3.41×10^2				
	32.10	3.01×10^2				
	37.03	1.26×10^2				
	32.76	4.01×10^2				
Rosário	30.54	8.12×10^2	90.6%	0.989	-3.57 0	40.935
	23.48	7.76×10^4				
	33.90	9.37×10^2				

0.989 and 0.992, respectively. The range of Cq and quantification values were 20.50-37.03 and $1.26 \times 10^1 - 1.77 \times 10^6$, respectively.

Discussion

In recent years, *M. suis* has been detected in pig farms in China (YUAN et al., 2009), Germany (RITZMANN et al., 2009; HOELZLE et al., 2010) and Brazil (GUIMARÃES et al., 2007; TOLEDO et al., 2016). In this sense, Yuan et al. (2009) reported the occurrence of *M. suis* of 86% in pigs and 49% in veterinarians and contact individuals with these animals in China, emphasizing the high occurrence of the agent in question and suggesting a possible zoonotic potential of this hemoplasma species. Interestingly, the high percentage of positivity for *M. suis* in pigs sampled in the present study (54.7%) was similar to that found in extensive pig farms in the state of Rio Grande do Norte (76.19%), northeastern Brazil (TOLEDO et al., 2016), where climatic and management conditions were similar to those of the present study.

The reuse of needles was incriminated as an important risk factor for the occurrence of *M. suis* in pig farms in China (YUAN et al., 2009). In the present study, although risk factors associated with *M. suis* infection were not investigated, syringe reuse and needle sharing were routine practices on farms where animals were sampled (data not shown) and may have contributed to transmission routes of *M. suis*. Further studies should be conducted to evaluate the risk factors associated with the high transmission of *M. suis* in swine in northeastern Brazil, in order to develop prophylaxis measures for swine hemoplasmosis.

According to Song et al. (2014), *M. suis* seroprevalence in sows in the Chinese province of Hubei was higher during the summer and fall than during the spring and winter seasons. These findings may be related to the period of greatest density and activity of arthropod vectors in the environment, which favor the transmission of the pathogen. This fact indicates that the transmission of *M. suis* seems to be more common in warmer weather, since mosquitoes thrive in higher temperatures. This may explain the high percentages of positivity for *M. suis* found in our survey, since high temperatures are found in the state of Maranhão all over the year.

The different rates of occurrence of *M. suis* in pigs reported in several studies may be related to the sensitivity of the molecular techniques used. In the present work, the qPCR technique was used, which demonstrates higher specificity and sensitivity when compared to the conventional PCR assays (GUIMARÃES et al., 2011). Guimarães et al. (2007) carried out a study in the State of Santa Catarina, southern Brazil, where 18.2% and 33.1% of swine blood samples were positive by conventional PCR and Southern blotting, respectively, emphasizing the low sensitivity of the former technique. Ritzmann et al. (2009) demonstrated in their study that of the 160 samples analyzed, 17 (10.6%) animals reacted as PCR-positive for *M. suis*, with a mean in-house prevalence of 41.5% and a mean bacterial load of 5.31×10^4 copies/µL.

In the present study, the absolute quantification of 16S rRNA-*M. suis* ranged from $1.26 \times 10^1 - 1.77 \times 10^6$ copies/µL, showing values lower than 10^3 in 20% of positive samples. These results show that low bacteremia may occur in quite a few animals, precluding accurate diagnosis using stained-blood smears and conventional PCR based methods. Therefore, the qPCR assay could be considered the gold standard for detection of hemoplasmas in swine herds. Despite the low bacteremia found in 20% of sampled pigs, estimated by qPCR absolute quantification, these animals may act as chronic carriers and play a role as important source of infection for other animals in a herd.

Finally, additional studies to verify the occurrence and risk factors associated with *M. suis* infection in herds in other regions of the country are of great importance, given the prominent position occupied by Brazilian swine production in the world. Additionally, the zoonotic potential of the *M. suis* genotypes found in Brazil should be further investigated.

Conclusion

This study reports the occurrence of *M. suis* in non-technical pig farms in the state of Maranhão, precisely in the municipalities of Itapecuru Mirim, Santa Rita and Rosario.

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