RESEARCH ARTICLE
Open Access

Journal of Veterinary and Animal Sciences
ISSN (Print): 0971-0701, (Online): 2582-0605
https://doi.org/10.51966/jvas.2022.53.4.596-601

Detection and molecular characterisation of *Mycoplasma* spp. from respiratory tract infections in pigs#

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Citation: Anjitha, G.S., Sumod, K., Chintu, R., Anoopraj, R., Rajasekhar, R., Jishnu, H.P., Chandankar, V.D. and Koshy, J.2022. Detection and molecular characterisation of Mycoplasma Spp. from respiratory tract infections in pigs. *J. Vet. Anim. Sci.* **53**(4): 596-601
DOI: https://doi.org/10.51966/jvas.2022.53.4.596-601

Received: 28.04.2022 *Accepted: 20.06.2022* *Published: 31.12.2022*

Abstract

Achieving food security involves the maintenance of intensive production systems and large animal populations where infectious diseases are the most important challenge that need to be addressed. Respiratory infections in pigs are a formidable threat to swine farmers worldwide. It is multifactorial and is caused by the interplay between bacterial pathogens, viral agents and environmental factors. The present study aimed to identify mycoplasmal agents causing respiratory infections in pigs. A total of 43 samples, comprising of nasal swabs, and necropsy samples with pneumonic lesions were collected. The DNA extracted from the samples were subjected to Mycoplasma genus-specific, followed by species-specific PCR. Twenty five samples were positive for Mycoplasma spp., but further molecular detection performed through species-specific primers, revealed that 12 samples were positive for M. hyorhinis. None of the samples were positive for *M. hyopneumoniae. The identity of the amplicons were confirmed by nucleic acid sequencing and BLAST analysis. The evolutionary relationship between the detected organisms was also studied by phylogenetic analysis. This study hints at the significance of M. hyorhinis in causing respiratory infections in swine and also suggests that M. hyopneumoniae may not be a significant health hazard for swine populations of north Kerala.*

Keywords: *PRDC, Mycoplasma hyorhinis, 16S rRNA, pneumonia*

Respiratory infections cause hefty financial constraints to swine farmers worldwide and reduced welfare to pigs (Sorensen *et al*., 2006). Porcine respiratory disease complex (PRDC)

#Part of MVSc thesis submitted to Kerala Veterinary and Animal Sciences University, Pookode, Wayanad, Kerala

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refers to the clinical manifestation of respiratory distress and classical pathological lesions on necropsy due to multifactorial aetiologies. The interaction of bacterial pathogens, viral agents and non-infectious factors such as improper management practices and unfavourable climatic conditions contributes to PRDC (Brockmeier *et al*., 2002). Chinju *et al*. (2007) investigated on the bacterial aetiologies causing porcine pneumonia eventually leading to higher mortality rates in Thrissur district of Kerala (India). Several organisms have been described as determinants of PRDC. Among them, *Mycoplasma hyopneumoniae* and *M. hyorhinis* are the organisms involved in porcine mycoplasmosis (Friis, 1971; Brockmeier *et al*., 2002; Lin *et al., 2006; Makhanon et al., 2012;* Siqueira *et al*., 2017; Lee *et al*., 2018).

Mycoplasma hyopneumoniae elaborated a chronic form of respiratory infection in herd and thus had been deemed as the primary agent of PRDC. The clinical manifestation of *Mycoplasma hyopneumoniae* infection included the uncommon epidemic form and the common endemic form, referred to as swine enzootic pneumonia. The infected herd also suffered from respiratory distress, lethargy, pyrexia, decreased growth rate and in severe cases, death (Garcia-Morante *et al*., 2016; Maes *et al*., 2018; Pieters and Maes, 2019). *Mycoplasma hyorhinis eventually came to be considered as a potential porcine pathogen causing swine enzootic pneumonia even though M. hyopneumoniae was initially considered as the only mycoplasmal agent causing the same. It was noticed that M. hyorhinis can cause infection alone or along with M. hyopneumoniae (Lin et al., 2006). M hyorhinis*, being an important bacterial agent in respiratory infection, enhances viral pneumonia and has been isolated from co-infection with diverse pathogens (Lee *et al*., 2018). *Mycoplasma hyorhinis* infection could potentially cause abortion, otitis, pneumonia, conjunctivitis, eustachitis, laboured breathing and meningitis (Bunger *et al*., 2021).

