

# Molecular characterisation of *Mycoplasma hyorhinis* isolated from pigs using pulsed-field gel electrophoresis and 16S rRNA sequencing

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## ABSTRACT

Economic loss in pig breeding is common due to respiratory disorders, and *Mycoplasma hyopneumoniae* and *Mycoplasma hyorhinis*, namely, are the most common infectious agents. The aim of this study is to recover these mollicutes and detect their genotypic variations by pulsed-field gel electrophoresis (PFGE) and sequencing the 16 s rRNA gene. One hundred and twenty-six swabs from tonsil and nasal mucus of pigs with respiratory disorders were analysed. A total of 78 lungs were sampled, as well as two trachea and two tonsils obtained from animals with respiratory disorder. A total of 59 isolates were obtained: 1 (1.70 per cent) of *M hyopneumoniae*, 2 (3.40 per cent) of *Mycoplasma flocculare* and 56 (94.90 per cent) of *M hyorhinis*. The PFGE for *M hyorhinis* showed 10 profiles with enzyme *Ava*I and 9 profiles with *Xho*I. A low polymorphism of the 16sRNS gene was detected in *M hyorhinis* isolates compared with the type strain in the GenBank. *M hyorhinis* isolates of different herds showed a large heterogeneity with enzymes *Ava*I and *Xho*I. The sequencing of the 16S rRNA gene allowed for analysing the interspecific and intraspecific variations of isolated mycoplasmas.

## INTRODUCTION

Respiratory disorders in pig breeding are economically important worldwide (Stakenberg and others 2006). Therefore, infectious agents in farms must be monitored. Respiratory disorders are multifactorial and the diseases depend on environmental conditions and the host–parasite relationships. *Mycoplasma hyosynoviae*, *Mycoplasma hyorhinis* and *Mycoplasma hyopneumoniae* are the most important species in swine farming (Armstrong and Friis 1981). *M hyopneumoniae* is the primary agent of swine enzootic pneumonia (SEP), which causes

high morbidity and low mortality in pigs (Simionatto and others 2013, Stärk 2000). However, *M hyorhinis* was also associated with SEP by Dinter and others (1965). However, for many decades, it was considered a secondary pathogen of SEP or a commensal of the upper respiratory tract.

Different molecular techniques may prove useful in future epidemiological studies to trace strains or to discern infection patterns. To perform such epidemiological studies, the choice of typing technique is critical. However, in the case of mycoplasmas, the value of different typing techniques has never been assessed. As long as whole genome sequencing is not easily attainable, typing techniques, which ideally represent the true phylogenetic relation between strains, are bound to their own intrinsic limitations (Stakenberg and others 2006). Concerned with diversity of mollicutes and their importance to cause respiratory disorders in pigs, the aim of this study was to recover mollicutes from respiratory tracts of pigs in herds and characterise the isolates using pulsed-field gel electrophoresis (PFGE) and 16S rRNA sequencing methodologies.

## MATERIALS AND METHODS

### Sample collection

Samples were collected from nasal mucus and tonsils of swine. These samples were obtained from four pig farms in São Paulo and Paraná/Brazil. In addition, the lungs of pigs were from two slaughterhouses in São Paulo. The samples of nasal mucus and tonsil were from 63 animals aged 8–16 weeks exhibiting coughing and sneezing. The swabs



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were rubbed onto the nasal cavity and tonsil, and then transferred to 2 ml of Friis medium and transported on ice to the laboratory. Fragments of 02 tracheas, 02 tonsils and 78 pig lungs were collected. Samples of trachea, tonsil and lung were obtained from two animals. The lungs with pneumonia lesions were selected during the inspection. The fragments of the trachea and tonsil were from animals with macroscopic lesions in all pulmonary lobes. The clinical samples were stored in sterile plastic bags and transported on ice to the laboratory. Briefly, swabs of chosen fragments were homogenised in the broth, filtered through 0.45 µm nitrocellulose membranes and diluted to 10<sup>-4</sup> in fresh Friis broth. The agar plates were inoculated with 200 µl of each sample dilution. The cultures were incubated at 37°C in 5 per cent CO<sub>2</sub> for up to 30 days (Tully 1993).

