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First molecular detection of *Mycoplasma suis* in the pig louse *Haematopinus suis* (Phthiraptera: Anoplura) from Argentina

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ABSTRACT

Porcine haemoplasmosis caused by *Mycoplasma suis* affects the global pig industry with significant economic losses. The main transmission route of *M. suis* is through the blood and some haematophagous arthropods, like flies and mosquitoes, could be the vectors to this pathogen. However, the presence of *M. suis* in pig haematophagous ectoparasites in natural conditions has not yet been studied. The most frequent ectoparasite in pigs is the blood-sucking louse *Haematopinus suis*, an obligate and permanent parasite. Therefore, this work aims to study the occurrence of *M. suis* in *H. suis* samples from both domestic and wild pig populations from Argentina; using the 16S rRNA gene. A total of 98 sucking lice, collected from domestic and wild pigs from Buenos Aires Province in central Argentina, were examined. We found *M. suis* DNA in 15 *H. suis* samples (15.30%). Positive lice were detected from all studied populations. This is the first report of *M. suis* presence in *H. suis*, being also the first detection in a pig ectoparasite species. We conclude that *H. suis* could serve as a mechanical vector for *M. suis*. This information not only extends the knowledge about the pathogen spectrum potentially transmitted by *H. suis*, but may be also useful in epidemiological studies about *Mycoplasma*.

1. Introduction

Haemotropic mycoplasmas or haemoplasmas are highly specialized hemotrophic bacteria which lodge in erythrocytes and cause deformity and damage to these blood cells (Neimark et al., 2001). In particular, *Mycoplasma suis* is the etiological agent of the “porcine haemoplasmosis” (porcine eperythrozoonosis), an infectious anaemia that affects domestic and wild pigs (*Sus scrofa*: Suidae) (Messick, 2004; Hoelzle, 2008; Felder et al., 2011). Their symptoms include anaemia and jaundice, general deficient development and growth retardation. Also, in females of reproductive age, decreased reproductive efficiency was observed, generating irregular cycles, anestrus, failures in conception and abortions (Messick, 2004; Felder et al., 2011; Pintos, 2016).

M. suis-infections have been reported worldwide and cause important economic losses in swine production (Hoelzle, 2008; Pintos, 2016). Besides, recent studies have reported that humans in close contact with *M. suis*-infected pigs can also be infected with these organisms (Yuan et al., 2009), suggesting *M. suis* as a potential zoonotic pathogen.

In Argentina, the swine production has increased about 80% in the last decade, becoming one of the main components of livestock production (Brunori, 2013). In this country, the largest area of swine farming is Buenos Aires province, where there is a strong interaction between domestic populations and feral pigs (Merino and Carpinetti, 2003). The main swine production system in Argentina is the extensive system “to field”, where the poor hygiene conditions, precarious facilities and poor sanitary controls result in a higher ectoparasites frequency, such as the louse *Haematopinus suis* (Phthiraptera: Anoplura: Haematopinidae) (Prieto et al., 1991). This blood-sucking louse is an obligate and permanent parasite in pigs (Florence, 1921). Regarding its epidemiologic role, several viruses and bacteria have been detected in *H. suis* samples, such as *Poxvirus variolae*, etiological agent of the "swine pox", *Erysipelothrix rhusiopathiae*, causative bacteria of the "bad red" and *Rickettsia slovaca*, a spotted fever group *Rickettsia* (Thibault et al., 1998; Zeroual et al., 2018).

The reported transmission routes of *M. suis* in pigs are through blood and placenta (Pintos, 2016). Concerning the blood route, a common scenario is the mechanical transmission by needles and surgical instruments contaminated with infected blood (Pintos, 2016). In addition, haematophagous arthropods, such as mosquitoes and flies, were shown to contribute to the *M. suis* infection spread (Song et al., 2014). Although the association between *S. scrofa* and the blood-sucking louse *H.*

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suis is recorded worldwide (Durden and Musser, 1994), to date the presence of *M. suis* in this louse, in natural conditions, has not been registered. Therefore, this work aims to investigate the occurrence of *M. suis* in sucking lice from domestic and feral pig populations from Buenos Aires province, Argentina, using the 16 s rRNA gene.

