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SERO-PREVALENCE OF *MYCOPLASMA CAPRICOLUM* SUBSP. *CAPRIPNEUMONIAE* IN GOATS THROUGH cELISA IN DIFFERENT DISTRICTS OF PUNJAB, PAKISTAN

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ABSTRACT

A cross-sectional study was conducted to determine the sero-prevalence of CCPP, in five districts of Punjab that is Okara, Faisalabad, Lahore, Bahawalpur and Pakpatan and three Govt. livestock research institutes that is Research and Development Center, Rakh Khare Wala (District Layyah), Barani Livestock Production Research Institute, Kherimorat (District Attock) and Livestock Production Research Institute, Bahadurnagar, Okara. A total of 364 serum samples were collected from July, 2012 to July, 2013, from clinically respiratory distressed and unvaccinated goats of different breeds, age and sex. Samples were subjected to monoclonal antibody-based competitive enzyme-linked immunosorbent assay (cELISA) for the specific measurement of antibodies to *Mycoplasma capricolum* subsp. *capripneumoniae* (Mccp) bacterium. Thirty one out of **364** samples were positive by cELISA indicating overall sero-prevalence of CCPP as 8.52 %. Statistically the proportional prevalence of CCPP in male and female Beetal goats was significantly higher in Faisalabad district and also at Research and Development Center, Rakh Khare Wala (District Layyah) rather than other districts and research centers. The findings of this survey revealed the evidence of goat exposure to Mccp in different districts and research centers in Punjab province, although at a low prevalence. This sero-survey was conducted for the first time in Pakistan by applying the latest cELISA technique, urging the need of control of this economically important disease for resource poor livestock keepers in Pakistan.

Keywords: *Mycoplasma capricolumn* subsp. *capripneumoniae*, contagious caprine pleuropneumoniae, goats, antibodies

INTRODUCTION

Contagious Caprine Pleuropneumonia (CCPP), an economically important and an Office International des Epizootics listed disease, can cause significant levels of morbidity and mortality in goats in developing countries like Pakistan. 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). This species of animal in the country is facing diversified problems including poor management practices, underfeeding and diseases including infectious and non-infectious one (Shahzad *et al*., 2012a). Among the infectious diseases, contagious caprine pleuropneumonia (CCPP) is an important economical, classical trans-boundary animal disease which can cause mortality rates up to 80 % in susceptible flocks of goats (OIE, 2008; Shahzad *et al*., 2012a). The disease is caused by the smallest fastidious bacteria, member of the *Mycoplasma* genus- usually *Mycoplasma capricolum* subspecies *capripneumoniae* (*Mccp*); taxonomically grouped as one of the member of the *Mycoplasma mycoides* cluster (Swai *et al*., 2013). Clinical signs of the disease include mild to severe cough, purulent nasal discharge, weakness, emaciation, dullness, anorexia, exercise intolerance and pyrexia (Shahzad *et al*., 2012a). The lesions at necropsy are sero-fibrinous pleuritis, accumulation of straw-colored pleural fluid, and a varying degree of lung consolidation or necrosis with marble appearance (Nicholas *et al*., 2008). The transmission of the disease within and between flocks occurs from direct and repeated contacts between sick and healthy animals, and the principal route of infection is by the inhalation of infective droplets from active or carrier animals to healthy animals. Factors such as overcrowding especially during confinement, stress due to extreme weather or weather change, lack of vaccination against *Mccp*, poor management and concurrent infections contribute to the occurrence and spread of the disease (OIE, 2008).

The prevalence of *Mycoplasma capricolum* subspecies *capricolum*, *Mycoplasma putrefaciens* and *Mycoplasma capricolum* subspecies *capripneumoniae* have been reported in goats in Pishin district of Balochistan (Awan *et al*., 2009a, 2009b). Shahzad *et al*. (2012a) has also confirmed the prevalence of *Mycoplasma mycoides* subspecies *capri* by Polymerase Chain Reaction (PCR) in different districts of four provinces of Pakistan. Similarly Shahzad *et al*. (2012b) has also observed 45.70 % prevalence of Mccp antibodies

in goats by using "CapriLAT" latex agglutination kit in three districts of Punjab province.

Since the immunogeno-protective response is associated with production of antibodies specific to Mccp in animals within 7 to 33 days post-infection; therefore, detection of antibody against Mccp in animals is one of the indicators of exposure (March *et al*., 2002; OIE, 2008). The presence or prevalence of Mccp antibodies in unvaccinated goats may indicate not only subclinical or in apparent infection but also nonlethal clinical infection or recovered animals, which could be of epidemiological importance (Swai *et al*., 2013).

