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Prevalence and Molecular Characterization of *Mycoplasma* Species Causing Contagious Caprine Pleuropneumonia in Goats from Sindh, Pakistan

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Key words
***Mycoplasma*, CCPP, Goat, Prevalence, Pakistan**

ABSTRACT

The contagious caprine pleuropneumonia (CCPP) is a complex respiratory syndrome causing high morbidity and mortality in goats resulting in the huge economic losses in the goat farming industry. The present study aimed to investigate the prevalence with molecular characterization of the *Mycoplasma* species causing infections in goats from the Sindh province of Pakistan. A total of 800 nasal samples from goats from four representative districts (Thatta, Tharparkar, Jamshoro and Larkana) were collected using swabs for bacteriological investigations. A total of 48 (6%) nasal samples out of 800 were found positive for the presence of either species of *Mycoplasma* in the study area. Data indicates a higher *Mycoplasma* infection rate from the positive sample of goats in Tharparker district (35%) followed by Thatta (27%), Jamshoro (21%) and Larkana (17%). Further, the PCR amplification using different genus and species specific primer sets were employed to identify species infecting goats. The results showed that two sets of genus specific primers targeting 30S ribosomal S7 proteins (31 samples) and 16S rRNA gene (17 samples) successfully amplified all the culture positive samples indicating the presence of *Mycoplasma* in the isolated samples. On the basis of molecular identification, species wise prevalence were detected as 64.58% *Mycoplasma mycoides* subsp. capri and 35.41% *Mycoplasma capricolum* subsp. capripnuemoniae. Moreover, the sequencing of amplified fragments showed that the former primer only amplified the targets from *Mycoplasma mycoides*, while the later amplified targets only from *Mycoplasma capricolum*. The sequence analysis revealed that both of the identified species shows a highest sequence similarity with isolates from Turkey and also clustered with same species during phylogenetic analysis. Taken together, the current study reports for the first time the prevalence and molecular characterization of the *Mycoplasma* species infecting goats in Sindh Province. The baseline data generated will further help in strategic control and vaccine development against *Mycoplasma* infecting goats in Pakistan.

INTRODUCTION

Contagious caprine pleuropneumonia (CCPP) is an infectious disease of caprine caused by a tiny gram negative bacterium belonging to the genus *Mycoplasma* (Cottew *et al*., 1987; Fischer *et al*., 2012). The infection is characterized by acute respiratory distress accompanied by coughing with purulent discharges, fever and anorexia (Radostitis *et al*., 2009; OIE, 2014). The disease results in the huge economic losses to the livestock industry due to its high morbidity and mortality rate in the endemic areas especially in Asia and Africa (Nicolas *et al*., 2008; OIE, 2014; Sandip *et al*., 2014).

Several *Mycoplasma* species are known to be associated with the diseases including CCPP and contagious agalactia (CA) in small ruminants and contagious bovine pleuropneumonia (CBPP) in large ruminants (Quinn *et al*., 2002; OIE, 2008). While, the small ruminants are known

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to be affected by the *Mycoplasma* species belonging to the *Mycoplasma mycoides* cluster including *Mycoplasma mycoides* subsp. *capri* (MMC), *M. capricolum* subsp. *capripneumoniae* (MCCP) (Cottew *et al*., 1987; Manso-Silván *et al*., 2007; Fischer *et al*., 2012). However, a variety of less clinically important species are also being involved in the infections such as *M. arginine, M. ovipneumoniae, M. agalactiae* and *M. putrifaciens* (Ayling and Nicholas, 2007; Manso-Silván *et al*., 2007).

These *Mycoplasma* species are highly contagious in nature which makes them easy spread from animal to animal through direct contact or aerosols (Thiaucourt *et al*., 1996; Lignereux *et al*., 2018) thereby achieving almost 100% morbidity with mortality can reach up to 80% in affected herds (DaMassa *et al*., 1997; OIE, 2014; Yatoo *et al*., 2018). Several factors are considered for the continuous persistence of the infection including environmental conditions, breed type, immunity of individual host, infection burden and interaction of herds (Thiaucourt *et al*., 1996; Wesonga *et al*., 2004). However, the cold and moist weather further adds up in the persistence and reoccurrence of the infection in endemic areas (OIE, 2009).

Clinically, CCPP is manifested by respiratory sign with acute to chronic forms. The symptoms may become evident with pyrexia followed by coughing along with nasal discharges (Radostitis *et al*., 2009; OIE, 2014). In addition to the respiratory infections, *Mycoplasma* can spread to the other organs causing arthritis, mastitis, ocular lesions and reproductive disorders (Bergonier *et al*., 1997; Kumar *et al*., 2013; Semmate *et al*., 2022). Further, the respiratory infections caused by the *Mycoplasma* species can pave the way for other microbes including bacterial and viral pathogens which further can complicate the disease and leads an animal to the death (Chu *et al*., 2011; Kgotlele *et al*., 2019).

Recent development in the molecular diagnostic tools are of great importance due to several advantages over traditional tools. The formerly used culture-based techniques require a long procedural time due to complexity and slow growth of *Mycoplasma* species which results in the delay in diagnosis and treatment of the disease (Nicholas *et al*., 2008). While, the serological tests widely used for the diagnosis of mycoplasmas are rapid but mostly avoided because of cross reactivity due to similarity among *Mycoplasma* species that again restrict the use of such techniques when species specific identification is required (Thiaucourt *et al*., 1994; Nicholas and Baker, 1998; Al-Harthi *et al*., 2018). The PCR based molecular tools can provide a more precise and sensitive detection and identification of specific *Mycoplasma* species infecting animals. Up to date, these molecular tools remained rapid and robust method for species specific detection of *Mycoplasma* which further can provide the basis for treatment and prevention of infections (Dominique *et al*., 2004; Halium *et al*., 2019; Semmate *et al*., 2022).

