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RESEARCH ARTICLE

Molecular Characterization of Local Isolates of *Mycoplasma capricolum* Sub Specie *Capripneumoniae* in Goats (*Capra hircus*) of Khyber Pakhtunkhwa, Pakistan

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ABSTRACT

Caprine mycoplasmosis is an important infectious respiratory complication lead to significant health issue and causes heavy economic losses in small ruminant population throughout the world. The study was designed to identify and characterize the pathogenic member of mycoplasma cluster the *Mycoplasma capricolum* sub specie *capripneumoniae* (*Mccp*) isolated from goat suffering from respiratory syndrome in the natural outbreak. The study was carried out during November, 2013 to April, 2015 in the Khyber Pakhtunkhwa (KPK), Pakistan. A total of 825 samples from nasal discharge, tracheal swab, pleural fluid and lung tissue were collected from goat clinically suspected for Contagious Caprine Pleuro pneumonia (CCPP). The samples taken in PPLO transport media were cultured on modified Hay Flick media and incubated at 37° C with 5% CO_2 for 7-12 days. Out of total 267 (32.36%) were positive for mycoplasma growth showing mass turbidity, whirling movement in culture broth and typical fried egg colonies in agar media. The positive culture was identified through biochemical assay and confirmed as *Mccp* through PCR by using cluster and specie specific primers. Out of 267 positive samples 55 (20.59%) were confirmed as *Mycoplasma mycoides* cluster and 23 (8.61%) were identified as *Mccp*. Sequencing of the 16-S rRNA gene blast search revealed 99% sequence homology with *Mccp* compared with eight available sequences at NCBI. The presence of *Mccp* was for the first time reported in small ruminants of Khyber Pakhtunkhwa.

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INTRODUCTION

Mycoplasmosis is the most important respiratory disease poses a serious threat to the small ruminant population by causing heavy economic losses in Northern and Southern regions of the country (Saddique *et al.*, 2012; Samiullah, 2013; Banaras *et al*., 2016). Among the different pathogenic species of mycoplasma, contagious caprine pleuropneumonia (CCPP) is highly fetal disease caused by *Mycoplasma capricolum* sub specie *capripneumoniae* (*Mccp*) (OIE, 2012). The CCPP was first time reported in Algeria in 1873 and later in many countries of Africa, Europe, Middle East and Asia (Tigga *et al*., 2014; Atim *et al*., 2016). In Pakistan the disease was considered to be caused by *Mycoplasma mycoides* sub specie *capri* till the molecular confirmation as *Mccp* in Baluchistan by Awan *et al*. (2010). Recently in the international collaborative study *Mccp* was confirmed in the northern Pakistan and Tajikistan (Peyraud *et al*., 2014). The disease also prevalent in different areas and livestock research centers of Punjab, Pakistan (Shahzad *et al*., 2016). The disease is widely spread in many countries of the globe and considered to be caused by six different pathogenic species called as *Mycoplasma* cluster. However, the classical form of disease is caused by *Mycoplasma capricolum* sub specie *capripneumoniae* that mainly confined to the thoracic cavity (Thiaucourt and Bolske, 1996; Manso-Silvan *et al*., 2007).

The classical signs of CCPP are characterized by respiratory distress, high fever (41-43°C), high morbidity and mortality in susceptible herds irrespective of age and sex. There is dyspnea accompanied by grunting and snoring,

continuous nasal discharges, anorexia and occasionally abortion (Zinka *et al*., 2013). The disease mainly confined to the thoracic cavity characterized by unilateral sero-fibrinous pleuropneumonia with severe pleural effusion and hepatization (Mondal *et al*., 2004). In some acute cases the pleural cavity contains an excessive straw colored fluid with fibrin flocculations (Sadique *et al*., 2012). In per acute cases, minimal clinical signs are noted with mortality within 1-3 days (Samiullah, 2013). Histo-pathological examination of affected lungs shows sero-fibrinous necrotic pneumonia, polymorph nuclear neutron-phil infiltration in alveolar spaces. The susceptible species of animals are domestic sheep (*Ovis aries*), goat (*Capra hircus)* and wild ruminants including wild goats (*Capra aegagrus*), Gerenuk (*Litocranius walleri*), Nubian Ibex (*Capra ibex nubiana*) and Lasristan mouflon (*Ovis orientalis lasristanica*). It causes significant losses in these animals with high morbidity and mortality (Arif *et al*., 2007).