2. Materials and methods

2.1. Sites of study and lice collection

Between 2014 and 2016 lice were collected from 74 domestic and feral pigs (*S. scrofa domestica*) in the following four locations from Buenos Aires province, Argentina: three domestic pig populations ($n_{pigs} = 58$), 1- Acevedo (33°43′00″ S; 60°25′00″ W), 2- General Viamonte (34°56′00″ S; 61°10′00″ W) and 3- Junín (34°34′10″ S; 60°57′35″ W), and a feral pig population ($n_{pigs} = 16$) from Bahía Samborombón (36°00′ S; 57°12′ W).

The lice were removed using combs, tweezers or by hand. All lice collected from the same infested pig were stored in 96% ethanol into pre-labeled vials for subsequent identification and molecular study in the Laboratory of the Centro de Bioinvestigaciones from CIT NOBA (Pergamino, Argentina). The lice were identified observing its morphology in an optic microscope (Primo Star ZEISS Axio) and stereoscopic microscope (Bestscope, Bs-3040), according to standard morphological keys (Durden and Musser, 1994) and original descriptions (Florence, 1921).

2.2. DNA extraction

The pig lice collected were rinsed twice in sterile water for 15 min and then dried on sterile filter paper. Then, genomic DNA was extracted from individual louse using a dissecting needle and following the CTAB protocol (Doyle and Doyle, 1987). The DNA from each specimen was eluted in 50 µl of Tris-EDTA buffer solution and stored at −20 °C under sterile conditions.

2.3. Detection of 16S rDNA gene of M. suis in H. suis and sequence analysis

Identification of *Mycoplasma* was performed by nested PCR for 16S ribosomal RNA gene (16S rRNA), based on the primers descripted by Pintos (2016). These primers integrate the hypervariable region V3, nucleotide site that provides more useful information for phylogenetic and taxonomic studies in bacteria. In the first reaction, a 444 bp fragment was amplified by external primers MSuisEP1f, 5′-AAWGGAGGC-TGCCGMAAGGT-3′ and MSuisEP1r, 5′-CACCGCGAACACTTGTTAAGC-AARTA-3′, while a fragment of 366 bp was amplified for the second reaction, using the internal primers MSuis-EP2f, 5′-AGRTMGTTGGAG-AGGTAADGGCT-3′ and MSuis-EP2r, 5′-AYTTTTAACAAKGRATACACA-YTTCA-3′.

PCR reaction was set to a final volume of 20 µL containing: 25–100 ng of template DNA, 1.5 mM Cl2Mg, 0.2 µM of each primer, 0.2 mM of each dNTP, 1X reaction buffer, 0.5U of Taq T-Plus DNA polymerase, and ultra-pure sterile water to complete the final volume. Thermocycling conditions were set at 92 °C for 5 min, followed by 35 cycles at 92 °C for 30 s, 45 °C for 30 s, and 74 °C for 40 s, with a final extension of 74 °C for 4 min. In the first round of amplification, 2 µl of genomic DNA were used, and in the second round 1 µl of DNA from the first reaction was used. All amplifications were performed in conjunction with a positive (DNA of *M. suis* provided by Servicio Central de Laboratorio del Hospital de Clínicas (IGEVET), Universidad Nacional de La Plata) and a negative (distilled water) control. In those samples in which the PCR was positive, we proceeded to purify using 10U of Exonuclease I and 1U of FastAp thermosensible alkaline phosphatase, incubating at 37 °C for 15 min and then at 85 °C for another 15 min to stop the reaction, and finally sequenced by Macrogen Co. Ltd. (South Korea). The prevalence of *Mycoplasma* in pig lice was calculated as the percentage of individuals in which we detected *Mycoplasma* DNA.

The sequences obtained were analyzed and edited manually using the BioEdit program (Hall, 2004). Subsequently, a comparison for each sequence was made using the BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi), in order to determine the identity with the sequences located in the GenBank nucleotide database (https://www.ncbi.nlm.nih.gov/genbank/). Then, we compared our amplifications products with *Mycoplasma* sequences from the Genbank database, where 29 GenBank sequences were taken from other Haemoplasma species (*M. wenyonii, M. ovis, M. haemocervae, M. erythrodidelphis, M. haemominutum, M. coccoides, M. haemofelis, M. iowae, M. pneumoniae, M. pirum, M. hominis, H. muris, H. canis*) and *Bacillus subtilis* used as an outgroup, in order to build a phylogenetic tree based on Neighbor-Joining (NJ) and Maximun-Likelihood (ML) algorithms implemented in MEGA v.6 (Tamura et al., 2013). For the NJ phylogeny, the degree of confidence assigned to the nodes was assessed by boot-strapping with 1000 replicates. These analyses were done to visualize the relationship between the sequences of *M. suis* and other Haaemoplasma species, as well as to discern the taxonomic level of the sequences studied.

