Pakistan J. Zool., pp 1-10, 2023. **DOI**: https://dx.doi.org/10.17582/journal.pjz/20230413100417

Molecular Surveillance Revealed Increasing Trend of Mycoplasmosis in Respiratory Infections of Goats (*Capra hircus*) in Northern Pakistan

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Article Information
Received 13 April 2023
Revised 25 July 2023
Accepted 11 August 2023
Available online 16 November 2023 (early access)

Authors' Contribution
FA and FAK designed and conceived the study. FA, HUK and MS carried out the research. FA, FAK and MU analysed the data. FA and FAK wrote the manuscript. FAK and HUK critically reviewed and revised the manuscript.

Key words
Goats, Mccp, Mmc, Mcc, Mycoplasma mycoides, Mycoplasmosis, contagious caprine pleuro-pneumonia (CCPP)

ABSTRACT

In many regions of Pakistan goat is a staple livestock for the livelihood of poor farmers. The goat is also known as the poor man's cow in the sub-continent. Mycoplasmosis impacts the poorest farmers most gravely. Currently, diagnostics and vaccines for *Mycoplasma* causing infections are lacking due to the uniqueness of strains endemic to Pakistan. *Mycoplasma* is responsible for causing several socio-economically important infectious diseases (including WOAH-listed CCPP) in goats. Therefore, to unveil the status of Mycoplasmosis in goats, a total of 2,400 samples consisting of nasal discharges, tracheal swabs, lung tissue, and pleural fluid were collected from four different zones of northern Pakistan. Out of 2400 samples 512 (21.3%) samples showed gross turbidity and typical whirling movement of mycoplasma in PPLO broth. The PCR revealed 284 (11.8%) of the Mm cluster, including six cases of mixed infection with *M. capricolum* subsp. *capricolum* (*Mcc*) and *M. capricolum* subsp. *capripneumoniae* (*Mccp*). The region-wise prevalence of the Mm cluster was 105 (17.5%) in the Northern region, followed by 66 (11%), 59 (9.8%), and 54 (9%) in the Central, Southern, and Tribal regions, respectively. The screening of the Mm cluster isolates by species-specific primers found 110 (4.5%), 92 (3.8), and 88 (3.6%) *Mcc, Mccp*, and *M. mycoides* subsp. *capri* (*Mmc*), respectively. Of the 284 *Mm* cluster positive samples, the highest number was obtained from pleural fluid 75 (18.8%), followed by lungs, tracheal swabs, and nasal swabs 53 (13.3%), 83 (10.4%) and 73 (9.1%), respectively. This study showed that the causes of mycoplasmosis in the northern region of Pakistan include *Mcc, Mmc,* and *Mccp*, and the lungs and pleural fluid samples could be used for the isolation of the causative agent.

INTRODUCTION

Pakistan is an agricultural country and livestock, a sub-sector of agriculture, plays a vital role in uplifting the economy of the country as it contributes 62.68% to the agricultures gross domestic product (GDP) and 14.36% to the national GDP. The population of goats in Pakistan is estimated as the highest (84.7 million) among the livestock population and is ranked as the 3rd country in the world that has the largest number of goats and adding 15% of the total meat production in the country (Economic survey of Pakistan 2022-23).

Small ruminants (sheep and goats) are reared mainly as a source of income, food security in the form of milk and meat, and cultural functions. They are also a direct source of cash to the farmer community when needed on an emergency basis (Peacock, 1996; Shiferaw *et al*., 2006). In poor countries, small ruminants are susceptible to various infectious diseases, including mycoplasmosis (Regassa *et al*., 2010).

Caprine mycoplasmosis has been reported in different regions of the globe. However, the incidence

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0030-9923/2023/0001-0001 $ 9.00/0

of the disease is more frequent in Asia and Africa, where it is a major constraint to goat production causing huge economic losses (Tigga *et al*., 2014; Ongor *et al*., 2011). Among the various mycoplasmal infections, contagious caprine pleuropneumonia (CCPP) is a severe threat to the goat population and their production performance (Bascunana *et al*., 1994; Lorenzon *et al*., 2002). Many different microorganisms cause various outbreaks of respiratory diseases including CCPP, resulting in heavy mortality and morbidity in northern and southern parts of the country (Fauzia *et al*., 2016; Awan *et al*., 2012). CCPP is a fatal respiratory disease affecting domestic goats and some wild ruminants. The etiological agent of CCPP is *Mycoplasma capricolum* subsp. *capripneumoniae* (*Mccp*). The lesions of CCPP are mainly confined to the thoracic cavity, causing up to 80% mortality and 100% morbidity in susceptible flocks (McMartin *et al*., 1989; Rahman *et al*., 2018; WOAH, 2018).

