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Characterization, Molecular Diagnosis and Prevalence of Caprine Mycoplasmosis in Different Areas of Pakistan

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Abstract.- A study was conducted to characterize caprine mycoplasma species and to know its prevalence in different areas of Pakistan during 2006 to 2007.A total of 1440 samples (including nasal discharge, pleural fluid, lung tissue, synovial fluid, and milk (1180), and 260 sera) were collected from clinically affected goats of different age and sex. These samples were subjected to cultural isolation, growth inhibition test (GIT) using rabbit polyclonal antiserum against *Mycoplasma mycoides* subspecies *capri*, latex agglutination test (LAT) for the detection of *Mycoplasma capricolum* subspecies *capripneumoniae* and polymerase chain reaction (PCR). 121 samples out of 1180 showed turbidity in PPLO broth and 58 of these grew on PPLO agar, showed positive reaction to GIT. No serum sample showed positive reaction with LAT kit. Thirty five were positive for *Mycoplasma mycoides* cluster through PCR and identified as *Mycoplasma mycoides* subsp. *capri* (Mmc) through DNA sequencing. Mycoplasmosis is more prevalent in hilly and plain areas (5.8 and 4.5%) than in semi-desert and sub-hilly areas (3.3 and 2.9%).

Keywords Mycoplasmosis, goats, polymerase chain reaction, *Mycoplasma mycoides* subsp. *capri*, prevalence.

INTRODUCTION

In Pakistan, livestock contributed approximately 55.1 percent of the agriculture value added and 11.5 percent to national GDP during 2010-2011 (Anonymous, 2011). The role of livestock in the economy of the country can be gauged from the fact that 30–35 million (M) rural population is engaged in livestock raising, as every family has 2-3 cattle/ buffaloes and 5-6 sheep/goats, thus leading to derive 30-40% of their income from these animals (Anonymous, 2011). Goat rearing carries tremendous importance in rural economy particularly for non-agricultural low lying class of people. This animal is recognized as the poor man's cow in Indo-Pak subcontinent (Rahman *et al*., 2006). The population of goats has been estimated as 61.5 M in Pakistan and it yielded 0.759 M tons of milk for human consumption, 0.616 M tons of mutton and 23.685 M skins during 2010-2011 (Anonymous, 2011).

This species of animal in the country is facing diversified problems including poor managemental practices, underfeeding and diseases including infectious and non-infectious one. Among the infectious diseases, caprine mycoplasmosis poses a significant threat to production capacities of this animal (Rahman *et al.*, 2006). It is a disease of major economic importance in developing countries, causing a major constraint to the goat production (Srivastava *et al*., 2010). The direct losses of the disease result from its high mortality, reduced milk and meat yield, cost of diagnosis, treatment and control. In addition to this, there are indirect losses due to the imposition of trade restrictions. Currently, the majority of control measures focus on practical animal husbandry factors such as maintaining a clean environment, flock monitoring and the removal of diseased/contagious animals due to increased occurrence of antimicrobial resistance.

Mycoplasmosis causes respiratory infection, mastitis, arthritis, conjunctivitis and occasionally abortion in goats. Many mycoplasma species are important pathogens for caprine; these include *Mycoplasma mycoides mycoides*, *Mycoplasma capricolum* subsp. *capripneumoniae* (Mccp), *Mycoplasma mycoides* subsp. *capri* (Mmc), and *Mycoplasma capricolum* subsp. *capricolum* (Mcc). All of these belong to a group of very closely related

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mycoplasmas known as the *Mycoplasma mycoides* (Mm) cluster (Manso-Silvan *et al.*, 2007).

The fact that several members of the *M. mycoides* cluster produce similar clinical signs in caprine with different virulence indicates that precise identification of pathogens is of paramount importance. It was therefore important to investigate that how many species of mycoplasma are causing the disease/s in goats in Pakistan. For this purpose selective areas of the country have been probed. Little work has been done on the isolation and molecular diagnosis of these organisms in Pakistan. Therefore, this project has been designed to study the prevalence of caprine mycoplasmosis in different areas of Pakistan and to isolate and identify the etiological agent/s of caprine mycoplasmosis using culturing and biochemical tests. Also primary diagnosis of caprine mycoplasmosis through polymerase chain reaction (PCR) was performed to confirm the culturally isolated organisms through this molecular technique.