A lot of work has been carried out for the diagnosis and identification of mycoplasma species by using different conventional techniques in Pakistan. But little work has been conducted on molecular characterization of the local isolates of mycoplasma species. Mostly the conventional methods of identification failed to address the issue properly because of its shortcoming. The isolation of mycoplasma is difficult due to its fastidious nature and needs special media and proper conditions for growth (OIE, 2012). The serological and biochemical tests are usually fail due to sharing of common antigenic epitopes by many species of mycoplasma. Therefore, the advanced molecular techniques like PCR and sequencing is the most accurate tool for identification and confirmation of different mycoplasma species (Woubit *et al*., 2004). It can confirm the exact specie of microorganism even in mixed infection and directly from clinical samples like nasal discharge and pleural fluids. The mycoplasma having 16-S rRNA gene allowed the identification of variable regions with both genus and species specific primers to identify the particular species of mycoplasma cluster (Hotzel *et al*., 1996; Manso-Silvan *et al*., 2007; Kumar *et al*., 2011). A new technique the Recombinase Polymerase Amplification Assay is developed for the rapid and accurate detection of different strains of *Mccp* in Kenya. However, this technique is costly and not easily accessible in the developing countries (Liljander *et al*., 2015). Looking at the paucity of the scientific literature on mycoplasma in Pakistan the present work is carried out to isolate, identify and characterize the never reported *Mycoplasma capricolum* sub specie *capripneumoniae* in small ruminants of Khyber Pakhtunkhwa. This study will have paved a way for researcher and planner to design strategies for curbing this fatal disease.

MATERIALS AND METHODS

Sampling: A total of 825 samples consisted of nasal, tracheal, pleural fluid and lung tissue were collected from goats exhibiting the signs of respiratory distress suspected for CCPP in Khyber Pakhtunkhwa Pakistan. The samples were taken by sterile swab and then transfer to the transport media. The collected samples were kept under refrigeration and transported to the Pathobiology Lab Department of Animal Health, the University of Agriculture, Peshawar, Pakistan for onward processing.

Isolation and identification: The samples were taken aseptically by sterile swab and transferred to the PPLO broth as a transport media. All the collected samples were incubated in anaerobic incubator (New Brunswick, Galaxy 48-S UK) with 5% CO_2 at 37°C for 5-10 days. The test tubes were examined daily for presence of mass turbidity, whirling movement and change in color with decreased in pH. The positive growths were cultured on Hay Flick agar for the appearance of nipple like or fried egg typical mycoplasma colonies. The positive colonies were re-cultured three time for obtaining pure culture as per standard protocol of (OIE, 2012).

Biochemical assay: Biochemical assay of the local isolates was carried out for identification of the specie of the mycoplasma cluster as per standard protocol of (Adehan *et al*., 2006). A volume of 0.5µl from each isolate were diluted in 5ml of Hay Flick broth and subjected to different biochemical tests like glucose fermentation, serum digestion, Tetrazolium reduction (aerobically and anaerobically), casein digestion and arginine hydrolysis test for the identification of desired mycoplasma species.

Polymerase chain reaction: The biochemical identified samples of the specie of mycoplasma *Mccp* were subjected to DNA extraction for confirmation through PCR. The polymerase chain reaction (PCR) was performed for the detection of *mycoplasma* species by using two set of primers the *Mycoplasma mycoides* cluster and specie specific as described by Hotzel *et al*. (1996). These primers targeted the 16-S rRNA gene of *mycoplasma* with an amplicon size of 548 and 316 bp for *Mycoplasma mycoides* cluster and *Mycoplasma capricolum* sub specie *capripneumoniae* respectively (Table 1).

