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Research Article

MOLECULAR DETECTION AND DIFFERENTIATION OF MYCOPLASMA SPECIES IN SWINE LUNG TISSUES

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ABSTRACT: *Mycoplasma* species are ubiquitous and cause both primary and secondary infections in swine. Most of these *Mycoplasma* species are fastidious and require stringent growth requirements. Molecular detection and differentiation of these *Mycoplasma* species directly from field samples have greater diagnostic value. A total of 57 porcine tissue samples collected from different regions of Tamil Nadu suspected of respiratory tract infections were screened with a PCR assay targeting *the 16SrRNA* gene of Mycoplasma and a multiplex PCR to identify and differentiate *M. hyopneumonia, M. focculare,* and *M. hyorhinis* infections. The PCR-based screening with *16SrRNA* genus-specific primer confirmed Mycoplasma infection in four (7%) of the 57 samples screened with a specific amplicon of 717 bp. Multiplex PCR analysis of all 57 samples showed a similar positivity rate of 7% (n=4), demonstrating the presence of three 1129 bp and one 754 bp amplicons specific to *M. hyorhinis* and *M. flocculare* genome respectively in pneumonic lesions of swine from Tamil Nadu. The specificity of the *M. hyorhinis* genome was further confirmed by sequencing and BLAST analysis. A BLAST homology search revealed 99% sequence identity with European Mycoplasma isolates. Phylogenetic analysis in MEGA X, through the Maximum Likelihood tree (MLT) method revealed specific clustering along with *M. hyorhinis* species. The assembled partial contig sequence *M. hyorhinis* was deposited in the GenBank database under the accession number MW822751.

Key words: *Mycoplasma* spp, Swine, Detection, Multiplex-PCR, Phylogenetic analysis.

INTRODUCTION

Mycoplasma species are the smallest known self-replicating prokaryotic organisms that lack cell walls and belong to the class *Mollicutes* (Tully 1992, Uphoff *et al.* 1992). Mollicutes are heterogeneous and distributed in all living things (humans, animals, insects, and plants) with known as well as unknown pathogenic potential (Baseman *et al.* 1997). *M. hyopneumoniae, M. hyorhinis*, and *M. focculare* are fastidious bacteria, commonly associated with the porcine respiratory tract with varying clinical outcomes. *M. hyopneumoniae* primarily causes zoonotic pneumonia and is also associated with the porcine respiratory disease complex. It is one of the most chronic diseases in pigs associated with low mortality and high morbidity (Frey *et al.* 1994).

M. hyorhinis is the normal habitant in the mucosa of the upper respiratory tract and tonsils of healthy adult and growing pigs, often without clinical disease and rapidly transmitted through nasal secretions in group-housed pigs and typically no clinical disease otherwise animals are stressed (Thacker and Minion 2012).

However, some strains can cause polyserositis and polyarthritis in weaned piglets, pneumonia, otitis media in adults, as well as neurologic disorders and abortion (Goiš *et al.* 1971, Friis and Feenstra 1994, Palzer *et al.* 2008). Apart from animal infections, *M. hyorhinis* was also detected as a cell culture contaminant (Namiki *et al.* 2009). *M. hyorhinis* frequently reported along with porcine respiratory disease complex (PRDC) conditions caused by *M. hyopneumoniae* (Lin *et al.* 2006). It can, also able to invade immune cells and be detected in gastric

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ulcer patients of humans with unexplored pathogenic potential via the consumption of porcine products (Tocqueville *et al.* 2014, Nascimento Araujo *et al.* 2021).

M. flocculare is widely distributed in swine, in normal and pneumonic lungs, and in nasal cavities, but the pathogenic potential and clinical outcome of *M. flocculare* unknown and needs to be explored further (Thacker and Minion 2012). Because of its close resemblance of *M. hyorhini and M. focculare* to *M. hyopneumoniae* has attracted great interest to discriminate between these three mycoplasma species (Gomes Neto *et al.* 2014).