### Pulsed-field gel electrophoresis

The isolates and the American type culture collection (ATCC) strains of *Mycoplasma hyorhinis* were subcultured in 12 ml Friis medium at 37°C. Just after pH shifting, each broth was centrifuged for 20 minutes at 16,300 g, washed twice with PBS and pelleted for 20 minutes at 16300 g before being homogenised in 400 µl of Tris-EDTA (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). DNA from the pelleted cells was extracted in agar blocks as described by Marois and others (2001) and each block was digested with *Ava*I and *Xho*I enzymes (Life Technologies, Carlsbad, California, USA). The electrophoresis of the digested DNA was performed in 1.2 per cent ultrapure agarose gel (Bio-Rad) in 0.5 per cent Tris-borate-EDTA (TBE) buffer (90 mM Tris-borate and 1 mM EDTA) at 14°C in a CHEF Mapper DR-III (Bio-Rad). The program was standardised and carried out in one step for PFGE of products digested with *Ava*I and *Xho*I enzymes. The products were exposed to an electrical pulse time of 1–13 seconds for 16 hours. The gels were stained with ethidium bromide (0.1 µg/ml) for 60 minutes and photodocumented with UV light. The size of the fragments was measured using the Vilber Lourmat photodocumentation program. Dendrograms were constructed using the BioNumerics program. The Unweighted Pair Group Method with Arithmetic Mean (UPGMA) was used to determine the genetic relationship between isolates and strains, applying the Dice similarity coefficient (per cent).

### Sequencing of 16S rRNA and phylogenetic analyses

DNA of cultured *Mycoplasma* in 2 ml of Friis medium was extracted according to the method described by Boom and others (1990). The specific primer sets F16S and R16 to amplify 16S rDNA sequence were used as described by Harasawa and Cassell (1996). Primer sets RCO3 and RCO4 were used to better amplify internal regions (unpublished data). PCR products (10 µl) were then electrophoresed in 1 per cent agarose gel with 10 µg/ml of ethidium bromide and visualised with UV

light (Vilber Lourmat, Germany). After confirmation of the only band, the PCR products were purified with GFX PCR DNA and Gel Band Purification Kit (GE Life Science, Brazil) and quantified by comparing with a Low Mass DNA Ladder (Invitrogen). These products were sequenced according to the MegaBACE 1000 protocol, using the DYEnamic ET Dye Terminator Kit (with Thermo Sequenase II DNA Polymerase). Sequences were analysed in the Sequence Analyzer software, Base Caller Cimarron 3.12. Phylogenetic analyses were performed using a BioNumerics program. Briefly, a multiple sequence alignment of the DNA sequences was constructed using the ClustalW (Thompson and others 1997). A phylogenetic tree was obtained using the neighbour-joining method with Tajima-Nei correction. The data set was resampled 1000 times to generate bootstrap values.