Therefore, this study has been designed to determine, through a sero-survey, the extent of goat exposure to Mccp by using competitive enzyme- linked immunosorbent assay (cELISA) and to explore factors associated with its infection in five goat populated districts and three Govt. livestock research institutes in the Punjab province.

MATERIALS AND METHODS

Study area: During a period of one year (July, 2012 to July, 2013) five districts including Okara, Faisalabad, Lahore, Bahawalpur and Pakpatan and three Govt. livestock research institutes such as Research and Development Center, Rakh Khare Wala (District Layyah), Barani Livestock Production Research Institute, Kherimorat (District Attock) and Livestock Production Research Institute, Bahadurnagar, Okara were selected for sero-prevalence study of Mccp antibodies in goats. The districts under study are thickly goat populated areas of Punjab province as per Livestock Census (2006).

Study Design, Population & Sampling Strategy: The study design was a cross sectional survey of all age, breed and sex groups of goats in the districts and research centers. Field veterinary staff of respective districts/research centres were contacted for goat flocks and sampling. A random sampling technique was used and blood samples were collected. The samples were collected from affected animals of different goat flocks mainly kept on traditional grazing of weeds, trees and seasonal green fodder. The affected animals were suspected through history, clinical signs and symptoms of the disease, and also by post mortem lesions. A pre-designed questionnaire was also recorded at the time of each sampling regarding the species, breed, sex, age, vaccination schedule, clinical signs observed, morbidity, mortality due to respiratory disease, purchased or sold animals and history of previous medication etc. All samples were obtained prospectively for the purpose of study from animals showing signs and symptoms of disease such as muco-purrulant or watery nasal discharge, more or less coughing, rapid shallow or difficult breathing, increased body temperature, abnormal lung sounds on auscultation, off fed, dull, depressed or lying on ground etc.

Sample Size: In a previous study, about 46 % prevalence of antibodies against CCPP was observed in goats in Punjab by Latex Agglutination Test (Shahzad *et al*., 2012a). Therefore, the assumption of 46 % prevalence is kept as hypothesis to investigate the prevalence of antibodies against CCPP in goats by cELISA test. In this regard we calculated a sample size as 380; keeping 95 % level of confidence (CL), 5 % desired level of precision and 46 % prevalence of CCPP as per the hypothesis. The sample size was determined using the formula given in Thrustfield (2005):

$$n = \frac{1.96^2 \times Pexp\ (1-pexp)}{d^2}$$

Where:

n = Required sample size
Pexp = Expected prevalence
d = Desired absolute precision

However, due to certain constraints, only 364 blood samples were collected from goats for further lab investigation.

Serum Samples Collection: Animals were restrained by two assistants, area around the mid Jugular vein was disinfected by 70 % ethanol and then 5 ml blood sample was collected from the Jugular vein using 10 ml vacutainer tubes (BD Vacutainer®, NJ, USA). The samples were kept under the shade in a slant position for two hrs or centrifuged. Serum was decanted into sterile serum tubes, numbered and then brought to lab (Livestock Production Support Labs of Livestock Production Research Institute, Bahadurnagar, Okara) on ice packs within 24 h of collection. In the lab the sera samples were tested immediately or stored at – 20 °C until tested. The detail of different number of samples in different districts/research centers with respect to sex has been given in Table 1.

Competitive enzyme-linked immunosorbent assay (c**ELISA**) **Test:** Collected goat serum samples were examined for the presence of specific antibodies against Mccp by competitive enzyme-linked immunosorbent assay (cELISA). This kit was prepared by CIRAD-Montpellier France and its pilot batch was donated by Dr. F. Thiaucourt (Director, Reference lab for CCPP at CIRAD-Montpellier, France) for this research project. This kit has been designed for the detection of antibodies directed against *Mycoplasma capricolum* subspecies *capripneumoniae* in individual caprine serum samples. This test was performed at Quality Control Lab, University of Veterinary and Animal Sciences, Lahore according to the Manual: cELISA-CCPP-V02 (Provided along the kit). Briefly, the ELISA plates (Nunc Immuno1-Maxisorb, Cat. A39454) were coated with a purified lysate, a 1:3,000 dilution of *M. capricolum*