Homology and Phylogenetic analysis: The gel product of specific amplicon size was taken and submitted for sequencing. The obtained sequence was subjected to NCBI BLAST to screen for homologous sequences for phylogenetic relation of the local isolates of *Mycoplasma capricolum* sub specie *capripneumoniae* with available sequences. Sequences of the isolates were downloaded from NCBI and were multiple aligned through BioEdit version 7.0.5.2 (Hall, 1999). Furthermore, Phylogenic tree topology was constructed for the obtained sequences using software MEGA version 7.1 for evolutionary study and to build correlation with other strains of different regions (Tamura *et al*., 2011).

Table 1: Primer sequence, annealing temperature and expected amplicon size of 16-S rRNA gene for the confirmation of mycoplasma species in infected goats.

Mycoplasma specie	Primer designation	Oligonucleotide sequence 5'-3'	Annealing Tm (°C)	Expected amplicon size (bp)	Reference
Mycoides cluster	Mm-F	(CGA AAG CGG CTT ACT GGC TTG TT)	52	548	Hotzel *et al*., 1996
	Mm- R	(TTG AGA TTA GCT CCC CTT CAC AG)	56		
Mccp	Mccp.spe-F	(ATC ATT TTT AAT CCC TTC AAG)	54	316	Woubit *et al*., 2004
	Mccp.spe-R	(TAC TAT GAG TAA TTA TAA TAT ATG CAA)	54		

Statistical analysis: Data was compiled in Microsoft Excel and analyzed through Chi- square test using SPSS 19.0 SOFTWARE to check statistical difference between different sources of samples.

RESULTS

Isolation of mycoplasma: Out of 825 samples 267 (32.36%) were positive on culture for mycoplasma showing mass turbidity and change in color in PPLO broth media. A typical nipple like and fried egg colonies were appeared on day 4th and 7th post incubation in Hay Flick agar media (Fig. 1 & 2). Highest culture was obtained from pleural fluid followed by lung tissue, tracheal and nasal discharge. However, on statistical analysis of the data by Chi- square test ($\chi 2$) non-significant association ($P>0.05$) was found between the four different sources of samples obtained from goats suspected for CCPP. The results of growth of *Mycoplasma capricolum* sub specie *capripneumoniae* on culture media are presented in the (Table 2).

The positive culture of mycoplasma was sub cultured on modified Hay Flick agar till the characteristic typical nipple like and fried egg colonies were obtained. On biochemical analysis 143 (17.33%) was positive for glucose fermentation, serum digestion, tetrazolium reduction test and casein digestion test while negative for arginine hydrolysis test (Table 4).

Molecular characterization: Based on PCR analysis, out of total 267 cultured samples 55(20.59%) were identified as *Mycoplasma mycoides* cluster with an amplicon size of 548 bp. On analysis of data ($\chi 2$) significant ($P<0.001$) result was obtained from pleural fluids (43.33%) followed by lungs tissue (27.14%) among the sources of samples (Table 3). Out of 55 PCR confirmed mycoides cluster, 23(8.61%) were positive for *Mccp* with an amplicon size of 316 bp (Fig. 3 & 4). The *Mycoplasma capricolum* sub specie *capripneumoniae* was identified for the first time in small ruminants of Khyber Pakhtunkhwa, Pakistan. Ten PCR confirmed local isolates were processed for sequencing and the sequence of the PCR product obtained through specie specific primers showed maximum sequence homology 99% of 16-S rRNA gene of *Mycoplasma capricolum* sub specie *capripneumoniae* with the strains of neighbor countries. The phylogenetic tree was constructed by using software Mega version 7.0.5.2 and compared with 08 available sequences in NCBI gene data bank. The constructed tree indicate that the local isolated field strain is different from the strains of USA and France but closely related with the strain of neighbor countries like India and China (Fig. 5).