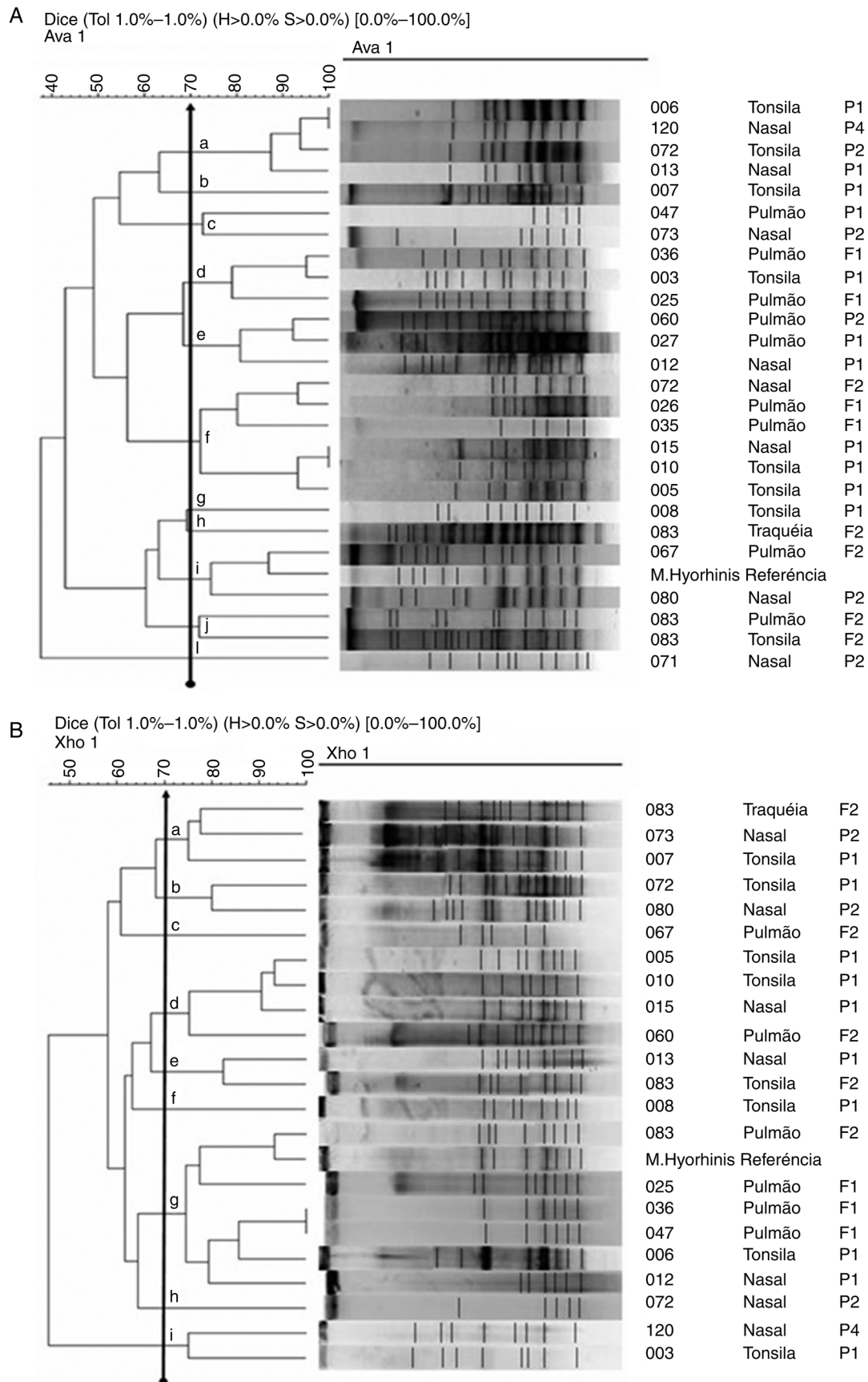
## RESULTS

### Isolation

In the present study, 40 positive cultures (31.74 per cent) of *Mycoplasma* species were obtained in 126 samples of nasal mucus (63 animals) and tonsil (63 animals) with 20 isolates each. Ten animals presented *Mycoplasma* subspecies in both anatomical sites. Nineteen isolates (23.17 per cent) were obtained in lung fragments (78 samples), trachea (2 samples) and tonsil (2 samples). Of these 19 isolates, 17 (89.47 per cent) were obtained from the lung, one was obtained from the trachea and one from the tonsil. One animal showed recovery of *Mycoplasma* species in the lung, trachea and tonsil simultaneously. Among the 59 isolates of *Mycoplasma* species, 56 (94.91 per cent) were identified by PCR methodology. For *M. hyorhinis*, two (3.39 per cent) were identified in two isolates as *Mycoplasma flocculare* and one (1.70 per cent) was identified as *M. hyopneumoniae*. *M. hyopneumoniae* was identified in a fragment lung sample.

### Pulsed-field gel electrophoresis

For PFGE analysis, among the 40 isolates of *M. hyorhinis*, 26 (65 per cent) showed fragments after digestion with enzyme *Ava*I. For the enzyme *Xho*I, 23 isolates (57.50 per cent) showed DNA bands. The dendrogram obtained from *Ava*I digestion allowed grouping the *M. hyorhinis* isolates in 11 clusters of similarity ranging between 70 per cent and 100 per cent (Fig. 1a). There was no formation group according to anatomical sites. Isolates 'Lung 083', 'Trachea 083' and 'Tonsil 083' were obtained from the same animal, but showed different profiles and were grouped into different clusters. The dendrogram obtained from the enzymatic profile using *Ava*I allowed for grouping the *M. hyorhinis* isolates in nine clusters of similarity ranging between 70 per cent and 100 per cent (Fig. 1b). The dendrogram with *Xho*I and *Ava*I did not allow grouping *M. hyorhinis* according to the site recovery of isolates.