Mycoplasma capricolum subsp. *capripneumoniae* belongs to the *Mycoplasma mycoides* cluster, which is pathogenic to small ruminants, especially goats. The cluster comprises *Mycoplasma mycoides* subsp. *mycoides* Small Colony (*MmmSC*) and *M. mycoides subsp. mycoides* Large Colony biotype (*MmmLC*), *M. mycoides* subsp. *Capri* (*Mmc*), *M. capricolum* subsp. *capricolum* (*Mcc*), *Mccp*, and *Mycoplasma* sp. *bovine group 7* of Leach (*MBG7*) as described by Cottew *et al*. (1987). Recently, the *MmmSc* and *MmmLc* were placed in a single group, e.g., *Mycoplasma mycoides* subsp. *mycoides* (*Mmm*). *Mycoplasma* sp. *bovine* group 7 has been renamed *Mycoplasma leachii* (Fischer *et al*., 2012). *Mccp* and *Mmm* are responsible for inducing CCPP and contagious bovine pleuropneumonia (CBPP), respectively. Both the CCPP and CBPP are life-threatening diseases of ruminants, enlisted by the World Organization for Animal Health (WOAH) formerly OIE, as notifiable diseases.

Other *M. mycoides* cluster members, such as *Mcc* and *Mmc*, influence a wide range of pathological conditions known as "MAKePS" (mastitis, arthritis, keratoconjunctivitis, pneumonia, and septicemia) in small ruminants (Thiaucourt and Bolske, 1996). In Pakistan, Mmc was first diagnosed by applying various biochemical tests on clinical samples of goats infected with CCPP (Khan *et al*., 1989). Later on, many species of mycoplasma (*Mcc, M. putrefaciens, Mmc*) has been reported in Pakistan elsewhere through various diagnostic techniques (Awan *et al*., 2010; Shahzad *et al*., 2012; Sadique *et al*., 2012). However, *Mccp* was reported for the first time in Balochistan province (Awan *et al*., 2010). Subsequently, the presence of *Mccp* was exposed to respiratory infections in goats by many scientists through copious testing methods in Pakistan (Shah *et al*., 2017; Wazir *et al*., 2016; Shahzad *et al*., 2016; Rahman *et al*., 2018; Ahmad *et al*., 2021).

The goat population of the country is at risk of mycoplasmal infection which causes economic losses in terms of mortality and morbidity and ultimately food insecurity (Awan *et al*., 2010; Shah *et al*., 2017; Rahman *et al*., 2018). To prevent high losses regular monitoring system for mycoplasmal infection is in dire need (Ayling *et al*., 2004; Chazel *et al*., 2010). Surveillance helps in identifying infected animals and implementing control measures promptly. Vaccination can help to reduce the severity and spread of the disease. The most efficient approach is vaccination combined with antibiotic therapy, and antibiotics including tetracyclines, fluoroquinolones, and the macrolide family are typically clinically beneficial if given early on (Ozdemir *et al*., 2006). Therefore, the present study has been designed to study the molecular surveillance of mycoplasma mycoides cluster members prevalent in the vicinity to plan the control strategy to prevent the economically important diseases and the risk of food insecurity.

MATERIALS AND METHODS

Study area

This study was carried out in selected districts of Khyber Pakhtunkhwa and Gilgit-Baltistan (The Northern region of Pakistan). The study region was divided into four different zones, namely: the northern zone, the central zone, the southern zone and the tribal zone. The northern zone includes Gilgit-Baltistan, Chitral, Swat, Buner and Hazara. The southern zone includes Dera Ismail Khan, Bannu, Kohat, Karak and Laki Marwat districts. The central zone includes Charsadda, Mardan, Swabi, Peshawar and Nowshera districts. The tribal zone includes the tribal districts of Khyber, Bajaur, and Mohmand.

Sample size

A total of 2,400 samples (n=600 from each zone) were collected from October 2017 to March 2020 mostly in the winter season. Equal samples were collected from both sexes of goats. The samples were composed of the nasal swab, tracheal swab, lung tissue and pleural fluid (Table I). All the samples were collected from the goats irrespective of age and breed. The samples were collected from goats exhibiting respiratory signs in the living stage and lungs showing lesions and pleural fluid from dead and sacrificed animals.

Sample collection

Nasal swabs were collected from infected animals showing respiratory signs, and tracheal swabs collected at postmortem were immediately transferred to modified

Table I. Sample collection for the molecular prevalence of mycoplasma mycoide cluster members in Khyber Pakhtunkhwa and Gilgit Baltistan.