subsp. *capripneumoniae* antigen (50 µl/well) derived from Madin–Darby bovine kidney cell culture, and the plates were incubated at 37 °C for 1 h with constant agitation. Unbound antigen was washed away using washing buffer (0.01 M, pH 7.4±0.2 plus 0.05%Tween 20), followed with the addition of 45 µl of blocking buffer to each of the wells (PBS containing 0.5 % *M. capricolum* subsp. *capripneumoniae* negative control serum and 0.05 % Tween 20). Eleven micro-liters of the test and control serum samples (negative, weak positive, and strong positive) were then added (in duplicate), followed with the addition of 110 µl of MAb (except to the conjugate control wells) at a concentration of 1:100 in blocking buffer. The plates were then incubated for 1 h±5 min at 37 °C (±3 °C) with a gentle agitation. All of the wells were washed with a 300 µl of washing solution two times. Anti-mouse IgG horse radish per oxidase conjugate, diluted 1:100 in blocking buffer, was added to each well (100 µl/well), and the plates were incubated for 30min (±3min) at 37 °C (±3 °C). Substrate solution (TMB-9) was added to each well (100 µl/well) and incubated for 20 min at 37 °C (±3 °C) in a dark place. Stop solution (100 µl) was then added into each well with a gentle agitation allowing 60 min for a color reaction to develop. The ELISA microplates were read with an immunoscan reader (Flow Laboratories, UK) with an inference filter of 450 nm. The reader was connected to a computer loaded with ELISA Data Interchange software, which was used to automate the reading and calculation of the percentage inhibition (PI) values. The optical density (OD) values were converted to percentage inhibition using the following formula: PI=100−(OD control or test serum/OD MAb control)×100. The samples with PI≥60 % (cut off) were considered positives for *M. capricolum* subsp. *capripneumoniae* infection.

Table 1. Details of serum samples collected in different districts with respect to sex

Districts/ Research centres	No. of Male	No. of Female	Total
Okara	24	47	71
Faisalabad	7	11	18
Lahore	37	13	50
Bahawalpur	67	30	97
Pakpatan	17	10	27
Research and Development Center, Rakh Khare Wala (District Layyah)	23	24	47
Livestock Production Research Institute, Bahadurnagar, Okara	10	21	31
Barani Livestock Production Research Institute, Kherimorat (District Attock)	13	10	23
Total	**198**	**166**	**364**

Data Analysis: The collected data were stored in Microsoft Office Excel 2007 spreadsheet. Lab investigation results were analyzed using descriptive statistics by STATA version 6. A statistical test of Chi-square was used. In all the analysis, confidence level was at 95 % and $p < 0.05$ was taken for significance. The significant risk factors were further subjected to multivariable stepwise logistic regression analysis to determine the major risk factors. The major risk factors were used for the model construction to predict the occurrence of the disease in the study area.

RESULTS

In this study, a total of 364 goat sera samples were collected from selected districts and livestock research centers present in Punjab **p**rovince. All samples were tested for the presence of serum antibodies against CCPP infection using cELISA test with *Mccp* antigens. The overall sero-prevalence of CCPP was proved to be 8.52 % using cELISA test (Table 2)**.**

The highest (33.33 %) sero-prevalence of CCPP was observed in district Faisalabad and lowest sero-prevalence was recorded in district Lahore. Since the P-value (0.0001^{**}) of the calculated (χ^2) statistic value is < than the level of significance i.e. α: 0.05 and thus concluded that the proportional prevalence of CCPP in goats is significantly higher in Faisalabad district and also at Research and Development Center, Rakh Khare Wala (District Layyah) rather than other districts and research centers.

Sero-prevalence of CCPP in different sexes in different districts/research areas: The sero-prevalence of CCPP tested by cELISA in different sexes in different districts/research areas has been shown in Table-3.

The highest (28.57 %) sero-prevalence of CCPP was observed in male goats in district Faisalabad and lowest (2.70 %) in district Lahore. Similarly the highest (36.36 %) sero-prevalence of CCPP was observed in female goats in district Faisalabad and lowest (0 %) in Lahore and Pakpatan. Since the P-value (0.0169^{**}) of the calculated (χ^2) statistic value is < than the level of significance i.e. α: 0.05 and thus concluded that the proportional prevalence of CCPP in male goats has significantly higher difference in field area of district Faisalabad than Okara, LPRI (Okara) and Layyah (Research and Development Center, Rakh Khare Wala). Similarly the P-value (0.0053^{**}) of the calculated (χ^2)

statistic value is < than the level of significance i.e. α: 0.05 and thus concluded that the proportional prevalence of CCPP in female goats has significantly higher difference in field area of district Faisalabad and Layyah (Research and Development Center, Rakh Khare Wala) rather than other districts.

Sero-prevalence of CCPP by cELISA in different age groups: The detail of sero-prevalence of CCPP tested by cELISA in different age groups has been shown in Table-4.

