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Short Communication

The Predominant Incidence of *Mycoplasma mycoides* subsp. *capri* in Suspected Cases of Contagious Caprine Pleuropneumonia in Sheep and Goats of Northern Pakistan

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HuR, US, ZuH, SA and SuR designed the study. MKS, SSAS, FR, AH, AR and IA collected and processed the samples. HuR performed the PCR and wrote the manuscript.

Key words
Small ruminants, Mycoplasmosis, PCR, *Mycoplasma mycoides* subsp. *capri*, *Mycoplasma capricolum* subsp. *Capripneumoniae.*

ABSTRACT

Contagious Caprine Pleuropneumonia (CCPP), a highly contagious and economically devastating disease, is caused by *Mycoplasma mycoides* (MM) - a cluster of six member species. However, literature is not available regarding the causative agents of CCPP in sheep and goats in Northern Pakistan. The current study was thus aimed to identify relative abundance of members of MM in small ruminants suspected of CCPP in Northern region of Pakistan. A total of 300 samples, (150 sheep and 150 goats) were randomly collected from nasal discharge, pleural fluid and lung tissues, and streaked into Pleuropneumonia like organism (PPLO) medium for isolation and identification of *Mycoplasma* species. Confirmed mycoplasma isolates were subjected to species specific PCR to identify members of MM cluster. Results revealed that 79/300 (26.3%) samples were found positive for the growth of *Mycoplasma*. MM cluster specific PCR showed 49 (16.3%) samples were found positive, of which 34 (11.3%) were found positive for *Mycoplasma mycoides* subsp. *capri* (Mmc) and 15 (5%) were identified as *Mycoplasma capricolum* subsp. *capripneumoniae* (Mccp) by species specific PCR. Three samples were found positive both for Mmc and Mccp suggesting mixed infection. To the best of our knowledge, we report for the first time on the molecular identification of Mccp and Mmc, and on predominance of Mccp as etiological agent of CCPP in sheep and goats of Northern Pakistan. The results have implications in the epidemiology and vaccine strategy against CCPP infection in small ruminants in the Northern region of Pakistan and therefore, a comprehensive country wide surveillance should further investigate the overall prevalence of members of MM cluster in suspected cases of CCPP in small ruminants.

Mycoplasmosis in small ruminants is considered an economically important respiratory infection resulting huge economic losses. Small ruminant mycoplasmosis is witnessed worldwide, while it remains prevalent in many countries of Africa, Asia, and is widespread in Pakistan (Sadique *et al*., 2012; Samiullah, 2013; Shahzad *et al*., 2013). The Office International des Epizootic has declared Contagious Caprine Pleuropneumonia (CCPP) as a noticeable disease. Pakistan is ranked as 3rd largest goat and 12th largest sheep producing country in the world. Thus sheep and goat farming provides not only quality protein, milk and hides etc., but also remains a main source of income for a considerable population and contributing in the national gross domestic product (GoP, 2015-16).

CCPP is one of the most severe and contagious respiratory syndrome associated with high morbidity and mortality rates in sheep and goat. The disease is caused by members of a group of six closely related bacteria species known as *Mycoplasma mycoides* (MM) cluster (Manso-Silván *et al*., 2007). Literature study shows that *Mycoplasma capricolum* subspecies *capripneumoniae* (Mccp) has been mainly responsible of causing CCPP in sheep and goats (Manso-Silván *et al*., 2007; Awan *et al*.,

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2010). However, *Mycoplasma myoicode*s subsp. *capri*, a member of MM cluster, has also been shown reported to cause CCPP in addition to secondary complication such as urogenital infection, arthritis, mastitis and occasional abortion in ewes in Asia and Africa (Madanat *et al*., 2001; Manso-Silván *et al*., 2007; Nicholas *et al*., 2008; Sadique *et al*., 2012). Notably, CCPP caused by Mccp affects thoracic cavity mainly (Thiaucourt *et al*., 2000; OIE, 2014), while Mmc has been also isolated from organs other than lungs suggesting more generalized and ubiquitous form of infection (Sadique *et al*., 2012; Shahzad *et al*., 2012; Shah *et al*., 2017).

In Pakistan, most of the population of sheep and goats are maintained in the northern rural area of Khyber Pakhtunkhwa, District Swat and District Buneer, mainly due to available pasture. The majority of nomadic and local farmers skip vaccination, and thus their flocks are highly susceptible to infections. Seroprevalence studies of CCPP indicate circulation of mycoplasma species in the suspected cases of sheep and goat in Pakistan (Sadique *et al*., 2012; Shahzad *et al*., 2012), however, due to close resemblances of member species of MM, cross reactivity makes it hard to identify mycoplasma specie. So far, to the best of our knowledge, no study is available that reports on the causative agent of natural outbreaks of CCPP in sheep and goats in Northern Pakistan. Here we report on the isolation, occurrence rate and molecular identification of clinical isolates of members of MM by specie specific PCR from suspected cases of CCPP.

