Detection, quantification and genetic variability of *Mycoplasma hyopneumoniae* from apparently healthy and pneumonic swine

Detecção, quantificação e variabilidade genética de Mycoplasma hyopneumoniae a partir de suínos pneumônicos e aparentemente saudáveis

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Abstract

Molecular differences among *Mycoplasma hyopneumoniae* strains present in pneumonic lungs of swine have been largely studied. However, no comparative studies concerning the strains present in apparently healthy pigs have been carried out. This study aimed to detect, quantify and perform molecular analysis of *M. hyopneumoniae* strains in pig lungs with and without pneumonic lesions. The detection of *M. hyopneumoniae* was performed using multiplex PCR (YAMAGUTI, 2008), real-time PCR (STRAIT et al., 2008) and multiple VNTR amplification (VRANCKX et al., 2011). Molecular characterization of the strains was achieved by analysis of the VNTR copy number in P97R1, P146R3, H2R1 and H4. *M. hyopneumoniae* was detected in samples from healthy and pneumonic pigs and the amount of *M. hyopneumoniae* positive samples detected varied with the type of assay. The greater number of positive samples was identified by the multiple VNTR amplification combined with capillary electrophoresis. Using real-time PCR, $4.9*10^4$ *M. hyopneumoniae* genome copies/mL was detected in apparently healthy lungs. A mean quantity of $3.9*10^6$ *M. hyopneumoniae* genome copies/mL was detected in pneumonic lungs. The analysis of VNTR copy number demonstrated a high genetic variability of the *M. hyopneumoniae* strains present in apparently healthy and pneumonic lungs. Strains having 3 VNTR copy number in P97R1, were detected only in pneumonic lungs and strains having 40 and 43 VNTR copy number in P146R3 were detected only in apparently healthy lungs. Despite the genetic variability of *M. hyopneumoniae*, predominant strains in the swine farms could be identified.

Keywords: *M. hyopneumoniae*. Enzootic pneumonia. Healthy. Quantification. VNTR.

Resumo

As diferenças moleculares entre as estirpes de *Mycoplasma hyopneumoniae* presentes em pulmões de suínos com pneumonia têm sido estudadas. Porém, estudos comparativos relativos às estirpes presentes nos suínos aparentemente saudáveis não foram levados a cabo. O objetivo do estudo foi a detecção, quantificação e análise molecular de *M. hyopneumoniae* nos pulmões suínos com e sem lesões pneumônicas. Para a detecção de *M. hyopneumoniae* usaram-se o PCR Múltiplo (YAMAGUTI, 2008), o PCR a Tempo Real (STRAIT et al., 2008) e a amplificação de múltiplo VNTR (VRANCKX et al., 2011). A caracterização molecular das estirpes foi realizada mediante a análise do número de cópias de VNTR em P97R1, P146R3, H2R1 e H4. O *M. hyopneumoniae* foi detectado em amostras de suínos saudáveis e pneumônicos e a quantidade de *M. hyopneumoniae* nas amostras positivas variou com o tipo de ensaio. O maior número de amostras positivas foi identificado pela amplificação de múltiplas VNTR combinado com a eletroforese de capilares. Usando o PCR a Tempo Real, $4.9*10^4$ cópias de genoma/mL de *M. hyopneumoniae* foram detectadas em pulmões aparentemente saudáveis. Uma quantidade média de $3.9*10^6$ cópias de genoma/mL de *M. hyopneumoniae* foi detectada em pulmões pneumônicos. A análise do número de cópias de VNTR demonstrou uma elevada variabilidade genética das estirpes de *M. hyopneumoniae* presentes nos pulmões pneumônicos e aparentemente saudáveis. As estirpes com três cópias de VNTR em P97R1 só foram detectadas em pulmões pneumônicos, enquanto as estirpes contendo 40 e 43 cópias de VNTR em P146R3 só foram detectadas em pulmões aparentemente saudáveis. Apesar da variabilidade genética de *M. hyopneumoniae*, foi possível identificar estirpes predominantes nas granjas de suínos.

Palavras-chave: *M. hyopneumoniae*. Pneumonia enzoótica. Saudável. Quantificação. VNTR.

