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Determination of Anti-*Mycoplasma capricolum* Subsp. *capripneumoniae* Antibodies for the Sero-Prevalence of Contagious Caprine Pleuropneumonia in North-Western Pakistan

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ABSTRACT

Contagious caprine pleuro-pneumonia (CCPP) is a fatal disease of goats and causes enormous economic losses due to high morbidity and mortality. CCPP is enlisted as a notifiable animal disease by the World Organisation for Animal Health. The causative agent of CCPP is *Mycoplasma capricolum* subsp. *capripneumoniae* (Mccp). The present study aimed to investigate the seroprevalence of CCPP in north-western Pakistan. The study areas were divided into three zones: the northern zone, the central zone, and tribal zone. A total of 1300 serum samples were collected from November 2017 – April 2019 from goats of different ages and sex and were analyzed by monoclonal antibody-based cELISA. The analyses revealed two hundred twenty-seven (17.5%) samples positive for anti-Mccp antibodies. The zone-wise distribution of CCPP in goats was significantly different ($P<0.05$), indicated by positive sera for Mccp of 23% animals from the northern zone followed by 15% and 13% from tribal and central zones, respectively. The data analysis showed non-significant values in the seroprevalence among bucks and doe(s), indicated by anti-Mccp sera from 16.6% bucks and 18.3% doe (s). Moreover, among different age groups, the prevalence of disease in adult goats (20%) was significantly ($P<0.05$) higher than in kids (10.8%). It is evident from the present study that CCPP caused by Mccp is prevalent in Pakistan and both sexes of animals are equally susceptible to Mccp infection. Furthermore, the disease is more prevalent in the northern zone.

INTRODUCTION

Livestock plays an important role in the economy of northwestern Pakistan, especially in the province of Khyber Pakhtunkhwa. The region has a long tradition of pastoralism, and livestock is an important source of income and livelihood for many people. The most common livestock species in the region are cattle, buffalo, goats, and sheep. These animals are kept for milk, meat, and hides, and are also used for ploughing fields and as a form of transportation.

Goats farming in the developing countries like Pakistan face several problems including poor husbandry practices, extreme environmental stresses, and deficiency of good quality feed stuff and various types of infectious, parasitic and various other non-corrosive diseases. Among the infectious bacterial diseases, the mycoplasmal diseases are well known all over the world for its pathogenicity and high economic loss directly affecting farmers (Regassa *et al*., 2010). The prevalence of caprine mycoplasmosis has been documented from different region of the globe, however the occurrence of these diseases is more frequent in Asia and Africa and are considered the major constraint to goat industry in terms of high morbidity and mortality

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(Tigga *et al*., 2014; Srivastava *et al*., 2010). Among various mycoplasmal infections, contagious caprine pleuro-pneumonia (CCPP) is serious threat to goats and their production performance in developing countries (Bascunana *et al*., 1994; Lorenzon *et al*., 2002). CCPP was first reported in 1873 in Algeria (McMartin *et al*., 1980). The causative agent *Mycoplasma capricolum* subsp. *capripneumoniae* (Mccp) was first isolated in 1976 (MacOwan and Minette, 1976). Mccp belongs to *Mycoplasma mycoides* cluster, a group of five closely related species, pathogenic to ruminant population that includes *Mycoplasma mycoides* subsp. *mycoides* (Mmm), *Mycoplasma mycoides* subsp. *capri* (Mmc), *Mycoplasma capricolum* subsp. *capricolum* (Mcc), Mccp and *Mycoplasma leachii* (Fischer *et al*., 2012). Two members of the group, Mccp and Mmm are responsible for economically dangerous diseases including CCPP and contagious bovine pleuropneumonia (CBPP) respectively. Both are listed by World Organization for Animal Health (WOAH, founded as *OIE*) as notifiable diseases. Other members of the *M. mycoides* cluster (Mcc, and Mmc) and non-cluster specie *M. putrefaciens* are involved to induce manifold pathological condition in small ruminants like mastitis, arthritis, keratoconjunctivitis, pneumonia and septicaemia, which is usually termed as 'MAKePS' (Thiaucourt and Bolske, 1996).

