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Seroprevalence of Antibodies to Peste Des Petits Ruminants at Various Governmental Livestock Farms of Punjab, Pakistan*

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Abstract: The main purpose of this study was to look into the prevalence of peste des petits ruminants (PPR) in some governmental livestock farms in northeast Pakistan, to ascertain differences in seroprevalence between imported verses local breeds and to find age and sex predisposition to PPR. A total of 280 serum samples from different unvaccinated breeds of sheep (198) and goats (82) were collected from January to July 2007. These samples were subjected to monoclonal antibodies-based competitive ELISA (cELISA) for the specific measurement of antibodies to PPR virus. Findings suggested that the seropositive cases were significantly higher in sheep (38.8%) than in goats (25.6%) ($p = 0.034$). The overall seroprevalence of PPR in small ruminants was 35.0%. There was no evidence ($p = 0.098$) of differences in seroprevalence between samples from three different locations. Based on the seroepidemiology, an insignificant difference ($p = 0.056$) was found in goat breeds while a significant difference ($p = 0.023$) was observed in sheep breeds. As regards to age, PPR antibodies were significantly ($p = 0.016$) higher in three age groups (young, adult and mature) of sheep while there was an insignificant difference ($p = 0.385$) in goat age groups. The influence of sex on PPR prevalence was estimated to be insignificant ($p = 0.062$) in all the breeds of sheep and goats. Given the high prevalence for PPR among all breeds (local and imported), it is advisable to include PPR serology in the sero-monitoring program to give a better indication of herd immunity and to establish appropriate PPR control measures in small ruminants, especially at governmental livestock farms.

Key words: Competitive ELISA, governmental livestock farms, ppr, seroprevalence, sheep, goats

INTRODUCTION

Following India and China, Pakistan has the third largest sheep and goat population over one third of which are kept in the province Punjab, characterized by irrigated crop production. About half are reared in governmental livestock farms. Despite a large population of sheep and goats, the production performance is low in the country. Among other limiting factors, infectious diseases such as contagious caprine pleuropneumonia, pasteurellosis, peste des petits ruminants (PPR) and contagious ecthyma are significant impediments to the economical rearing of small ruminants. Out of these, PPR causes immense economic losses in the small ruminant industry (Hussain *et al*., 2002).

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PPR is a viral disease of goats and sheep characterized by erosive stomatitis, enteritis and pneumonia. The morbidity rate is 100% and during a severe outbreak, mortality can reach 100%, which has a substantial economic impact on developing countries (Radostits *et al*., 2007). The PPR virus (a member of the genus *Morbillivirus*) is antigenically related to rinderpest virus, which infects cattle and other large ruminants (Anderson and McKay, 1994). As of today, PPR-virus isolates are grouped into four distinct lineages on the basis of partial sequence analysis of the fusion (F) protein genes; lineage III is the lineage found in Eastern Africa (Ethiopia) (Shaila *et al*., 1996) and lineage IV was reported in Asia (Ozkul *et al*., 2002).

The Arabian Peninsula, the Middle East and remaining parts of Sub-continents were swept by an epidemic of PPR in 1993-1995 (Shaila *et al*., 1996). PPR was confirmed for the first time in Pakistan in 1994 when samples collected during a suspected outbreak were sent to the Institute of Animal Health, Pirbright, UK (Amjad *et al*., 1996). Since then, the disease has been recorded in all four provinces in Pakistan, either on the basis of virus isolation, antigen detection, or by the measurement of group-specific antibodies against the virus (Zahur *et al*., 2006; Munir *et al*., 2008). Because the disease is new, it is often confused with contagious caprine pleuropneumonia, pasteurellosis or contagious ecthyma (Hussain *et al*., 2002). Large flock sizes, visiting live animal markets and inadequate veterinary services have been identified as risk factors for PPR seropositivity (Majali *et al*., 2008).

Despite the profound immunosuppression, PPR virus may induce, this effect is transient and recovery from the disease is usually followed by the establishment of a strong, specific and long-term protective immune response of the host (Cosby *et al*., 2005). Anti-PPRV antibodies generated by vaccinated animals last for at least 3 years, the effective economic life of the animal (Diallo *et al*., 2007).

