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Article

Frequency of *M. hyopneumoniae*, *M. hyorhinis* and *M. hyosynoviae* in nasal and lung samples from pigs with symptoms of porcine enzootic pneumonia

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Abstract:

M. hyopneumoniae, *M. hyorhinis* and *M. hyosynoviae* are genetically related species of the genus *Mycoplasma* that affect pig production. The objective of this work was the isolation and identification by PCR of *M. hyopneumoniae*, *M. hyorhinis* and *M. hyosynoviae* from nasal swabs and lung samples of pigs from different regions of Mexico in order to determine the frequency of these species and to evaluate PCR as a diagnostic tool for PEP. Pigs aged 4 to 8 weeks with clinical diagnosis of PEP were included. Lung samples and nasal swabs were obtained for the isolation of the *Mycoplasma* in liquid Friis medium and identified by species-specific PCR based on the 16S rRNA subunit. Isolation was achieved in 37.11 % (36/97) of the samples. The three *Mycoplasma* species were identified in lung and nasal swab samples. *Mycoplasma* co-infection was identified in 27.77 % (10/36). The bacterial genera associated with *Mycoplasma* infections were *E. coli*, *Bordetella*, *Enterobacter*, SCN, *Corynebacterium*, *Pasteurella*, *Streptococcus*, *Shigella* and *Klebsiella*. Mixed infection was present in 26 nasal swabs (45.61 %) and absent in the lungs. It was concluded that the frequency of *Mycoplasma* on production farms was higher than expected (40.27 %). It was also identified other *Mycoplasma* species involved in the development of PEP. Therefore, surveillance through isolation and molecular techniques can be of great help to breeding stock providers, as well as for removing *Mycoplasma* from pig farms.

Key words: Mycoplasmosis, *M. hyopneumoniae*, *M. hyorhinis*, *M. hyosynoviae*, Porcine enzootic pneumonia.

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Introduction

The Swine Respiratory Disease Complex (PRDC) is a major health problem for the pig industry worldwide[1]. It is caused by the association of infections such as *Mycoplasma*, porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus type 2 (PCV2), *Pasteurella multocida*, *Actinobacillus pleuropneumoniae*, *Streptococcus suis*, *Haemophilus parasuis*, *Bordetella bronchiseptica*, and *Arcanobacterium pyogenes*[1,2]. A predisposing factor is porcine enzootic pneumonia (PEP), primarily caused by *Mycoplasma hyopneumoniae*[3], which adheres to the respiratory epithelium, damages the ciliated cells of the trachea, bronchi and bronchioles[4], and suppresses the immune response of the upper respiratory tract that favors the development of PRDC[5,6].

PEP is a high-prevalence chronic respiratory disease with high morbidity and low mortality. between 30 to 80 % of the pig programmed for slaughter exhibit typical consolidation lesions[7,8]. Throughout the pig's productive life, the prevalence of *M. hyopneumoniae* increases until it reaches the age of slaughter, even in vaccinated animals[9]. Reproductive females are a reservoir that perpetuates the continuous circulation of respiratory pathogens associated with PEP[10,11].

The severity of the disease differs among herds, with a high prevalence in conventional pig farms[12]. The most significant clinical sign of PEP is a chronic, dry, non-productive cough that occurs in fattening pigs aged 16 to 22 wk. The main macroscopic lesion is cranio-ventral pulmonary consolidation[5], which is histologically characterized by broncho-interstitial pneumonia with hyperplasia of the bronchus-associated lymphoid tissue (BALT)[13]. The main risk factor for PEP is vertical transmission from sow to piglet during lactation, given that vaccination does not guarantee protection[14] since *M. hyopneumoniae* can circulate in vaccinated animals[15] and in free-living animals such as wild boar, with which vulnerability to *M. hyopneumoniae* is shared, and which can be a reservoir of these bacteria[16]. The severity of the disease at the time of slaughter may be predicted of the initial prevalence at weaning, based on the variables indicative of infection (average of lung lesions, percentage of lung tissue affected, presence of *M. hyopneumoniae* in the bronchial epithelium and seroconversion), as there is a positive correlation between these two variables[17].