In Pakistan, Mmc was first detected among Mm cluster by applying various tests on clinical samples from infected goats suspected for CCPP (Khan *et al*., 1989). Subsequently from district Pishin of Balochistan, goats flocks were investigated for CCPP based on clinical examinations and two *Mycoplasma* species (Mcc and MP) were found prevalent in the affected goats flock (Awan *et al*., 2009). The prevalence of respiratory mycoplasmal infection in different region of Pakistan was reported by late latex agglutination test (LAT) for Mccp, growth inhibition test (GIT) for Mmc and PCR for *Mm cluster* by (Shahzad *et al*. (2012). Similarly, Mmc was found in samples collected from goats having similar signs of CCPP infection in Khyber Pakhtunkhwa (Sadique *et al*., 2012). However, the first report where the presence of Mccp has been confirmed by PCR in the country was published from district Pishin of Balochistan (Awan *et al*., 2010). Later, the molecular prevalence of Mccp was also reported in goats from Khyber Pakhtunkhwa elsewhere (Shah *et al*., 2017). The sero-prevalence of Mccp was also showed in selected districts of Khyber Pakhtunkhwa and Punjab, Pakistan, by monoclonal antibody-based competitive enzyme-linked immunosorbent assay (cELISA) previously (Wazir *et al*., 2016; Shahzad *et al*., 2016). The current distribution of CCPP in many regions of Africa and Asia is not very well known because of scarcity of diagnostic facilities. Furthermore, for prevalence of CCPP at herd level the World Organization for Animal Health (OIE) prescribed cELISA and CFT (Jores *et al*., 2020).

Due to the survival of the Mccp pathogens in treated animals, which serve as disease carriers, treatment with antibiotics as the only therapeutic strategy for CCPP is not a realistic choice (Thiacourt *et al*., 1996; Yatoo *et al*., 2019). In addition, extensive and repetitive antibiotic treatments of herds put farmers in a difficult financial position because of the cost and worries about antibiotic resistance (Parray *et al*., 2019; Yatoo *et al*., 2018; Manso-Silvan *et al*., 2011). In order to prevent CCPP outbreaks and stop its spread, prophylactics like vaccines are therefore becoming essential in the current situation (Thiacourt *et al*., 1996) Additionally, disease prevention eliminates the necessity for mass animal culling in endemic regions, issues with trading affected animals, and other impractical preventive measures (Lipner and Brown, 1995). With 0.15 mg of freeze-dried Mccp protein and 3 mg of saponin in a dose of 1 mL per goat, the World Organisation for Animal Health (OIE) has recommended this saponin-inactivated vaccination for CCPP (WOAH, 2021).

Increased interest and view of the above facts there is a dire need for monitoring of mycoplasma related outbreaks and distribution of these economically important diseases. This project was designed to investigate the serological prevalence of CCPP in Khyber Pakhtunkhwa Province and northern areas of Pakistan for effective therapeutic interventions and control strategy to prevent CCPP outbreak in goat's population.

MATERIALS AND METHODS

Study area

This study was performed across the Khyber Pakhtunkhwa, Gilgit-Baltistan, Pakistan. The selected districts along with tribal districts were divided into three different zones namely, northern zone, central zone, tribal zone. Northern zone includes Gilgit-Baltistan, Chitral, Swat, Buner, and Hazara. Central zone includes Charsadda, Mardan, Swabi, Peshawar, and Nowshera districts. The tribal zone includes the tribal districts, Khyber, Bajour, and Mohmand. The climatic condition of the northern zone is extremely cold with heavy rainfall and snowfall in winter season. The weather is pleasant in summer days and extremely cold in winter season. The tribal region containing federally administrated areas (Now part of Khyber Pakhtunkhwa, Province) has borders with Afghanistan and has extreme climate in summer and winter. The climatic condition of the Central zone is hot humid.

Blood samples

A total of thirteen hundred serum samples were collected from goats during November 2017 to April 2019. All the samples were collected based on clinical signs i.e., mucopurulent nasal discharges, productive cough, deep abdominal respiration, pyrexia (40-41°C) and history of respiratory infection and no vaccination record against CCPP. The samples were collected from different age and sex groups of animals. The number of samples collected from each zone is presented in Table I. The lab work and analysis of the results were performed in Pathology Laboratory, College of Veterinary Sciences, Faculty of Animal Husbandry and Veterinary Sciences, The University of Agriculture, Peshawar.

Table I. Sample collection for the serological prevalence of Mccp in goats of Khyber Pakhtunkhwa and northern areas of Pakistan.

Area/zones of KP	Goats				Total
	Young		Adult		
	Buck	Doe	Buck	Doe	
Northern zone	50	50	200	200	500
Central zone	50	50	150	150	400
Tribal districts	50	50	150	150	400
Total	150	150	500	500	1300

Young age: day 0-180; adult: age above 180 days

The blood samples were collected from the jugular vein of animal's adopting aseptic condition. The blood was poured to gel containing tube and centrifuged for 5 min at 5000 rpm. The serum was transferred to sterile eppendorf tube in aseptic environment and was stored at -20 °C until further processing.