Materials and methods

The current study was approved by the ethical committee of the University of Agriculture Peshawar, and all experimental work including sample collection was carried out according to the national and institutional guidelines of animal ethics.

Samples were collected from two districts, District Buneer and Swat, of Khyber Pakhtunkhwa province. District Swat and Buneer are located in the North of Khyber Pakhunkhwa Province Pakistan. Nomads of these districts usually bring their animals down to the plain areas during winter (November to February) while return back to the hilly mountains for pastures during summer (March-October).

Samples were collected between October 2013 and April 2014. A total of 300 samples (the nasal discharge, pleural fluid and lung tissues) were collected at farms, herds and abattoirs from those suspected of CCPP, and were transferred to DifcoTM PPLO broth (Becton Dickinson, Sparks, MD, USA), and transported in ice box to our *Mycoplasma* reference laboratory at the Faculty of Animal and Veterinary Science, University of Agriculture, Peshawar.

All samples were streaked into PPLO media and incubated in anaerobic incubator (New Brunswick, Galaxy 48-S UK) with 5% CO2 at 37°C for 3-7 days for the isolation of mycoplasma species as described previously (Shahzad *et al*., 2012; OIE, 2014). The identification of the *Mycoplasma* was made by the appearance of typical fried egg or nipple live of 0.1 to 1 mm in diameter with a dense raised center in the middle.

Bacterial DNA was extracted using TIANamp Bacteria DNA Kit (TIANGEN, Neijing, China) according to the manufacturer's instructions. The extracted DNA was then subjected to cluster specific PCR amplification with a set of primer MmF 5'-CGA AAG CGG CTT ACT GGC TTG TT-3' and MmR 5'-TTG AGA TTA GCT CCC CTT CAC AG-3', followed by specie specific PCR for Mccp with primer set Mccp.spe-F: 5'-ATCATTTTTAATCCCTTCAAG-3', and Mccp. spe-R: 5'-TACTATGAGTAATTATAATATATGCAA-3 and Mmc P4-F: 5'-ACTGAGCAATTCCTCTT-3' P6-R: 5'- TTAATAAGTCTCTATATGAAT-3' was performed as described previously (Hotzel *et al*., 1996; Manso-Silván *et al*., 2007). A PCR product of 548 bp for MM cluster, 194 bp for Mmc and 316 bp for Mccp was considered positive.

Table I.- occurrence and species-wise distribution of *Mycoplasma* isolates from CCPP suspected cases of sheep and goats.

Region / Species	Total samples (n=25)	Primers					
		Mycoides cluster		Mccp		Mmc	
		+ve	-ve	+ve	-ve	+ve	-ve
Buner							
Sheep	Nasal swab	2	23	Nil	Nil	2	Nil
	Pleural fluid	4	21	1	3	3	Nil
	Lung tissue	3	22	Nil	Nil	3	Nil
Goats	Nasal swab	8	17	3	5	5	Nil
	Pleural fluid	5	20	2	Nil	3	Nil
	Lung tissue	4	21	2	2	2	Nil
Swat							
Sheep	Nasal swab	5	20	1	4	4	Nil
	Pleural fluid	4	21	Nil	4	4	Nil
	Lung tissue	2	23	1	1	1	Nil
Goats	Nasal swab	4	21	2	2	2	Nil
	Pleural fluid	5	20	1	4	4	Nil
	Lung tissue	3	22	2	Nil	1	Nil
Total confirmed samples		49	251	15	25	34	Nil
Overall occurrence		16.33	-	5%	-	11.3%	-

Mccp, *Mycoplasma capricolum* subsp. *Capripneumoniae*; Mmc, *Mycoplasma mycoides* subsp. *capri.*

Results

Of 300 samples, 79 (26%) resulted mycoplasma like growth. A total of 100 samples were collected from nasal swab, and 25 (25%) were found positive for mycoplasma growth containing 10/50 samples (20%) from sheep, while 15/50 (30%) were from goats (See Supplementary Fig. S1). Similarly, a total of 100 samples (50 from sheep and 50 from goats) from pleural fluid with 33% indicated positive growth such as 15/50 (30%) were found positive from sheep and 18/50 (36%) from goats. Finally, of the 100 samples obtained from lung tissue, a total of 21 were found positive such as 9/50 (18%) were found positive for growth of mycoplasma from sheep, while 12/50 (24%) were derived from goats. Interestingly, 28% of samples from District Buneer were cultured positive, while 33% from District Swat. Overall, our study shows that pleural fluid is relatively preferred for mycoplasma isolation from suspected cases.