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Introduction

M. hyopneumoniae is the etiological agent of porcine enzootic pneumonia, a chronic respiratory disease that leads to important economic losses in the swine industry worldwide (CHARLEBOIS et al., 2014). This microorganism adheres to the host cilia and colonizes the porcine respiratory epithelia causing degenerative changes in the ciliated epithelial cells. The organism modulates the immune system of the respiratory tract and interacts with other pathogen virus and bacteria leading to a more complicated disease (THACKER; THACKER; JANKE, 2001).

M. hyopneumoniae is transmitted vertically and also horizontally by direct contact with respiratory tract secretions from infected pigs. Transmission by aerosols it has been also described (RAUTISINEN, 2001). Porcine enzootic pneumonia shows different degrees of severity occurring from an acute to a chronic form. At a chronic or latent stage of infection, clinical signs can rarely be observed (MAYOR et al., 2008). When pigs are exposed to *M. hyopneumoniae,* some individuals may become immune, while others remain in a carrier state, having no clinical signs of disease (GOODWIN; WHITTLESTONE, 1973; RAUTISINEN, 2001). Infection of pigs with *M. hyopneumoniae* depends on environmental factors (management conditions), animal factors (health and immunological status) (WALLGREN et al., 1998; RAUTISINEN, 2001) and pathogen factors (*M. hyopneumoniae* load in the herd, differences in virulence and molecular features (VICCA et al., 2003; MAROIS et al., 2010).

Molecular methodologies based on DNA amplification in clinical samples have become the method of choice for routine detection and molecular characterization of *M. hyopneumoniae*, due to the difficulties in isolation of the agent (FRIIS, 1975; MAROIS et al., 2007) and to the frequent co-isolation of *Mycoplasma hyorhinis,* a normal inhabitant of the upper respiratory tract of young pigs (KOBISCH; FRIIS, 1996). Genetic heterogeneity between *M. hyopneumoniae* strains recovered from clinical samples of different or the same farms has been shown using different molecular methodologies (CALUS et al., 2007; VICCA et al., 2003; MAYOR et al., 2007; NATHUES et al., 2011; STAKENBORG et al., 2005; VRANCKX et al., 2011). The multiple loci variable number of tandem repeats (VNTR) analysis (MLVA) (VRANCKX et al., 2011), overcome the limitations of other typing techniques like random amplified polymorphic DNA (RAPD) (ARTIUSHIN; MINION, 1996), amplified fragment length polymorphism (AFLP) (KOKOTOVIC et al., 1999) and pulsed-field gel electrophoresis (PFGE) (STAKENBORG et al., 2005). MLVA is reproducible, have high discriminatory power, do not require previous isolation of the agent and can be easily performed (VRANCKX et al., 2011, CHARLEBOIS et al., 2014). Analysis of the genetic variability of field strains by means of VNTR copy number comparison has been performed (VRANCKX et al., 2011; CHARLEBOIS et al., 2014), but no relation between VNTR copy number and severity of pneumonic lesions has been established.

M. hyopneumoniae has been detected not only in pigs with clinical symptoms, but also in the asymptomatic animals (GUZMÁN et al., 2008); however, no comparative studies concerning the strains found in symptomatic and asymptomatic pigs have been carried out. Knowledge of *M. hyopneumoniae* load and molecular features in swine showing no clinical sings in regard to symptomatic animals is important to control the disease in a more efficient way.

In this study, PCR assays previously reported in literature were used to detect *M. hyopneumoniae* in respiratory samples from apparently healthy pigs as well as from animals with clinical symptoms. Also, *M. hyopneumoniae* was quantified and molecular characterization was carried out by means of VNTR copy number analysis in order to compare the strains present in apparently normal and pneumonic lungs of swine.

Materials and methods

Mycoplasma and walled bacteria

Reference strains *M. hyopneumoniae* J, *M. hyorhinis* BTS7 and *M. flocculare* Ms42 *Mycoplasma hyosynoviae* S16, *Acholeplasma granularum* BTS39, *Acholeplasma laidlawii* PG8 and *Ureaplasma diversum* A417 were obtained from the bank of mycoplasma strains belonging to the Laboratory of Mycoplasmas, University of São Paulo, Brazil.

Sample collection

One hundred fifty-five respiratory samples taken from growing pigs of five farms from the western region of Cuba were recovered at a slaughterhouse. The samples included 15 nasal swabs from pigs with respiratory disturbances, 98 swabs and six tracheo-bronchial lavages from pneumonic lungs and 36 tracheo-bronchial lavages from lungs without pneumonic lesions. The animals were under a controlled environment and preventive treatments with medicated food in order to limit the occurrence of pneumonia in the farms. The pigs were not vaccinated against *M. hyopneumoniae*.