IDEXX CCPP antibody test

The serum was subjected to cELISA test for the detection of Mccp specific antibodies. The procedure was performed following the manufacturer instructions (IDEXX). The optical density (OD) values of samples and controls were measured and record at 450 nm wavelength using the ELISA plate reader.

The absorbance values of controls, were calculated by the following formulas:

Control mean absorbance (CCx) = (CC1 A (450) + CC2 A (450)/2

Monoclonal antibody (Mab) Control mean absorbance (MabCx) = (MabC1 A (450) + MabC2 A (450) + MabC3 A (450) + MabC4 A (450))/4

Percentage of inhibition (S PI%) = 100X ((MabCx- S A (450))/(MabCx- CCx)

Validity criteria

$0.500 \leq \text{MabCx} \leq 2.00$

$\text{CCx} < 0.300$

Mean NC PI ≤ 35%

50%≤ Mean PC PI ≤ 80%

60% ≤ Mean SPC PI ≤ 90%

Interpretation

Negative S PI% < 55%

Positive S PI% > 55%

Statistical analysis

All the data obtained was compiled into the Microsoft Excel spread sheet and analysed through Chi square statistical test. Confidence level was taken at 95% and p value less than 0.05 for significance in all analysis.

RESULTS

Prevalence of CCPP in different zones

The screening of the samples through monoclonal antibody based cELISA test revealed 227 (17.5%) samples positive for CCPP. The region wise prevalence of the disease in goats was recorded as 23% in northern zone followed by 15% tribal zone, and 13% central zone. The analysis of the data through Chi square statistical test significantly revealed difference ($P<0.05$), in the prevalence of disease, among different zones of the studied areas (Table II).

Gender based prevalence of CCPP in different zones

Out of total thirteen hundred samples, 650 serum samples were collected from bucks and 650 from Doe(s). The processing of the samples by cELISA revealed 108 (16.6%) male and 119 (18.3%) female goats positive for anti-Mccp antibodies. The analysis of the data by Chi square test revealed non-significant difference ($P>0.05$), in the prevalence of disease, between different sexes of goats (Table III).

Table II. Prevalence of Mccp in different zones of Khyber Pakhtunkhwa and Northern Pakistan.

Area	Mccp		Total No's of samples	Chi-sq	P. value
	Positive sample	Negative samples			
Northern region	115	385	500	17.85	0.001
Central region	52	348	400		
Tribal region	60	340	400		
Total	227	1073	1300		

Data revealed significant deference ($P<0.05$) among the different zones and prevalence of Mccp.

Table III. Gender age based sero-prevalence of CCPP in four zones of Khyber Pakhtunkhwa and Northern Pakistan.

Gender	Positive sample	Negative samples	Total samples	Chi-sq	P. value
Gender based seroprevalence					
Male	108	542	650	0.646	0.422
Female	119	531	650		
Total	227	1073	1300		
Age based seroprevalence					
Adult	184	716	900	18.06	0.001
Young	43	357	400		
Total	227	1073	1300		

Age wise prevalence of CCPP in goats

Out of nine hundred samples collected from adult goats having age above 180 days, the anti-Mccp antibodies by cELISA were observed in 184 (20%) samples. While in young animals (age: day1 up to 6 months), 43(10.8%) samples revealed reactive antibodies against Mccp pathogen. The prevalence was significantly ($P<0.05$) different recorded between different age group of animals (Table III).

Table IV. Frequency of occurrence of Contagious caprine pleuropneumonia (CCPP) in goats flock from northern, central and tribal zones.

Name of district	Total No's of sample	Positive samples	%age of positive sample
Northern zone			
Chitral	120	29	24
Gilgit Baltistan	135	37	27
Swat	83	14	17
Buner	71	14	20
Hazara	91	21	23
Total	500	115	23
Central zone			
Charsadda	80	14	17.5
Mardan	80	12	15
Swabi	80	11	13.7
Peshawar	80	6	7.5
Nowshera	80	9	11.2
Total	400	52	13
Tribal zone			
Khyber	150	21	14
Bajour	150	28	18.6
Mohmand	100	11	11
Total	400	60	15

District wise prevalence of CCPP in studied area

In the northern zone the highest prevalence of the disease was found in animals from Gilgit Baltistan (27.4%), followed by animals from Chitral, Hazara, Buner and Swat 24.4%, 23%, 19.7%, and 16.8% respectively (Table IV).