Most *Mycoplasma* infections remain subclinical[18] and may involve other species of the same bacterial genus such as *M. hyorhinis*, a commensal inhabitant of the upper respiratory tract mucosa and tonsils[19]. *M hyosynoviae*, a species mainly associated with acute arthritis and, to a lesser extent, with suppurative pneumonia with severe pulmonary consolidation, and pleurisy[20,21,22]. *M. hyopneumoniae, M. hyorhinis* and *M. hyosynoviae* are genetically related species of porcine interest[23], which can be discriminated by PCR based on the hypervariable regions of the 16S subunit of the genus[23,24].

The objective of this work was the isolation and identification by PCR of *M. hyopneumoniae, M. hyorhinis* and *M. hyosynoviae* from nasal swabs and samples from pigs of different regions of the Mexican Republic, in order to determine the frequency of these species and to evaluate PCR as a diagnostic tool for enzootic swine pneumonia.

Material and methods

Animals and sampe collection

Pigs aged 4 to 8 wk diagnosed with PEP according to clinical signs and with gross lesions in the lung (purple to gray areas of tissue consolidation in the cranio-ventral lung lobe) were included in this study. 40 lung samples and 57 nasal swabs were aseptically obtained by pressing against the structural wall of the tissue[25]. Sample collection was conducted on farms in four regions of Mexico, from May 2015 to January 2016 (Table 1). Each sample was collected in duplicate for *Mycoplasma* isolation and for traditional bacteriology. All animal procedures were approved by the Institutional Committee for the Care and Use of Experimental Animals (CICUAE) of the National Autonomous University of Mexico, following international ethical standards.

Table 1: Regions of origin of the lung samples and nasal swabs included in this work

Samples	Geographic region	Number of samples
40 lungs	Mexico	12
	Veracruz	28
57 nasal swabs	Hidalgo	25
	Guanajuato	32
Total		97

Mycoplasma isolation

For *Mycoplasma* isolation, nasal swabs were resuspended in 2 ml of Friis medium. Lung samples were frozen at -20°C until they were followed up in the laboratory. Lung samples were routinely processed by maceration in 3 ml of Friis medium for isolation[18,26,27]. 200 µl of the suspension of each sample in Friis medium were inoculated in 1.8 ml of Friis medium supplemented with pig serum (10 %), horse serum (10 %), and penicillin (100 µg/mL) to optimize the recovery of *M. hyopneumoniae*[28], and supplemented with L-arginine (0.05 %) for the recovery of *M. hyosynoviae*[29]. Subsequently, up to 10^{-6} serial dilutions were made, and, finally, 10 µL were plated onto Friis agar[27]. Tubes were incubated at 37 °C until a color change was observed in the medium, or up to 30 d, before being discarded. Positive samples were those that developed at least one unit of color change, while samples that had no color change after 30 d were considered negative. The agar plates were incubated at 37 °C with 5 % CO_2 for 1 to 2 wk. Each isolated colony was further inoculated into 2 ml of Friis medium and incubated. After observing the color change, the cultures were evaluated to confirm their purity and subsequent use until PCR discrimination of the species.

Species-specific PCR for the identification of *Mycoplasma*

PCR based on the 16S rRNA subunit for the identification of the three *Mycoplasma* species was applied to each of the isolates. The reference strains *M. hyopneumoniae* ATCC 25617, *M. hyorhinis* ATCC 17981, *M. hyosynoviae* strain S-16, and *M. bovis* Donetta PG45—all kindly donated by Aarhus University, Aarhus, Denmark—were cultured in 50 ml medium, concentrated by centrifugation for DNA extraction according to the protocol with guanidinium thiocyanate[30]. Each isolate was also processed for DNA extraction and stored at -70 °C until further analysis.

Amplification of the 16S rRNA subunit was performed in a total reaction volume of 25 µL containing 0.25 µL of Taq PCR Reaction Mix (Sigma-Aldrich, Austria), 10 pmol of each sense and antisense initiator (Table 2)[24], and 10 µl of DNA[31]. The reaction conditions were: initial denaturation at 96 °C, for 5 min, followed by 30 denaturation cycles at 94 °C for 45 s, alignment at 72 °C for 2 min, and extension at 72 °C for 4 min. DNA from pure cultures of *M. hyopneumoniae* ATCC 25617, *M. hyorhinis* ATCC 17981 and *M. hyosynoviae* strain S-16 were applied as positive controls, and *M. bovis* Donetta PG45, as negative control.