Trachea bronchial lavages were obtained. Briefly, the lung was extracted by an incision in the trachea and a sodium chloride 150 mM solution was added through it. Manual massages were done in the organ and the resulting liquid was placed in a sterile recipient, avoiding the introduction of macroscopic particles.

Lung swabs were obtained as follows: a piece of pulmonary lobule containing sick and healthy tissue was introduced into 70°C alcohol and flamed. An incision was made using a flamed scalpel to reach the pulmonary parenchyma with a sterile swab, thus avoiding contamination due to manipulation. Swabs were introduced into Eppendorf tubes containing 500 µL of sterile PBS and centrifuged at 12 000 rpm for 2 min. The liquid obtained was then collected.

DNA extraction

Genomic DNA from mycoplasma reference strains was extracted using the PureLinkTM Genomics DNA Mini Kit (Invitrogen TM). Quality and concentration of DNA extracted from all reference mycoplasma strains and from supplied DNA of walled bacteria was determined by spectrophotometry using a NanoDrop ND-1000, Witec Ag, and Littau, Switzerland. Microbial DNA from clinical samples was extracted as follows. Five hundred microliters of collected lung swabs and 1 mL of tracheo-bronchial washing lavages were spun down (10 min, 12 000 rpm). The pellets were resuspended in 1 ml of sterile phosphate-buffered saline (PBS), vortex and spun down again (10 min, 12 000 rpm). The pellets were then resuspended in 100 ul of sterile nuclease free water. After boiling for 10 min, the samples were cooled on ice and kept at -20°C prior to use in further analysis.

PCR assays

All samples were firstly analyzed using a *Mollicutes*-PCR (KUPPEVELD et al., 1994). Afterwards, all *Mollicutes* positive samples were used for *M. hyopneumoniae* detection using the following DNA amplification assays: A multiplex PCR described by Yamaguti (2008), directed to the multiple detection of *M. hyopneumoniae, M. hyorhinis* and *M. flocculare* (data not published), a real-time PCR (STRAIT et al., 2008) and an assay based in the amplification of 4 VNTR containing regions (VRANCKX et al., 2011).

Mollicute-PCR

A *Mollicute*-PCR was carried out using primers MGSO and GPO-3 described by Kuppeveld et al. (1994). The mix contained PCR buffer 1X (Invitrogen), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 1 U of Platinum Taq DNA polymerase (Invitrogen) 0.3 µM of each primer and 1 µL of the targeted DNA were added to the reaction mixture in a final volume of 25 µL. The reaction was set to 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 57.7°C for 30 s and 72°C for 30 s, and 72°C for10 min.

Multiplex PCR

A multiplex PCR was performed in volumes of 30 µL according to Yamaguti (2008). Briefly, 0.6

uM of primers sense was added (MHYOP for: TTC AAA GGAGCC TTCAAGCTTC, MHYOR for: CGG GAT GTA GCA ATA CAT TCA G and MFLOC for: GGG AAG AAA TTA GGT AGG G) and 1 uM of primer antisense (MYrev: GGGTTCCCGTCAATTCCTTTA), PCR Buffer 1X, 2.5 mM MgCl2, 0.3 mM each dNTP, 2.5 U of Platinum Taq DNA polymerase (Invitrogen) and 1 µL of DNA template. Amplification program was set at 94°C for 4 min, followed by 30 cycles of 94°C for 30 s, 54.6°C for 30 s and 72°C for 1 min, and a final step at 72°C for 10 min. The PCR products were analyzed by electrophoresis in 2% agarose gels stained with ethidium bromide and visualized with ultraviolet light.

Real-Time PCR

A real-time PCR was performed according to Strait et al. (2008). For constructing DNA standards for absolute quantitation, the genomic DNA copy number was calculated by spectrophotometry (NanoDrop ND-1000, Witec Ag, and Littau, Switzerland). Ten-fold serial dilutions (10^7–10 copies/ml) of the *M. hyopneumoniae* DNA standard were prepared and analyzed. The threshold was manually adjusted within the logarithmic curve, above the background level and below the linear and plateau phase.