**FIG 1:** Dendrogram obtained by Unweighted Pair Group Method with Arithmetic Mean (UPGMA) cluster analysis of DNA fragments from *Mycoplasma hyorhinis* digested by the enzymes *Ava* (a) and *Xho* (b). F, refrigerator; P, farm; Nasal, Nasal swab; Tonsila, Tonsilar swab or tonsilar fragment; Pulmão, Lung. Arrow: cut-off 'Cluster'

## 16S SEQUENCING

The PCR products of *M. hyorhinae* isolates mapped 1438 bases to the ATCC 17981 and the 16 isolates of *M. hyorhinae* showed variations in 31 positions (data not shown). The *M. hyorhinae* isolates were compared with other pathogenic porcine mycoplasmas (Fig. 2). Each group (A, B and C) was related to the species *M. hyopneumoniae*, *M. flocculare* and *M. hyorhinae*, respectively. In group C, isolates of *M. hyorhinae*, the reference ATCC 17981 strain and US 714234 strain showed similarity greater than 98 per cent.

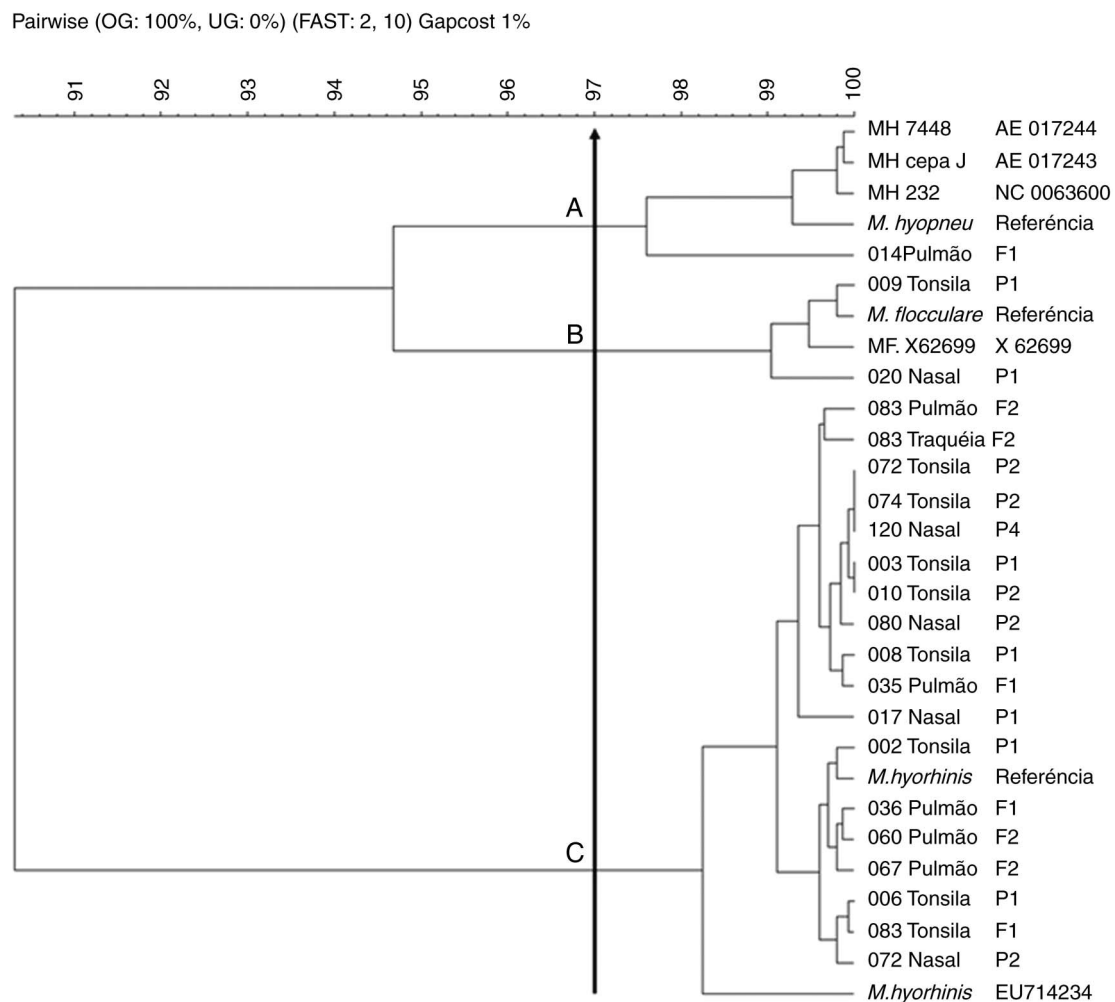
## DISCUSSION

Several methodologies are used to detect mollicutes in infections, and culture remains the gold standard method. However, recovering these bacteria from clinical samples may be laborious and time consuming as occurs with *M. hyopneumoniae*. Although the clinical signs of a respiratory infection in swine helps the diagnosis of this mycoplasmosis (Marchioro and others 2014), the

molecular diagnosis techniques are very helpful to detect these infectious agents in clinical samples.

