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PATHOLOGICAL AND MOLECULAR DETECTION OF MYCOPLASMA CAPRICOLUM SUBSPP. CAPRIPNEUMONIAE IN SMALL RUMINANTS OF DISTRICT DERA ISMAIL KHAN, KHYBER PAKHTUNKHWA

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Abstract

Background: *Contagious caprine pleuropneumonia is an infectious respiratory disease of sheep and goat which is caused by Mycoplasma capricolum subspp. capripneumoniae (MCCP). MCCP is a major challenge for livestock production globally.*

Objective: *The aim of the study was Pathological and molecular detection of Mycoplasma capricolum subspp. capripneumoniae in small ruminants of district Dera Ismail Khan, Khyber Pakhtunkhwa*

Methodology: *This study was carried out at molecular pathology laboratory, department of Pathology, University of Veterinary & Animal Sciences Lahore Pakistan from December 2016 to January 2017. A total of 50 blood and tissue samples (25 sheep and 25 goats) were collected from CCPP suspected animals at government slaughter house for molecular assay. Blood and tissue samples were collected for serology, hematology, histopathology and PCR identification. Samples were properly labeled, packed and transported to pathology laboratory, Department of Pathology, University of Veterinary and Animals Sciences (UVAS), Lahore. All the data analysis was done by using SPSS version 20.0*

Results: *Based on histopathology, animal lungs showed interstitial emphysema with bronchopneumonia. The Epithelial lining of the alveoli and bronchi was extensive interrupted and interlobular area was thickened. Congestion, inflammation and bleeding scattered cells were also observed. A significant association was observed between hematological parameters and cases of MCCP in goat. ($p<0.05$). Among all 50 samples only 2 goats samples were considered positive based on PI values ≥ 56 by using C-*

ELISA. Based on PCR only two samples were positive for MCCP in goat.
Conclusion: *The study concluded that MCCP prevalence is 4% in goat on the basis of cELISA in district Dera Ismail Khan. The cELISA and PCR for MCCP detection in blood is useful diagnostic tool to detect MCCP by using blood sample.*

INTRODUCTION

Contagious caprine pleuropneumonia is an infectious respiratory disease of sheep and goat which is caused by *Mycoplasma capricolum* subspp. *capripneumoniae* (MCCP). MCCP is a major challenge for livestock production and has a potential for rapid spread. Countries with CCPP infected animals are now excluded from international trade of live animals. This disease is widely distributed with high rates of morbidity and mortality in Asian and African continents. The clinical signs and symptoms exhibited by the animal in case of CCPP varies differently in different parts of the world (1). In infected goats the true lesions of CCPP are only limited to the alveolar tissues which can distinguish CCPP from other respiratory diseases in small ruminants, caused by members of the *Mycoplasma mycoides*. The prominent signs of CCPP are pleurisy, unilateral hepatinization adhesions, the gathering of pleural fluid and straw color fluid in the pleural cavity. Unilateral and bilateral pneumonia is present (Thiaucourt et al. 1996) which clearly differentiates *Mycoplasma mycoides subspp. capri* from "MAKePS" syndrome. So the exact geographic and epidemiological distributions remain un-clarified. It is very difficult to diagnose this disease due to lack of advance techniques in Asian and African continents. So, around 30 to 40 nations have confirmed the contamination in their general area. The MCCP affirmation in China, Mauritius and Tajikistan has been reported (2). Across the Asia, the spread of disease has been confirmed. On the other hand, the disease free zones highlight the epidemiological risks reported by CCPP, especially for those countries which are being considered endemic. In the past few years, there were numerous nations where its presence suspected was affirmed (3). From some recent studies, the extent and significance of the infection has been elaborated that it is relative to other diseases in some endemic areas of Pakistan, Ethiopia, Kenya and Tanzania (4-8). The infection is still maintaining equally while in some areas its morbidity is recorded very high. The current outbreaks in Iran, India and Turkey (OIE, 2012) are reported as high sero-prevalence (Hadush et al. 2009, Mbyuzi et al. 2014).