Area/zones of KP	Types of sample from goats								
	Nasal swab		Tracheal swab		Lungs tissue		Pleural fluid		Total
	M	F	M	F	M	F	M	F	
Northern zone	100	100	100	100	50	50	50	50	600
Central zone	100	100	100	100	50	50	50	50	600
Southern zone	100	100	100	100	50	50	50	50	600
Tribal districts	100	100	100	100	50	50	50	50	600
Total	400	400	400	400	200	200	200	200	2400

PPLO broth. The lungs and pleural fluid samples from goats at necropsy died from a natural outbreak and at the slaughterhouse were collected. Pleural fluids were collected in a clean sterile syringe and were transferred immediately to screw-capped sterile falcon tubes (Kartal, Italy). A lung sample was taken from the area between consolidated and normal tissue. Whole lungs showing massive hepatization were collected. All samples collected were immediately transported to the laboratory in a cool chain for further processing. The samples processing and analysis were carried out in the Pathology Lab, College of Veterinary Sciences, The University of Agriculture Peshawar, and the Veterinary Diagnostic Laboratory, University of Minnesota, USA.

Culturing of sample

Nasal swabs and pleural fluid samples were cultured in PPLO broth. The tissue samples were minced, and 1 g of the sample was poured into the 10 ml PPLO (20% horse serum, 0.2% glucose, 0.4% sodium pyruvate, 2.1% PPLO broth base, benzylpenicillin, thallium acetate) . All the tubes containing samples were incubated at 37°C in a humid environment with 5% CO_2 in the carbon dioxide incubator (New Brunswick, Galaxy 48S UK) and were observed every day after the first 3 days of incubation for 14 days. One tube containing PPLO broth with no sample was also run in parallel to this procedure as a negative control. Color change, turbidity, and flocular movement at the bottom upon shaking were considered indicators of mycoplasma growth.

PCR amplification of genes of Mycoplasama mycode cluster members

A commercially available GeneJET genomic DNA kit (Thermo-scientific) was used for the extraction of DNA from the broth culture following the manufacturer's protocol. A total of 3 mL of broth from each sample was used for the genomic DNA extraction. The extracted DNA was quantified with a Nanodrop (MultiSkane Go, Thermo-Fisher Finland). The concentration of the extracted DNA was diluted according to the desired level for PCR as described by Manso-Silván *et al*. (2007).

For polymerase chain reaction 25 µl volume of the PCR reaction was prepared, which contained 10 µl master mix (Dream Taq Green, Thermo Scientific), 1.75 µl of forward primer and 1.75 µl reverse primer, 3 µl of extracted DNA, and 8.5 µl of nuclease-free water. A total of 34 amplification cycles were run in a Bio-Rad T100 thermal cycler for each primer pair. Reaction conditions in the machine were initial denaturation of 95°C for 5 min, followed by the cyclic denaturation at 94 ºC for 30 sec, annealing at 53 and 57 ºC (according to primer applied), polymerization at 72 ºC for 90 sec, and a final step at 72 ºC for 5 min to extend the single-strand DNA fragments. Table II shows a list of primers used.

Statistical analysis

Data were compiled into the Microsoft Excel spreadsheet and analyzed through the Chi-square statistical test on SPSS version 19 at a significant level of 0.05 %. The statistical difference between the mycoplasma isolates and different sources of samples and gender were also determined by the Chi-square test. The Z-test was applied for comparison of the different sources of samples and mycoplasma isolates recovered.

RESULTS

Clinical signs and postmortem examination of animals

The most prevalent clinical signs were watery or mucopurulent nasal discharge, mucus plug in nasal cavities, productive cough, pyrexia (40ºC and above), and

Table II. List of primers, oligonucleotide sequence, annealing temperature and expected product size of 16S-rRNA gene for confirmation of *Mycoplasma species and FusA* core gene of *Mycoplasma mycoides* cluster.

Species	Primer name	Oligonucleotide sequence 5'-3'	Tm (°C)	Product size (bp)	Reference
Mycoplasma mycoides cluster	*Mm*-F	CGAAAGCGGCTTACTGGCTTGTT	52	548	Azevedo *et al.*, 2006
	Mm- R	TTGAGATTAGCTCCCCTTCACAG	54		
FusA	FusA-F	TGAAATTTTTAGATGGTGGAGAA	56	781	Manso *et al.*, 2007
	FusA-R	GGTAATTTAATAGTTTCACGATATGAA	56		
Mycoplasma mycoides subsp. *capri*	P4-F	ACTGAGCAATTCCTCTT	56	196	Hotzel *et al.*, 1996
	P6-R	TTAAATAAGTTTGTATATGAAT	56		
Mycoplasma capricolum subsp. *capripneumoniae*	Mccp.spe-F	ATCATTTTTAATCCCTTCAAG	54	316	Woubit *et al.*, 2004
	Mccp.spe-RMccp	TACTATGAGTAATTATAATATATGCAA	54		
Mycoplasma capricolum subsp. *capricolum*	P4	ACTGAGCAATTCCTCTT	54	192	Hernandez *et al.*, 2006
	P8	GTAAACCGTGTATATCAAAT	53		

deep abdominal respiration with grunting sounds. A postmortem examination revealed frothy discharges in the lower region of the trachea, various degrees of lung hepatization, fibrinous pleuropneumonia, and adhesion of the lungs to the thoracic wall and accumulation of straw-colored pleural fluid in the thoracic cavity (Fig. 1).