Table 2: PCR initiators based on 16S rRNA from *M. hyopneumoniae*, *M. hyorhinis* and *M. hyosynoviae*

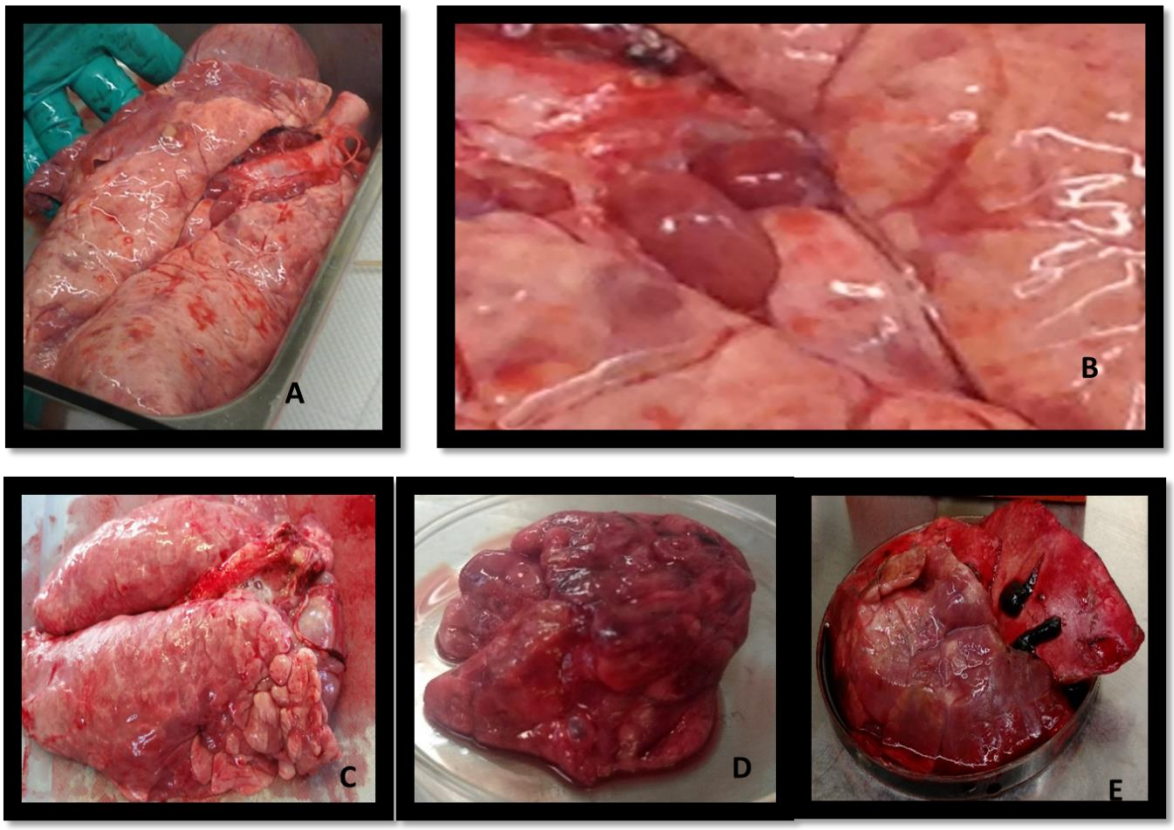
***Mycoplasma* species**	**Sequence (5 -3)**	**Product (bp)**	**Reference**
M. hyopneumoniae	F 5'-TTC AAA GGA GCC TTC AAG CTT C-3' R 5'-GAC GTC AAA TCA TCA TGC CTC T-3'	1000	30
M. hyorhinis	F 5' CGGGATGTAGCAATACATTCAG 3' R 5' GACGTCAAATCATCATGCCTCT 3'	1129	30
M. hyosynoviae	F 5' CAGGGCTCAACCCTGGCTCGC 3' R 5' GACGTCAAATCATCATGCCTCT 3'	585	This work Gen Bank Access No. NR029183.1

Results

Mycoplasma isolation

97 samples were collected: 40 from lungs with typical *Mycoplasma* lesions suggestive of PEP (Figure 1) and 57 nasal swabs from pigs from different geographical regions of Mexico (Table 1). From the lung samples, 22.5 % (9/40) were positive to the isolation of *Mycoplasma* spp and 77.5 % (31/40) were negative. Of the nasal swabs, 47.36 % (27/57) were positive, and 52.63 % (30/57) were negative. In the positive samples, the color change of the culture medium was observed as early as the 5th d or until the 12th d. On average, the color change was observed on the 7th d. The remaining samples were considered as negative after 30 d without color change.