MATERIALS AND METHODS

During a period of 2 years (2006-2007), twelve districts including Mansehra, Peshawar, Swabi, Kohat, Abbottabad, Dera Ghazi Khan, Quetta, Pishin, Jhang, Sargodha, Lahore and Faisalabad with 6 union councils (UC) in each district were selected for screening goat Mycoplasmosis. Using random sampling technique, a total of 1440 morbid materials of different nature such as nasal discharge, pleural fluid, lung tissue, synovial fluid, milk and serum samples were collected from clinically affected goats of different breeds, age groups and either sex showing symptoms of nasal discharge, pneumonia, arthritis and mastitis. The affected animals were identified through clinical signs and symptoms of the disease, by taking the history of the affected animals from the owners at the time of sampling and also by post mortem lesions. All samples were obtained prospectively for the purpose of study and were collected in the transport medium (Miles and Nicholas, 1998) except serum. The samples thus collected were processed culturally for isolation and identification as described by OIE (2009). In order to obtain pure culture, a single colony was picked and sub-cultured to broth which was passed through a cellulose acetate membrane filter of 0.45µ (Orange Scientific, Braine-I' Alleud, Belgium).Dilutions of the filtrate were then plated onto PPLO agar (Acumedia, Michigan, USA) to obtain isolated colonies. Each colony represented the progeny of a single cell that passed through the filter and was therefore a clone. Clones were picked and transferred to PPLO broth medium (Acumedia, Michigan, USA) for storage till further use. These clones were subjected to different biochemical and serological tests such as digitonin sensitivity test, film and spot production test, tetrazolium reduction test, glucose breakdown test, urea hydrolysis test, phosphatase activity test and growth inhibition test for *Mycoplasma mycoides* subsp. *capri*. Serum samples were subjected to latex agglutination test for *Mycoplasma capricolum* subsp. c*apripneumoniae* (Kits donated by Dr. J. B. March, Moredun Research Institute, UK).

PCR was employed directly to the field samples, suspected for mycoplasma species as well as to primary broth culture showing whorl formation and purified colonies of mycoplasma in broth medium obtained after culturing on agar plates. DNA was extracted from the entire samples and colonies using commercially available reagent (TrireagenT®) (Molecular Research Center, Ohio, USA) according to the manufacturer's protocol. During DNA extraction ultra centrifugation was achieved by using ultra speed centrifuge machine (Sorvall®, Thermo Electron Corp. USA).

The primers described by van Kuppeveld *et al.* (1992) targeting highly conserved 16S rRNA genes, were used for the genus-specific detection of mycoplasma. Then cluster and species specific primers as shown in Table I were employed for the detection of different mycoplasmas.

All PCR amplification reactions were carried out in a final volume of 25 µl containing DNA template and 12.5 µl commercially available PCR master mix (Fermentas, Ontario, Canada). The primers were used at concentration of 10 ρmols μl^{-1}. Amplification was carried out in a thermocycler (PEQLAB Biotechnologies GmbH, Erlangen, Deutschland) under the following conditions. A denaturation step of 4 min at 94 °C was followed by

Table I.- Primer name, sequence, product, target gene, annealing temperature and specificity of different primers used for PCR.