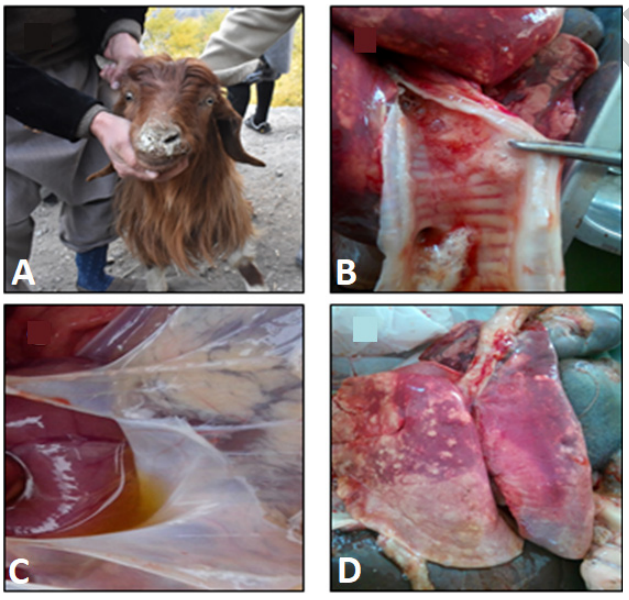

Fig. 1. Clinical signs and post mortem examination of naturally infected goats Suspected for CCPP. (A) Mucus plug in nasal cavities, (B) frothy discharges in lower tracheal portion, (C) straw coloured pleural fluid in thoracic cavity, (D) various degree of bilateral hepatisation of lungs.

Characteristics of Mycoplasma mycoides *cluster members*

Of the 2400 samples, a total of 512 (21.3%) showed gross turbidity and typical whirling movement upon shaking in PPLO broth medium after the incubation process of 3–7 days in a CO_2 incubator (Fig. 2). Among the positive samples, the highest growth rate was recorded in the northern region at 149 (24.8%), followed by the tribal region, southern region, and central region at 129 (21.5%), 119 (19.8%), and 115 (19.1%), respectively. The statistical analysis of the data revealed non-significant differences among the climatic zones and the prevalence of mycoplasma based on positive culture media. The positive cultured broth where the growth of mycoplasma appeared was re-cultured on PPLO agar medium and the growth of typical mycoplasma colonies was recorded on days 3 to 7 post-incubation process, as shown in Figure 3. Each cultured plate was examined under a compound

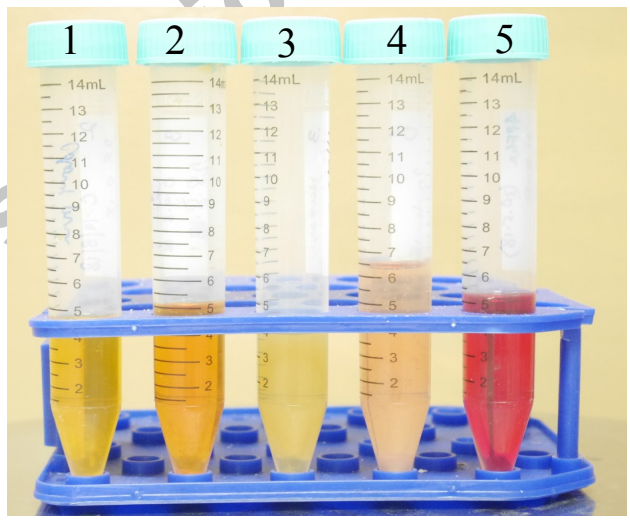

Fig. 2. PPLO broth showing the growth of mycoplasma based on colour change of broth medium (1-4) and 5 negative control.

microscope at 4X and 10X objectives and a single colony having a typical nipple-like or fried egg appearance was marked and transferred to PPLO broth. This procedure has been repeated a minimum of four times and has obtained purified culture (Fig. 4).