MCCP is epidemic, can spread in a short distance in a short duration of time. As it is directly transmitted without earlier contact with the agent in the population. So, for the rapid detection and confirmation of infected animals, an exact and dependable symptomatic method is basically required (McGuire et al. 1987). Although, it has been isolated once but due to common antigenic epitopes, it is extremely hard to recognize a specific strain. Similarly a cross response is normally seen among various species on serological examinations (Bölske et al. 1994, Shompole et al. 1997). Many reports have been published for the identification and isolation of MCCP through different serological and biochemical techniques at a very large scale (9, 10). Reports for the isolation of Mycoplasma species like MCCP, MP and MMC using DNA from the suspected goat lung tissues of *pleuropneumonia* are available (11) So keeping in mind that complex pathogenesis of mycoplasma, its composite properties and similarities with other mycoplasma organism. The present study is designed to characterize the infectious organism in both goats and sheep through molecular, histo-pathological and hematological examination. Ordinary techniques may neglect to recognize correct species or subspecies because of the presence of high antigenic heterogeneity among various species or subspecies like *Mycoplasma mycoides* and *Mycoplasma capricolum subspp. capripneumoniae*.

Materials and methods

This study was carried out in molecular pathology laboratory, department of Pathology, University of Veterinary & Animal Sciences Lahore Pakistan from

December 2016 to January 2017. The present project was planned for the pathological study of *Contagious caprine pleuropneumonia* in district Dera Ismail Khan Khyber Pakhtunkhawa. For this purpose, a total of 50 blood and tissue samples (25 sheep and 25 goats) were collected from CCPP suspected animals at government slaughter house for molecular assay. Blood and tissue samples were collected for serology, hematology, histopathology and PCR identification. Samples were properly labeled, packed and transported to pathology laboratory, Department of Pathology, University of Veterinary and Animals Sciences (UVAS), Lahore. Sample for bacterial identification and for histopathological examination were collected separately from clinical suspected animals. Blood samples were collected in 5ml EDTA vacutainer tubes. They were directly put in the cool chain box where the common temperature was 8°C. The samples were further preserved under refrigeration at - 4°C. Tissue samples were collected for histo-pathological examination in sterile bottle and were preserved in 10% buffered formalin. During sample collection, detailed history was recorded related to the diseased animals. Clinical parameters including temperature, coughing, nasal discharge, lacrimation and breathing of the animals were recorded. Postmortem was performed on slaughtered animals and lesions in lungs organ were noted. The sample was taken between the healthy and unhealthy region from the lungs for histopathology according to described protocol OIE (2008). The gross pathological lesions of sheep and goats were recorded and noticed during the postmortem examination of the animals at slaughter house. In postmortem examination, the samples were collected from suspected animal lung organs and fixed in 10% formalin buffer. The review samples were handled by standard procedures as described previously by (12) at molecular laboratory UVAS Lahore. The blood samples were immediately processed for hematology (13). The samples were processed for hematology in Dera Ismail Khan at SEENA Lab through a hematological analyzer DYMIND DH36 Analyzer.

C-ELISA test was done for a total of 50 sera (25 sheep and 25 goats) in Veterinary Research Institute (VRI) at FMD Section Peshawar KPK according to the protocol as described in the IDEXX CCPP 06-56231-01 kit. DNA was extracted through GeneAll® kit method by Exgene™ Method following manufacturer instructions. Already reported set of primers were used to amplify the *Mycoplasma* (14). Details of both forward and reverse sets of primers are given in (Table 1). The gene targeted through these primers was 16s rRNA gene.

Table 1: Details of species specific PCR primer targeting 16s rRNA gene of *Mycoplasma capricolum* subspp. *capripneumoniae*

S. No.	Species	Primer Sequence	Product size	Reference
1	MCCP	(5′ATCATTTTTAATCCCTTCAAG-3′ Forward	316 bp	(14)
2	MCCP	(5'TACTATGAGTAATTATAATATATGCAA-3′ Reverse	316 bp	(14)

PCR Amplification:

The extracted DNA of *Mycoplasma capricolum subspp, capripneumoniae* (MCCP), and the bands were identified and visualized on UV illuminator. The PCR containing specific primers were used from 5' and 3'end of the base pair 316. In the PCR reaction we used 2µl forward primer and 2µl reverse primer and the Master Mix 10µl, DNA template 2µl and 4µl distilled water (Taylor et al. 1994).

PCR Programming:

The initial denaturation stage of PCR was at 95°C for 5 minute. The next stage was denaturation at 95°C for 30 sec. The annealing temperature was at 58°C for 30 sec. The extension temperature was 72°C for 30 sec. Final extension temperature was 72°C for 10 minute. The final elongation stage is optional and their optimum temperature was 70-74°C. The final stage for the PCR product is the final hold and their temperature was 4°C and the total numbers of cycles in the PCR reaction were 35 cycles.