Livestock population in Pakistan is always dominated by goat population with currently almost 82.5 million heads are being domesticated in the country (GOP, 2022). Despite the huge investment and employment opportunities created, the goat production system is still facing several constraints particularly lack of vaccination and treatment against commonly prevalent diseases. Keeping in mind, the present study aimed to determine the prevalence and molecular characterization of *Mycoplasma* species infecting goats from Sindh Pakistan which can help in generating baseline data for prevention of the disease.

MATERIALS AND METHODS

Study area

The study area targeted during to sample collection comprised four different districts of Sindh Province including Thatta, Tharparkar, Jamshoro and Larkana. Geographically these area are located between 27.8514 to 24.4498° N latitude and 68.2944 to 70.5315° E longitude (Fig. 1). The areas were selected as representative zones of the province having humid to dry, warm to moderate cool, and with rainfall fall during summer months. These areas comprises various agro-climatic zones and are rich in goat population with nomadic behavior also observed during dry seasons.

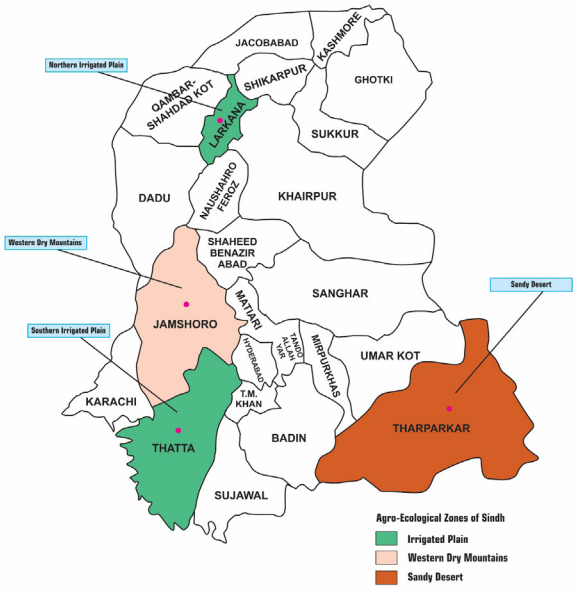

Fig. 1. Geographic distribution of the sampling sites for the detection of contagious caprine pleuropneumonia (CCPP) in goats from different zones of Sindh province of Pakistan.

Animals and samples collection

A total of eight hundred (800) mix breed goats (6 months to 4 years) from different goat farms located in different zones of Sindh province of Pakistan (Thatta, Tharparkar, Jamshoro and Larkana) were examined and random nasal samples were collected during the period from September 2018-April 2019. During the sampling, clinical signs of the disease were also observed for further characterization of the clinical disease. The nasal swabs were collected using sterile swabs in duplicate. The swabs used for culture and isolation of organism were transported to the laboratory in PPLO broth (Oxoid Limited, UK). While, swabs used for DNA extraction were transported in ice box in order to avoid deterioration.

Phenotypic characterization of Mycoplasma

For isolation and phenotypic characterization of *Mycoplasma*, the nasal swabs inoculated PPLO broth (supplemented with 0.5% glucose, 0.5g/L thallium acetate, 0.9g/L yeast extract, 0.03% penicillin G and 20% inactivated horse serum) was filtered through 0.22 µM pore size. Afterwards, the filtered broth was inoculated initially in PPLO broth and cultured at 37 °C for 3–7 days followed by streaking on PPLO agar medium and incubated at 37 °C for 14 days in a humidified incubator supplemented with 5% CO_2. The positive colonies were identified through the typical "fried egg" colonies in the specified time. Further, the growth inhibition test was employed using the protocol described by OIE (2008) and Hernandez *et al.* (2006) to differentiate the *Mycoplasma* with other related organisms. Briefly, the tenfold dilutions of the bacterial culture in the broth were prepared. Each dilution of culture was spread on the *Mycoplasma* agar plates. After even spreading, the excessive material was removed. The plates were incubated at 30° C for 50 min for drying. Disks impregnated with polyclonal rabbit anti-sera against *Mycoplasma mycoides* subsp. *capri* (VPU, Central Veterinary Diagnosis Laboratory, Tandojam) were carefully placed on the agar plates. The plates were incubated at 37° C for 2-6 days. The zones of inhibition were recorded.

Genomic DNA extraction

The genomic DNA from the nasal swabs/bacterial pellet was collected through phenol-chloroform method (Sambrook and Russell, 2006). Briefly, the swabs/pellet was taken into a fresh 1.5 ml Eppendorf tube and mixed with 200 µl of lysis buffer (10 mM NaCl, 20 mM Tris-HCL (pH:8.0), 1mM EDTA, 1% SDS) through vertexing followed by the addition of 50 µg of proteinase K. The samples were then incubated at 56°C for overnight. Afterwards, 500 µl of a solution of phenol-chloroform-isoamyl alcohol (25:24:1) was added, vortexed and centrifuged at 12000 rpm for 10 min. After centrifugation, clear supernatants were carefully collected and transferred to a new tube. The DNA precipitation was performed using 40 µl of 3 M sodium acetate (pH 5.0) and 0.8 ml ethanol through incubating samples at -80°C for 1 h. Then samples were centrifuged and supernatant was discarded and washed once with 1 ml each of 100% ethanol and then by 75% ethanol. The supernatants were discarded and pellets were air dried. Finally, 100 ul of TE buffer was added to dissolve pellet and then the extracted DNA was stored at -20°C until further use.