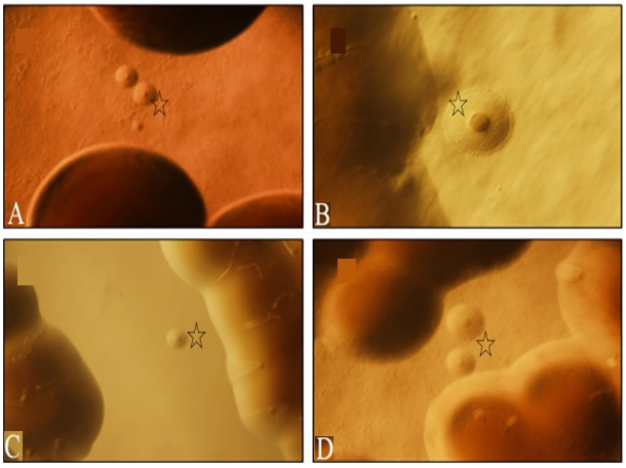

Fig. 3. (A, B, C, D) Growth of mycoplasma colonies (colonies with star) on PPLO agar plate on day 4th-7th after the incubation.

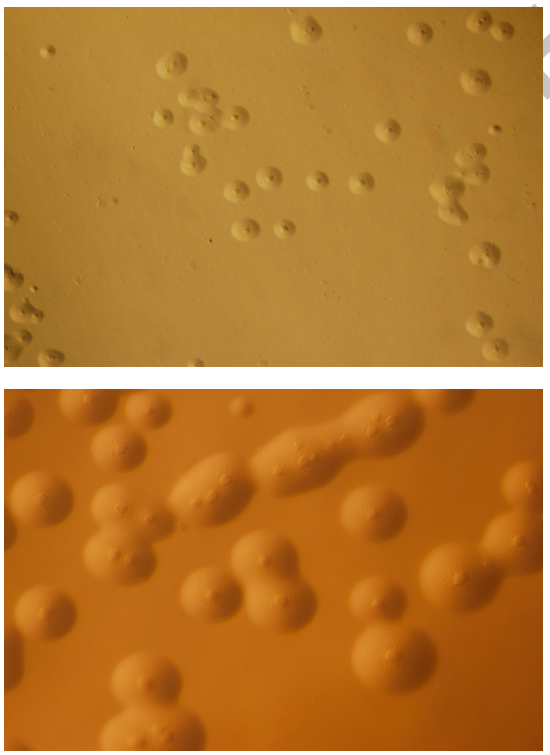

Fig. 4. Pure single type of colonies of *Mycoplasma mycoides* cluster members after 4th passage of the primary culture.

Molecular confirmation with PCR

The DNA was extracted from all the single purified colony cultures. The growth of mycoplasma, particularly *Mccp*, is very difficult and often subsided by the growth of other bacteria. Due to the said reason, DNA was also extracted from all the positive culture isolates to find out the exact prevalence of mycoplasmosis. The screening of the DNA by PCR revealed 284 (11.8%) out of a total of 2,400 samples was confirmed as *Mycoplasma mycoides* cluster (*Mm* cluster), including six cases detected of mixed infection of *Mcc* and *Mccp* in the Northern zone. The highest prevalence percentage of the *Mm* cluster was recorded in the northern region 105 (17.5%), followed by 66 (11%), 59 (9.8%), and 54 (9%) by the central, southern, and tribal regions, respectively (Table III). The analysis of the data by the Chi-square test revealed a significant association ($P<0.001$) between the PCR-confirmed *Mm* cluster isolates and different climatic regions of the study area (Table IV). The primer set used for the *Mm* cluster targeted the 16S rRNA gene produced an amplicon size of 548 bp on agarose gel.

Table III. Growth of mycoplasma in broth culture and PCR identification of *Mycoplasma mycoides* cluster in different zones.

Area	No's of samples	CC of PPLO	Percentage of CC in PPLO	Mm cluster	Percentage of Mm cluster
Northern	600	149	24.8	105	17.5
Central	600	115	19.1	66	11
Southern	600	119	19.8	59	9.8
Tribal	600	129	21.5	54	9
Total	2400	512	21.3	284	11.8

CC, colour change; PPLO, pleuro pneumonia like organism (growth medium of mycoplasma); Mm, mycoplasma mycoide cluster.

Table IV. Confirmation of *Mycoplasma mycoides* cluster through PCR in four different zones from naturally infected goats' samples suspected for CCPP.

Area	PCR confirmed Mm cluster		Percent-age	Chi-sq	P. value
	Positive	Negative			
Northern	105	495	17.5	25.78	0.0001
Central	66	534	11		
Southern	59	541	9.8		
Tribal	54	546	9		
Total	284	2116	11.8		

Statistical analysis by ($\chi 2$) showed significant association ($P<0.001$) among different climatic zone and Mycoplasma isolates, df=3.

The result of the sex-based prevalence of the *Mm* cluster obtained was 148 (12%) in males and 136 (11%) in females, respectively. Statistical analysis of the results showed a non-significant association ($P > 0.05$) between different sexes of an animal. It is evident from the results that both sexes of animals are equally susceptible. For the prevalence of different cluster members, species-specific primers were applied. The different sets of primers used for specie specific were *Mccp, Mcc,* and *Mmc*. The positive PCR product of these primers developed an amplicon size of 316, 192, and 194 bp, respectively.