In-vitro cultivation and characterization of *Mycoplasma* species are laborious, require specific nutritional supplementation and all the Mycoplasma species cannot be isolated using a common isolation medium (Friis 1972). Therefore diagnostic methods with high sensitivity like genome detection by PCR, qPCR, and genome sequencing are promising options (Calsamiglia *et al.* 1999). Stakenborg *et al.* (2006) developed a multiplex PCR assay with three different forward primers in the species-specific region, while the reverse primer was based on a highly conserved region of 16S rRNA gene common to all mycoplasmas to distinguish *M. hyopneumoniae, M. focculare,* and *M. hyorhinis* in the broth culture and the specificity of this multiplex PCR was also tested using each forward primer together with the common reverse primer in individual PCR and proved its specificity. At present infectious causes are crucial in swine husbandry associated with the loss of swine population and contribute to economic loss, but as of date, reports of *Mycoplasm*a species-associated infections in livestock are scanty and there is no detailed study on swine *Mycoplasma* prevalence in the entire India due to the difficulties in isolation and characterization. Keeping the above points, the present study has been undertaken for the identification and differentiation of major Mycoplasma species-associated lung lesions in swine.

MATERIALS AND METHODS

Collection and extraction of nucleic acid

A total of 57 tissue samples (pool of all visceral organs and lymphoid tissues) were collected from different parts of Tamil Nadu during 2020-2021 with a history of respiratory tract infections were used in this study (Fig. 1). All the samples were collected from dead pigs and piglets as postmortem samples with the history of respiratory tract infections following standard sampling procedures and the study sampling don't involve any invasive procedures for sample collection, hence this study doesn't require any institutional animal ethical committee approval. The collected samples were immediately transported to the laboratory under refrigeration conditions and processed as per the standard procedures and stored at - 40 °C until analyzed by following the standard method (Rakesh Kumar *et al.* 2021, Parthiban *et al.* 2022). All the tissues were subjected to extraction of DNA using DNAeasy Blood and Tissue Kit (Qiagen, Germany, Cat. No. 69504) and screened for Mycoplasma screening.

Genus-specific detection of mycoplasma nucleic acid

All fifty-seven porcine tissue samples were screened for the mycoplasma genome through genus-specific 16S rRNA gene-based PCR assay (Dussurget and Roulland-Dussoix 1994). The forward and reverse primer sequences are 5'ACTCCTACGGG AGGCAGC AGT3'and 5'TGCACCATCTGTCAC TCTGTTAAC CTC3' respectively. The reaction mixture includes 12.5 µl of 2x GoTaq Green master mix (Promega, USA), 1µl of each forward and reverses primer with 10 pmol/µl concentration, 4 µl of the template DNA, and nuclease-free water was added to make up the 25 µl PCR reaction mixture. PCR cycling condition includes initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 2 min and final extension step at 72°C for 10 mins. PCR products were analyzed by 1 % agarose gel electrophoresis and gel documented.

Multiplex PCR for detection and differentiation of Mycoplasma species

Sensitive detection and differentiation of mycoplasma species can be made through screening of suspected culture grown in Mycoplasma broth culture medium with supplements (Himedia) by multiplex PCR with three forward primers 5'TTCAAAGGAGCCTT CAAGCTTC3', 5'CGGGATGTAGCAATA CATTCA G3'and 5'GGGAAGAAAAAAATTAGGTAGGG3' specific for the *M. hyopneumoniae, M. hyorhinis,* and *M. flocculare* respectively and one common reverse primer 3'AGAGGCATGATGATTTG ACGTC5'conserved in all three mycoplasma species was developed by Stakenborg *et al.* (2006). The specific PCR amplicons comprise 1000 bp for *M. hyopneumoniae*, 754 bp for *M. focculare*, and 1129 bp for *M. hyorhinis*. This approach is slightly modified to screen the Mycoplasma genome in tissue samples directly without cultivation to minimize the detection time. The optimized PCR reaction conditions include initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec,

annealing at 55°C for 30 sec, extension at 68°C for 1 min, and the final extension at 72°C for 10 mins and the PCR was carried out in Thermocycler (Mastercycler® model nexus, Eppendorf, Germany). PCR reaction mixture includes 12.5 ml of 2x GoTaq Green master mix (Promega, USA), 0.5µl of each forward primer with 50 pmol concentration (total volume of 1.5µl/for three primers), and 150 pmol (1.5 ul) of one common reverse primer,4 µl of DNA template and finally nuclease-free water was added to make up the 25 µl reaction. PCR-positive samples were randomly selected and sequenced at a commercial DNA sequencing facility.