The central zone is divided into 05 districts (Charsadda, Mardan, Swabi, Peshawar, Nowshera). The prevalence of Mccp recorded in the present study was higher in Charsadda 17.5% followed by Mardan, Swabi, Nowshera and Peshawar 15%, 13.7%, 11.2% and 7.5%, respectively. The lowest occurrence of the disease was recorded in District Peshawar (Table IV). In tribal zone the maximum frequency of occurrence of the CCPP was recorded in tribal district Khyber (18.6% and tribal district Bajour (14%), while the lowest occurrence of the disease was noted in tribal district Mohmand (11%).

DISCUSSION

Among Pakistan's livestock population, goats have a highest population with 84.7 million numbers (Pakistan Economic Survey, 2022-23). Most of the small poor farmers are solely depended on goats population where rearing of cattle is difficult, due to its great influence on the livelihood of poor farmers, it is also known as poor's man cow in Pakistan (Rahman *et al*., 2003). Among the fatal infectious diseases mycoplasmosis is a severe threat to small ruminant directly impacting farmer's economy (Regassa *et al*., 2010). Caprine mycoplasmosis has been documented across the globe; however, the frequency of the infection is higher in underdeveloped countries of Africa and Asia and cause huge economic losses (Srivastava *et al*., 2010; Tigga *et al*., 2014). CCPP is severe threat to goat population and their production performance in developing countries (Bascunana *et al*., 1994; Lorenzon *et al*., 2002) The serological and molecular prevalence of CCPP in the northern areas and various other region of the country have been previously reported by several researchers (Awan *et al*., 2010; Sadique *et al*., 2012; Peyraud *et al*., 2014; Wazir *et al*., 2016; Shah *et al*., 2017). The present study was designed to investigate the seroprevalence of CCPP in goat's population in North-western Pakistan. The result of the present study is in coordination with the statements of various other scientists who studied the serological prevalence of CCPP caused by Mccp an international collaborative study showed the prevalence of Mccp as 2.7% and 44.2% through cELISA test in two districts of northern region of Pakistan (Peyraud *et al*., 2014). Similar experiment used by (Wazir *et al*., 2016) in specified 4 districts of KP with 3.91% prevalence of Mccp while 8.52% prevalence of Mccp was reported in various regions of Punjab province (Shahzad *et al*., 2016).

The results of the present study revealed that Mccp is present in Pakistan. However, these findings are in contrast with the previous reports (Shahzad *et al*., 2012; Sadique *et al*., 2012; Rahman *et al*., 2013) who reported that Mmc is responsible for CCPP in Pakistan. The findings of the present study are further justified by reports on the molecular prevalence of Mccp in Pakistan (Awan *et al*., 2010; Shah *et al*., 2017; Ahmad *et al*., 2020).

The results of the region wise distribution of the disease obtained in the present study were 115 (23%) in northern region followed by tribal region and central region 60 (15%) and 52 (13%), respectively. The highest frequency of the disease in the present study was recorded in northern region of Pakistan. This could be due to pastoral practices, extreme cold environmental condition, improper management system for large flock (up to 2000 animals in single flock), and movement of animals in winter season (last of October to start of April) for grazing. These factors may lead to stress and make the goats susceptible for the proliferation of pathogenic *Mycoplasma* to cause infection. It has been reported that the flock size, management and production system of the small ruminants, carrier animals in the vicinity play a key role in extent of the disease in small ruminants (Sherif *et al*., 2012). Several other reports (Mekuria and Asmare, 2010; Shahzad *et al*., 2012; Ahmad *et al*., 2020) documented that the occurrence of the disease is more frequent in hilly areas and cold climatic condition.