Materials and methods

A total of 43 samples were collected for the study. Twenty seven necropsy samples with pneumonic lesions and nasal swabs from sixteen animals showing respiratory difficulties were considered. The total DNA was extracted from the samples by Qiagen DNeasy Blood & Tissue Kit as per manufacturer's protocol. The polymerase chain reaction (PCR) for genus level detection of *Mycoplasma* spp. was carried out as described by Botes *et al*. (2006) and species level detection of *M. hyopneumoniae* and *M. hyorhinis* was performed as detailed in Lin *et al*. (2006). The primer details are listed in the Table 1. The 25 µL reaction mix consisted of 12.5 µL of 2X Emerald Amp GT PCR Master Mix containing Taq polymerase (TaKaRa), 2µL each of 20 pmol *Mycoplasma* genus specific forward (GPO3) and reverse primers (MGSO), 3 µL of DNA, and NFW. The cycling conditions for amplification of *16S rRNA* gene of *Mycoplasma* were 94°C for 2 min (initial denaturation), 35 cycles of 94°C for 15 sec (denaturation), 59.3°C for 15 sec (annealing) and 72°C for 15 sec (polymerisation) followed by single cycle at 72°C for 5 min (final extension). A no-template control (NTC), which does not contain a template DNA, was included in each run. The cycling conditions for *M. hyopneumoniae* and *M. hyorhinis* were 95°C for 5 min (initial denaturation), 35 cycles of 94°C for 1 min (denaturation), 60°C for 30 sec (annealing) and 72°C for 1 min (polymerisation) followed by single cycle at 72°C for 10 min (final extension). A no template control (NTC) was also included.

The PCR products were purified using Gene JET Gel Extraction Kit (Thermo Fisher Scientific, Massachusetts, USA) and sent to AgriGenome Lab Private Limited, Cochin, India for sequencing.

With the help of Chromas Lite v2.01 software (http://www.technelysium.com.au) the chromatograms of the sequences were analysed for their proper assembly. To confirm the presence of gene specific to *M. hyorhinis*, BLAST was conducted (http://www.ncbi.nlm.nih.gov/BLAST). Subsequently, sequences of other bacterial isolates available in the GenBank database were retrieved and compared with those obtained in the study. Phylogenetic analysis of the sequences obtained in this study was carried out using MEGA X software (Kumar *et al*., 2018). For phylogenetic analysis, *Mycoplasma hyorhinis*

Table 1. Details of PCR primers used for detection of *Mycoplasma* spp.

Set	Organism	Primer Name	Sequence 5'-3'	Target region/ gene	Product size (bp)	References
1	*Mycoplasma* spp.	GPO3 FP	5' -TGGGGAGCAAACAG GATTAGATACC-3'	*16S rRNA*	270	Botes *et al*. (2006)
		MGSO RP	5'-TGCACCATCTGTCAC TCTGTTAACCTC-3'			
2	*Mycoplasma hyopneumoniae*	NMF FP	5'-ACTAGATAGGAAATG CTCTAG-3'	*16S rRNA*	413	Lin *et al*. (2006)
		N2 RP	5'-ATACTACTCAGGCGG ATCATTTAAC-3'			
3	*Mycoplasma hyorhinis*	R1 FP	5'-TATCGCATGATGAGT AATAG-3'	*16S rRNA*	707	Lin *et al*. (2006)
		N2 RP	5'-ATACTACTCAGGCGG ATCATTTAAC-3'			

isolates from different countries were downloaded from GenBank (www.ncbi.nlm.nih.gov/genbank/). Using ClustalW program of MEGA 10.2, alignment of the downloaded sequences was done followed by trimming of the same to match sequence lengths obtained in this study. The evolutionary history was inferred by using the Maximum Likelihood tree method (Yang, 1997). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein, 1985) was taken to represent the evolutionary history of the sequences analysed. Determination of evolutionary distances were done Tamura-3-parameter method (Tamura, 1992) as they had the lowest Bayesian Information Criterion scores (BIC).