Genetic characterization

Multiplex PCR-specific amplicons were sequenced at a commercial sequencing facility and sequence reads were annotated and aligned in the SnapGene tool (Feng *et al.* 2019) and contig sequences were further subjected to phylogenetic studies along with reference and established *Mycoplasma* species sequences in Molecular Evolutionary Genetics analysis-X (MEGA X) tool using Maximum Likelihood tree (MLT) with 1000 bootstrap replicates to find evolutionary relatedness (Kumar *et al.* 2018). The sequence obtained in this study was characterized based on BLAST homology in the NCBI database, and phylogenic studies.

RESULTS AND DISCUSSION

Molecular detection for mycoplasma genome in swine

Genus specific PCR based screening for the Mycoplasma genome in 57 field samples revealed, 7% (n=4) positivity by producing a specific amplicon of 717 bp (Fig. 2). The details of Mycoplasma screening results for all samples are displayed in Table 1. All four samples which are found positive for the Mycoplasma genome in this study had pneumonic lesions from adult pigs and none of the samples from piglets were found to be positive for the Mycoplasma genome. A mycoplasma screening study conducted by Pulgarón *et al.* (2015) in Western Cuba with 155 samples collected from pigs with respiratory tract infection and or pneumonic lesion revealed 68% (n=106) positivity for the Mollicutes by genus-specific PCR. More than 80% prevalence of Mollicutes was documented in pigs with pneumonic lesions whereas, apparently healthy pigs evidenced less than 20% prevalence of Mollicutes which supports the present study. A study by Makhanon *et al.* (2012) in Thailand strongly evidenced that direct PCR-based screening yielded a higher detection rate of mycoplasma positivity when compared to culture-based detection from field samples.

Table 1. Details of tissue samples screened for mycoplasama of Tamilnadu state, India.

Sample source (District)	Farm size	Vaccination History	Year of sample	No of samples collected	No of samples positive by PCR	Percent Positivity	Mycoplasma species detected
Chennai (LWY)	120	Vaccinated for CSF; non vaccinated for PPV, PCV2.	2021	17	3	17.64	*M. hyorhinis* - 2 *M. flocculare* -1
	30	Non vaccinated for CSF, PPV, PCV2.	2021	3	0	-	-
Chengalpattu (LWY and KPMG)	320	Vaccinated for CSFV, PCV2, non vaccinated for PPV	2021	19	0	-	-
Tirunelveli (LWY)	40	Vaccinated for CSF, non vaccinated for PPV, PCV2	2020	3	1	33.3	*M. hyorhinis* -1
Vellore (ND)	20	Non vaccinated for CSF, PPV, PCV2	2021	01	0	-	-
Villupuram (LWY, ND)	100	Non vaccinated for CSF, PPV, PCV2	2020-2021	14	0	-	-
Total				57	4	7.01	

*LWY- Large white Yorkshire, KPMG-Kattupakkam gold, ND- Non-descript.
*CSF-Classical swine fever; PPV- Porcine parvovirus; PCV2- Porcine circovirus-2.

Table 2. Details Mycoplasama sequences used in Phylogenetic study.