VNTR amplification

The amplification and analysis of VNTR containing regions was carried out using fluorescently labeled primers targeting VNTRs P97R1, P146R3, H2R1 and H4 as described by Vranckx et al. (2011), followed by capillary electrophoresis. GeneMapper software version 4.0 (Applied Biosystems) was used to estimate amplicon sizes and the values were imported into Bionumerics software version 7 (Applied Maths) for VNTR copy number determination. Additionally, VNTR amplification products were analyzed by gel electrophoresis in 2% agarose gel stained with ethidium bromide and visualized with ultraviolet light.

Results

Detection of Mollicutes and porcine mycoplasmas in respiratory samples

Of the 155 analyzed samples from the respiratory tract of pigs, 68% (106/155) were positive by the *Mollicutes*-PCR. This included 100% of the nasal swabs, 75% (77 lung swabs and 1 TBL) of the 104 samples taken from pneumonic pigs and 36% of the tracheo-bronchial lavage samples obtained from lungs without pneumonic lesions (13/36). *M. hyopneumoniae* was detected in samples from healthy pigs as well as in pigs with clinical symptoms and the amount of positive *M. hyopneumoniae* samples detected varied with the type of assay used (Table 1). In general, the greater number of positive *M. hyopneumoniae* samples (78%) was detected by amplifying the four VNTR containing regions P97R1, P146R3, H2R1 and H4 with labeled primers, followed by capillary electrophoresis. Seventy-two percent of the samples were detected by the real-time PCR, 55% were detected by amplifying the P97R1, P146R3, H2R1 and H4 VNTR regions followed by gel electrophoresis and the lowest number of positive samples was detected by the multiplex PCR (17%). Regarding the type of samples analyzed, the greater number of tracheo-bronchial lavage and nasal swabs positive to *M. hyopneumoniae* was detected by the multi-target amplification assay (VNTR containing regions) followed by capillary electrophoresis, whereas the higher number of positive pneumonic lungs was detected with the real-time PCR.

In addition, the multiplex PCR detected *M. hyorhinis* in 30% of samples positive to *Mollicutes* (32/106), specifically, in 81% (26/32) of pneumonic lungs, 13% (4/32) of nasal swabs taken from pigs with respiratory symptoms and 6% of tracheo-bronchial washing of normal lungs (2/32). *M. flocculare* was detected in one sample of nasal swab from a pig with respiratory symptoms.

Table 1 – *M. hyopneumoniae* positive samples obtained from the porcine respiratory tract of apparently healthy and pneumonic animals – Cuba – 2012-2015

***M. hyopneumoniae* positive samples**					
	Apparently healthy		**Pneumonic**		
	TBL	**Lung swab**	**Nasal swab***	**TBL**	**Total**
Multiplex PCR	3 (23%)	15 (19%)	0	0	18
VNTR Multiplex amplification/ capillary electrophoresis	13 (100%)	56 (73%)	13 (87%)	1	83
VNTR Multiplex amplification/ gel electrophoresis	0	42 (55%)	9 (60%)	1	52
RT-PCR	5 (38%)	61 (79%)	9 (60%)	1	76
Minimal quantity (genome copies/mL)	0	0	0	-	-
Maximal quantity (genome copies/mL)	$4.9*10^4$	2.3*108	$4.7*10^5$	-	-
Mean quantity (genome copies/mL)	-	3.9*106	$6.3*10^4$	-	-

TBL: tracheobronchial lavage
*Nasal swab obtained from symptomatic animals

Quantification of M. hyopneumoniae

The amount of *M. hyopneumoniae* DNA in respiratory tract samples from healthy and pneumonic pigs is shown in table 1. In the tracheo-bronchial lavage from normal lungs, the DNA load ranged from 0 to $4.9*10^4$ genome copies /mL. The mean quantity of *M. hyopneumoniae* in pneumonic lungs was $3.9*10^6$ genome copies/mL and $6.3*10^4$ genome copies/mL in nasal swabs.