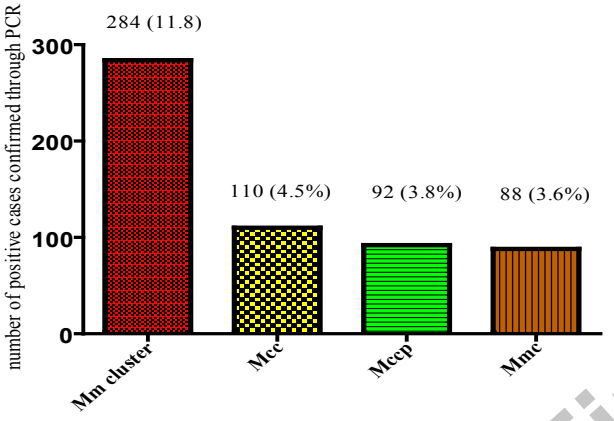

Fig. 5. Overall prevalence of the *Mycoplasma mycoides* cluster and its members in four different zones.

Table V. Molecular detection (PCR) of various pathogenic *Mycoplasma mycoides* cluster member in different climatic zones.

Area	No's of samples	PCR confirmed results of Mm cluster members		
		Mcc (%)	Mccp (%)	Mmc (%)
Northern zone	600	47 (7.8)	37 (6.1)	27 (4.5)
Central zone	600	26 (4.3)	21 (3.5)	19 (3.1)
Southern zone	600	16 (2.6)	21 (3.5)	22 (3.6)
Tribal zone	600	21 (3.5)	13 (2.1)	20 (3.3)
Total	2400	110 (4.5)	92 (3.8)	88 (3.6)

Mcc, *Mycoplasma capricolum* subsp.; *Capricolum:* Mmc, *Mycoplasma mycoides* subsp. *Capri*; Mccp, *Mycoplasma capricolum* subsp. *capripneumoniae.*

Prevalence of different Species of mycoplasma and isolates recovered from various source of samples

Among the 284 (11.8%) of the *Mm*, cluster isolates, 110 (4.5%) were confirmed as *Mcc*, 92 (3.8%) *Mccp*, and 88 (3.6%) *Mmc* through PCR (Fig. 5). The summarized result of the different region-wise distribution of *Mcc*, *Mccp*, and *Mmc* is displayed in Table V. Among the total 284 *Mm* cluster samples, the highest number of isolates were obtained from the pleural fluid 75 (18.8%), followed by lung tissue, tracheal swab, and nasal swab 53 (13.3%), 83 (10.4%), and 73 (9.1%), respectively. The percentage of *Mm* cluster isolates and the samples collected from a different source of origin is shown in Figure 6. The data were analyzed statistically by the Chi-Square test, which revealed a significant association ($P<0.001$) between the mycoplasma isolates and the site of the sample collection (Table VI). The highest mycoplasma isolates can be recovered from the pleural fluid, and this proved the best site for sample collection for accurate diagnosis of CCPP. The analysis of the data by Z-test for comparison of the different types of samples is presented in (Table VII). There was no significant difference between nasal swabs and tracheal swabs. However, significantly more cases were detected in lung tissue and pleural fluid samples compared to nasal swabs. By comparing the tracheal swab and lung samples, non-significant results were recorded. However, the number of *Mm* clusters detected in pleural fluid samples was significantly higher compared to tracheal and lung tissue samples.

Table VI. Confirmation of *Mycoplasma mycoides* cluster through PCR in four different types of samples from naturally infected goats suspected for CCPP.

Source of sample	Mm cluster/ Total samples	Per-centage	Chi-sq	P. value
Nasal swabs	73 / 800	9.1	26.37	0.0001
Tracheal swabs	83 / 800	10.4		
Lungs tissue	53 / 400	13.3		
Pleural fluid	75 / 400	18.8		
Total	284 / 2400	11.8		

Statistical analysis by ($\chi 2$) showed significant association ($P<0.001$) among different source of samples and Mycoplasma isolates, df=3.

Table VII. The PCR results of *Mycoplasma mycoides* cluster and proportional difference among different types of samples collected from diseased goats suspected for CCPP.