Sl. No.	Accession No.	Country	Year	Sequence details
1.	KC737044.1	UK	2012	Uncultured bacterium clone WB50 16S ribosomal RNA gene, partial sequence
2.	NR_117469.1	USA	2009	Mycoplasma hyorhinis ATCC 17981 16S ribosomal RNA, partial sequence
3.	NR_113686.1	USA	2011	Mycoplasma hyorhinis strain NBRC 14858 16S ribosomal RNA, partial sequence
4.	GU227386.1	Brazil	2009	Mycoplasma hyorhinis strain USP72N 16S ribosomal RNA gene, partial sequence
5.	MK554801.1	Austria	2003	Mycoplasma sp. strain 480 16S ribosomal RNA gene, partial sequence
6.	MK789493.1	Turkey	2019	Mycoplasma ovipneumoniae strain MYCO19 16S ribosomal RNA gene, partial sequence
7.	NR_117470.1	USA	2009	Mycoplasma hyopneumoniae J 16S ribosomal RNA, partial sequence
8.	MK615080.1	Austria	2018	Mycoplasma sp. strain 3151_1 16S ribosomal RNA gene, partial sequence
9.	KY307829.1	Cuba	2016	Mycoplasma hyopneumoniae isolate YBPM302 16S ribosomal RNA gene, partial sequence
10.	NR_025185.1	USA	2008	Mycoplasma lagogenitalium strain 12MS 16S ribosomal RNA, partial sequence
11.	FJ226566.1	USA	2019	*Mycoplasma procyoni* strain LR5794 16S ribosomal RNA gene, partial sequence
12.	DQ000588.1	China	2009	Mycoplasma ovipneumoniae strain XJ-3f 16S ribosomal RNA gene, partial sequence
13.	NR_025182.1	Sweden	2019	Mycoplasma dispar strain 462/2 16S ribosomal RNA, partial sequence
14.	NR_121731.1	USA	2019	Mycoplasma bovoculi M165/69 16S ribosomal RNA, partial sequence
15.	MT735182.1	Austria	2020	Mycoplasma sp. strain Z92 16S ribosomal RNA gene, partial sequence
16.	KY264124.1	Cuba	2016	Mycoplasma hyopneumoniae isolate F1.12 16S ribosomal RNA gene, partial sequence
17.	MK343461.1	Brazil	2019	Uncultured Mycoplasma sp. clone E53 16S ribosomal RNA gene, partial sequence
18.	GU227387.1	Brazil	2009	Mycoplasma hyorhinis strain USP80P 16S ribosomal RNA gene, partial sequence
19.	NR_041845.1	USA	2019	Mycoplasma hyorhinis strain BTS7 16S ribosomal RNA, partial sequence
20.	EU265779.1	USA	2008	Mycoplasma ovipneumoniae strain 06OR03 16S ribosomal RNA gene, partial sequence
21.	EF687778.1	China	2007	Mycoplasma ovipneumoniae strain MoGH3-3 16S ribosomal RNA gene, partial sequence
22.	MK789475.1	Turkey	2019	Mycoplasma ovipneumoniae strain MYCO1 16S ribosomal RNA gene, partial sequence
23.	KJ433280.1	Norway	2014	Mycoplasma ovipneumoniae strain Dovre 16S ribosomal RNA gene, partial sequence
24.	MW280113.1	Germany	2020	Mycoplasma sp. strain 338_4 16S ribosomal RNA gene, partial sequence

PCR differentiation and characterization of mycoplasma species in swine

All 57 samples were further re-subjected to the multiplex PCR assay for detection and differentiation of *M. hyopneumonia, M. focculare*, and *M. hyorhinis* species. Three samples (5.2 %) out of 57 samples screened yielded specific amplicon of 1129 bp specific to *M. hyorhinis* (Fig. 3a) and one sample (1.7 %) yielded 754 bp specific to *M. flocculare* (Fig. 3b). The *M. hyorhinis* detection was further confirmed by sequencing and NCBI-BLAST analysis of one specific amplicon (1129 bp) specific to sample INDTNCHN-L17. BLAST homology analysis of sequence revealed 99% sequence identity with European *M. hyorhinis* isolates and the assembled contig sequence was deposited to the GenBank database under the accession number MW822751. Phylogenetic analysis of obtained 16S rRNA sequence of INDTNCHN-L17 sequence along with 24 published different Mycoplasma sequences (Table 2) by MLT in MEGA X yielded specific clustering along with *M. hyorhinis* species.