Determination of VNTR copy number in M. hyopneumoniae positive samples

As shown in table 2, eight genotypes differing in the VNTR copy number were found during analysis of locus P97. A total of 14 genotypes were found while analyzing locus P146R3, 20 genotypes with locus H2R1 and 15 with locus H4. Strains with 3, 8, 9 and 10 copies of P97R1 and 10 and 17 copies of P146R3 were predominant, while strains with five copies

Table 2 – P97R1, P146R3, H2R1 and H4 VNTR copy numbers and its frequencies in *M. hyopneumoniae*-positive samples from pneumonic and healthy pigs – Cuba – 2012-2015

VNTR	**Number of samples analyzed**	**VNTR copy number**	**Frequency %**	**Symptomatology**
P97R1	87	9	29	A,P
		3	22	P
		10	18	A,P
		8	16	A,P
		5, 12,13	<10	A,P
P146R3	118	17	30	A,P
		10	22	A,P
		12, 19, 20, 21, 22, 23, 26, 27, 31, 37	<10	A,P
		40, 43	<10	A
H2R1	181	8,11	10	A,P
		15	11	A,P
		9 , 10,12, 13, 14, 16,17,18,19,20,21, 22, 25, 27, 28, 30, 31	<10	A,P
H4	128	6	10	A,P
		4, 11	13	A,P
		8	15	A,P
		5	18	A,P
		3, 7, 9, 10, 12, 13, 14, 16, 18, 19	<10	A,P

A: Asymptomatic, (apparently healthy)
P: Pneumonic

of H4 were slightly predominant. No predominant genotypes could be identified by analyzing the H2R1 VNTR.

Different genotypes were found among herds, in the same herd and even co-infecting the same animal. The same genotypes were present in apparently healthy and pneumonic lungs, except for strains having 3 VNTR copy number in P97R1, that were detected only in pneumonic lungs and strains having 40 and 43 VNTR copy number in P146R3 that were detected only in six and five apparently health lungs, respectively.

Discussion

Molecular detection of *Mycoplasma hyopneumoniae* in porcine samples is influenced, among other factors, by the diagnostic method used. In the present study, four different molecular methodologies were used to detect *M. hyopneumoniae* in porcine respiratory samples, a conventional multiplex PCR (YAMAGUTI, 2008), a real-time PCR (STRAIT et al., 2008), an amplification approach, namely MLVA (VRANCKX et al., 2011), based in the multiple amplification of four VNTR containing regions of *M. hyopneumoniae* followed by a capillary electrophoresis and the last methodology used was the same MLVA assay, but using gel electrophoresis to visualize the PCR products. Real-time PCR has several advantages; it is highly sensitive, fast and requires no post-PCR manipulations (MAROIS et al., 2010). However, in our study, the real-time PCR detected six samples less than the multiplex VNTR amplification methodology followed by capillary electrophoresis. According to Marois et al. (2010), because of the great variability reported among *M. hyopneumoniae* strains, PCR assays using only one DNA target per reaction could have reduced performance. Nevertheless, a triplex RT-PCR developed by these authors showed approximately the same detection limit as other RT-PCR assays (DUBOSSON et al., 2004; STRAIT et al., 2008), which detect four targets independently. In our study, including more than one DNA target of the microorganism in the assay increased the number of positive samples detected, which is in agreement with Marois et al. (2010).

Also, in the present study, capillary electrophoresis and gel electrophoresis were carried out in parallel in order to see the number of positive samples obtained after applying both methods of DNA visualization. This was done taking into account that contrary to gel electrophoresis, capillary electrophoresis is a methodology that is not always present in diagnostic laboratories and obviously it is not recommended for the routine diagnosis of a great number of samples. When using gel electrophoresis instead of capillary electrophoresis to visualize the amplified fragments after the multiplex VNTR amplification methodology, the assay detected 24 less samples than the real-time PCR. Our results showed that the gel electrophoresis can be used after multiple VNTR amplification in samples obtained from pneumonic lungs but in those obtained from apparently healthy lungs or in samples with a low load of *M. hyopneumoniae* DNA, results obtained must be carefully analyzed.

Using the multiplex PCR described by Yamaguti (2008), we detected *M. hyopneumoniae* and *M. hyorhinis* in a higher number of pneumonic lungs samples than in healthy ones, which is in agreement with other studies (CARON; OUARDANI; DEA, 2000; PALZER et al., 2008). *M. hyorhinis* was detected in a higher number of samples than *M. hyopneumoniae* and *M. flocculare*, the latest was identified in only one sample. These findings differ from Charlebois et al. (2014), who detected *M. flocculare* in 8.8% of the lungs with lesions suggestive of enzootic pneumonia and always in coinfection with the other two mycoplasmas. In our study, the presence of *M. hyopneumoniae* was not detected in several pneumonic lungs. Thus, the pneumonic lesions observed in these samples were probably caused by other pathogenic virus or bacteria present in the porcine respiratory tract. Charlebois et al. (2014) reported loads of *M. hyopneumoniae* in