Table 2: Results of culture of mycoplasma obtained from different sources of samples on modified Hay Flick media collected from goats suffering from respiratory diseases suspected for CCPP. Statistical analysis of the data showed non-significant association ($P>0.05$) between the different sources of samples.

Nature of sample	Culture Media		Total	Pearson Chi-square	P-value
	Positive (%)	Negative (%)			
Nasal Swab	151 (30.2)	349 (69.8)	500	5.766	0.124
Tracheal swab	74 (32.9)	151(67.1)	225		
Lung Tissue	28 (40)	42 (60)	70		
Pleural fluid	14 (46.7)	16 (53.3)	30		
Total	267 (32.4)	558 (67.6)	825		

Fig. 1: Typical *Mycoplasma capricolum* sub sp. *capripneumoniae* colony with fried egg appearance obtained on day 7th post incubation on modified Hay Flick agar, isolated from lungs tissue of naturally infected goat at 10X.

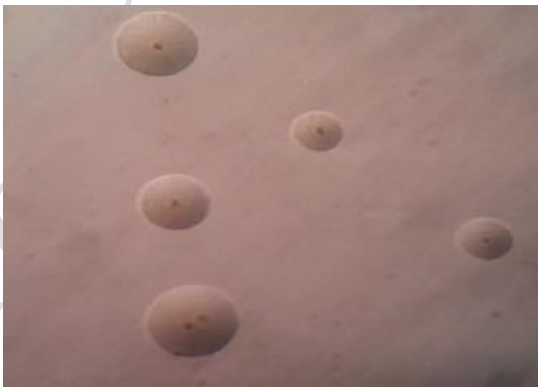

Fig. 2: *Mycoplasma capricolum* sub sp. *capripneumoniae* colonies with nipple like appearance on day 4th post incubation on modified Hay Flick agar, isolated from the pleural fluid of naturally infected goats at 4X.

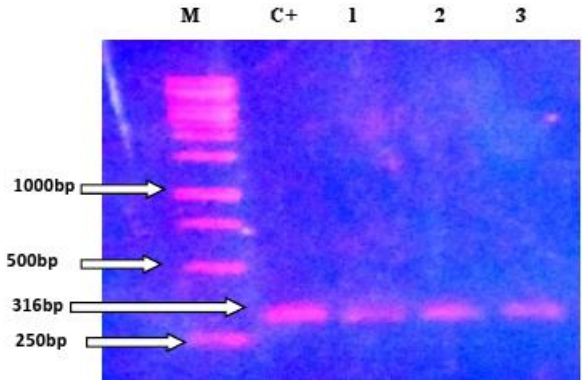

Fig. 3: PCR product of *Mycoplasma capricolum* subsp. *capripneumoniae* amplicon size 316 bp in samples collected from goats exhibiting the sign CCPP. M=1kb DNA ladder, Samples = 1, 2, 3, C+ = Positive control.

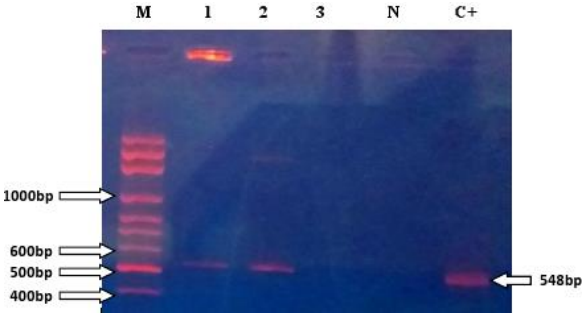

Fig. 4: PCR product of *Mycoplasma mycoides* cluster with an amplicon size of 548 bp in samples collected from goat exhibiting the sign of CCPP. M = 1KB DNA ladder, Sample = 1, 2, 3, N = Negative Control, C+ = Positive Control.

Table 3: PCR based identification of *Mycoplasma mycoides* cluster from different clinical samples of goats suspected for CCPP in Khyber Pakhtun Khwa, Pakistan. Statistical analysis ($\chi 2$) showed significant association ($P<0.001$) between the isolates and different sources of samples.