The serological-based Mycoplasma species differentiation methods are not always appropriate due to the cross-reactivity between *M. hyopneumoniae*, *M. hyorhinis*, and *M. flocculare* species (Petersen *et al*. 2016). Hence the popular and potential PCR-based assay was used for the differentiation of Mycoplasma species (Calsamiglia *et al.* 1999). Initially, *M. hyopneumoniae* was considered to be the sole mycoplasma species associated with swine enzootic pneumonia (SEP) in Taiwan but later *M. hyorhinis* associated cases of porcine respiratory disease complex (PRDC) and SEP were also evidenced. This increased incidence of *M. hyorhinis* associated infections suggests its significant role in swine respiratory diseases (Lin *et al.* 2006). Whole genome sequencing and phylogenetic analysis were followed in many countries for molecular differentiation of mycoplasma species (Trüeb *et al.* 2016).

Makhanon *et al.* (2012) screened 160 samples in Thailand by species-specific primers and revealed 10% (16/160) and 8.8% (14/160) genome positivity for *M. hyorhinis* and *M. flocculare,* respectively whereas, the

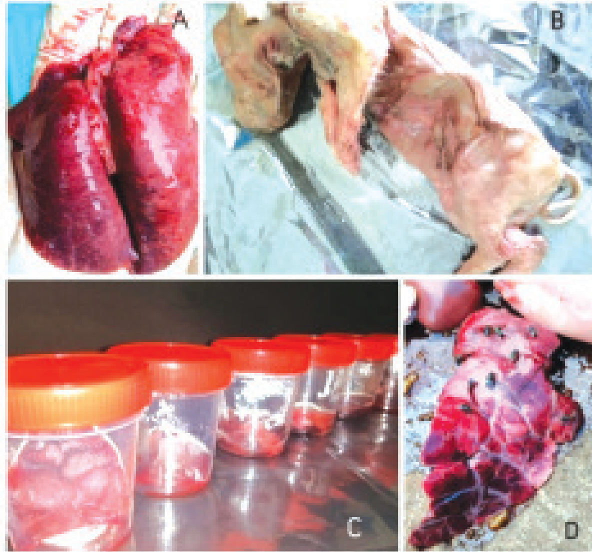

Fig. 1. Porcine tissue samples.
[A. Lung tissue from piglet showing pneumonic lesions, B. Piglet died of respiratory failure, C. Aseptically collected tissue samples, D. Lung tissue from adult pigs with pneumonic lesions].

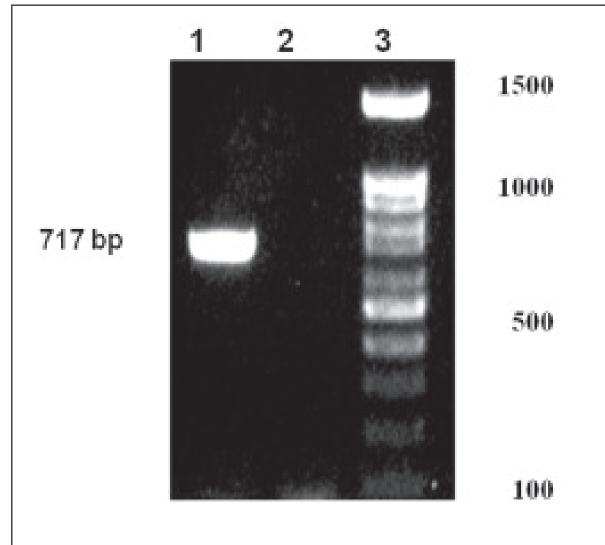

Fig. 2. Screening of Mycoplasma genome by PCR assay targeting 16srRNA gene.
[Lane 1- Field sample (CUL17); Lane 2 – Non template control; Lane 3- ladder (100 bb). The amplified product is 717 bp and labeled separately].