In the present study, *M. hyorhinae* was the most frequent mollicute recovered from the samples of tonsillar and nasal mucus, and organs of pigs with clinical signs of respiratory infection. These findings are consistent with the findings of a study conducted by Palzer and others (2008) to detect *M. hyorhinae*. Lin and others (2006) inoculated *M. hyorhinae* in pigs and reproduced a SEP-like disease. Only recently has *M. hyorhinae* been considered the primary agent of SEP (Hansen and others 2010).

The PFGE methodology characterised the field isolates of *M. hyorhinae* by their DNA profiles and allowed for grouping them in clusters. The recovered *M. hyorhinae* from different geographic regions and anatomical sites of studied pigs explains the heterogeneity described herein. In fact, Stakenborg and others (2006) and Kokotovic and others (2002) reported high DNA heterogeneity of *M. hyopneumoniae* and *M. hyosynoviae* recovered from pigs from different herds. The role and the consequences of genotypic variations continue to be almost



**FIG 2:** Dendrogram obtained from the sequences of 16S rRNA gene of *Mycoplasma* isolates from pigs and sequences deposited in the GenBank. Arrow: cut-off at least 97 per cent (suitable for intraspecific similarity analysis). F, refrigerator; P, farm; Nasal, Nasal swab; Tonsila, Tonsillar swab or tonsillar fragment; Pulmão, Lung

unknown in mollicutes (Marais and others 1996). The antigen variation due to variable surface lipoprotein genes was detected in isolates of *M. hyorhinis* (Yogev and others 1995).

A number of 16S rRNA gene polymorphisms found between isolates and reference strains of studied *Mycoplasma* were variable, and intraspecific variation was more frequent than interspecific variation (Johansson and others 1998). Königsson and others (2002) evaluated the sequencing of the 16S rRNA gene of 17 isolates of *Mycoplasma agalactiae* and 8 isolates of *Mycoplasma bovis*, finding 21 and 12 polymorphic positions, respectively. However, the interspecific analysis showed only four polymorphic positions. This analysis for *Mycoplasma capripneumoniae* isolates ranged from 11 to 17 nucleotides (Heldtander and others 2001, Pettersson and others 1998).

The genomic subtyping of infectious agents is important to recognise the epidemiology of an outbreak, cross-transmission of a nosocomial pathogen, the source of infection, recognition of virulence and monitoring of vaccination programmes (Savic and others 2010). The PFGE methodology used herein for *M. hyorhinis* was helpful and reproducible, although this technique presents challenging issues during its execution. The 16S rRNA gene sequencing differences found in the present study mainly showed the genomic diversity of *M. hyorhinis* in different anatomical sites of pigs and geographical regions. The different techniques described are useful for modelling the epidemiology of mycoplasmas isolated from pigs, which may be helpful for developing precautionary measures to control enzootic pneumonia in future.

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#### REFERENCES

Armstrong C. H., Friis N. F. (1981) Isolation of *Mycoplasma flocculare* from swine in the United States. *American Journal of Veterinary Research* 42, 1030–1032

Boom R., Sol C. J., Salimans M. M., Jansen C. L., Wertheim-Van Dillen P. M., Van Der Noordaa J. (1990) Rapid and simple method for purification of nucleic acids. *Journal of Clinical Microbiology* 28, 495–503

Dinter Z., Danielsson D., Bakos K. (1965) Differentiation of porcine *Mycoplasma* strains. *Journal of General Microbiology* 41, 77–84

Hansen M. S., Pors S. E., Jensen H. E., Bille-Hansen V., Bisgaard M., Flachs E. M., Nielsen O. L. (2010) An investigation of the pathology and pathogens associated with porcine respiratory disease complex in Denmark. *J Comp Pathol* 143, 120–131

Harasawa R., Cassell G. H. (1996) Phylogenetic analysis of genes coding for 16S rRNA in mammalian ureaplasmas. *International Journal of Systematic Bacteriology* 46, 827–829