It is necessary to diagnose and control PPR in the minimum possible time to avoid economic crisis in small ruminants. Furthermore, because of the transboundary nature of Rinderpest (RP) and the hinderence of an eradication campaign in Pakistan and bordering countries, early diagnosis and control are crucial. There have been no seroprevalence reports of PPR in governmental livestock farms, although veterinarians have previously observed a PPR like disease in some farms of Punjab. There is an unrestricted flow of livestock from neighbouring villages where PPR is reported to be endemic into common grazing places. The major objective of this study was to investigate the prevalence of PPR in some governmental livestock farms in northeast areas of Pakistan. Emphasis was also given to ascertain whether there are differences in seroprevalence between herds with imported and local breeds and to find age and sex predisposition to PPR.

MATERIALS AND METHODS

Clinical History

A total of 280 serum samples from different unvaccinated breeds of sheep (198) and goats (82) were collected. None of the animals were known to have been vaccinated against RP or PPR before, or at the time of sampling. The sex, breed, species and number of samples collected are presented in Table 1. During the period from January to July 2007, the Sheep and Goat Research center at Khariwala, District Layyah, the Livestock Production and Research Institute, Bahadurnagar, District Okara and the Livestock Experimental Station, Qadarabad, District Sahiwal, Punjab, Pakistan were visited and tested. A comprehensive history of species, breed, age, vaccination schedule, clinical signs, mortality, morbidity and purchased or sold animals and evidence of previous medication were recorded on a pre-designed questionnaire.

Table 1: The detailed sketch of collection of samples from various breeds of sheep and goats at three livestock farms

Source	Species	Breeds	Total No. of animals at farms	No. of samples collected		
				Male	Female	Total[b]
Sheep and Goat Research Center at Khariwala, District Layyah	Sheep	Karakul	503	4	46	50
		Thalli	82	1	7	8
		Kachhi	105	2	8	10
	Goat	DDP[a]	118	3	8	11
		Nachi	54	1	4	5
		Angora	119	2	9	11
		Teddy	314	4	26	30
		Beetal	158	4	21	25
Livestock Production and Research Institute, Bahadurnagar, District Okara	Sheep	Awassi	483	5	43	48
		Lohi	379	4	34	38
Livestock Experimental Station Qadarabad, District Sahiwal	Sheep	Awassi	241	3	20	23
		Lohi	176	3	18	21

[a]DDP = Daera Din Panah; [b]Samples are collected from about 10% of the herd at three farms from different sheep and goat breeds

Farm Management

On all three farms, there were three (Thalli, Kachhi and Lohi) and four (DDP, Nachhi, Teddy and Beetal) local breeds of sheep and goats, respectively. At all farms, the imported Karakul and Awassi breeds of sheep and Angora breed of goats were introduced at least 5 years ago. Animals were farmed mainly on grazing for research and meat production and were generally kept in small herds of 20-100 animals. Natural breeding was the sole mean of reproduction and outside breeding stock was rarely purchased. Goats browsed extensively with little supplementary feeding. A simple questionnaire was employed at each sampling site to investigate the above-mentioned management practices.

Collection and Transportation of Samples

Blood was collected by jugular vein puncture using vecutest Kima (Italy) tubes from about 10% of all herds (Table 1). The blood was left to clot overnight at 4°C. Serum was decanted into sterile tubes and brought to the laboratory on ice packs within 24 h of collection. In the laboratory, the sera samples were centrifuged at 4000 x g for 5 min to remove the remaining red blood cells before being transferred to 1.8 mL cryovials and stored at -20°C until testing.

Competitive Enzyme Linked Immunosorbent Assay (cELISA)

Competitive ELISA was performed as laid down by Anderson *et al*. (1991), by using Biological Diagnostic Supplies LTD (BDSL, UK) kit.

A solid phase microtitre competitive ELISA was used for the qualitative assay of the test sera. Flat-bottomed polystyrene micro-ELISA plates (Nunc-immuno Maxisorb plate, Copenhagen, Denmark) were used. Fifty microliter of each reagent were used throughout the test, unless otherwise indicated. The incubation temperature was 37°C for 1 h at each step and the plates were washed three times after each step.

The procedure started with adsorption onto the plate of the reconstituted pre-titrated antigen diluted 1:100 in Phosphate-buffered saline (PBS 0.01 M, pH 7.2-7.4) and incubated in orbital shaker. Then, 1:5 dilutions of the test antigen and controlled sera were added. The dilution was made by adding 10 µL of the test sera following the addition of 40 µL of blocking buffer (0.1% v/v polyoxyethylene sorbitol monolaurerate (Tween-20) and 0.3% (v/v) negative anti-PPR virus serum). This step was followed by the addition of 50 µL of a 1:100 dilution of the reconstituted pre-titrated MAb. Monoclonal antibody control wells received 50 µL at the same concentration in addition to 50 µL of 1:1000 dilution of rabbit anti-mouse HRPO conjugate. Finally, freshly prepared

orthophenylenediamine (OPD) containing 0.004% (v/v) H_2O_2 was added. The plates were then incubated at room temperature for 10 min to allow the color to develop and then the reaction was stopped by the addition of 50 μL 1.0 mol L^{-1} H_2SO_4.