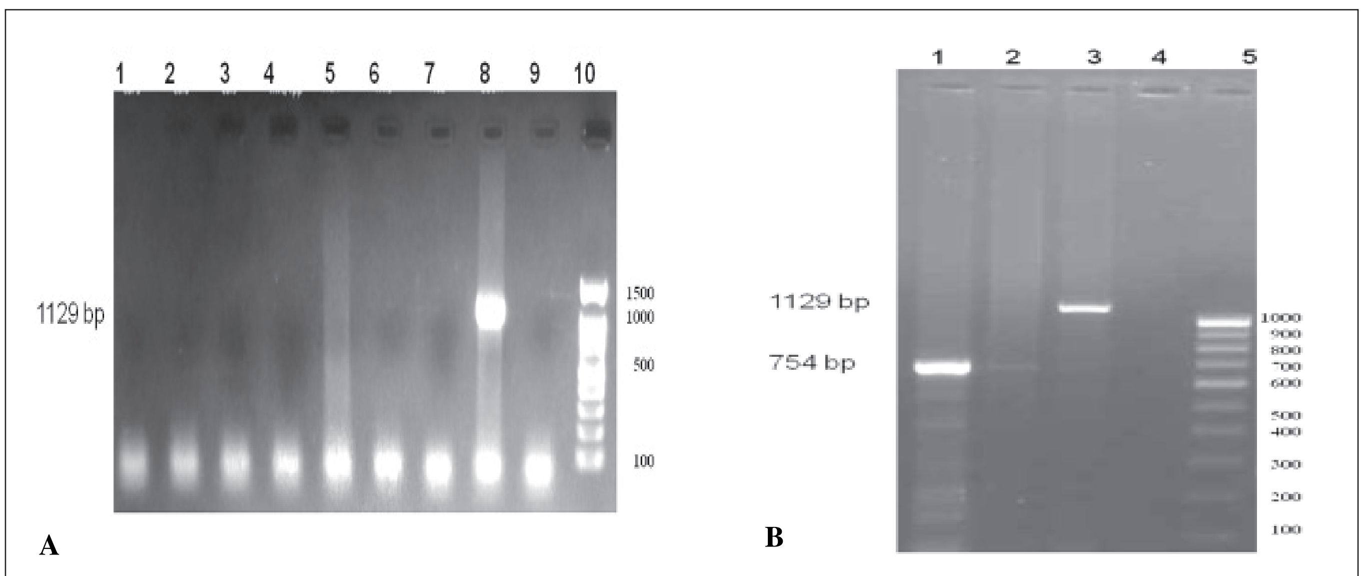

Fig. 3. Mycoplasma species detection and differentiation by Multiplex PCR assay.
[A. *M. hyorhinis* positivity: Lane 1, 2, 3, 4, 5, 6 & 7- field samples, Lane 8- Field sample (CUL-17) positive for *M. hyorhinis,* Lane 9-Non-template control, Lane 10- 100 bp ladder; B. *M. hyorhinis* and *M. flocculare* –positivity: Lane 1, 2, 3, 4, 5, 6 & 7- field samples, Lane 8- CUL-17 positive for *M. hyorhinis,* Lane 9-Non-template control, Lane 10- 100 bp ladder].

present study conducted in pigs with respiratory problems also showed less than 10% positivity for *Mycoplasma* sp which needs to be compared with healthy slaughtered animals screening for its clinical association with respiratory problems. Another study based on Multiplex PCR-based Mycoplasma species differentiation in 106 mycoplasma-positive samples from west Cuba revealed 30% (32/106) positivity of *M. hyorhinis* genome and 80% of it detected from pneumonic lungs. *M. flocculare* was detected in one sample out of 106 positive samples from a pig with respiratory symptoms which is in concordance with the present study (Pulgarón *et al.* 2015).

Molecular characterization and phylogenetic analysis of mycoplasma in India are scarce. This study documents the incidence of *M. hyorhinis and M. flocculare* for the first time in Tamil Nadu. The *M. hyorhinis* isolate