Statistical Analysis

The research data was put on the SPSS version 20.0 and analyzed by student t-independent test for the descriptive mean result of hematology in CCPP in sheep and goats. The Comparison of CCPP positive/negative of Sheep and goats population on the basis of cELISA was checked through chi square test by using the SPSS version 20.0. While, level of significance was set at 0.05 ($p<0.05$).

Results

Clinical Signs

Before collecting the blood samples, clinical signs of high fever, nasal discharge, lacrimation, difficult breathing, forceful cough and eventually death were observed in suspected sheep and goats at Dera Ismail khan slaughter house Khyber Pakhtunkhawa**,** Pakistan.

Gross Lesions

On postmortem examination of suspected animals, lungs tissue had marbled appearance. The appearance of reddening and consolidation of the lungs (Figure No. 1). Further examination showed there was tinged pleural fluid in the chest cavity (Figure No 2).

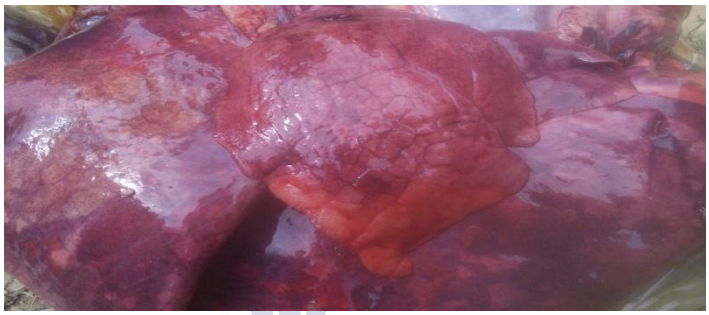

Figure No 1: Effected Lung suffering from CCPP shows congestion and marbled appearance

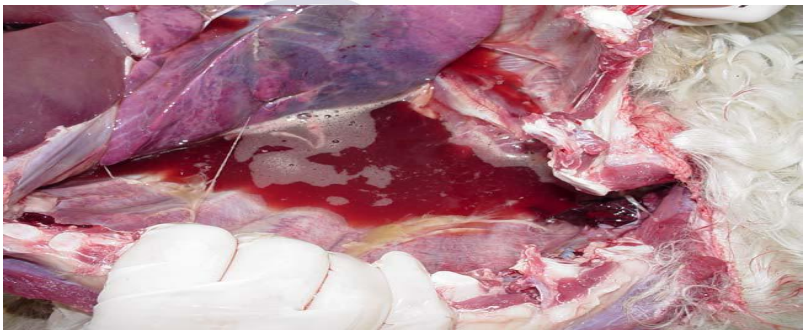

Figure. No 2: Tinged straw color pleural fluid in the chest cavity of goat suffering from CCPP

Histopathology

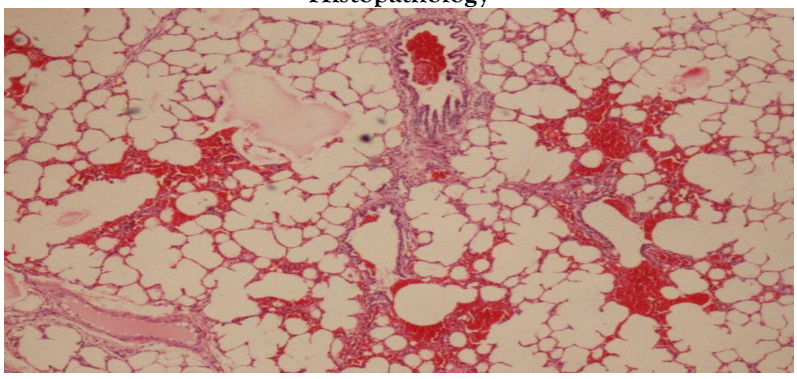

Figure. No. 3: Emphysema of Alveoli and Hemorrhages in lung of Goat suffering from CCPP

The Animal lungs showed interstitial emphysema with bronchopneumonia (Figure No 3). The Epithelial lining of the alveoli and bronchi was extensive interrupted and interlobular area was thickened. (Figure No. 4). Congestion, inflammation and bleeding scattered cells were also observed (Figure No. 3).