Table 2. Sero-prevalence of CCPP among goats in selected districts and research centers of Punjab province

Districts/ Research centres	No. Examined	No. Positive	Sero-prevalence %
Okara	71	7	9.86
Faisalabad	18	6	33.33
Lahore	50	1	2.00
Bahawalpur	97	5	5.15
Pakpatan	27	1	3.70
Research and Development Center, Rakh Khare Wala (District Layyah)	47	7	14.89
Livestock Production Research Institute, Bahadurnagar, Okara	31	2	6.45
Barani Livestock Production Research Institute, Kherimorat (District Attock)	23	2	8.69
Total	364	31	8.52

Table 3. Sero-prevalence of CCPP in different sexes

Districts/Research centres	Male			Female		
	Total No. of samples (n=198)	No. of positive samples	Prevalence %	Total No. of samples (n=166)	No. of positive samples	Prevalence %
Okara	24	3	12.50	47	4	8.51
Faisalabad	7	2	28.57	11	4	36.36
Lahore	37	1	2.70	13	0	0
Bahawalpur	67	2	2.98	30	3	10
Pakpatan	17	1	5.88	10	0	0
Research and Development Center, Rakh Khare Wala (District Layyah)	23	3	13.04	24	4	16.66
Livestock Production Research Institute, Bahadurnagar, Okara	10	1	10.00	21	1	4.76
Barani Livestock Production Research Institute, Kherimorat (District Attock)	13	1	7.69	10	1	10
Total	198	14	7.07	166	17	10.24

Since the P-value (0.0001^{**}) of the calculated (χ^2) statistic value is < than the level of significance i.e. α: 0.05 and thus concluded that the proportional prevalence of CCPP among goats of group 1 (Day one to 180 days) has significantly higher difference in field area of district Faisalabad than Okara and Layyah. Similarly the P-value (0.1492^{**}) of the calculated (χ^2) statistic value is < than the level of significance i.e. α: 0.05 and thus concluded that the proportional prevalence of CCPP among goats of group 2 (181 to 365 days) has significantly higher difference in field area of district Faisalabad and Attock than Okara, Bahawalpur and Layyah (Research and Development Center, Rakh Khare Wala). Similarly the P-value (0.0113^{**}) of the calculated (χ^2) statistic value is < than the level of significance i.e. α: 0.05 and thus concluded that the proportional prevalence of CCPP among goats of group 3 (365 days and above) has significantly higher difference in field area of district Faisalabad than Attock (Barani LPRI Kherimorat), Okara (LPRI Bahadurnagar) and Layyah (Research and Development Center, Rakh Khare Wala).

Sero-prevalence of CCPP by cELISA in different breeds of goat: The sero-prevalence of CCPP by cELISA in different breeds has been shown in Table-5.

Table 4. Sero-prevalence of CCPP by cELISA in different age groups of goats

Districts/ Research centres	Total No. of samples (n=83)	No. of positive samples	Prevalence %	Total No. of samples (n=91)	No. of positive samples	Prevalence %	Total No. of samples (n=190)	No. of positive samples	Prevalence %
Okara	9	2	22.22	17	2	11.76	45	3	6.66
Faisalabad	3	2	66.66	9	2	22.22	6	2	33.33
Lahore	7	0	0	13	0	0	30	1	3.33
Bahawalpur	27	1	3.70	16	2	12.5	54	2	4.44
Pakpatan	7	0	0	3	0	0	17	1	5.88
Research and Development Center, Rakh Khare Wala (District Layyah)	10	2	20	17	2	11.76	20	3	15
Livestock Production Research Institute, Bahadurnagar, Okara	9	0	0	11	0	0	11	2	18.18
Barani Livestock Production Research Institute, Kherimorat (District Attock)	11	0	0	5	1	20	7	1	14.28
Total	83	7	8.43	91	9	9.89	190	15	7.89

Table 5. Sero-prevalence of CCPP by cELISA in different breeds of goats

Name of District	Breeds of Goats	Total No. of Samples	No. of Positive samples	Prevalence %
Okara	Beetal	37	3	8.1
	Beetal Teddy Cross	23	2	8.69
	Teddy	11	2	18.18
Faisalabad	Beetal	6	2	33.33
	Beetal Teddy Cross	12	4	33.33
Lahore	Beetal	30	-	-
	Beetal Teddy Cross	20	1	5
Bahawalpur	Rohi	47	2	4.25
	Beetal Teddy Cross	30	2	6.66
Pakpatan	Beetal	20	1	5
	Beetal Teddy Cross	17	1	5.88
	Teddy	10	-	-
Research and Development Center, Rakh Khare Wala (District Layyah)	Angora	23	2	8.69
	Nachi	17	2	11.76
Livestock Production Research Institute, Bahadurnagar, Okara	Beetal	7	3	42.85
	Beetal Teddy Cross	16	1	6.25
Barani Livestock Production Research Institute, Kherimorat (District Attock)	Beetal	15	1	6.66
	Beetal Teddy Cross	11	1	9.09
	Phari	12	1	8.33