Results of the MM cluster specific PCR revealed a total of 49 (16.33%) samples were positive (Table I). All samples that were found positive for MM cluster PCR were then subjected to specie specific PCR for the identification of Mmc and Mccp (not shown). Results indicated that only 15 (5%) samples were found positive for Mccp, while 34 (11.3%) samples were PCR amplified against Mmc. Interestingly, three of the samples were found positive both for Mmc and Mccp suggesting mixed infection. Overall, the isolation of both Mmc and Mccp from suspected cases of CCPP suggests their involvement in the development of the diseases.

Discussion

Mycoplasmosis is an important respiratory disease of small ruminants, causing heavy economic losses throughout the country (Sadique *et al*., 2012; Abbas *et al*., 2018). Members of the *Mycoplasma mycoides* could lead to a severe respiratory syndrome called as CCPP. Mccp is the principal cause of CCCP in small ruminants; however, there are reports that other members of MM cluster may potentially cause CCPP. There is often outbreak of CCPP in small ruminants in the Northern region of Pakistan; however, the nature of the etiological agent confirmed by PCR has never been reported. The current study was thus aimed to identify members of MM involve in the development of CCPP.

Although, this is established that the principal cause of CCPP is known to be Mccp, however in Asia and Africa, isolation of Mmc from suspected cases of CCPP suggests its involvement and an unknown role in pathogenesis. Our first report on the isolation of Mmc as an etiological agent of CCPP in goats in central District of Peshawar in Khyber Pakhtunkhwa province prompted us to investigate further the prevalence of Mmc in suspected cases of CCPP mainly in the Northern region of Pakistan (Shah *et al*., 2017). Notably, in the current study Mmc was found significantly highly prevalent (11%) as compared to Mccp (5%). Furthermore, quite surprisingly, the incidence rate of Mccp was found significantly lower (5%) in the Northern regions as compared to our previous report of 24% in the central district of Peshawar-Khyber Pakhtunkhwa. Other reports from Pakistan indicate isolation and identification of *Mycoplasma mycoides* subspecies *Capri* (Awan *et al*., 2009), *Mycoplasma capricolum* subspecies *capricolum* (Awan *et al*., 2010; Shah *et al*., 2017), *Mycoplasma putrefaciens* (Awan *et al*., 2009) and *Mycoplasma capricolum* subspecies *capripneumoniae* (Awan *et al*., 2009; Shahzad *et al*., 2013).

In the present study Mycoplasma was successfully grown on modified PPLO media for isolation from samples originated from different sources of suspected animals including nasal discharges, pleural fluid and lung tissues. It is observed that better isolation was achieved from the pleural fluid of necropsied and sacrificed animals. These findings are in full agreement with previous studies (Thiaucourt *et al*., 2000; Sadique *et al*., 2012). Of the 49, only 15 were confirmed for Mycoplasma capricolum subspecies capripneumoniae through PCR from different sources such as nasal discharges 6 (12.24%), pleural fluid 4 (8.16 %) and lung tissues, 5 (10.20 %) respectively. While 34 out of 49 positive were confirmed as *Mycoplasma mycoides* subspecies Capri from different sources of samples such as 13 (26.53 %) from nasal discharges, 14 (28.57 %) from pleural fluid and 7 (14.28 %) from lung tissues, respectively. Possibly, this is more likely due to the fact that Mycoplasma is intracellular microorganism surrounded by cell membrane containing lypoglycan. This initiates the acute inflammatory response in host with severe exudation. The survival percentage of the Mycoplasma increases when it is present in the deep tissues of the host by evading immune system. Antimicrobial resistance is swiftly increasing all over the world including Pakistan (Ali *et al*., 2016, 2017; Khattak *et al*., 2018) and our current report suggested that mycoplasma species isolated from suspected cases of CCPP have been found resistant to tylosin, a favorite drug used against the condition (Shah *et al*., 2017), confirms this notion. Identification of the prevalent isolates could thus be used for determining susceptibility profile in order to avoid empirical treatment and emergence of antibiotic resistance. In conclusion, our study indicates that Mmc could be actively involved in the development of CCPP infections in sheep and goats in Pakistan, and thus an overall epidemiological survey of the Northern region should be initiated in the country.

Conclusion

The current study concluded a high prevalence of *Mycoplasma mycoides* subspecies *capri* followed by *Mycoplasma capricolum* subspecies *capripneumoniae* in the suspected cases of CCPP from sheep and goats of Northern region of Pakistan. The higher isolation rate of *Mycoplasma mycoides* subspecies *Capri* suggests its involvement in the disease, and a proper and comprehensive survey should be carried out in order to understand the frequency of members of MM cluster involved in the development of CCPP infection in Pakistan.

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Supplementary material

There is supplementary material associated with this article. Access the material online at: http://dx.doi.org/10.17582/journal.pjz/2018.50.5.sc9

Statement of conflict of interest

Authors have declared no conflict of interest.

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