Status	Nasal (%)	Tracheal (%)	Lungs tissue (%)	Pleural fluids (%)	Total (%)	Chi-square	P-value
Positive	17 (3.4)	06 (2.66)	19 (27.14)	13 (43.33)	55 (6.66)	126.35	0.001
Negative	483 (96.6)	219 (97.33)	51 (72.85)	17 (56.66)	770 (93.33)		
Total	500	225	70	30	825		

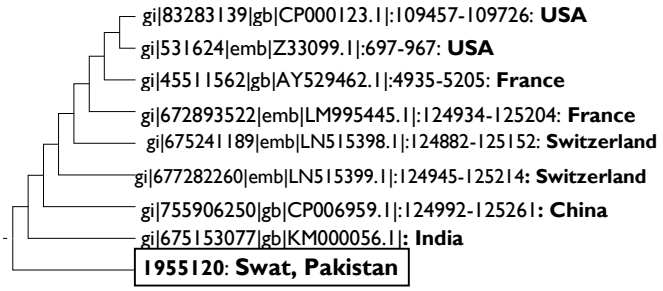

Fig. 5: Phylogenetic relationship of the *Mycoplasma capricolum* sub specie *capripneumoniae* sequence obtained (KPK, Pakistan) comparing with other eight isolates available sequences in NCBI. Sequences of the isolates were downloaded from NCBI and were aligned through Bio Edit multiple alignment. The phylogenetic tree was constructed by neighbor-joining algorithm using the software MEGA version 7.0.5.2.

Table 4: Results of different biochemical assay on culture of mycoplasma isolated from samples collected from goat suffering from respiratory syndrome for the confirmation of mycoplasma cluster and *Mccp*.

S/No	Biochemical assay	Sensitivity
1	Glucose fermentation	+
2	Serum digestion	+
3	Tetrazolium reduction	+
4	Casein digestion	+
5	Arginine hydrolysis	-

+ Positive**, -** Negative

DISCUSSION

Mycoplasmosis is important respiratory disease of small ruminant, causing heavy economic losses throughout the country, especially in Northern and Southern regions of Pakistan (Saddique *et al*., 2012; Hira *et al*., 2015; Banaras *et al*., 2016). Different pathogenic species are reported throughout the country, several conventional and nonconventional techniques are used for the identification of different pathogenic species of mycoplasma with various degree of success. The presence of *Mccp* and other cluster specie was investigated and successfully isolated from nasal, tracheal, lungs tissue and pleural fluid. The member of *Mycoplasma mycoides* cluster in small ruminants has wide range of tissue tropism and causes infections in various body organs. The *Mccp* is the ability to cause acute respiratory infection which mostly confined to the thoracic cavity (OIE, 2012). Maximum growth of *Mccp* was obtained from pleural fluid followed by lungs tissue, tracheal and nasal discharge of goats in the study area. Similar observations were also made by (Nicholas *et al*., 2002; Sadique *et al*., 2012; Liljander *et al*., 2015). It is revealed from the present findings that most desirable source of samples are lungs tissue and pleural fluids for the successful isolation of *Mccp*. The isolation of *Mccp* has also been reported from tracheal and nasal discharge (Kumar *et al*., 2011; Zinka *et al*., 2013). It is justified by the fact that as the disease progressed the purulent pulmonary discharge come through upper respiratory tract along with cough which contain the microorganism. The isolates were successfully grown on modified Hay Flick media from different clinical specimen of the infected animals. Apart from other member of mycoplasma cluster the *Mccp* has the characteristic to grow slowly on modified Hay Flick agar media. However, a typical fried egg and nipple like colonies were observed on modified Hay Flick agar medium on day 7th and 9th during first inoculation, while the same colonies were observed on day 4-7th in third passage. Similar observations were also reported by Hernandez *et al*. (2006).