3. Results

From the 74 examined pigs (58 domestic pigs and 16 feral pigs), a total of 98 sucking lice were recovered. The prevalence was 70% ($P_{domestic\ pigs}$ = 62%; $P_{feral\ pigs}$ = 100%). Lice were morphologically identified as *Haematopinus suis*. Out of 98 *H. suis* samples, 15 (15.30%) were found to be positive for *Mycoplasma* spp. in the reaction of nested PCR for the 16S rRNA gene (Table 1). Positive lice were detected from all study populations, both domestic and feral pigs.

The length of the 16S rRNA gene sequences amplified from *Mycoplasma* was 337 bp, and it is available in the GenBank nucleic acid sequence repository (http://www.ncbi.nlm.nih.gov/genbank). The BLASTn analysis of the obtained 16S rRNA gene fragment, showed identities between 98 and 99% with the species *M. suis* for the 100 best hits, with query cover values from 94 to 98%, confirming that the species detected in this study corresponds to *M. suis*.

Phylogenetic inferences, done both with NJ and MV presented identical topology, therefore only the NJ trees are shown (Fig. 1). The phylogenetic tree supports the previously reported results, the 15 sequences from Argentina were contained in a single “clade” along with those of *M. suis* taken from GenBank, while separated from the rest of the species of the Haemoplasmas genus. Our results showed that *M. suis* species is found within the "Haemominutum group", along with the *M. wenyonii*, *M. ovis* and *M. haemominutum* species and separated from the "Haemofelis group" (*M. haemocanis*, *M. haemofelis*) and "Pneumoniae group" (*M. iowae*, *M. pneumoniae*, *M. pirum*), finding the Haemominutum and Haemofelis groups closest to each other, in contrast to the more distant Pneumoniae group.

4. Discussion

To the best of our knowledge, this constitutes the first report of the molecular evidence of *M. suis* DNA in the *H. suis* louse in natural

Table 1
Mycoplasma suis in *Haematopinus suis* from Buenos Aires province, Argentina: Sample origin, nested PCR results and prevalence.

Localities of collection	Host	No. of lice *H. suis*	No. of PCR-Positive samples	Prevalence of *M. suis* (%)
Acevedo	Domestic pig	16	3	18.75
Junín	Domestic pig	36	5	13.88
General Viamonte	Domestic pig	17	5	29.41
Bahía Samborombón	Feral pig	29	2	6.89
Total		**98**	**15**	**15.3**