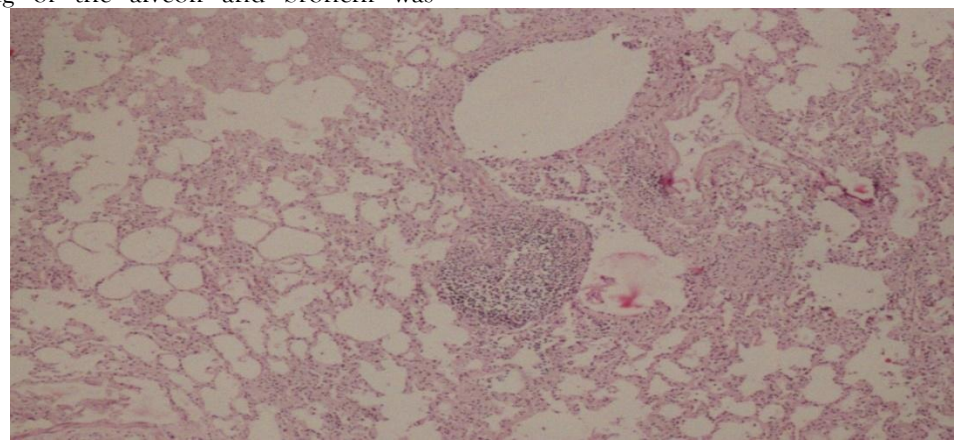

Figure No. 4 Inflammation and hemorrhages of the necrotic areas in the lung of goat suffering from CCPP

Effect of CCPP on hematology in suspected sheep and goats

RBCs values of positive and negative cases of CCPP in goats were 9.50±0.27 and 3.15±0.39 respectively. There is significant difference ($p<0.05$) between positive and negative cases of CCPP in goats regarding RBCs values. WBCs values of positive and negative cases of CCPP in goats were 8.31±0.38 and 17.19±1.22 respectively. There is significant difference ($p<0.05$) between positive and negative cases of CCPP in goats regarding WBCs values. PCV values of positive and negative cases of CCPP in goats were 4.03±.63 and 17.11±1.5 respectively. There is significant difference ($p<0.05$) between positive and negative cases of CCPP in goats regarding PCV values. The MCH values of positive and negative cases of CCPP in goats were 110±30.6 and 7.65±1.01. So there was significant difference ($p<0.05$) between positive and negative cases of CCPP in goats regarding the MCH values. The MCV values of positive and negative cases of CCPP in goats were 26.72±0.91 and 50.56±1.48. There was significant difference ($p<0.05$) between positive and negative cases of CCPP in goats regarding the MCV values. The MCHC values of positive and negative cases of CCPP in goats were 29.82±1.92 and 158.87±39.60. There was significant difference ($p<0.05$) between positive and negative cases of CCPP in goats regarding the MCHC values. The PLT values of positive and negative cases of CCPP in goats were 689.69±54.40 and 212.22±22.38. There was significant difference ($p<0.05$) between positive and negative cases of CCPP in goats regarding the PLT values. Details of each hematological parameters of goat suspected for CCPP and confirm positive cases are given in table 2.

Table 2: Descriptive mean result of sheep and goat Hematology

Parameters	Sheep		Goat		
	Normal Values (Range)	Calculated Values	Normal Values (Range)	Negative	Positive
RBC (× 10^6/µL)	9-5	7.16±0.65	8-18	9.50±0.27[a]	3.15±0.39[b]
WBC (× 10^3/µL)	4-8	13.21±1.19	4-13	8.31±0.38[a]	17.19±1.22[b]
PCV (%)	27-45	34.05±3.39	22-38	17.11±1.50[a]	4.03±0.63[b]
MCH (pg)	8-12	13.38±0.76	5.2-8.0	7.65±1.01[a]	110.00±30.60[b]
MCV (fL)	28-40	45.76±0.48	16-25	26.72±0.91[a]	50.56±1.48[b]
MCHC (g/dl)	31-34	28.11±1.30	30-36	29.82±1.92[a]	158.87±39.60[b]

PLT (× 10^3/µL)	330-650	240.76±30.75	310-690	689.69±54.40[a]	212.22±22.38[b]

*Within a row, values with superscripts [a/b] differ significantly ($p<0.05$) from each other.
Schalm's Veterinary Hematology (6th Edi); Page: 862

C-ELISA

In this study the total 50 serum samples were collected from sheep (n=25) and goats (n=25) from the government slaughter house of district Dera Ismail khan, K.PK. Samples for the presence of antibodies in the serum against CCPP infection were tested by using cELISA through (IDEXX CCPP). Among all these 50 samples only 2 goats samples were considered positive based on PI values ≥ 56. This OD values were converted to percentage values by using the following formula:

P1= 100-(OD control/OD M Ab. control) ×100.