Results and discussion

A total of 43 samples were subjected to amplification of *16S rRNA* gene for detecting *Mycoplasma* spp. and 25 samples showed positive results. These positive samples were then subjected to amplification with species-specific primers. Twelve samples were positive for *M. hyorhinis* and no samples were positive for *M. hyopneumoniae*. The three primer sets were targeting the *16S rRNA* gene of the bacteria. Percentage positivity was calculated to be 27.91 per cent for *M. hyorhinis*. Amplicons of 270 bp generated by the PCR reaction with *Mycoplasma* genus-specific primers, and 707 bp generated by the PCR reaction with *M. hyorhinis* primers were visualised in agarose gel (Fig.1). No band was observed in the NTC. The positivity rate of 27.91 per cent in samples with pneumonic lesions and absence of *M. hyopneumoniae*, suggests the potential of *M. hyorhinis* in causing respiratory infections in pigs. Similar results have been described in previous studies, which indicated the potential role of *M. hyorhinis* in causing swine pneumonia, even though *M. hyopneumoniae* was the most frequently isolated mycoplasma in enzootic pneumonia cases of pigs. These studies also mentioned *M. hyorhinis* as a pneumonic pathogen and reported the PCR technique as the quickest, easiest, most reliable, and sensitive approach to diagnose *M. hyorhinis* compared to culture and western blotting (Lin *et al*., 2006).

In the present study, clinical signs noticed in animals showing respiratory

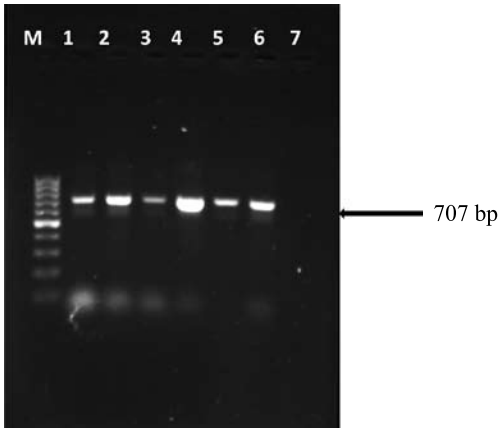

Fig.1. Agarose gel showing the 707 bp amplicons generated by targeting 16S rRNA gene of *M. hyorhinis*

distress were general weakness, weight loss, inappetence, pyrexia, anorexia, laboured breathing, lameness, dyspnoea, epiphora and mucopurulent oculonasal discharge. On necropsy, the major lesions noticed were bronchopneumonia, fibrinous pleuropneumonia, lobar pneumonia, haemorrhagic peripheral lymph node, blood-tinged froth in the trachea, epicardial haemorrhage, meningitis, cerebral oedema, epistaxis and pulmonary emphysema. Similar clinical signs and lesions were noticed in *M. hyorhinis* infection and in experimental studies (Gois *et al*., 1971; Lin *et al*., 2006; Lee *et al*., 2018; Fourour *et al*., 2019).

In the phylogenetic studies of *16S rRNA* gene sequence of *M. hyorhinis*, a 707 bp fragment was taken for analysis. Alignment of the sequences corresponding to the isolates in the study together with foreign reference isolates depicted in the phylogenetic tree revealed that the isolates in the study are clustered together except for one isolate (214/MIB/21). The majority of isolates considered in the study showed more relationship to strain ATCC VR-2580 and HUB-1 isolated from USA and China, respectively, even though they formed a separate branch in the phylogenetic tree. The isolate 214/MIB/21 showed more resemblance to strain USP17N (GU227402) from Brazil. There was no suitable