Pairs of types of samples	Prop. difference	Z value	P. value
Nasal vs Tracheal	-0.0125	-0.840	0.3994^{NS}
Nasal vs Lungs	-0.0413	-2.20	0.0280**
Nasal vs Pleural fluid	-0.0963	-4.78	0.000***
Tracheal vs Lungs	-0.02875	-1.48	0.1386^{NS}
Tracheal vs Pleural fluid	-0.0838	-4.04	0.0001***
Lungs vs Pleural fluid	-0.0550	-2.12	0.0339**

** Significant, NS: Nonsignificant, ***: Highly significant

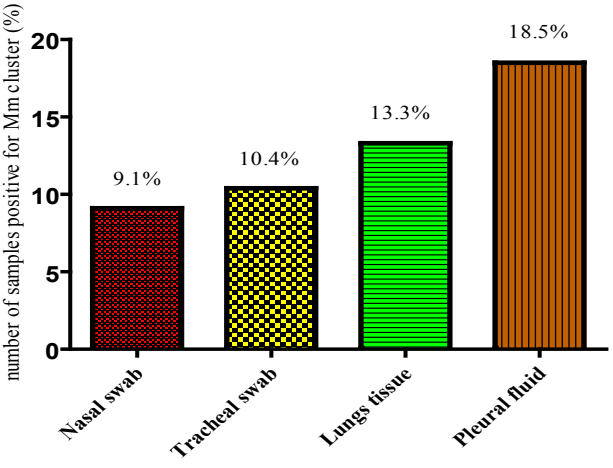

Fig. 6. *Mycoplasma mycoides* cluster recovered from different source of sample. Total number of samples was 2400. Nasal swab n=800, Tracheal swab n=800, Lungs tissue n=400, Pleural fluid n=400.

DISCUSSION

Of the 2400 samples, 512 (21.3%) showed gross turbidity and typical whirling movement upon shaking in PPLO broth. On agar medium, characteristic fried egg and nipple-like colonies were seen on days 5 to 10 post-incubation (Fig. 2). Similar results and procedures have also been reported previously (Shah *et al*., 2017; OIE, 2018; Rahman *et al*., 2018). PCR is a confirmatory test and can be applied to clinical samples like lung tissue and pleural fluid (OIE, 2018; Rahman *et al*., 2018). As some of the mycoplasmas are strenuous to grow, DNA was extracted from all positive broth cultures and screened by PCR. Of the 2400 samples, 284 (11.8%) were positive for the *Mm* cluster with amplicon size 548 bp. The maximum number of *Mm* cluster isolates were recovered from the northern region 105 (17.5%), followed by central and southern regions 66 (11%) and 59 (9.8%), respectively. The lowest prevalence rate of *Mm* cluster 54 (9%) was recorded in the Tribal region of the studied area of the country. It was believed up to now that *Mmc* is responsible for CCPP in Pakistan (Sadique *et al*., 2012; Shahzad *et al*., 2012).

The detection of *Mccp* through PCR in Balochistan has been reported earlier, whereas later on the prevalence of *Mccp* through cELISA was also recorded (Wazir *et al*., 2016). Very recently, the prevalence of *Mccp* in Khyber Pakhtunkhwa through PCR was reported (Shah *et al*., 2017; Rahman *et al*., 2018). In the present study among 284 (11.8%) *Mm* clusters, the prevalence of the various pathogenic *Mycoplasma* species recorded were 110 (4.5%), 92 (3.8%), and 88 (3.6%) of *Mcc, Mccp*, and *Mmc*, respectively. These results are in line with the findings of Shah *et al*. (2017) who reported the prevalence of *Mmc* (13.53%) and *Mccp* (5.5%). Similar results were documented in two districts of Khyber Pakhtunkhwa, where they found *Mmc* and *Mccp* 11.3% and 5% samples, respectively (Rahman *et al*., 2018).

Several other researchers also reported the different prevalence rates of *Mmc* across the country (Fauzia *et al*., 2016; Shahzad *et al*., 2012; Sadique *et al*., 2012). The isolation of *Mmc* from the respiratory infection of goats in various parts of the world has been documented by Wang *et al*. (2014) and Schumacher *et al*. (2011).

The molecular detection of *Mccp* in the present study reinforces the statements that CCPP in the country is caused by *Mccp* (Shah *et al*., 2017; Rahman *et al*., 2018). However, our findings are contrary to the findings about the causative agent of CCPP (Sadique *et al*., 2012). Our results also supported the reports regarding the presence of *Mccp* in Balochistan (Awan *et al*., 2010). In the present study, the prevalence percentage of *Mcc* recorded was 110 (4.5%) having a distribution pattern of 47 (7.8%) in the northern region followed by the central region, tribal region, and southern region 26 (4.3%), 21 (3.5%), 16 (2.6%), respectively. This is the first time molecular detection of *Mcc* in Khyber Pakhtunkhwa and northern areas of Pakistan. However, molecular detection was previously presented in the Balochistan province of Pakistan (Awan *et al*., 2009).