pneumonic lungs ranging from $1.17*10^5$ to $3.37*10^9$ genome copies/mL in Quebec, Canada. However, we found in pneumonic lungs a maximal load of *M. hyopneumoniae* of $2.3*10^8$ genome copies/mL. The lesser quantity of *M. hyopneumoniae* found in our samples, regarding the quantity previously reported, could be due to the fact that we sampled pigs subjected to a strongly controlled environment and management conditions, in a program directed to control pneumonia in pigs. In healthy lungs, loads of *M. hyopneumoniae* ranging from 0 to $4.9*10^4$ genome copies/mL were detected. However, even when this quantity is lesser than the load detected in pneumonic lungs, no relationship between the amount of *M. hyopneumoniae* and the severity of the lesions has been previously established (CHARLEBOIS et al., 2014). In adition, a load of *M. hyopneumoniae* similar to that found in pneumonic lungs ($1.6*10^9$ genome copy/mL) was reported in a lung sample with no macroscopic lesions during an experimental inoculation assay (MAROIS et al., 2010).

Analysis of VNTR copy number carried out on samples from the porcine respiratory tract showed the high genetic variability of *M. hyopneumoniae* strains detected in healthy and sick animals from different or the same herd. These results agree with those described previously for isolates obtained from pneumonic lungs (NATHUES et al., 2011; CHARLEBOIS et al., 2014). To our knowledge, this is the first study that shows MLVA genotypes of *M. hyopneumoniae* present in swine lungs without pneumonic lesions in regard to the MLVA genotypes present in pneumonic lungs. Although the four VNTR containing regions analyzed were highly polymorphic, the analysis of VNTR P97R1 and P146R3 revealed the existence of predominant genotypes distributed among all the farms analyzed. These results are in concordance with those reported by Mayor et al. (2008) who suggested that clones of *M. hyopneumoniae* are responsible for local outbreaks.

Differences in the number of aminoacidic repetitions due to the variability in the number of repetitive units contained in the VNTR could have implications in structural, physiochemical and antigenic characteristics of the corresponding proteins (CASTRO et al., 2006). However, there are no studies that show any relationship between such aminoacidic repetitions and the differences in pathogenicity of some *M. hyopneumoniae* strains. In the case of P97 adhesin, it has been suggested that at least seven repeats in the R1 region are needed for *M. hyopneumoniae* adhesion to cilia of swine trachea, a prerequisite for infection and pathogenicity (HSU; MINION, 1998; MINION; ADAMS; HSU, 2000). Interestingly, in the present study, 22% of the strains analyzed have three repeat units in P97R1 and all were identified in pneumonic lungs, but not in healthy ones. On the other hand, strains having 40 and 43 VNTR copy number in P97R1 were identified only in healthy lungs. The presence of nine repeats in *M. hyopneumoniae* strain J (CASTRO et al., 2006), known to be avirulent and have a low adherence activity, (VASCONCELOS et al., 2005), confirms the assumption that other determinants, like putative adhesins P76, P146 and P216, are involved in the adhesion process (CASTRO et al., 2006). Also, VNTR P97R2 is suggested as a required determinant for binding to the host respiratory surfaces, (JENKINS et al., 2006). However, previous studies carried out by Charlebois et al. (2014), concerning MLVA clustering of *M. hyopneumoniae* from abattoir pigs, including VNTR P97R1, P97R2 and P146 among others, revealed that no MLVA clusters were associated with virulent strains.

With the present study, it is impossible to demonstrate if the strains having three repeat units in P97R1 have, in fact, the capacity to adhere to cilia of swine trachea as a prerequisite for being pathogenic. Nevertheless, their presence in the lungs, even without having the minimal number of P97R1 repeats suggested as requisite for adhesion to swine cilia is a matter that needs to be studied further.

Conclusions

In conclusion, the study of apparently healthy lungs of swine showed that the number of *M. hyopneumoniae* positive samples, as well as the load of the microorganism in the samples, was lesser than in pneumonic lungs. Despite the high genetic variability of *M. hyopneumoniae* strains present in different farms in Cuba, predominant strains can be identified by means of VNTR copy number analysis in P97R1, P146R3 and H4. The majority of genotypes were present both in lungs with and without pneumonic lesions; however, a genotype was only detected in pneumonic lungs while other genotypes were only detected in apparently healthy lungs.

Competing interests

The authors declare that they have no competing interests.

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