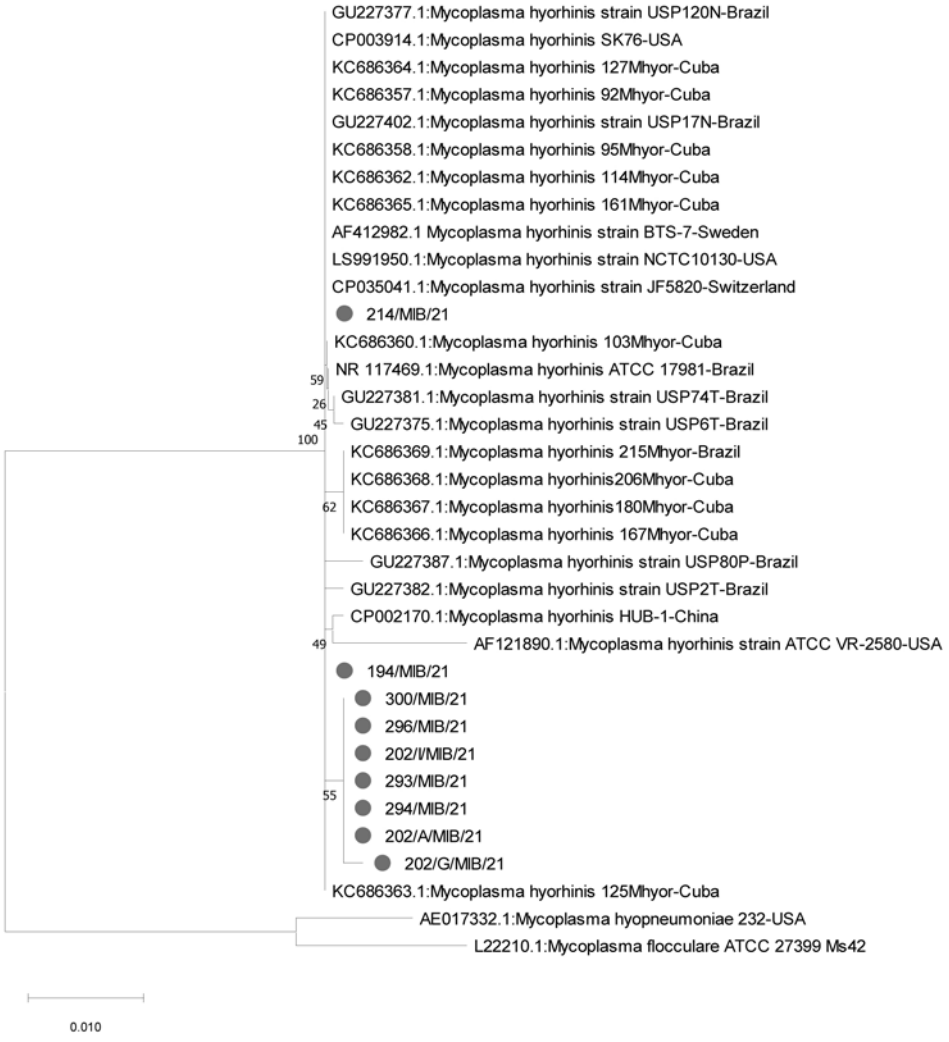

Fig.2. Phylogenetic tree based on the analysis of 707 nucleotides of 16S rRNA gene of *Mycoplasma hyorhinis* isolates of pig from various countries.

nucleotide sequence related to *16S rRNA* gene of *M. hyorhinis* Indian isolates available in the GenBank for phylogenetic studies. The *16S rRNA* genes of *M. hyopneumoniae* and *M. flocculare* were also considered in the analysis and branched out separately in three arms indicating the dissimilarity (Fig.2).

Conclusion

The present study could detect the predominant *Mycoplasma* species in respiratory infections in swine in north Kerala. *Mycoplasma hyorhinis*, and not *Mycoplasma hyopneumoniae*, plays a major role in porcine mycoplasmosis in north Kerala, although historically, more studies report *M. hyopneumoniae* as the major pathogen of enzootic pneumonia.

Acknowledgement

The authors thank the Dean, College of Veterinary and Animal Sciences, Pookode for providing the facilities for the conduct of the study.

Conflict of interest

The authors declare that they have no conflict of interest.

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