Polymerase chain reaction (PCR)

The PCR amplification was carried out using the extracted gDNA to accurately identify the *Mycoplasma* species. The reaction was set up with a total volume of 50 µL containing 25 µL 2× Absolute Master Mix (MOLEQULE-ON®, New Zealand), 1 µL (10 pmol/µL) of each primer, 2 µL (50 ng/µL) DNA with the remaining volume adjusted with double distilled water (ddH_2O). The PCR reaction was carried out using the primers Mm-F: 5′-CGAAAGCGGCTTACTGGCTTGTT-3′ along with Mm-R: 5′-TTGAGATTAGCTCCCCTTCACAG-3′ (Hotzel *et al.*, 1996), MM450: 5′-GTATTTTCCTTTCTAATTTG-3′ along with MM451: 5′-AAATCAAATTAATAAGTTTG-3′ (Bashiruddin *et al.*, 1994) in a thermal cycler (2720 Thermal Cycler, Applied Biosystems, USA). The reaction was performed with an initial denaturation at 94°C for 3 min, followed by 45 cycles of denaturation (94°C) for 30 sec, annealing (54°C and 58°C, respectively) for 30 sec, extension (72°C) for 45 sec and a final extension step at 72°C for 10 min. The PCR products were separated on 1.5% agarose gel and visualized under UV light in a gel documentation system.

Sequencing and phylogenetic analysis

The PCR products were further sequenced by Bionics Pvt. lmt. through Sanger dideoxy sequencing technology. The obtained DNA sequences were searched on National Center for Biotechnology Information (NCBI) database by using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) tool for comparison and identification of each species. Further, the sequences were analyzed by using Chromas Lite version 2.0 (http://www.technelysium.com.au). The genomic sequences of target genes from different *Mycoplasma* species were retrieved from NCBI database for comparison and alignments using Clustal W (http://www.clustalw.genome.jp). The sequence homology was searched used sequence identity and similarity (SIAS) tool (http://imed.med.ucm.es/Tools/sias.html). Phylogenetic tree was constructed for the gene sequences of *Mycoplasma* species through Neighbor-joining (NJ) analyses with

10000 bootstraps using MEGA 6 (Tamura *et al*., 2013).

Data submission

The DNA sequences obtained from the field isolates were submitted to the GenBank sequence database of NCBI for assigning the accession numbers.

RESULTS

Prevalence of Mycoplasma *species in goats*

A total of 800 randomly collected nasal samples during the study period were subjected to the bacteriological examination for confirmation of the *Mycoplasma* infection. Initially, the nasal samples were inoculated into *Mycoplasma* specific PPLO broth and subsequently after observing the bacterial growth, the broth were filtered through 0.22 µM pore size filter in order to achieve purity in the samples through filtering large sized bacteria. Further, the filtered media were streaked on PPLO agar plates. The growth of the *Mycoplasma* was observed and differentiated through typical "fried egg" with an average colony diameter of 0.1–0.5 mm on day 14 (Fig. 2). For further confirmation of the presence of *Mycoplasma* infection, the growth inhibition test was employed on all samples which shows any growth on PPLO media. Considering the typical fried egg colonies and growth inhibition test, a total of 48 nasal samples out of 800 were found positive for the presence of either species of *Mycoplasma* which indicates 6.0% prevalence in the study area. Furthermore, the data regarding the prevalence of *Mycoplasma* among positive samples indicates that the higher prevalence was recorded in Tharparker district (35%) followed by Thatta (27%), Jamshoro (21%) and Larkana (17%) (Fig. 3, Table I).

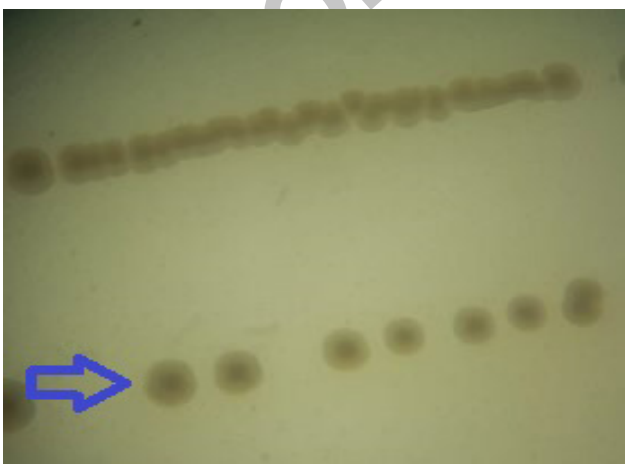

Fig. 2. Typical fried egg colonies of *Mycoplasma* on PPLO agar on 7th day of incubation.

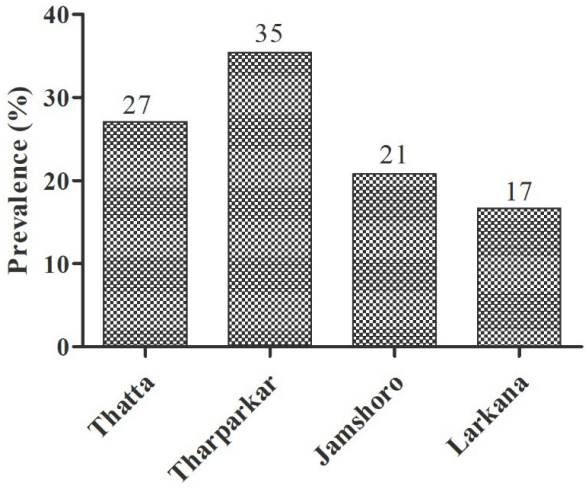

Fig. 3. The prevalence of *Mycoplasma* infection in goats among different districts of Sindh Pakistan.