Figure 1: Typical *Mycoplasma* lesions in the lungs collected for this study

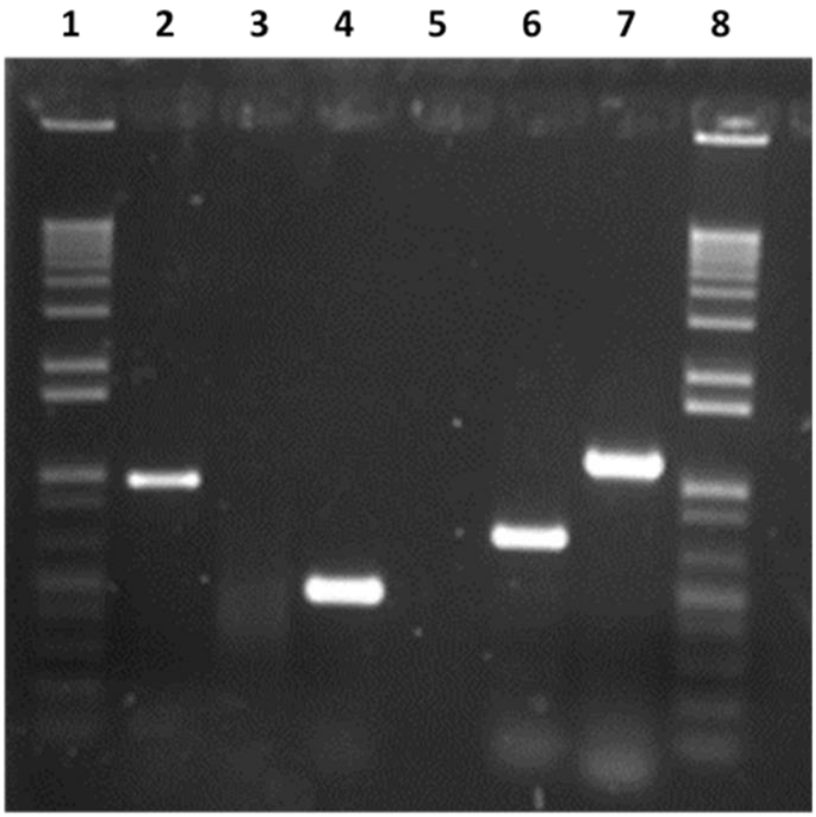


In (A) lung with typical PEP injury, distributed over all lobes of the lung, (B) approach to lung consolidation, (C) lung with higher degree of lung consolidation, (D) lung sequestration resulting from the evolution of the injury, (E) evidence of scarring in the lung tissue.

PCR results

Amplified fragments of 1,000 bp of M. *hyopneumoniae*, 1,129 bp of *M. hyorhinis*, and 585 bp of *M. hyosynoviae* using the reference strains (ATCC 25617, ATCC 17981, and *M. hyosynoviae* strain S-16), were visualized by 1.5% agarose gel electrophoresis at 80 V for 60 min, stained with ethidium bromide and displayed on a UV transilluminator, as shown in Figure 2. 22 % (2/9) of the lung sample isolates (LSIs) of *Mycoplasma* tested positive for *M. hyopneumoniae*; 55.5 % (5/9), for *M. hyorhinis*, and 44 % (4/9), for *M. hyosynoviae*. 44 % (4/9) of the LSIs tested negative with species-specific PCR. 7.40 % (2/27) of the nasal swab isolates (NSIs) tested positive for *M. hyopneumoniae*; 51.85% (14/27), for *M. hyorhinis*, and 33.3 % (9/27), for *M. hyosynoviae*. 22.22 % (6/27) of the NSIs tested negative with species-specific PCR (6/27) (Table 3). Despite having been successfully isolated, four LSIs and six NSIs remained unidentified with the species-specific PCR.

Figure 2: Electrophoretic profiles of the amplified fragments of *M. hyopneumoniae*, *M. hyorhinis* and 16S rRNA



Lane 1, Molecular Weight Marker (1 Kb plus Invitrogen), Lane 2, *M. hyopneumoniae* ATCC 25617, 1000 bp; Lane 3. *M. bovis* Donetta PG45, donated by the University of Aarhus, Denmark, Lane 4, *M. hyorhinis*, ATCC17981, 585 bp, Lane 6, Unrelated product with 685 bp of p97 protein from *M. hyopneumoniae*, ATCC25617, Lane 7, *M. hyosynoviae*, strain S-16, 1129 bp, also donated by the University of Aarhus, Denmark, Lane 8, Molecular Weight Marker (1 Kb plus Invitrogen).