Among the different sources of samples, the maximum number of isolates were confirmed from the pleural fluid at 18.5%, followed by lung tissue, tracheal swab, and nasal discharges at 13.3%, 10.4%, and 9.1%, respectively. A similar pattern of isolation of mycoplasma was shown by (Noah *et al*., 2011), who reported 83.78% of *Mycoplasma* isolates from the pleural fluid during his study. The same trend of isolation of *Mycoplasma* is presented previously, where stated that *Mycoplasma* has a high affinity to the lungs and lower respiratory tract where the receptors are present in enormous quantity for the attachment of antigenic epitope of *Mycoplasma* (Shah *et al*., 2017; WOAH, 2018). This antigenic protein of *Mycoplasma* possesses lipoglycan, which initiates an inflammatory response in infected host tissue, which develops increase exudation and pleural effusion (Gyles *et al*., 2008). These results are supported by the findings of various other researchers, who reported the highest isolation of *Mmc* from lung tissue (Sadique *et al*., 2012; Awan *et al*., 2010). However, the maximum isolates of *Mccp* recovered from the pleural fluid in the present study support the findings documented elsewhere (Samiullah, 2013). Various other researchers also reported the isolation and identification of various species of *Mycoplasma* from nasal discharges and

tracheal swabs (Fauzia *et al*., 2016; WOAH, 2018).

The nasal discharges represent respiratory infection and an easy site for sample collection for initial diagnosis and isolation of the causative agent. In the chronic form of the disease, the purulent pulmonary discharges having causative agent come to the upper respiratory tract with coughing and make it possible to isolate the causative agent from nasal discharge and tracheal swabs. However, the highest number of *Mycoplasma* isolates can be recovered from the pleural fluid and lung tissue but it can only be performed in the sacrificed and dead animal during postmortem examination.

In the present study, the prevalence of the *Mm* cluster confirmed through PCR was 12% in males and 11% in females. While the frequency of mycoplasmosis showed previously was slightly increased in females compared to the male animals of the flock (Sherif *et al*., 2012; Abegunde *et al*., 1981). The prevalence of 16.9 % in female Spanish Ibex and 8.4 % in male Ibex in Spain is reported elsewhere (Verbisck-Bucker *et al*., 2008). The increased prevalence of mycoplasmosis in female animals might be due to various factors, which develop stress in animals including pregnancy, lactation, and the estrus cycle. The stress induced by these various factors weakens the immune status of the animals and paves the way for the proliferation of opportunistic pathogenic *Mycoplasma* to cause infection in immune-compromised animals (Studdert *et al*., 2007). However, some of the researchers agree with our findings and reported a high prevalence in males 5.3% than 4.7% in female goats (Yousuf *et al*., 2012). Similarly, Ethiopia reported a lower prevalence of CCPP in female animals 6.7% as compared to bucks 24.1% (Regassa *et al*., 2010). Various other researchers reported from Ethiopia and Tanzania that sex does not play a significant role in the epidemiology of CCPP (Yousuf *et al*., 2012; Mekuria and Asmare, 2010; Abrhaley *et al*., 2019; Kipronoh *et al*., 2016). The difference in the prevalence rate of mycoplasmosis in male and female animals may be due to the immune status of the animal, differences in the locality of sampling, the male-female ratio in a herd, and biosecurity at the herd level.

CONCLUSIONS

Mycoplasmosis in the northern region of Pakistan is caused by *Mcc, Mmc,* and *Mccp*. We represent *Mcc* apparently first time in the studied region. We explore the baseline data which can be used for an effective control strategy of the disease in the respective region.

ACKNOWLEDGMENT

We are thankful to Livestock and Dairy Development Department (Extension and Research), Khyber Pakhtunkhwa for their help and support in sampling. We are grateful to Dr. Francois Thiaucourt, and Dr. Lucia Manso-Silvan, CIRAD-INRA ASTRE Animal, Sante, Territoires, Risques, Ecosyste`mes TA A-117 Campus de Baillarguet 34398, Montpellier Cedex 5, France, for their guidance and technical support. We are also thankful to the staff of the Veterinary Diagnostic Laboratory, University of Minnesota, St. Paul, MN 55108, USA for technical assistance.

Funding

This project was financially supported by the joint research work of The University of Agriculture, Peshawar and Sandia National Laboratories, New Mexico, USA under the Pak-US Science and Technology Cooperation Program, Phase 7, 2017 under the Higher Education Commission (HEC) of Pakistan.

IRB approval

The Advance Studies & Research Board (ASRB) in its 45th meeting held on 08-08-2019 approved, this study vide Notification No. 1528/ASRB/45/UAP dated 27-08-2019.

Ethical statement

The study was carried out according to standard animal rights and approved by the Animal Ethics Committee of the College of Veterinary Science (CVS), University of Agriculture Peshawar.

Statement of conflict of interest

The authors have declared no conflict of interest.

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