PP No	Primer name	F.P. S. (5'-3')	Primer name	R. P.S (5'-3')	P (bp)	T.G	A.T °C	Specificity	Described by
1	GPO-3	GGG AGC AAACAG GAT TAG ATACCCT	MGSO	TGC ACC ATC TGT CAC TCT GTT AAC CTC	270	16S rRNA	55°C	Genus Myco-plasma	van Kuppeveld *et al.* (1992)
2	FusA-F	TGAAATTTTT AGATGGTGGA GAA	FusA-R	GGTAATTTAATAG TTTCACGATATGA A	781	fusA	52°C	Members of *M.m* Cluster	Manso-Silvan *et al.* (2007)
3	GlpQ-L-F	GTGAATATAA TGATCTTAGT AGC	GlpQ-L-R	TCAGGATAATCTG AAATATAACC	695	glpQ	45°C	Members of *M.m* Cluster	Manso-Silvan *et al.* (2007)
4	GyrB-F	GTGTTGTTAA TGCTTTATCTT TATATG	GyrB-R	TTGTGGATCTGTAT GTCTAACTGATAA AA	635	gyrB	52°C	Members of *M.m* Cluster	Manso-Silvan *et al.* (2007)
5	LepA-L-F	AGAAATTTTA GTATTATTGC TCATA	LepA-L-R	CTTGGAGCAGTTG CAATTAGTTCAA	1097	lepA	52°C	Members of *M.m* Cluster	Manso-Silvan *et al.* (2007)
6	RpoB-F2	GCTCAATCTA ATGTTAATCA AGATG	RpoB-R2	TCTTCTGGAGAAA GTTGAACTTGTC	824	rpoB	52°C	Members of *M.m* Cluster	Manso-Silvan *et al.* 2007
7	LMF1	TGAACGGAAT ATGTTAGCTT	LMR1	GACTTCATCCTGC ACTCTGT	361	16S rRNA	55°C	*M. ovi-pneumoniae*	Besser *et al.* (2008)
8	MAGF	CCT TTT AGA TTG GGA TAG CGG ATG	MAGR	CCG TCA AGG TA TTC CTA C	360	16S rRNA	57°C	*M. agalactiae*	Azevedo *et al.* (2006)
9	Mput1	AAATTGTTGA AAAATTAGCG CGAC	Mput2	CATATCATCAACT AGATTAATAGTAG CACC	316	Arc B	52°C	*M. putrefaciens*	Peyraud *et al.* (2003)
10	MboF2	GAAGAAAAG TAGCATAGGA AAAAAATGAT	MboR2	CGTCGTCCCCACC TTCCTCCCG	734	16S rRNA	65°C	*M.bovis*	Das *et al.* (2003)

PP No, Primer pair No; FPS, Forward primer sequence; RPS, reverse primer sequence; P, product, T.G, target gene; AT, annealing temperature.

35 cycles consisting of 1 min denaturation at 94ºC, 1 min primer-template annealing from 45°C to 65°C depending upon primer pair (Table I) and 1 min polymerization at 72 ºC. A final extension step of 5 min at 72°C was performed to polymerize all remaining single strand DNA fragments. Twenty µl of the PCR product admixed with 6 X DNA loading dye™ (Fermentas, Ontario, Canada) were electrophorized on a 1% agarose gel (Gene Choice®, Maryland, USA) (1h at 90V) with a 100 bp ladder (Fermentas, Ontario, Canada) as size marker. The gels were stained with ethidium bromide (HP 47.1, Roth) (250 µg/ml @ one drop/25 ml of 1% agarose gel) and analyzed in a UV trans-illuminator (Wealtec, NV, USA) for visualization of different sized (270, 781, 695, 635, 1097, 824, 361, 360, 316 and 734 base pairs, specific for primer pair 1 through 10, respectively) PCR products.

FusA gene targeted by primer pair No 2 was sequenced from Centre of Excellence of Molecular Biology, University of Punjab, Lahore, Pakistan to confirm the species within *Mycoplasma mycoides* cluster.

RESULTS

Samples inoculated in mycoplasma growth medium (PPLO broth) showed whorl formation with turbidity which was detected in the presence of light. It was recorded at 37°C after 48 to72 hours of incubation in 5% CO_2 environment (Table II). The un-inoculated control tubes did not show such type of cloudiness. The plates inoculated with turbid material from the tubes were observed for 15 days and the positive plates showed small pin point colonies (less than one mm diameter) with slight pinkish colour on the surface of PPLO agar plate containing phenol red indicator. Typical fried egg appearance with central dark red coloured colonies (initially less then up-to 2mm diameter) was also

evident on the surface of agar. Out of 121 samples showed turbidity in broth culture, 58 samples showed colonial growth on the plates (Table II). The negative broths were also tested and confirmed by PCR for the absence of any mycoplasma.

Single colony was picked from 58 samples separately and after re-culturing on PPLO agar, all of these samples showed plate growth. Culturally (on agar plate) positive clones were subjected to various biochemical tests; results summarized in Table III.

Table II.- No. of samples showing turbidity on PPLO broth and plate growth on PPLO agar.

Nature of samples	A	B	C^+
Plural fluid	266	26	17
Nasal discharge	282	37	23
Lung tissue	284	28	18
Synovial fluid	136	13	-
Milk	212	17	-
Total numbers	1180	121	58

A, total No collected; B, No. of samples showing turbidity in PPLO broth; C^+, No. of samples showing plate growth (No. out of PPLO broth cultures)

Table III.- Results of different biochemical tests on plate growth.