Results were interpreted by fitting a multichannel spectrophotometric ELISA plate reader (Multiscan Plus MK 11, Flow Laboratories, UK) using an interference filter of 492 nm used to read the test. The reader was connected to computer loaded with ELISA Data Interchange (EDI) software (FAO/IAEA, Vienna, Austria), which was used to automate the reading and calculation of percentage of inhibition (PI) values. The results were expressed in term of PI by converting the optical density (OD) to PI according to the following formula:

$$PI\ (\%) = 100 - \left(\frac{\text{Mean OD of test wells}}{\text{Mean OD of cma wells}}\right) \times 100$$

where, OD is the optical density value and cma refers to the monoclonal antibodies control. Inhibition values greater than 50% were regarded as positive.

Statistical Analysis

Unvariable analysis was carried out by Chi-square analysis in SAS to test differences of PPR prevalence amongst age, sex, breed groups and locations.

RESULTS

The competitive ELISA was used to clearly differentiate the exposed (infected) population from the unexposed (not infected) population. The considerable differences were observed between the exposed versus unexposed sheep and goat populations and the results of the competitive ELISA were plotted as a frequency of the percent color inhibition. Among the samples considered negative for PPRV (% color inhibition of less than 50%) the greatest number of samples had a percent color inhibition of between 1 to 10 and then 11 to 20%. Alternatively, among the samples considered positive for PPRV, (% color inhibition of greater than 50%) a peak frequency distribution of between 81 to 90% color inhibition was observed for the sheep and goat population (Fig. 1).

Of the 198 sheep tested, 77 were found infected giving a seroprevalence of 38.8%, while in goats 21 out of 82 tested samples were observed positive giving a prevalence of 25.6%. Statistically, sheep showed a significantly higher seroprevalence than goats ($p = 0.034$). The study showed insignificant differences in the three tested localities ($p = 0.098$). An overall prevalence in both sheep and goats was found to be 35.0% (Table 2).

To investigate the influence of breed on antibody prevalence in sheep and goats, data from the three farms were analyzed in more detail. Amongst the sheep breeds, Lohi at Okara District farm was found to show the highest (57.8%) seroprevalence of PPR, followed by Karakul (46.0%) at Layyah District farm. The Awassi breed of sheep at Okara District farm and lohi at Sahiwal district farms showed the same level of prevalence (33.3%) while Kachhi showed the least (10.0%). In the case of goats, the highest prevalence of PPR through cELISA was recorded in Daera Din Panah (45.4%), followed by Beetal (40.0%), Nachi (20.0%), Angora (18.1%) with the least prevalent seen in the Teddy breed (10%). Based on the seroepidemiology, an insignificant difference ($p = 0.056$) was found in goat breeds while a significant difference ($p = 0.023$) was observed in sheep breeds (Table 3).

The effect of sex on the PPR prevalence was estimated in all of the breeds of sheep and goats and was shown not to differ significantly (data not shown) enabling prevalence estimates from different farms to be compared directly without adjustment for variation in sex structure.

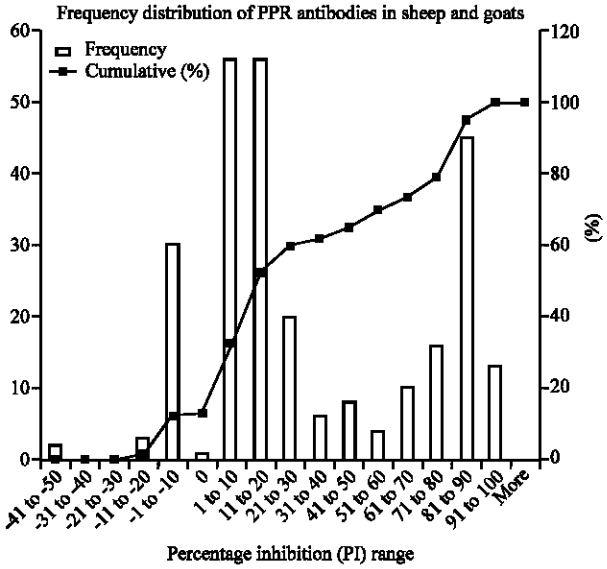

Fig. 1: Frequency distribution of antibodies against PPR as observed with competitive ELISA in sheep and goats. Among the samples considered negative for PPRV, (% color inhibition of less than 50%) the greatest number of samples had a percent color inhibition between 1 to 10 and then 11 to 20%. Alternatively, among the samples considered positive for PPRV, (% color inhibition of greater than 50%) a peak frequency distribution of between 81 to 90% color inhibition was observed for the sheep and goat populations