Table 3: List of isolates identified by species-specific PCR for *M. hyopneumoniae*, *M. hyorhinis*, and *M. hyosynoviae*

Sample	Positive isolation (%)			
	M. hyopneumoniae	***M. hyorhinis***	***M. hyosynoviae***	***Mycoplasma* spp isolates**
Lung	2/9 (22.0)	5/9 (55.5)	4/9 (44.0)	4/9 (44.0)
Nasal swab	2/27 (7.4)	14/27 (51.8)	9/27 (33.3)	6/27 (22.2)
Total	4/36 (11.1)	19/36 (52.7)	13/36 (36.1)	10/36 (27.7)

The coexistence of *M. hyopneumoniae*, *M. hyorhinis* and *M. hyosynoviae* was detected in ten samples representing 27.77 % (10/36): in two lungs all three species, in two other lungs and five nasal swabs *M. hyorhinis* and *M. hyosynoviae* were identified, and only one swab contained *M. hyopneumoniae* and *M. hyorhinis* (Complementary Table 1). Additionally, the associated bacterial genera identified by general bacteriology in nasal swabs were *E. coli*, *Enterobacter*, coagulase-negative *Staphylococcus*, *Klebsiella*, *Bordetella*, *Corynebacterium*, *Pasteurella*, *Shigella* and *Streptococcus*. No bacterial growth was identified in lung samples.

Supplementary table 1: Identification of *Mycoplasma* isolates by species-specific PCR

Number	Description	Type of sample*	*M. hyop*	*M. hyor*	*M. hyos*	Bacterial Genera
1	111	NS	-	+	-	CNS
2	112	NS	-	+	+	*E. coli* , *Shigella*
3	113	NS	-	-	-	*Pasteurella*
4	114	NS	-	-	-	*Klebsiella*, CNS
5	115	NS	-	+	+	*Klebsiella*, *E. coli*, CNS
6	116	NS	-	+	-	*E. coli*, CNS, *Bordetella*, *Corynebacterium*
7	117	NS	-	+	-	CNS, *Corynebacterium*
8	118	NS	-	+	-	CNS, *Corynebacterium*
9	119	NS	-	-	+	*Enterobacter*
10	120	NS	-	+	-	*Corynebacterium*
11	121	NS	-	-	-	*Klebsiella*, CNS
12	122	NS	-	+	+	*Corynebacterium*
13	123	NS	-	-	+	*Klebsiella*, CNS
14	124	NS	-	-	+	CNS
15	125	NS	-	+	-	*Corynebacterium*
16	126	NS	-	-	-	*Corynebacterium*
17	127	NS	-	-	-	*Klebsiella*, *Corynebacterium*
18	130	NS	+	-	-	CNS
19	133	NS	-	-	-	*E. coli*
20	148	NS	-	+	-	CNS
21	159	NS	+	+	-	No bacterial growth
22	160	NS	-	+	-	CNS
23	161	NS	-	-	+	*Pasteurella*
24	162	NS	-	+	+	*E. coli*, CNS

25	165	NS	-	+	-	*Streptococcus*, CNS
26	168	NS	-	+	-	*E. coli*
27	170	NS	-	+	+	CNS
28	182	L	-	+	+	No bacterial growth
29	183	L	-	+	+	No bacterial growth
30	186	L	-	-	-	No bacterial growth
31	188	L	-	-	-	No bacterial growth
32	194	L	+	+	+	No bacterial growth
33	206	L	+	+	+	No bacterial growth
34	207	L	-	+	-	No bacterial growth
35	208	L	-	-	-	No bacterial growth
36	210	L	-	-	-	No bacterial growth

M. hyop = M *hyopneumoniae: M. hyor= M. hyorhinis; M. hyos= M. hyosynoviae;* *NS= nasal swab, L= lung, CNS= Coagulase-negative *Staphylococcus*

Discussion

In Mexico there are few studies on the association of these three *Mycoplasma* species with PEP, mainly due to the difficulties for their isolation and to those inherent in the biological sample. The concentration of microorganisms is often below the detection limit as a result of the widespread use of antibiotics for the control of porcine mycoplasmosis. Therefore, isolation procedures are necessary to encourage their growth and identification for research and surveillance purposes. The procedure used allowed the association identification of the three species related to pig production.