Since the P-value (0.0004^{**}) of the calculated (χ^2) statistic value is < than the level of significance i.e. α: 0.05 and thus concluded that the proportional prevalence of CCPP among Beetal goats has significantly higher difference in field area of district Faisalabad and Layyah than Okara and Layyah (at Research and Development Center, Rakh Khare Wala) rather than other districts.

DISCUSSION

Pakistan has a large livestock population, well adapted to the local conditions, having a total of 37 goat breeds, some of which fall among the best varieties in the world (Afzal and Naqvi, 2004). The goat population in Pakistan is reported as 66.6 million (M) which yielded 0.822 M tons of milk for human consumption, 0.657 M

tons of mutton and 25.66 M No's skins during 2013-2014 (Anonymous, 2014). Pakistan has been ranked third in number of goats and ranked fourth in the amount of goat milk and meat produced in the world (Aziz, 2010). Punjab province is the home tract of different goat breeds and represents 37 % of the total goat population of the country (Livestock Census, 2006).

In the present study, there was evidence that a small proportion of goat populations of different breeds are exposed to *M. capricolum* subsp. *capripneumoniae* (Mccp) bacterium, indicating the importance of the disease in goat population in this developing country like Pakistan. This evidence has been obtained by the use of newly established cELISA kit, which was used during the present research work and is specific for the detection of antibodies against *Mccp* in the serum of goats. This test is particularly useful in identifying animals which developed the CCPP and survived and developed the antibodies against CCPP thus provide a definite diagnosis of CCPP caused by *Mccp*. This is a latest technique and this cELISA kit has been donated by Dr. F. Thiaucourt from CIRAD, France specifically for this research study.

During this study, 31 out of 364 (8.52 %) serum samples from goats were found positive to the antibodies against Mccp by using cELISA technique. Shahzad *et al*. (2012b) has found that 343 out of 750 (45.73 %) serum samples positive to Mccp antibodies by using the Latex agglutination test kits "CapriLAT" in three districts i.e Okara, Sahiwal and Faisalabad districts of Punjab. The higher prevalence (45.73 %) of Mccp antibodies in that study cannot be compared with the low prevalence (5.83) of Mccp antibodies in the present study due to difference in location of samples, difference in antibodies detection technique and a difference of time span. Furthermore, this relatively low sero-prevalence detected may be related to the low level of exposure to pathogen, low level of new animal introductions to the flock for crossbreeding or flock expansion purposes.

Sero-prevalence of CCPP in goats in Pakistan has been studied by very few researchers in Pakistan by using different lab techniques. Hussain *et al*. 2012 has observed 32.50 % sero-prevalence of CCPP in Beetal goats by using counter immunoelectrophoresis technique. Similarly Shahzad *et al*. (2012a) could not found positive samples by using Latex Agglutination Test, kits donated by J. B. March and were specific against Mccp. The reason for this discrepancy might be the validity of LAT kit or absence of Mccp in the study area.

The prevalence of CCPP in Pakistan during the present study was found 8.52 % by cELISA technique. The findings of this study are also in accordance with the findings of Swai *et al*. (2013), who found a 9.6 % prevalence of CCPP in goats in two districts of northern Tanzania by using the same type of cELISA kit.

It must be noted that the presence of Mccp antibodies in goats without clinical disease does not exclude these animal species from being susceptible to infection. The fact that the studied animal shared grazing, housing, marketing and watering with other goats implies that the opportunity for the spread of infection from diseased to healthy should not be underestimated. Uncontrolled movement of small ruminants due to various reasons, such as marketing, seasonal grazing, sacrificial purposes movements etc are common practices in different parts of the country, and therefore, the possibility of cross-infections cannot be ruled out.

Sero-prevalence is very subjective because it cannot differentiate between current and previous exposures to the organisms (Muuka *et al*., 2011; Schubert *et al*., 2011). The principle limitation of the wide use of CCPP seroprevalence studies is due to the occurrence of false-positive results, and that acute cases caused by *M. capricolum* subsp. *capripneumoniae* rarely show positive titers before death. Such tests are best used on a flock basis rather than for diagnosis in individual animals. Therefore, there is a need to do more isolation studies and characterize the microorganisms. This will help to understand its epidemiology and devise proper control measures.

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