Biochemical test	OIPF (n=17)	OIND (n=23)	OILT (n=18)
Digitonin sensitivity test	P	P	P
Film and spot production test	N	N	N
Tetrazolium Reduction test	P	P	P
Glucose breakdown test	P	P	P
Urea hydrolysis test	N	N	N
Phosphatase activity test	N	N	N

OIPF, organisms isolated from pleural fluid; OIND, organisms isolated from nasal discharge; OILT, organisms isolated from lung tissues; P, positive; N, negative

All 260 serum samples subjected to latex agglutination test for *Mycoplasma capricolum* subsp. *capripneumoniae*, gave negative reaction. The field isolates subjected to growth inhibition test using polyclonal rabbit anti-sera against the reference strain of *Mycoplasma mycoides* subsp. *capri* which produced a growth inhibition zone on agar plates greater than 4 mm diameter. Since p-value of (χ^2) test statistic is >α:0.05 hence the sources of experimental material (*i.e.* pleural fluid, nasal discharge, lung tissue) are homogeneous in showing the presence of *Mycoplasma mycoides* subsp. *capri* with equal proportional impact based on growth inhibition test.

Polymerase chain reaction (PCR) test

The results of field samples which have been directly subjected to different primers for PCR showed amplicons of various molecular weights (bp) which is shown in Figure 1, 2 and 3.

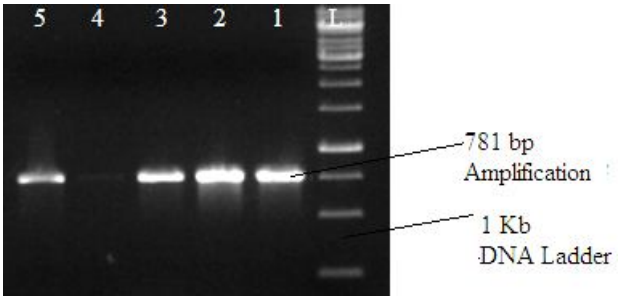

Fig. 1 Amplification of 3 field samples with primer pair FusA-F &FusA-R at 781 bp. L: Molecular Ladder; Lanes 1, 2, 3: test samples + ve for *Mycoplasma mycoides* cluster members; Lane 4: Negative control; Lane 5: Positive control.

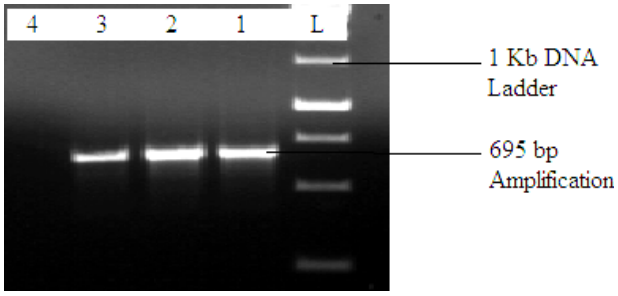

Fig. 2. Amplification of two field samples for members of *Mycoplasma mycoides* cluster at 695 bp with primer pair GlpQ-F &GlpQ-R. L: Molecular ladder; Lanes 1 & 2 test samples +ve for *Mycoplasma mycoides* cluster members; Lane 3: Positive control; Lane 4: Negative control.

PCR test employed directly on field samples

The results of PCR test employed directly on field samples and agar plate cultured samples is shown in Table IV. Based on PCR results, members

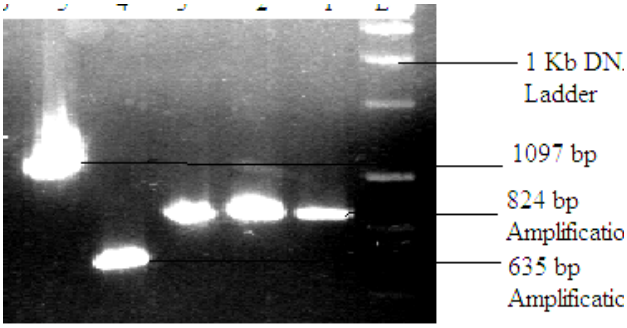

Fig. 3. Amplification of different samples with different primer pairs for the members of *Mycoplasma mycoides* cluster showing amplicon sizes of 635, 824 and 1097 bp. L: Molecular Ladder; Lanes 1, 2 & 3 : Test samples +ve for *Mycoplasma mycoides* cluster members at 824 bp with primer pair Rpob-F &Rpob-R; Lane 4: Test sample +ve for Mycoplasma cluster members at 635 bp with primer pair GryB-F & GryB-R; Lane 5: Test sample +ve for Mycoplasma cluster members at 1097 bp with primer pair Lep-A-F &Lep-A-R; Lane 6: Negative control

of *Mycoplasma mycoides* cluster were detected through primers employed during PCR and the specific species (*Mycoplasma mycoides* subsp. *capri*) was confirmed through DNA sequencing.