M. hyopneumoniae is the most frequently isolated species of *Mycoplasma* from pigs with clinical signs of pneumonia and has a low transmission rate. However, in association can increase the severity of infections caused by viruses and bacteria[32].

M. hyorhinis has gone from being a secondary pathogen[33,34], to being considered a causal agent of PEP and PRDC[35]. In this study, this species of *Mycoplasma* is the most prevalent in nasal swabs 51.85% (14/57); this observation can be explained by the success of the control measures that have been implemented in pig production farms. This study reports herein that *M. hyosynoviae* is in close interaction with the other two *Mycoplasma* species in lungs with typical PEP lesions. *M. hyosynoviae* was present in nasal swabs as a microorganism associated in a high percentage of the cases (33%, i.e. 9/27). Therefore, this commensal *Mycoplasma* species may have pathogenic potential, and further studies will be required to assess its role in the development of PEP.

Bacteriological culture is the "gold standard" for diagnosis. However, among its drawbacks, it is very laborious, it is seldom used as a routine method, and it does not distinguish between species associated with PEP. PCR based on the 16S rRNA subunit allowed to discern, quickly and precisely, between *M. hyopneumoniae* and *M. hyorhinis*. On the other

hand, 10 cases were identified in which the species evaluated in this work were not involved. This result raises the possibility that other *Mycoplasma* species may be involved.

The collection method (nasal swab, tracheobronchial mucus, deep *postmortem* swab, bronchoalveolar lavage or lung tissue) has a significant effect on the frequency of *M. hyopneumoniae*, since the reported frequency varies between 3 and 40 %, according to the method used[36]. Hitherto, nasal swabs have been the method of *antemortem* sample collection in piglets at herd level[28,37]. Pieters *et al*[38] suggest that laryngeal swabs are useful in the early stage of infection in piglets. Other authors state that the optimal sampling site for detection by molecular methods for *M. hyopneumoniae* is tracheobronchial mucus collection (TBMC), since its sensitivity is 3.5 times more sensitive in piglets aged under 25 d[36].

The upper respiratory tract (nasal cavity and pharynx) plays an important role in monitoring and cleansing pathogenic microorganisms and also in inducing the appropriate immune response. *M. hyopneumoniae* mainly colonizes the cilia of the respiratory tract of the pigs[39]. In adult animals from production farms, TBMC becomes difficult and expensive to obtain. Animal handling is restricted in keeping with current swine influenza prevention measures, and, based on this experience, it is recommend the use of nasal swabs as the appropriate sampling technique.

The prevalence of *M. hyopneumoniae* in naturally infected sows is 36.4 %[40]; in piglets, it can vary from 3.6 to 16 %. The frequency of *Mycoplasma* determined here was higher than expected, namely: 37.11 % (36/97). *Mycoplasma* was present in the lungs of 22.50 % of the animals, (9/40), and in nasal swabs, in 47.36 % (27/57). Infection by a single Mycoplasma species was 44.44 % (16/36): in LSIs 11.11 % (1/9) and in NSIs 55.55 % (15/27). The association of more than one *Mycoplasma* species was present in 27.77 % (10/36): the association of the three species represented 2 % (2/10), and the association of *M. hyorhinis* and *M. hyosynoviae* 5.15% (5/10). Co-infection of *M. hyopneumoniae* and *M. hyorhinis*, and *M. hyosynoviae* and *M. hyorhinis* have previously been associated with joint problems. In this work, both associations were identified in the respiratory tract of animals in pig farms with PEP.

The rate of *Mycoplasma* associated with PEP is variable, regardless of whether the rate of mixed infections remains constant[35]. In this study, the bacterial genera associated in mixed infections were similar to those previously reported[41]. PCR can be complementary or alternative to histopathological diagnosis and represents an option for epidemiological surveillance and research. In addition, it can assist in the elimination of *Mycoplasma* spp from swine production farms, as it is the best long-term control strategy, so far, for many swine producers and breeding stock suppliers[42].

Conclusions and implications

The frequency of *Mycoplasma* in pig farms in the states of Hidalgo, Guanajuato, Veracruz and Mexico was higher than expected (40.27 %). There are other *Mycoplasma* species that may be involved in the development of PEP, and this paper adds evidence of *M. hyorhinis* as a causal agent of PEP.

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