Table I. The prevalence of *Mycoplasma* infection in goats from different districts of Sindh Pakistan.

Risk factors	Districts			
	Thatta	**Tharparkar**	**Jamshoro**	**Larkana**
Sex				
Male	4.16%	8.33%	4.16%	2.08%
Female	22.91%	27.08%	16.66%	14.58%
Age				
<1 year	4.16%	6.25%	4.16%	2.08%
1-2 year	10.41%	12.50%	6.25%	8.33%
>2 year	12.50%	16.66%	10.41%	6.25%
Breeds				
Teddy	8.33%	8.33%	4.16%	2.08%
Tapri	12.50%	16.66%	6.25%	6.25%
Pateri	4.16%	6.25%	4.16%	4.16%
Kamori	2.08%	4.16%	6.25%	4.16%

Molecular detection of isolated Mycoplasma

Following the traditional techniques based identification, all the positive samples were subjected to the PCR amplification for molecular identification using different sets of primers targeting *Mycoplasma* species. The results shows that PCR successfully amplified the target fragments in all isolated samples with two sets of primers (Fig. 4). During the PCR, initially the universal sets of primers targeting all *Mycoplasma* species were used for confirmation of the presence of either species of *Mycoplasma*. The data indicates that the primer pair targeting 30S ribosomal protein S7 gene successfully amplified a 574 bp fragment from 31 out of 48 samples

while the primer pair targeting 16S rRNA gene found amplifying 548 bp fragment in 17 out of 48 samples. While, unfortunately no any species specific primer amplified any samples which makes more complicated the identification of specific *Mycoplasma* species involved in the infection (data not shown).