Since the P-value (0.171*) of (χ^2) test statistic is >α:0.05 hence the sources of experimental material (*i.e.* pleural fluid, nasal discharge, lung tissue) are homogeneous in showing the presence of Mmc with equal proportional impact**.** The results of different field samples (after primary culture showing whorl formation in PPLO broth) subjected to PCR have been shown in Table V.

Comparison between cultured technique and direct PCR

The comparison between cultured technique (58 PCR positive samples) and direct PCR (35 PCR positive samples) for the detection of mycoplasmosis in different nature of samples indicates that the p-value (0.000**) of (χ^2) test statistic is <α:0.05 hence there is significantly higher association between PCR direct test and cultured on agar plate technique for indicating the presence of Mmc by various resources such as pleural fluid, nasal discharge, lung tissue and all combined effect.

Sequencing of FusA gene

Five isolates (two from hilly areas and one each from sub- hilly, plain and semi-desert areas) were processed for sequencing. These isolates were recovered from different breeds of goats present in these areas, showing same type of clinical signs and disease pattern i.e respiratory distress. Sequence of the PCR product obtained through FusA gene showed 100% homology with FusA gene of *Mycoplasma mycoides* subsp. *capri.* The phylogenetic tree (Fig.4) constructed by using all 22 sequence available in NCBI gene data bank indicates that this field strain is different from other.

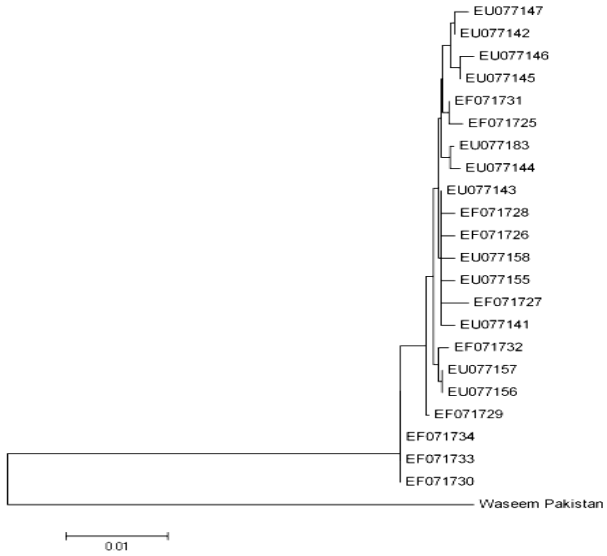

Fig. 4. Phylogenetic tree of the sequence obtained (Waseem Pakistan, {this study}) with 22 sequences available in NCBI

District and age group wise prevalence of mycoplasmosis

District and different age group wise detail of mycoplasmosis has been shown in the Table VI.

Since the P-value (0.015**) of the calculated (χ^2) statistic value is < the level of significance *i.e.* α: 0.05 and it falls in the critical region. Therefore, it is concluded that interaction between age groups (1, 2, 3) and districts are not homogeneous in showing the prevalence of Mmc with equal proportional impact.

Table IV.- Results of PCR test employed directly on field samples.

Primer name	Pf (n = 266)		Nd (n= 282)		Lt (n = 284)		Sf (n= 136)	M (n = 212)
	Fs	Apcs	Fs	Apcs	Fs	Apcs	Fs	Fs
GPO-3, MGSO	15	17	7	23	13	18	Nil	Nil
FusA- F, FusA-R	15	17	7	23	13	18	Nil	Nil
GlpQ-L-F, GlpQ-L-R	15	17	7	23	13	18	Nil	Nil
GyrB-F, GyrB-R	15	17	7	23	13	18	Nil	Nil
LepA-L-F, LepA-L-R	15	17	7	23	13	18	Nil	Nil
RpoB-F2, RpoB-R2	15	17	7	23	13	18	Nil	Nil
LMF1, LMR1	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
MAGF, MAGR	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Mput1, Mput2	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
MboF2, MboR2	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil

Pf, pleural fluid; N, nasal discharges; Lt, lung tissues; Sf, synovial fluid; M, milk; Fs, field samples; Apcs, agar plate cultured samples.