Both conventional and non-conventional techniques are used for diagnosis of CCPP with varying degree of success. Mostly the serological and biochemical techniques are failed due to sharing of antigenic epitopes of *Mycoplasma mycoides* clusters (Nicholas *et al*., 2002). The biochemical assay is still used to differentiate the pathogenic species of mycoplasma in developing countries (OIE, 2012). The digitonin sensitivity distinguishes Mycoplasma from Acholeplasma, serum digestion distinguishes *Mycoplasma cluster* members from all other species of mycoplasma of small ruminant (Samiullah, 2013). All the local isolates were positive for glucose fermentation, serum digestion, tetrazolium reduction test and casein digestion test while negative for arginine hydrolysis. The results are supported by the finding of (Nicholas *et al*., 2002). It is further justified by the findings that the *Mccp* lack the ability to digest arginine which differentiate it from *Mycoplasma capricolum* sub specie *capricolum* (Noah *et al*., 2011). In Pakistan the CCPP was consider to be caused by *Mycoplasma mycoides* subspecies *capri* by using conventional methods (Rehman *et al*., 2003). Later on molecular identification of the same specie was reported Shahzad *et al*. (2012) in Punjab, Awan *et al*. (2012); Hira *et al*. (2015) in Baluchistan and Sadique *et al*. (2012) in Khyber Pakhtunkhwa. However, the causative agent *Mccp* was for the first time isolated and confirmed through molecular diagnosis in district Pishin, Baluchistan Pakistan by Awan *et al*. (2010). The molecular detection has greatly improved CCPP diagnosis even in mixed culture and also directly from clinical specimen like nasal discharge and pleural fluids. PCR based on 16-S rRNA gene analysis allow the detection of *Mycoplasma mycoides* cluster and other species with accurate identification (Manso-Silvan *et al*., 2007; Kumar *et al*., 2011).

In the northern Pakistan the *Mycoplasma capricolum* sub specie *capripneumoniae* (*Mccp*) was for the first time isolated and confirmed through PCR by using species specific primers. Out of total 55 PCR confirmed mycoplasma cluster 23(8.61%) were positive for *Mccp* with an amplicon size of 316 bp. The study agrees with the findings of the prevalence of *Mccp* in Baluchistan, Pakistan by Awan *et al*. (2010). It is further supported by the findings that the seroprevalence of CCPP caused by *Mccp* was 2.7% and 44.2% in Gilgit and Diamer Districts of Norther Pakistan and 10.1% in the Shuro-Obod District of Tajikistan (Peyraud *et al*., 2014). Similar findings were also reported in different areas of Punjab, Pakistan reflecting the seroprevalence of *Mccp* 8.52% (Shahzad *et*

al., 2016). It is reported that CCPP is prevalent in 40 countries but *Mccp* has been isolated only in 17 countries (Manso-Silvan *et al*., 2011). However, now it has been reported that *Mccp* is prevalent in many country of the world including China and Tajikistan (Chu *et al.*, 2011). The Northern areas of Pakistan are adjacent with Afghanistan and Tajikistan hosted a large population of sheep and goat throughout the year. This influx of small ruminants from these neighbor countries is the major risk factor of cross boundary infectious diseases of livestock particularly the CCPP. In Pakistan only one specie specific vaccine (*Mycoplasma mycoides* sub specie *capri*) is available and used as prophylactic measures for the control of this disease. The failure of vaccine justifies the prevalence of disease caused by *Mccp* as the different species of mycoplasma having no cross immunity for protection. The sequence study of *Mccp* revealed that the local isolates showed close relation with isolates of China and India, but having evolutionary distance from strains of other countries like USA, France and Switzerland.

Conclusions: It is concluded that *Mycoplasma capricolum* sub specie *capripneumoniae* is wide spread pathogenic specie isolated from goat population in natural outbreak throughout Khyber PakhtunKhwa, Pakistan. The isolated specie of *Mccp* having close homology with the strains of neighboring countries like China and India. The successful isolation and characterization of local isolates of *Mccp* has provided an opportunity for the researcher to develop indigenous vaccine for the control of CCPP in Pakistan.

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