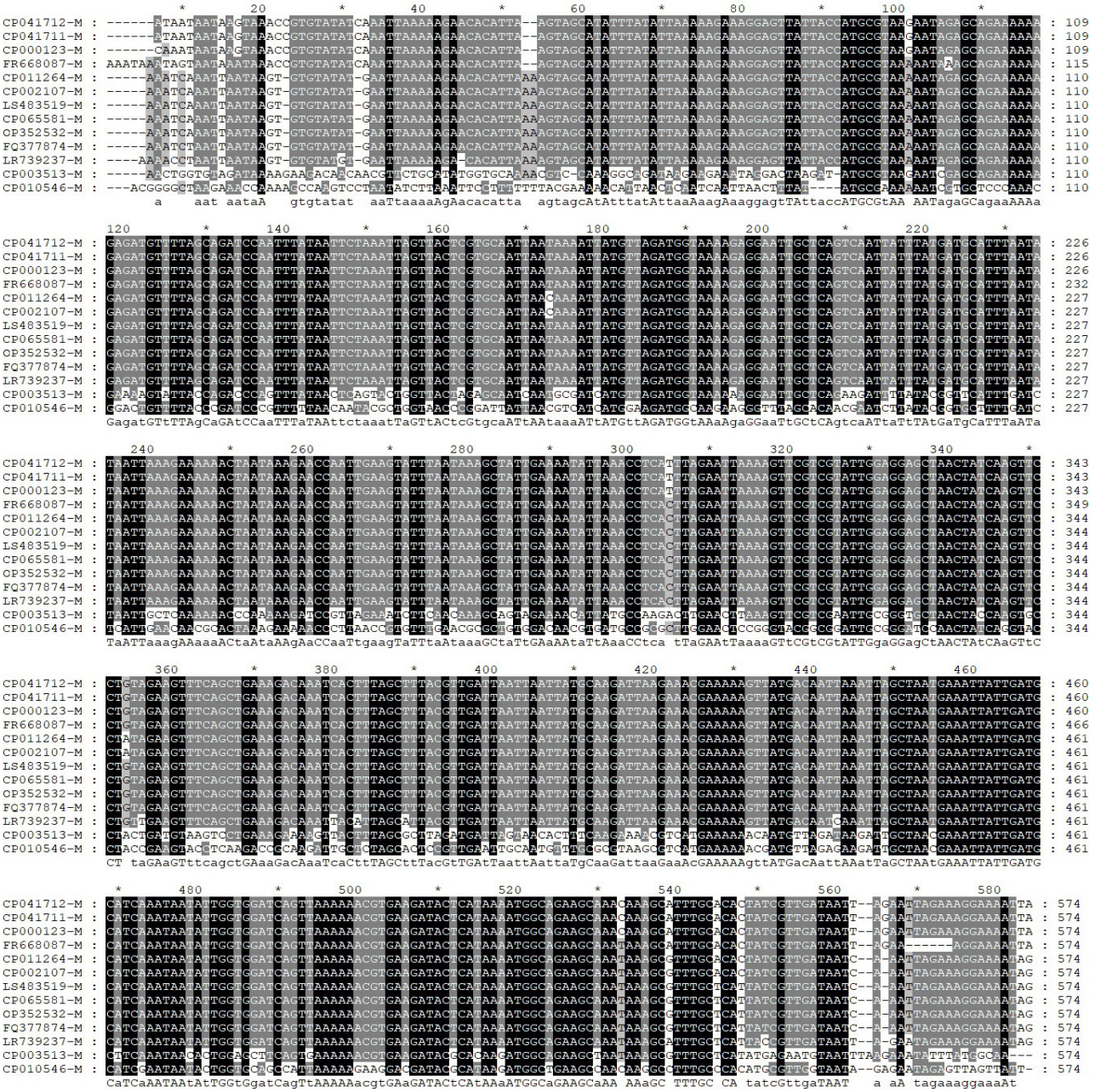

Fig. 4. Multiple alignment of 30S ribosomal protein S7 gene of *Mycoplasma* species. The conserved residues are specified in black. The GenBank accession number indicates reflects as: OP352532 (*M. mycoides* subsp. *capri* strain GM12 (This study)), LS483519 (*M. mycoides* subsp. *capri* strain GM12), CP011264 (*M. mycoides* subsp. *mycoides* strain Ben468), FQ377874 (*M. mycoides* subsp. *capri* LC str. 95010), CP002107 (*M. mycoides* subsp. *mycoides* SC str. Gladysdale MU), CP065581 (*M. mycoides* subsp. *capri* strain PG3), LR739237 (*M. feriruminatoris* 14/OD_0492), CP003513 (*M. gallisepticum* NC08_2008.031-4-3P), CP010546 (*M. pneumoniae* FH), CP041711 (*M. capricolum* subsp. *capripneumoniae* strain AMRC-C758), CP041712 (*M. capricolum* subsp. *capripneumoniae* strain 033C1), CP000123 (*M. capricolum* subsp. *capricolum* ATCC 27343), FR668087 (*M. leachii* 99/014/6).