Table V.- Results of PCR test on primary broth culture.

Primer name	Pf (n = 26)	Ns (n= 37)	Lt (n = 28)	Sf (n= 13)	M (n = 17)
GPO-3, MGSO	17	23	18	Nil	Nil
FusA- F, FusA-R	17	23	18	Nil	Nil
GlpQ-L-F, GlpQ-L-R	17	23	18	Nil	Nil
GyrB-F, GyrB-R	17	23	18	Nil	Nil
LepA-L-F, LepA-L-R	17	23	18	Nil	Nil
RpoB-F2, RpoB-R2	17	23	18	Nil	Nil
LMF1, LMR1	Nil	Nil	Nil	Nil	Nil
MAGF, MAGR	Nil	Nil	Nil	Nil	Nil
Mput1, Mput2	Nil	Nil	Nil	Nil	Nil
MboF2, MboR2	Nil	Nil	Nil	Nil	Nil

Pf, Pleural fluid; Ns, Nasal swabs; Lt, Lung tissues; Sf, Synovial fluid; M, milk

Table VI.- District and age wise prevalence of mycoplasmosis in goats.

District	NSC	NPS	P %	A*			B*			C*		
				TNS (n=525)	NPS	P %	TNS (n=360)	NPS	P %	TNS (n=555)	NPS	P %
Mansehra	120	10	8.3	47	6	12.7	27	2	7.4	46	2	4.3
Peshawar	120	5	4.2	43	3	6.9	30	1	3.3	47	1	2.1
Swabi	120	4	3.3	39	2	5.1	33	1	3.0	48	1	2
Kohat	120	3	2.5	47	1	2.1	29	1	3.4	44	1	2.2
Abbott-abad	120	4	3.3	43	1	2.3	31	1	3.2	46	2	4.3
D.G. Khan	120	4	3.3	47	2	4.2	27	1	3.7	46	1	2.1
Quetta	120	3	2.5	44	1	2.2	31	1	3.2	45	1	2.2
Pishin	120	3	2.5	42	1	2.3	30	1	3.3	48	1	2
Jhang	120	4	3.3	39	2	5.1	33	1	3	48	1	2
Sargodha	120	4	3.3	44	1	2.2	31	2	6.4	45	1	2.2
Lahore	120	6	5	43	3	6.9	31	2	6.4	46	1	2.1
Faisalabad	120	8	6.6	47	4	8.5	27	2	7.4	46	2	4.3
Total	1440	58	4	525	27	5.1	360	16	4.4	555	15	2.7

NSC, number of samples collected; NPS, number of positive samples; P, prevalence; A*, age group No.1 (Day one to 180 days); B*, age group No.2 (181 to 365 days); C*, age group No. 3 (One year and above); TNS, total number of samples.

Table VII.- Prevalence of mycoplasmosis in different sexes in different districts

Districts	Male			Female		
	TNS (n=708)	NPS	P %	TNS (n=732)	NPS	P %
Mansehra	63	4	6.3	57	6	10.5
Peshawar	56	2	3.5	64	3	4.6
Swabi	56	2	3.5	64	2	3.1
Kohat	61	1	1.6	59	2	3.3
Abbottabad	58	1	1.7	62	3	4.8
D.G. Khan	63	1	1.5	57	3	5.2
Quetta	59	1	1.6	61	2	3.2
Pishin	57	2	3.5	63	1	1.58
Jhang	56	2	3.5	64	2	3.1
Sargodha	58	2	3.4	62	2	3.2
Lahore	58	2	3.4	62	4	6.4
Faisalabad	63	3	4.7	57	5	8.7
Total	708	23	3.2	732	35	4.7

TNS, total number of samples; NPS, number of positive samples; P, prevalence.

Prevalence of mycoplasmosis in different types of areas

The prevalence of mycoplasmosis in plain areas (Lahore, Faisalabad, Sargodha, Jhang) is 4.5%, 2.9% in sub-hilly areas (Peshawar, Swabi, Kohat, Quetta, Pishin), 5.8% in hilly areas (Mansehra, Abbottabad) and 3.3% in semi desert area (D.G.Khan). Since the p-value (4.197*) of (χ^2) test statistic is > α: 0.05 hence the samples are homogeneous by area type in showing the prevalence of Mmc with equal proportional impact.