Through this formula the values of samples PI ≥ 56 were considered positives (table 3). On the basis of statistical analysis and odd ratio of data, we can conclude that there is 18.43% chances of CCPP occurrence in goat as compared to sheep in district Dera Ismail Khan. Data is highly non-significant ($p>0.05$), shows that there is no relationship between sheep and goat regarding occurrences of CCPP.

Table no. 3: Comparison of CCPP positive/negative of Sheep and goats population on the basis of cELISA

Sheep		Goat		Odd ratio	P value
Positive	Negative	Positive	Negative	0.1843	0.4898
0	25	02	23		

a. chi square significantly ($p< 0.05$)

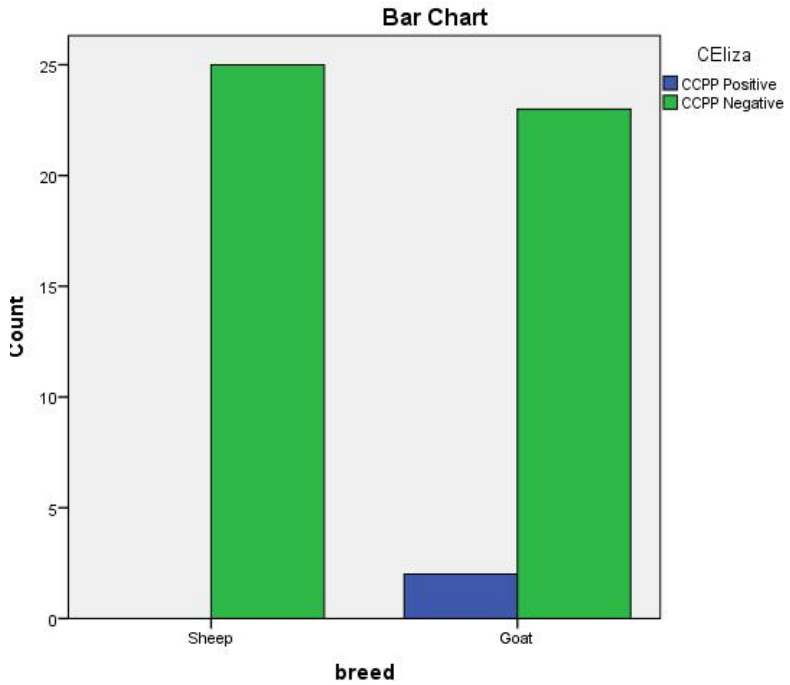

Figure No. 5: Bar Chart of cELISA CCPP positive/negative in Sheep and Goats population in District D.I.Khan

Molecular identification

DNA Extraction

The DNA was extracted from the samples which were confirmed positive for CCPP through cELISA. Extracted product was then run by 1 % agarose gel. Gel was visualized in UV illuminator (Trans Luma ™). Details of all samples are described in (Figure 6).

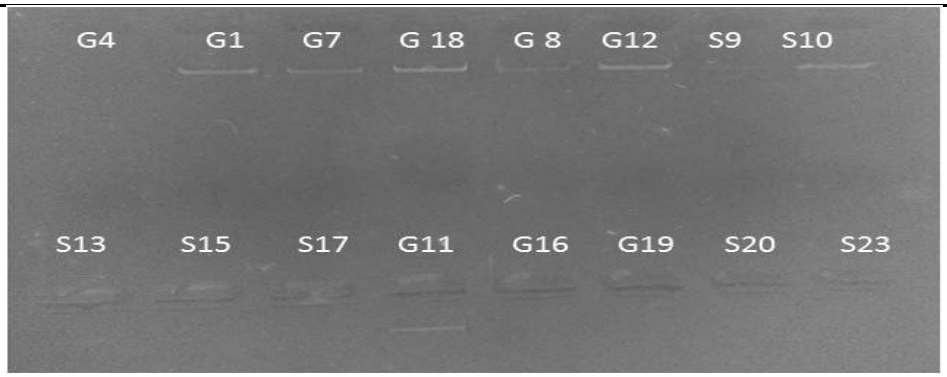

Figure No 6: Extracted DNA from MCCP infected blood samples.