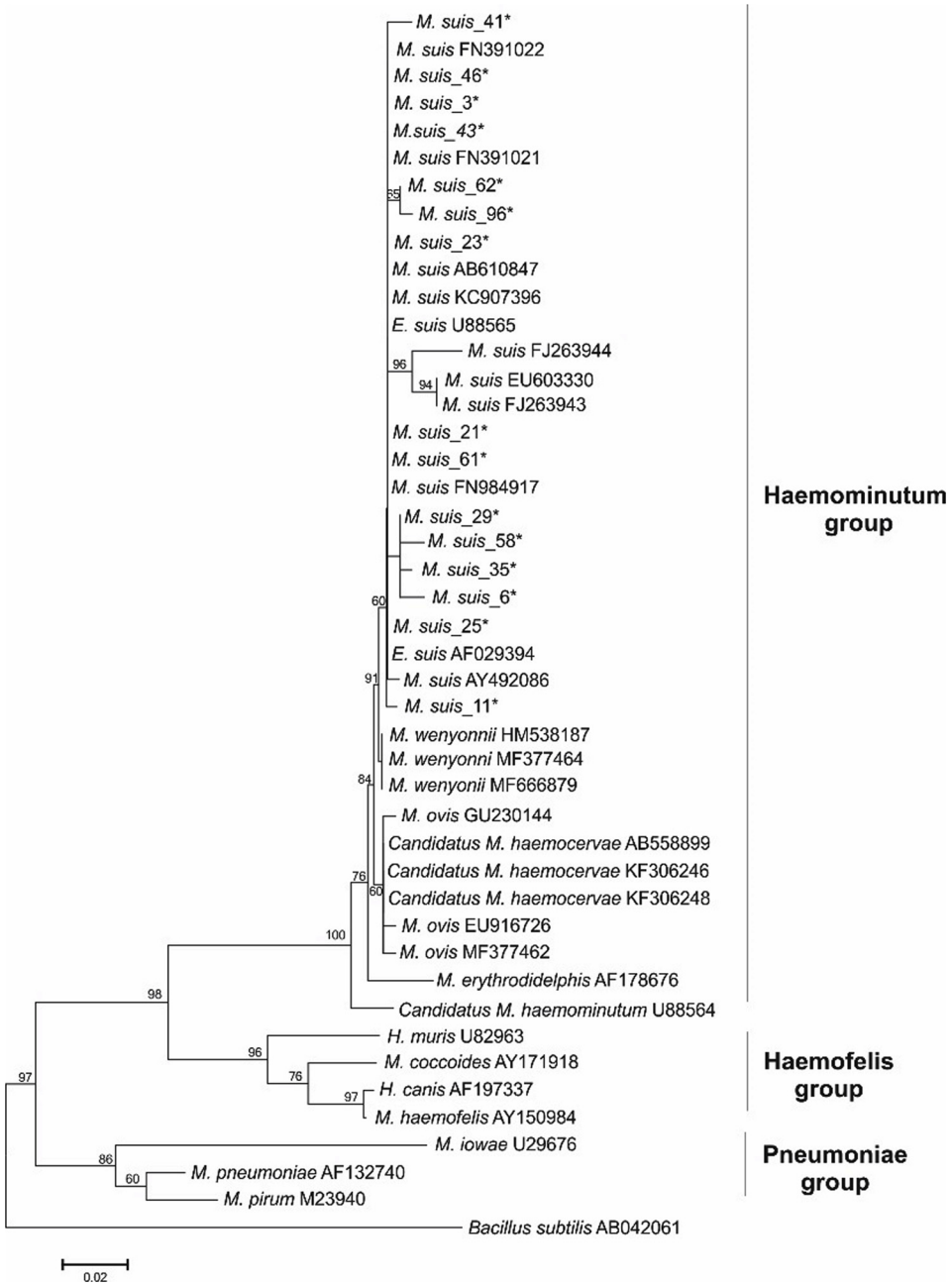

Fig. 1. Phylogenetic tree obtained by the NJ methodology for a 337 bp fragment of the 16S rRNA gene for the 46 sequences analyzed in this study (n = 15 from Buenos Aires, Argentina, n = 30 from GenBank). The sequences obtained in this study are marked with an asterisk. GenBank accession numbers are listed next to species names. Significant bootstrap values of 1000 replicates (> 50%) for the NJ tree are shown in brackets.

conditions, representing also the first detection in a pig-ectoparasite species. In this study, the presence of *M. suis* DNA was confirmed by conventional nested PCR for the 16S rRNA gene, followed by sequencing and bioinformatic analysis. In addition, the results of the phylogenetic analysis revealed the taxonomic classification to specific level of the obtained sequences and the relationship between *M. suis* and other Haemoplasma species (Fig. 1).

Mycoplasma suis is a haemotrophic prokaryote formerly classified as *Eperythrozoon suis* in the order Rickettsiales. Posteriorly, it was assigned to the *Mycoplasma* genus on the basis of 16S rRNA gene sequences and reclassified as a new species: *Mycoplasma suis* comb. nov. (Neimark et al., 2001; Messick, 2004). Respecting the phylogenetic relationship between Haemoplasma species, our results are in agreement with previous studies (e.g. Zhou et al., 2009), showing that *M. suis* is included within the "Haemominutum group", and the "Haemominutum" and "Haemofelis" groups are closest to each other, in contrast to the more distant "Pneumoniae" group (Fig. 1).

Regarding *M. suis* transmission paths, previous studies have demonstrated that blood-sucking arthropods, such as mosquitoes and flies, contribute to *M. suis* spread, acting as mechanical transmission vectors in natural conditions (Song et al., 2014). The presence of arthropods in the farm has been considered a risk factor for *M. suis* infection in pigs, reflecting a higher infection probability in farms with greater occurrence of blood-sucking insects (Song et al., 2014). In addition, there are reports about other *Mycoplasma* species mechanically transmitted by ectoparasites, such as lice and ticks (Hornok et al., 2011; Song et al., 2013). Based on our study and bibliography, we suggest that *H. suis* could serve as a mechanical vector for *M. suis*. This information extends the knowledge about the potential *H. suis*-transmitted pathogen spectrum. However, the contagion route for *M. suis* through

haematophagous ectoparasites should be integrally studied for the thorough understanding of the pathogen transmission and maintenance cycle in the vector.