Prevalence of mycoplasmosis in different sexes

The percent of positive samples in different sexes in different districts has been shown in Table VII.

Since the p-value (0.248*) of (χ^2) test statistic is > α: 0.05 hence the interaction between sex groups (Male, Female goats) and districts are homogeneous in showing the prevalence of Mmc with equal proportional impact.

DISCUSSION

In the present study, different areas were selected for sampling on the basis of their geographical locality and keeping in view the goat population (heavily populated) after Livestock Census (2006). Different scientists selected these areas to study on various aspects of goat mycoplasmosis during different time spans. Awan *et al*. (2009a,b) have surveyed Pishin district for isolation of *Mycoplasma capricolum* subsp. *capricolum, Mycoplasma putrefaciens* and *Mycoplasma capricolum* subsp. *capripneumoniae* from goats because the said district was caprine dense populated area having borders with Afghanistan. Similarly Rahman *et al*. (2006) surveyed district Faisalabad for isolation and sero-prevalence of *Mycoplasma mycoides* subsp. *capri.*

The major syndrome associated with mycoplasma spp. infection in small ruminants is pneumonia (Srivastava *et al*., 2010), however, *Mycoplasma* spp. do cause other disease syndromes such as poly-arthritis, mastitis and kerato-conjunctivitis. Different species of mycoplasma are involved in causing the above mentioned maladies. As purpose of this project was to study the prevalence of goat mycoplasmosis which include a variety of species, so a diversified nature of samples such as nasal discharge, pleural fluid, lung tissue, synovial fluid, milk and serum samples were collected and processed for the isolation and identification of different mycoplasmas by culturing as well as by PCR. Similar kind of sampling was also done by Azevedo *et al*. (2006), Awan *et al*. (2009a, b) and Srivastava *et al*. (2010) who also used milk, nasal swabs, lung piece, joint exudates and pleural fluid etc. for the isolation/recovery of different types of mycoplasma.

Out of 1180 clinical samples, including nasal discharge, pleural fluid, lung piece, synovial fluid and milk samples, 121 different samples showed whorl formation with turbidity (Table II). The samples (1059) which did not yield a whorl formation pattern indicated the absence of mycoplasma although at this stage without further tests it was hard to say that these samples were truly negative for mycoplasma. According to Awan *et al*. (2009a) and Srivastava *et al.* (2010) mycoplasma is very fastidious to grow as well as the widespread and indiscriminate use of antimicrobials, especially the macrolides, tetracycline and fluoroquinolones suppress the growth of organism in vitro. The over-zealous use of such antibiotics in present study might influence the mycoplasma growth.

Out of 121 turbid samples, 58 samples showed colony growth on PPLO agar when incubated at 37°C for up-to 14 days with 5% Co_2 with moist atmospheric condition. Small pin point pinkish colonies having diameter between less than one mm to 2mm diameter were observed. Colonies having typical fried egg appearance with dark centers were also evident on PPLO agar medium. *Hernandez* et al. (2006) and Srivastava *et al*. (2010) found typical fried egg colonies on agar plates after incubation at 37 °C for 48-72 hours in a humid atmosphere with 5% CO_2 while isolating *Mycoplasma mycoides* subsp. *capri* and *Mycoplasma capricolum* subsp. *capripneumoniae* from pleural fluid and lung piece samples from diseased goats. These findings fully support the findings of this study in which typical fried egg colonies were found on PPLO agar medium. Sixty three samples out of 121 PPLO broth positive samples did not grew on agar plates. These 63 samples showed false positive results with broth culture as it was confirmed through PCR of these samples, where no sample was found positive for mycoplasma.

The latex agglutination test (LAT) kit used during present work was specific for the detection of *Mycoplasma capricolum* subsp. *capripneumoniae* in the serum of goats. This test is particularly useful in identifying animals in the earliest stages of CCPP (March *et al.,* 2000). During this study, all 258 serum samples subjected to LAT, gave negative reaction, indicating that *Mycoplasma capricolum* subsp. *capripneumoniae* was not prevalent in the study areas of Pakistan. The absence of finding *Mycoplasma capricolum* subsp. *capripneumoniae* during this work was in agreement with Awan *et al.* (2009a) who also failed to detect the said species while surveying goat population in Pishin district, a common area of both studies.