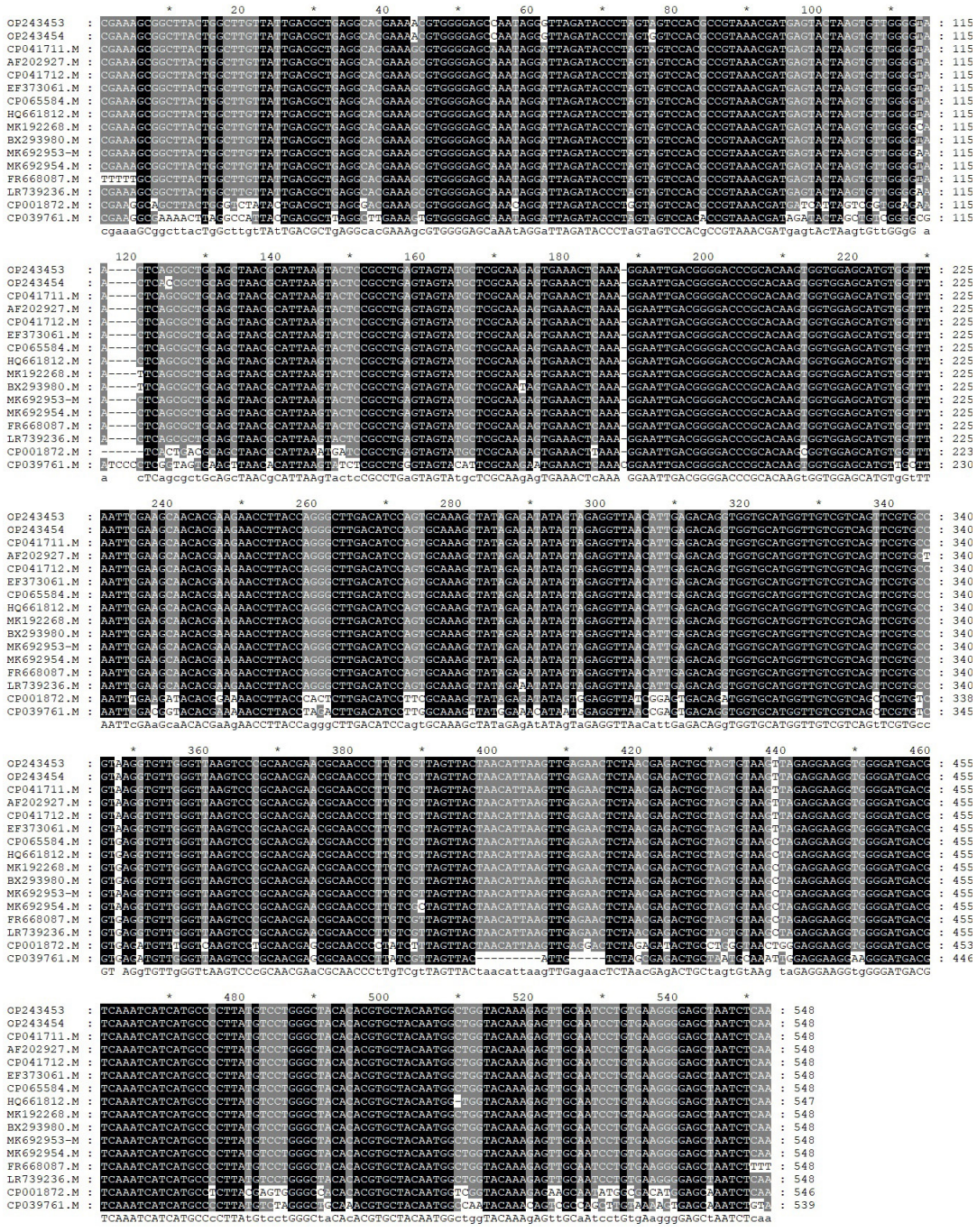

Fig. 5. Multiple alignment of 16S rRNA gene of isolated *Mycoplasma capricolum* and field strains of other related species of *Mycoplasma* genus. The conserved residues are specified in black. The GenBank accession number indicates reflects as: OP243453(*M. capricolum* subsp.*capripneumoniae* (This study)), OP243454 (*M. capricolum* subsp. *capripneumoniae* (This study)), CP041711 (*M. capricolum* subsp. *capripneumoniae* strain AMRC-C758), AF202927 (*M. capricolum* subsp. *capripneumoniae*), CP041712 (*M. capricolum* subsp. *capripneumoniae* strain 033C1), EF373061 (*M. capricolum* subsp. *capripneumoniae* strain TR01), CP065584 (*M. mycoides* subsp. *capri* strain 7730), HQ661812 (*M. mycoides* subsp. *capri* LC strain 37), MK192268 (*M. mycoides* subsp. *mycoides* isolate SC), BX293980 (*M. mycoides* subsp. *mycoides* SC strain PG1), MK692953 (*M. mycoides* subsp. *mycoides* isolate PAK/C354), MK692954 (*M. mycoides* subsp. *mycoides* isolate PAK/C499), FR668087 (*M. leachii* 99/014/6), LR739236 (*M. feriruminatoris* 14/OD 0535), CP001872 (*M. gallisepticum*), CP039761 (*M. pneumoniae* strain 16-734).

Sequence analysis

To further confirm the species involved in the infection in study area, the amplified fragments were further sequenced for DNA based identification. The obtained sequences shows that all the samples amplified using primer set targeting 30S ribosomal protein S7 gene resulted in the yield of identical sequences belonging to the *M. mycoides* subsp. *capri* (Fig. 4), while the primer set targeting 16S rRNA gene resulted in the two highly similar sequences belonging to *M. capricolum* subsp. *capripneumoniae* (Fig. 5). Due to identical sequences, one representative sequence from *M. mycoides* and two from *M. capricolum* were deposited in the GenBank of National Center for Biotechnology Information (NCBI) with the accession numbers; OP352532 (*M. mycoides*), OP243453 and OP243454 (*M. capricolum*).

The sequences obtained from the current study were aligned with the sequences of other isolates of *Mycoplasma* for comparisons. The multiple alignment of sequences shows that all the obtained sequences possess high sequence similarity with the strains isolated from different parts of the world (Figs. 4, 5). Further, the sequence similarity and identity analysis indicate that both the species identified in the current study found to have high nucleotide sequence similarity with *M. mycoides* and *M. capricolum* isolated from different sources and regions of the world. *M. mycoides* shows a high sequence similarity with the *M. mycoides* subsp. *capri* strain GM12 originally isolated from nasal samples of goat from Turkey and Switzerland (Table II). However, *M. capricolum* showed a high sequence similarity with *M. capricolum* subsp. *capripneumoniae* strain 033C1 isolated from lung samples of goat also from Turkey (Table III).

Phylogenetic tree analysis

The phylogenetic analysis indicate that the isolates belonging to the *M. mycoides* were sub-clustered in the same clade as with the isolates from *M. mycoides* subsp. *capri* isolated from nasal discharges of the goat (Fig. 6A). While, the isolates from the *M. capricolum* were clustered along with the isolates from *M. capricolum* subsp. *capripneumoniae* (Fig. 6B).

Table II. Sequence identity percentage of the 30S ribosomal protein *S7* gene of isolated *Mycoplasma mycoides* as compared to the reference and field strains of other related species.

		1	2	3	4	5	6	7	8	9	10	11	12	13
OP352532-*M. mycoides* subsp. *capri* strain GM12 (This study)	1	100.0												
LS483519-*M. mycoides* subsp. *capri* strain GM12	2	100.0	100.0											
CP065581-*M. mycoides* subsp. *capri* strain PG3	3	100.0	100.0	100.0										
FQ377874-*M. mycoides* subsp. *capri* LC str. 95010	4	99.8	99.8	99.8	100.0									
CP011264-*M. mycoides* subsp. *mycoides* strain Ben468	5	99.3	99.3	99.3	99.1	100.0								
CP002107-*M. mycoides* subsp. *mycoides* SC str. Gladysdale MU	6	99.3	99.3	99.3	99.1	100.0	100.0							
CP041712-*M. capricolum* subsp. *capripneumoniae* strain 033C1	7	96.3	96.3	96.3	96.5	96.3	96.3	100.0						
CP041711-*M. capricolum* subsp. *capripneumoniae* strain AMRC-C758	8	96.3	96.3	96.3	96.5	96.3	96.3	100.0	100.0					
CP000123-*M. capricolum* subsp. *capricolum* ATCC 27343	9	96.3	96.3	96.3	96.5	96.3	96.3	99.7	99.7	100.0				
FR668087-*M. leachii* 99/014/6	10	96.0	96.0	96.0	96.2	96.0	96.0	97.9	97.9	97.6	100.0			
LR739237-*M. feriruminatoris* 14/OD_0492	11	97.6	97.6	97.6	97.7	96.9	96.9	95.1	95.1	95.1	95.1	100.0		
CP003513-*M. gallisepticum* NC08_2008.031-4-3P	12	67.1	67.1	67.1	67.1	66.7	66.7	66.6	66.6	66.4	66.6	66.7	100.0	
CP010546-*M. pneumoniae* FH	13	57.3	57.3	57.3	57.5	57.8	57.8	57.8	57.8	57.8	57.3	58.0	60.5	100.0

Table III. Sequence Identity percentage of the *16S rRNA* gene of isolated *Mycoplasma capricolum* as compared to the reference and field strains of other related species.