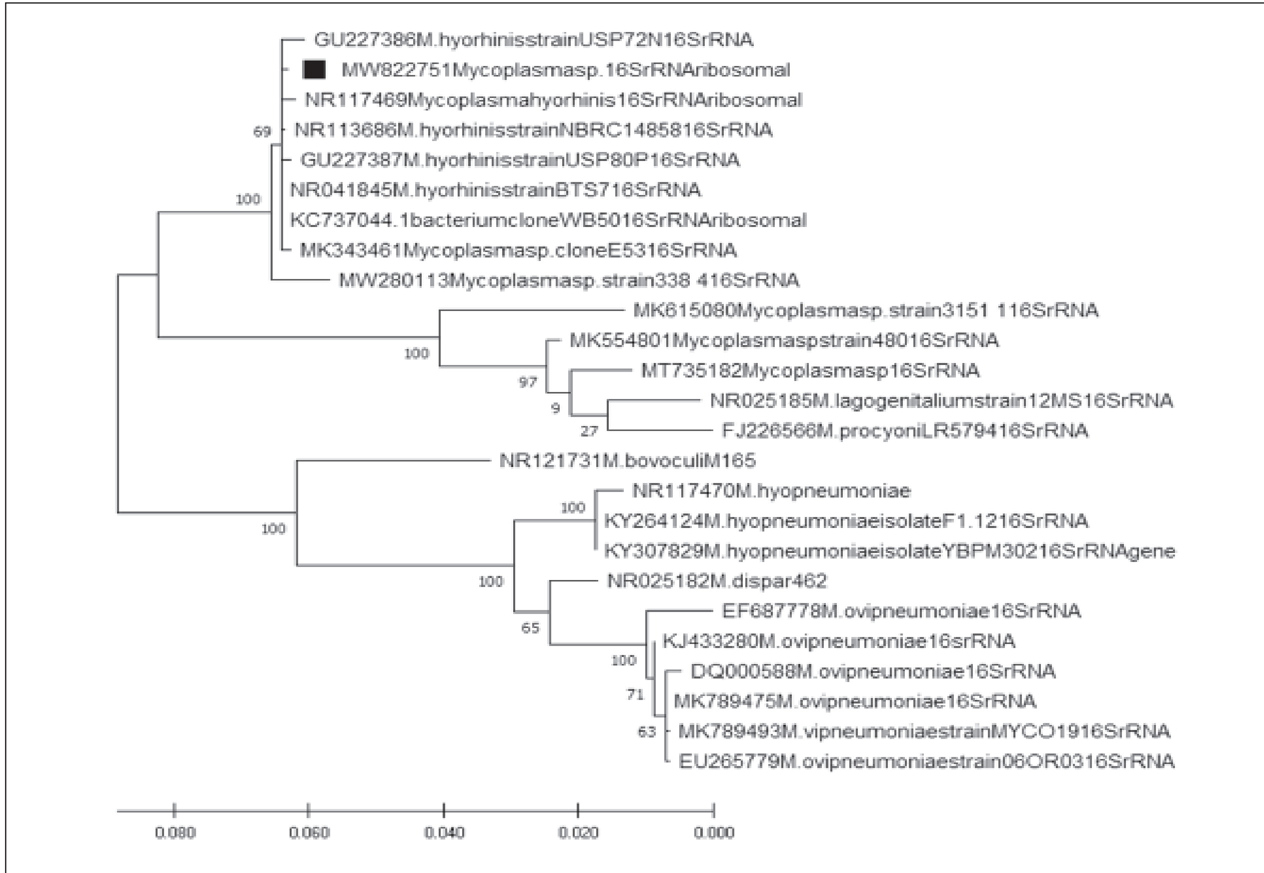

Fig. 4. Phylogenetic analysis of *Mycoplasma hyorhinis* in MEGAX (Maximum Likelihood Tree).
[The phylogenetic trees were drawn with one *M.hyorhinis* sequences from this study (labeled by black Colored Square along with 24 Mycoplasma sequences from the GenBank. The analyses were conducted in MEGA X with Bootstrap replicates of 1000].

(GenBank accession number MW822751) in this study showed homology to European Mycoplasma isolates which needs further extensive analysis and one possible reason may be due availability of imported pigs from Western countries. To determine the prevalence, transmission, molecular epidemiology, and impact of Mycoplsma in commercial swine husbandry it is necessary to extend this study to larger populations. Mycoplasma may occur as a primary pathogen or co-pathogen in the swine community and can cause diverse clinical outcomes which need close monitoring and surveillance to explore its fullest pathogenic potential.

CONCLUSION

In conclusion, this is the foremost report on the molecular detection of porcine mycoplasma genomes in postmortem tissues. This study reports molecular evidence of *M. hyorhinis* and *M. flocculare* associated pneumonic infection in swine for the first time in Tamil Nadu. Extensive genome-based screening and isolation of mycoplasma species from swine populations of southern India are required to understand its pathogenic potential.

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