G1, G7, G18, G8, G12, G10 and G11 shows the positive bands of DNA while G4, S9, S13, S15, S17, G16, G19, S20 and S23 shows the negative bands of DNA. G: goat and S: sheep

PCR Amplification

The DNA was extracted from 20 goat sample among 2 positive sample 16 and 17 (figure no 7) were present which were detected positive through c-ELISA and then were run by PCR. Primer reported by (14) were used for amplification of 16s rRNA gene and details are given in (Table No. 1).

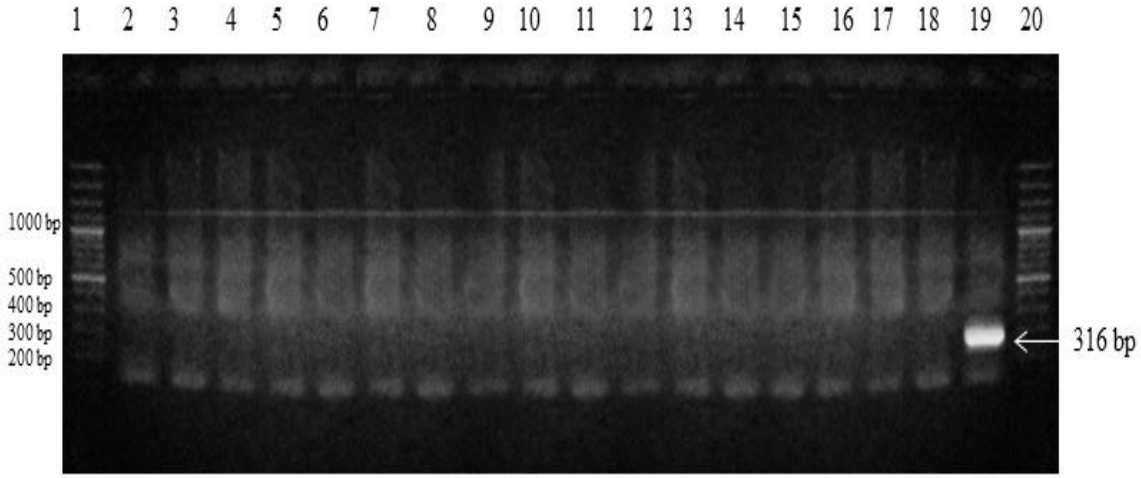

Figure No 7: PCR profile of *Mycoplasma capricolum* subspecies *capripneumoniae* (*MCCP*)

(Amplicon size of 316bp is negative) for blood samples of goats (9-17) and sheep (2-8). 1 and 20, 100 bp molecular weight marker, lanes 2 to 17 tested samples for MCCP, 18 –ve control, 19 +ve control. Stared shows c-ELISA positive samples

Discussion

As caprine pleuropneumonia and other respiratory sign and symptoms related with Mycoplasma species are common in Khyber Pakhtunkhawa. The clinical signs and symptoms effects shown by the animals in the event of CCPP vary differently in various part of the world (1). This distinctions in the signs and symptoms effects of CCPP is because of the way that CCPP is caused by Mycoplasma clusters having diverse species and sub species which at last helping in the events of various lesions. So the clinical study of disease is further complex by action of secondary infection like bacteria and virus especially *Pasteurella* (1). The clinical indications of CCPP are characterized by high fever, nasal discharged, lacrimation, difficult breathing, forceful cough and eventually death (13). In the terminal stages, the animals were not able to move and remain with snatched forelimbs. In any case, high rate of disease was seen in the northern region. This most

noteworthy rate of disease in the region was because of extreme climatic condition and migrant nature of the goat's owner. The travelers play essential part in the spreading of the disease to the area from neighboring nation i.e. Afghanistan, through trans-boundary transmission. The serious climatic condition joined by long traveling causes stress and declines the immune status of the animals. This make the animal more helpless against infection and cold climatic condition expands the intensity of disease. The classical type of CCPP caused by *Mycoplasma capricolum subspp capripneumoniae* is absolutely respiratory in nature (15).