Organism	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
OP243453-*M. capricolum* subsp. *capripneumoniae* (This study)	1	100													
OP243454-*M. capricolum* subsp. *capripneumoniae* (This study)	2	99.6	100												
CP041712-*M. capricolum* subsp. *capripneumoniae* strain 033C1	3	99.5	99.1	100											
EF373061-*M. capricolum* subsp. *capripneumoniae* strain TR01	4	99.5	99.1	100	100										
CP041711-*M. capricolum* subsp. *capripneumoniae* strain AMRC-C758	5	99.5	99.1	100	100	100									
AF202927-*M. capricolum* subsp. *capripneumoniae*	6	99.3	98.9	99.8	99.8	99.8	100								
MK692954-*M. mycoides* subsp. *mycoides* isolate PAK/C499	7	99.1	98.7	99.6	99.6	99.6	99.5	100							
MK692953-*M. mycoides* subsp. *mycoides* isolate PAK/C354	8	99.1	98.7	99.6	99.6	99.6	99.5	99.6	100						
CP065584.*M. mycoides* subsp. *capri* strain 7730	9	99.1	98.7	99.6	99.6	99.6	99.5	99.6	99.6	100					
HQ661812-*M. mycoides* subsp. *capri* LC strain 37	10	93.2	92.9	93.8	93.5	93.8	93.6	93.8	93.8	94.2	100				
BX293980-*M. mycoides* subsp. *mycoides* SC strain PG1	11	98.7	98.4	99.3	99.3	99.3	99.1	99.3	99.3	99.6	93.8	100			
LR739236-*M. feriruminatoris* 14/OD 0535	12	98.7	98.4	99.3	99.3	99.3	99.1	99.3	99.6	99.6	93.8	99.3	100		
FR668087-*M. leachii* 99/014/6	13	97.6	97.3	98.2	98.2	98.2	98	98.2	98.2	98.5	93.1	98.2	98.2	100	
CP001872-*M. gallisepticum*	14	24	24.3	24	24	24	24	24	23.6	24	24	24.3	23.6	24.3	100
CP039761-*M.pneumoniae* strain 16-734	15	22.8	23.2	22.5	22.5	22.5	22.5	22.5	22.1	22.5	22.5	22.8	22.1	22.8	89.1	100

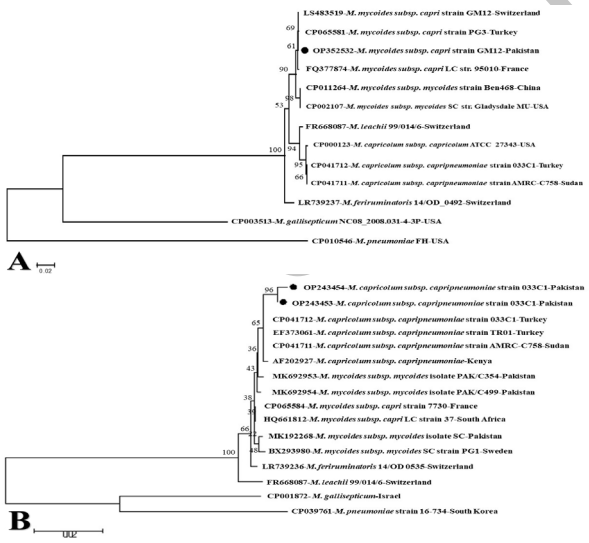

Fig. 6. Phylogenetic and molecular evolutionary analysis of 30S ribosomal protein S7 gene (574 bp) (A) and the 16S rRNA gene of *Mycoplasma* species (548 bp) (B) conducted using Neighbor-joining (NJ) analyses with 10000 bootstraps using MEGA 6 software. The marked sequences along with related sequences with accession numbers are indicated to infer an evolutionary relationship among different isolates.

DISCUSSION

CCPP is a multifactorial disease of the goats that can cause acute disease and mortality especially in kids which results in economic losses in goat industry (Nicolas *et al*., 2008; OIE, 2014). The highly contagious nature, chronic and persistent nature of infection and high resistance to antibiotics make this pathogen more successful in causing outbreaks in the host animals (Thiaucourt *et al*., 1996; Wesonga *et al*., 2004; Lignereux *et al*., 2018). Among the goat diseases, CCPP has a long history of affecting goats throughout the Pakistan with outbreaks in each season thus leading to the severe consequences for the farmers (Rahman *et al*., 2006; Awan *et al*., 2009; Sadique *et al*., 2012; Banaras *et al*., 2016). Due to lack of available data regarding the genetic diversity of *Mycoplasma* species infecting goats, the current study was designed to explore the species diversity infecting goats in different zones of southeastern province of Pakistan.

During the study, out of 800 randomly collected samples from different districts, only 48 (6%) samples showed growth on PPLO culture media which were further confirmed as either species of *Mycoplasma* through growth inhibition test and typical fried egg colonies observed on day 7th post incubation. These typical characteristics of the

colonies on the similar time duration was also observed by several researchers with the identical colony shape observed for *M. mycoides* subsp. *capri, M. capricolum* subsp. *capripneumoniae, M. putrefaciens* (Houshaymi *et al*., 2002; Wang *et al*., 2014; Kabir and Bari, 2015). The lower prevalence rate may be attributed due to random sampling where most of the animals were apparently healthy with no clinical signs. Similarly, another study in cattle from Nigeria also showed 6.27% infection rate of *Mycoplasma mycoides* small colony (Nwankpa *et al*., 2008).

During the data analysis, it was found that the highest infection rate was observed in Tharparker and Thatta districts which are supported by the statements from the previous researchers that harsh weather and nomadic nature in these areas may contribute to the persistence of the infection (Mekuria *et al*., 2008; Shahzad *et al*., 2012).

Despite the limitations, still culture-based techniques including colony morphology analysis, several biochemical tests are widely used for the identification of *Mycoplasma* species (OIE, 2004; Adehan *et al*., 2006). However, arriving at the conclusion that which specific species is responsible for the infection in an area, it is imperative to determine the accurate species level identification in order to properly treat the infection or vaccination against prevalent species.