Heldtander M., Wesonga H., Bölske G., Pettersson B., Johansson K.-E. (2001) Genetic diversity and evolution of *Mycoplasma capricolum* subsp. *capripneumoniae* strains from eastern Africa assessed by 16S rDNA sequence analysis. *Veterinary Microbiology* 78, 13–28

Johansson K. E., Heldtander M. U., Pettersson B. (1998) Characterization of mycoplasmas by PCR and sequence analysis with universal 16S rDNA primers. *Methods in Molecular Biology* 104, 145–165

Kokotovic B., Friis N. F., Nielsen E. O., Ahrens P. (2002) Genomic diversity among Danish field strains of *Mycoplasma hyosynoviae* assessed by amplified fragment length polymorphism analysis. *Veterinary Microbiology* 85, 221–231

Königsson M. H., Bölske G., Johansson K. E. (2002) Intraspecific variation in the 16S rRNA gene sequences of *Mycoplasma agalactiae* and *Mycoplasma bovis* strains. *Veterinary Microbiology* 85, 209–220

Lin J. H., Chen S. P., Yeh K. S., Weng C. N. (2006) *Mycoplasma hyorhinis* in Taiwan: diagnosis and isolation of swine pneumonia pathogen. *Veterinary Microbiology* 115, 111–116

Marais A., Bové J. M., Renaudin J. (1996) *Spiroplasma citri* virus SpV1-derived cloning vector: deletion formation by illegitimate and homologous recombination in a spiroplasmal host strain which probably lacks a functional *recA* gene. *Journal of Bacteriology* 178, 862–870

Marchioro S. B., Fisch A., Gomes C. K., Jorge S., Galli V., Haesebrouck F., Maes D., Dellagostin O., Conceição F. R. (2014) Local and systemic immune responses induced by a recombinant chimeric protein containing *Mycoplasma hyopneumoniae* antigens fused to the B subunit of *Escherichia coli* heat-labile enterotoxin LT<sub>B</sub>. *Veterinary Microbiology* 173, 166–171

Marois C., Dufour-Gesbert F., Kempf I. (2001) Comparison of pulsed-field gel electrophoresis with random amplified polymorphic DNA for typing of *Mycoplasma synoviae*. *Veterinary Microbiology* 79, 1–9

Palzer A., Ritzmann M., Wolf G., Heinritz K. (2008) Associations between pathogens in healthy pigs and pigs with pneumonia. *Veterinary Record* 162, 267–271

Pettersson B., Bölske G., Thiaucourt F., Uhlén M., Johansson K. E. (1998) Molecular evolution of *Mycoplasma capricolum* subsp. *capripneumoniae* strains, based on polymorphisms in the 16S rRNA genes. *Journal of Bacteriology* 180, 2350–2358

Savic B., Ivetic V., Milicevic V., Pavlovic I., Zutic M., Gagrcin M. (2010) Genetic diversity of *Mycoplasma hyopneumoniae* isolates from conventional farrow-to-finish pig farms in Serbia. *Acta Veterinaria Hungarica* 58, 297–308

Simionatto S., Marchioro S. B., Maes D., Dellagostin O. A. (2013) *Mycoplasma hyopneumoniae*: from disease to vaccine development. *Veterinary Microbiology* 165, 234–242

Stakenborg T., Vicca J., Maes D., Peeters J., De Kruijff A., Haesebrouck F., Butaye P. (2006) Comparison of molecular techniques for the typing of *Mycoplasma hyopneumoniae* isolates. *Journal of Microbiological Methods* 66, 263–275

Stärk K. D. (2000) Epidemiological investigation of the influence of environmental risk factors on respiratory diseases in swine – a literature review. *The Veterinary Journal* 159, 37–56

Thompson J. D., Gibson T. J., Plewniak F., Jeanmougin F., Higgins D. G. (1997) The CLUSTAL\_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 25, 4876–4882

Tully J. G. (1993) Current status of the mollicute flora of humans. *Clinical Infectious Diseases* 17 Suppl. 1, S2–S9

Yogev D., Watson-McKown R., Rosengarten R., Im J., Wise K. S. (1995) Increased structural and combinatorial diversity in an extended family of genes encoding VLP surface proteins of *Mycoplasma hyorhinis*. *Journal of Bacteriology* 177, 5636–5643