On postmortem examination, lungs showed congestion and marbled appearance. There was present straw color pleural fluid. In this study the spread of CCPP is seen more in the goat than sheep. As *Mycoplasma mycoides capri* cause infection systemically (16). The infection of CCPP is usually a chronic nature and give fatal result (15). Affected animal became a permanent carrier and a big source for the spreading of disease to other animals. These finding are similar with the result reported by (17). The straw colored pleural fluid was found in about 10 percent of the clinically affected animals in slaughter house. Fluid was tinged with yellow appearance and these finding are similar with the result reported by (17). The lesions were mostly in the middle lobe and apical portion of the lungs. These observations show resemblance with the findings of (6). RBCs values of positive and negative cases of CCPP in goats were 9.50±0.27 and 3.15±0.39 respectively. There is significant difference ($p<0.05$) between positive and negative cases of CCPP in goats regarding RBCs values. WBCs values of positive and negative cases of CCPP in goats were 8.31±0.38 and 17.19±1.22 respectively. There is significant difference ($p<0.05$) between positive and negative cases of CCPP in goats regarding WBCs values. PCV values of positive and negative cases of CCPP in goats were 4.03±.63 and 17.11±1.5 respectively. There is significant difference ($p<0.05$) between positive and negative cases of CCPP in goats regarding PCV values. The MCH values of positive and negative cases of CCPP in goats were 110±30.6 and 7.65±1.01. So there was significant difference ($p<0.05$) between positive and negative cases of CCPP in goats regarding the MCH values. The MCV values of positive and negative cases of CCPP in goats were 26.72±0.91 and 50.56±1.48. There was significant difference ($p<0.05$) between positive and negative cases of CCPP in goats regarding the MCV values. The MCHC values of positive and negative cases of CCPP in goats were 29.82±1.92 and 158.87±39.60. There was significant difference ($p<0.05$) between positive and negative cases of CCPP in goats regarding the MCHC values. The PLT values of positive and negative cases of CCPP in goats were 689.69±54.40 and 212.22±22.38. There was significant difference ($p<0.05$) between positive and negative cases of CCPP in goats regarding the PLT values. Details of each hematological parameters of goat suspected for CCPP and confirm positive cases are agree with the result reported by (18). Samples for the presence of antibodies in the serum against CCPP infection were tested by using cELISA (IDEXX CCPP) kit with MCCP antigens. So out of 50 samples, 2 goats samples were detected positive on cELISA with 04% overall seroprevalence in the selected district Dera Ismail Khan. The same study was conducted in Khyber Pakhtunkhawa in 2016 with the 3.91% seroprevalence (19). A cross section study was also conducted in five different districts in Punjab province with the seroprevalence of 8.52 % (20). Previous research work and are not in accordance with the current research work due to differences in sample size and sampling time and sampling area. A zero percent incidence was observed of mycoplasmosis in small ruminants at Baluchistan, Pakistan (21). Because of the different values may extend this research with the use of different tests based on different principles. Zero percent incidence by Latex Agglutination test in MCCP ' 2006-2009 ' in different areas of Pakistan did not support the study (22). The difference may be due to the sample size and have a great time gape between the sample size and various technologies used in previous research. In the present study, the prevalence was recorded 4% in district Dera Ismail Khan. The results of this study are agree with the results of (19, 23, 24). Different types of tissue and organs are involved in the Mycoplasma infection and developed different lesions in different organs (1, 25). The histopathological lesions in the CCPP caused by MCCP are limited to the respiratory tract (15). Similarly, in this study the lesions were found in the

lungs of mostly goats. The most common lesions of the lung were characterized as emphysema and hemorrhages and the results resemble with the findings reported by (17).The epithelium of bronchi and alveoli were also sloughed off. In this study, it was shown that MCCP caused interstitial pneumonia and hemorrhages in the lungs of affected goat. The results of this study are in accordance with the results reported by (26). Many reports have been published for the identification and isolation of MCCP through different serological and biochemical techniques at very large scale (9, 10). Reports of the isolation of Mycoplasma species like MCCP, MP and MMC using DNA from the suspected goat lung tissue of pleuropneumonia are available (11).

Conclusion

The study concluded that MCCP prevalence is 4% in goat on the basis of cELISA in district Dera Ismail Khan. The cELISA and MCCP detection in blood through PCR is useful diagnostic tool to detect MCCP by using blood sample. Further study is required to uncover the deadly disease by increasing the sample size and geographical region from Khyber Pakhtunkhawa, Pakistan.

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