Due to sophisticated nature and low sensitivity of the serological tests used for the detection of *Mycoplasma* species in the nasal secretions with cross reactivity makes more difficult to diagnose the infection caused by different species (Samiullah, 2013). The development of DNA based detection of *Mycoplasma* infection by PCR is used as an accurate and feasible tool for monitoring the infection in goats (Abraham *et al*., 2015).

In the current study, the culture positive samples were further subjected to the PCR based molecular identification of individual species prevalent in the study area. Herein, all the cultured samples were confirmed by PCR using universal primer sets targeting different genes. The data revealed that only two universal primers targeting 30S ribosomal protein *S7* gene and 16S rRNA gene successfully amplified target fragments. Using the universal sets of primers specified for *Mycoplasma* genus, a total of 31 samples found positive for one primer pair yielding 574 bp fragment targeting 30S ribosomal protein S7 gene, while the 17 samples were successfully amplified through another primer pair yielding a band of 548 bp through targeted amplification of 16S rRNA gene. The sequence analysis revealed that all the samples amplified through the targeted amplification of 30S ribosomal protein S7 gene are of *M. mycoides* subsp. *capri*, while the primer set targeting 16S rRNA gene belong to *M. capricolum* subsp. *capripneumoniae*. Interestingly, both of the primer pairs are genus specific but the results indicated that these primers amplified only targeted species. The data showed a higher abundance of *M. mycoides* in goats from the study area. However, the findings of the some researchers confirmed through PCR based identification in the country indicates a higher prevalence rate (20.59%) of *Mycoplasma* in different zones of Khyber Pakhtunkhwa (Shah *et al*., 2016). While, the molecular detection of infection in goats from Balochistan indicates almost similar pattern of prevalence (maximum of 9%) in different districts of the province (Awan *et al*., 2012; Ejaz *et al*., 2015). Moreover, another study focusing on different districts of Punjab and Khyber Pakhtunkhwa provinces reported only 2% infection rate through PCR based confirmation with the goats being infected by *M. mycoides* subsp. *capri* (Shahzad *et al*., 2012). Furthermore, the similar study focusing on molecular diagnosis of mycoplasmosis in goats from Gilgit-Baltistan and Khyber Pakhtunkhwa provinces indicates 8% prevalence rate (Ahmed *et al*., 2021). In contrast to the present study, previous reports indicates the prevalence of *M. putrefaciens* in Khyber Pakhtunkhwa and Balochistan provinces which was although very low (~1-8%) but at least shown a presence of infection in the country (Awan *et al*., 2012; Ejaz *et al*., 2015; Shah *et al*., 2017). The variation in the prevalence rate may be attributed to the different geographic locations, climatic conditions, sampling seasons, breeds and husbandry practices.

Further, the molecular analysis based on sequence similarity showed 99% similarity of identified *Mycoplasma mycoides* with different strains of *M. mycoides* isolated from different regions of the world, while *M. capricolum* shares almost 96% similarity with the identified *M. mycoides* isolates. However, *16S rRNA* gene sequences amplified from the *M. capricolum* from the present study showed a highest similarity (99%) among both *Mycoplasma* species infecting goats. This may be due higher sequence identity of the target gene amplified.

Although, lack of available data regarding DNA sequences obtained from different regions of the country, this is rather difficult to infer an evolutionary relationship among isolates from different vicinities. However, the obtained sequences from *Mycoplasma mycoides* showed a close relationship with the isolates from Turkey and Switzerland (accession no. LS483519, CP065581 and CP041712). In addition, *M. capricolum* isolates obtained during the current study showed a close relationship with the *M. mycoides* (accession no. MK692953 and MK692953) isolated of from the Punjab province as these are clustered in the same clade (Anjum *et al*., 2020). These data indicates that both of these species might

be originated from the same species and geographical location. However, this may be confirmed through the availability of more sequences from different geographical origins. The PCR based assay followed by sequencing made more precise identification of different strains of a variety of *Mycoplasma* species which will further add in the development of vaccine against commonly prevalent species.

In addition, the goats in the study area are usually kept in open entities with grazing in different pastures which further predispose them to the infection due to interaction with different herds during grazing. Further, the nomadic nature of the farmers and animal trade play a key role in the maintenance and spread of the infection in the endemic area. Such type of findings are also observed by different researchers that the husbandry practices play a key role in the transmission of the infection among herds (Mekuria *et al*., 2018; Sadique *et al*., 2012).

Up to date, there is only one vaccine against *M. mycoides* subsp. *capri* is available in Pakistan which maybe the reason of failure to protect infection as the other members of the group may cause infection (Sadique *et al*., 2012). This was also confirmed by sequencing of the organism from the killed vaccine available in the province.

CONCLUSION

Taken together, data revealed that CCPP is an endemic disease of goats in the Sindh Province. The disease in the studied population is caused by two species namely *M. mycoides* subsp. *capri* and *M. capricolum* subsp. *capripneumoniae*. The results indicate that there are multiple genotypes and species associated with this disease in the study areas as well as in the country which may vary depending on region as well as other risk factors such as husbandry practices or environmental conditions which can influence its spread or severity within an area or population group.

DECLARATIONS

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Ethical statement and IRB approval

All the experiments conducted were pre-approved through Ethical Committee of Directorate of Advanced Studies, Sindh Agriculture University Tandojam, Pakistan through letter no. DAS/1884/of 2021 dated: 18-06-2021.

Data availability

The datasets generated and submitted during the current study are included in this published article. While, the raw data can be made available by the authors upon request.

Statement of conflict of interest

The authors have declared no conflict of interest.

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