The prevalence of CCPP being reported in 40 countries through various diagnostic techniques (Manso-Silvan *et al*., 2011). The causative agent has recently been reported in Tajikistan and China (Chu *et al*., 2011). In Pakistan, Gilgit is bordering with China, and Chitral shares long boundary line with Afghanistan and adjacent to Tajikistan. The northern zone of Pakistan has large population of small ruminants and additionally the entrance of sheep and goats from neighbouring countries could be a considerable threat of transmitting/ carrying this transboundary disease (CCPP). The presence of Mccp is reported across the world in many countries however the frequency of occurrence is more in poor countries of Asia, Africa, Middle East and Europe (Woubit *et al*., 2004; Ozdemir *et al*., 2005; Manso-Silvan *et al*., 2011; Chu *et al*., 2011; Peyraud *et al*., 2014; OIE, 2014). The climatic condition of the southern zone is hot humid with minimum rain fall and sandy terrestrial condition. During the winter season the nomads shift their animals to the southern region and thus the carrier animals disseminate the disease in the respective area. Secondly the non-availability of the fodder and harsh climatic condition of the southern region contribute to weakness of the health status of the animals and resultantly immunosuppression of the animals which becomes vulnerable to the infection. Similar findings were also reported that the intensity of the disease increases with cold climatic condition (Mekuria *et al*., 2008). Similarly, during the extensive movement of animals during Eid Ul Adzha (Muslim festival), seasonal movement of nomads and transportation of animals to markets round the year are hypothesized for the spread of the disease across the country. Similar trend of CCPP spreading is reported previously (Bekele *et al*., 2011; Sadique *et al*., 2012). Additionally, it has been observed that in Pakistan only a single specie vaccine based on Mmc is massively used and not provide any cross protection to Mccp which usually fails to control the CCPP out breaks in the country.

In Pakistan most of the farmers keeps mixed herd of different sex, age, and breed. Both sex and ages of animals are equally susceptible to CCPP infection. In the present study the prevalence of the Mycoplasmosis was 119 (18.3%) in female and 108 (16.6%) in male. Previously, 33.03% prevalence of mycoplasmosis in female was reported compared to 29.2% in male (Sherif *et al*., 2012). Similar observations were also reported with the prevalence of CCPP in female animals was higher (39%) as compared to male (Shah *et al*., 2017). These results are also in accordance with the findings reported about 16.9% prevalence in female Spanish Ibex and 8.4% in male Ibex in Spain elsewhere (Verbisck *et al*., 2008). The increase prevalence of CCPP in female animals might be due to various factors which develop stress in animals including pregnancy, lactation, and oestrus cycle. The stress induce by these various factors weakens the immune status of the animals and pave the way for the proliferation of opportunistic Mycoplasma to cause infection in immune compromised animals (Blood *et al*., 2007). However, some researchers reported high prevalence in male 5.32% comparable to female goats 4.67% (Yousuf *et al*., 2012). Similarly, in Ethiopia prevalence of CCPP in female animals (6.66%) was found lowered compared to bucks 24.08% (Regassa *et al*., 2010). Various other researchers from Ethiopia and Tanzania reported that sex does not play significant role in epidemiology of CCPP (Kusiluka *et al*., 2000; Hadush *et al*., 2009; Mekuria and Asmare, 2010; Yousuf *et al*., 2012). The difference may be due to multifactorial consequences, due to immune status, sampling from different locations, close association and sharing of ration among male and female and failure in biosecurity measure may lead to the prevalence of disease dissemination.

CCPP can affect goat's population at all stages of their life, however the increase morbidity and mortality are reported in lower age of goat's population. In present study minimum prevalence was observed in kids 43 (10.8%) compared to adult goats 184 (20%). Our results agreed with the finding of high prevalence of CCPP 30% in adult goats

(Regassa *et al*., 2010). Similarly, it has also been reported that goats at adult age are more affected than young stage elsewhere (Sherif *et al*., 2012). However, these finding are in contrast with reports documented previously from Pakistan (Shah *et al*., 2017; Shahzad *et al*., 2016). The increase percentage of mortality in young kids might be due the weak immune system of the kids who cannot fight with invading microbe affectively. The lymphoid organs develop when animal grows and strengthen the immune system of the host, and with the advancement of age the animals may encounter many pathogens in their life which further strong the immune system. Thus, the adult goats are more capable to encounter the invading Mycoplasma effectively. Furthermore, the antibody can be detected in serum after the infection has been cleared (Rurangirwa *et al*., 1987).

CONCLUSION

We explored the prevalence of contagious caprine pleuro-pneumonia (CCPP) caused by *Mycoplasma capricolum* subsp. *capripneumoniae* (Mccp) across the Khyber Pakhtunkhwa and Gilgit Baltistan. In comparison to all other zones, the northern zone has the highest prevalence of CCPP. This study offers baseline information that can be used to develop an efficient plan for CCPP outbreak control in the area.

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IRB approval

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

Ethics statement

This study was approved by the ethical committee of The University of Agriculture Peshawar. All experimental procedures were performed under the institutional guidelines and animal ethics.

Statement of conflict of interest

The authors have declared no conflict of interest.

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