The growth inhibition test (GIT) often fails with low passage heavily capsulated cultures even if they are grown in the presence of type specific antiserum, and if the number of passages is increased *in vitro*, the same cultures start showing zone of inhibition (personal communication by Professor Dr. Rosenbusch, Iowa State University, USA). The results of this study are in contrast with the above statement, as all58 isolates with only three to four passages produced a growth inhibition zone of greater than 4 mm diameter on agar plates having polyclonal rabbit antiserum raised against *Mycoplasma mycoides* subsp. *capri*.

Members of *Mycoplasma mycoides* cluster share genomic and antigenic features, which result in common biochemical and serological properties, complicating species identification (Woubit *et al*., 2004). Some of these mycoplasmas are very fastidious pathogens (Srivastava *et al.,* 2010) and is difficult to differentiate with conventional techniques. PCR is relatively a newer technique based on molecular biology. According to McAuliffe *et al.* (2003) in near future molecular technology will be used not only to identify mycoplasma species, but also to detect them without the need for culture. In present studies, using PCR, field samples were confirmed for the presence of mycoplasma in less than 9 hours. During present studies PCR did not prove itself a more sensitive method as compared to culture method, in course of screening the field samples prior to culture. Only 35 samples out of total 1180, gave positive reaction with this test as compared to 58 samples positive through culture, thus yielding a difference of 23 more samples with the latter technique. All of these 23 samples were negative with PCR when this test was applied directly to the samples whereas all of these samples showed a positive reaction with PCR after culturing. This finding clearly indicated that PCR was less sensitive as compared to culture test. The number of mycoplasma was probably low in the

field samples at a level which was increased by culturing whereas PCR failed to amplify the specific genome targets. During the present research work, primers used were targeted against members of mycoplasma cluster as well other species which included *Mycoplasma ovipneumoniae, Mycoplasma agalactiae, Mycoplasma bovis* and *Mycoplasma putrefaciens*. Out of all these oligonucleotides, only the cluster targeting primers yielded the specific amplicons. At this stage of study it was confirmed that the isolated organisms belonged to *Mycoplasma mycoides* cluster. To reach at a definite confirmation about the species of the cluster, DNA sequencing was done using amplicon targeted by FusA oligonucleotide. Using a basic alignment search tool (BLAST) the sequence was aligned and the sequencing data matched a highly conserved region of the *Mycoplasma mycoides* genome, corresponding to *Mycoplasma mycoides* subsp. *capri*. Manipulation of *FusA* gene may be a rapid tool for identification and phylogenetic positioning by PCR and sequencing (Manso-Silvan *et al*., 2007).

The highest prevalence (8.33%) of mycoplasmosis was in district Mansehra, followed by (6.6%) in district Faisalabad, whereas lowest percentage (2.5) was in districts Quetta, Pishin and Kohat. As the sampling was made purely on the basis of symptoms, therefore the difference in prevalence of mycoplasmosis in different districts is biased one, because the symptoms (respiratory distress, temperature, arthritis etc) could be due to some other infections or non infectious agents. The results of present study has a little part to coincide with the findings of Awan *et al.* (2009a), in terms of presence of caprine mycoplasma in Pishin district, but the prevalence of this organism in the area was much higher (56.67%) in previous study as compared to prevalence noted during this study. The variation in results might be due to different time periods of two surveys. There was a huge gap between sample sizes of two studies. Also primer sets (for PCR) were different in both projects.

On the basis of PCR assay on cultured samples, more cases of mycoplasmosis were recorded in goat kids (5.1%) of age group 1 (1 to 180 days). Whereas 4.4 was the percentage of *Mycoplasma mycoides* subspecies *capri*, recorded in goats of age group 2 (181 to 365 days) and 2.7% in goats of age group 3 (one year and above). The adult group 3 having age above one year has significantly lowest prevalence of Mmc as compared to other two age groups (1 and 2) as per statistical evidence. This difference may be due to chance but not areal difference. These findings correlate to the work of Verbisck-Bucker *et al*. (2008) who also has found higher prevalence of mycoplasmosis with *Mycoplasma agalactiae* in young free-ranging Spanish ibex in Spain than adult ones.

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