In the studied area, Buenos Aires province, there is an intense swine livestock activity (Brunori et al. 2013). In this study, we record a high prevalence of *H. suis* parasitism (> 60%), both in domestic and feral pig populations. Thereby, the finding of *M. suis* in a frequent pigs' ectoparasite, represents a risk factor for this livestock activity. Future studies, about bacterial genetic markers analysis, both from lice and pig blood samples, will allow to reveal epidemiological aspects of *M. suis*.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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References

Brunori, J.C., 2013. Producción de cerdos en Argentina: situación, Oportunidades, Desafíos. on subordinate document. (Accessed 18 Feb 2018). http://inta.gob.ar/proyectos/pncar-011121.

Doyle, J.J., Doyle, J.L., 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. 19, 11–15.

Durden, L.A., Musser, G.G., 1994. The sucking lice (Insecta, Anoplura) of the world: a taxonomic checklist with records of mammalian hosts and geographical distributions. Bull. Am. Mus. Nat. Hist. 218, 1–90.

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.

Florence, L. (Ed.), 1921. The hog louse, Haematopinus suis Linné: Its biology, anatomy and histology. Cornell University Agricultural experiment station 106 pp.

Hall, T., 2004. BioEdit 6.0.7. Department of Microbiology, North Carolina State University.

Hoelzle, L.E., 2008. Haemotrophic mycoplasmas: recent advances in *Mycoplasma suis*. Vet. Microbiol. 130, 215–226.

Hornok, S., Micsutka, A., Meli, M.L., Lutz, H., Hofmann-Lehmann, R., 2011. Molecular investigation of transplacental and vector-borne transmission of bovine haemoplasmas. Vet. Microbiol. 152, 411–414.

Merino, M.L., Carpinetti, B.N., 2003. Feral pig *Sus scrofa* populations estimates in Bahía Samborombón Conservation Area, Buenos Aires province, Argentina. Mastozool. Neotrop. 10, 269–275.

Messick, J.B., 2004. Hemotrophic mycoplasmas (hemoplasmas): a review and new insights into pathogenic potential. Vet Clin Path. 33, 2–13.

Neimark, H., Johansson, K.E., Rikihisa, Y., Tully, J.G., 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'*. Int. J. Syst. Evol. Micr. 51, 891–899.

Pintos, M.E., 2016. Diagnóstico de Mycoplasma suis con técnicas convencionales y de biología molecular, su relación con circuvirus porcino tipo 2. Tesis doctoral. Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata.

Prieto, O.H., Cicchino, A., Abrahamovich, A., Nuñez, J.L., 1991. Piojos (Phthiraptera) parásitos del bovino y porcinos. Estado actual de su conocimiento y propuestas para su manejo y control. Parte III: las enfermedades parasitarias causadas por Phthiraptera. conclusiones, propuestas básicas y aplicadas para su manejo y control. Rev. Med. Vet. 72, 264–282.

Song, Q.Q., Wang, L.X., Fang, R., Khan, M.K., Zhou, Y.Q., Zhao, J.L., 2013. Detection of *Mycoplasma wenyonii* in cattle and transmission vectors by the loop-mediated isothermal amplification (LAMP) assay. Trop. Anim. Health Prod. 45, 247–250.

Song, Q.Q., Zhang, W., Song, W., Liu, Z., Khan, M.K., He, L., Fang, R., Li, P., Zhou, Y.Q., Hu, M., Zhao, J.L., 2014. Seroprevalence and risk factors of *Mycoplasma suis* infection in pig farms in central China. Prev. Vet. Med. 1, 215–221.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729.

Thibault, S., Drolet, R., Alain, R., Dea, S., 1998. Congenital swine pox: a sporadic skin disorder in nursing piglets. J. Swine Health Prod. 6, 276–278.

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.

Zeroual, F., Leul, H., Benakhla, A., Raoult, D., Parola, P., Bitam, I., 2018. Molecular evidence of *Rickettsia slovaca* in wild boar lice, in northeastern Algeria. Vector Borne Zoonotic Dis. 18, 114–116.

Zhou, R.Q., Nie, K., Huang, H.C., Hu, S.J., Zhou, Z.Y., Luo, H.L., 2009. Phylogenetic analysis of *Mycoplasma suis* isolates based on 16S rRNA gene sequence in China. Vet. Res. Commun. 33, 855.