Table 2: Overall serological results of peste des petits ruminants in sheep and goats at all farms

	Sheep			Goats			Sheep and goats	
Farms	Total	Positive	Positive (%)	Total	Positive	Positive (%)	Overall positive	Overall positive (%)
Sheep and Goat Research Center at Khariwala, District Layyah	68	26	38.2	82	21	25.6	–	–
Livestock Production and Research Institute, Bahadurnagar, District Okara	86	38	44.1	–[a]	–	–	–	–
Livestock Experimental Station, Qadarabad, District Sahiwal	44	13	29.5	–	–	–	–	–
Total	198	77	38.8	82	21	25.6	98/280	35.0

[a]= Animals are not present in this group; For overall populations = 95% CI for sheep 69.30-93.70, CI for goats 28.70-53.30 (p = 0.034); For all three localities = 95% CI for district Layyah 52.50-97.50, CI for district Okara 30.10-55.90, CI for district Sahiwal 15.40-28.60 (p = 0.098); CI = Confidence Interval

The assessment of seroprevalence of disease in diverse age groups of the goats and sheep gave the same trend at all three farms. Highest seroprevalence was recorded in young animals (≤6 months) while the least was found in mature animals aged ≥12 months. The adults aged between 7-12 months were seen in between the young and mature. Statistically, PPR antibodies were significantly (p = 0.016) higher in all three age groups (young, adult and mature) of sheep while there was an insignificant difference (p = 0.385) in goat age groups (Table 4).

Table 3: Breed-related prevalence of PPR antibodies in sheep and goats at three governmental livestock farms

Source of sampling	Species	Breed	Total No. of animals	No. of animals examined	No. of samples positive by cELISA
Sheep and Goat Research Center at Khariwala, District Layyah	Sheep	Karakul	503	50	23 (46.0)
		Thalli	82	8	2 (25.0)
		Kachhi	105	10	1 (10.0)
	Goats	DDP[a]	118	11	5 (45.4)
		Nachi	54	5	1 (20.0)
		Angora	119	11	2 (18.1)
		Teddy	314	30	3 (10.0)
		Beetal	158	25	10 (40.0)
Livestock Production and Research Institute, Bahadurnagar, District Okara	Sheep	Awassi	483	48	16 (33.3)
		Lohi	397	38	22 (57.8)
Livestock Experimental Station, Qadarabad, District Sahiwal	Sheep	Awassi	214	23	4 (17.3)
		Lohi	176	21	7 (33.3)

[a]DDP = Daera Din Panah; Values in parenthesis are shown in percentage; 95% CI average for all sheep breeds 03.03-44.11 (p = 0.023); 95% CI average for all goat breeds 01.28-22.32 (p = 0.056)

Table 4: Age-related occurrence of PPR antibodies in sheep and goats at three governmental livestock farms

Source	Species	Age	Total No. of animals	No. of animals examined	No. of samples positive by cELISA
Sheep and Goat Research Center at Khariwala, District Layyah	Sheep	<6 months[a]	98	10	6 (60.0)
		7-12 months[b]	128	12	7 (58.3)
		>12 months[c]	464	46	13 (28.2)
	Goats	<6 months[a]	124	12	5 (41.6)
		7-12 months[b]	186	18	4 (22.2)
		>12 months[c]	453	52	12 (23.0)
Livestock Production and Research Institute, Bahadurnagar, District Okara	Sheep	<6 months[a]	92	9	5 (55.5)
		7-12 months[b]	118	11	6 (54.5)
		>12 months[c]	670	67	27 (40.2)
Livestock Experimental Station, Qadarabad, District Sahiwal	Sheep	<6 months[a]	53	5	3 (60.0)
		7-12 months[b]	74	7	2 (28.5)
		>12 months[c]	263	31	6 (18.1)

[a]: Young, [b]: Adult, [c]: Mature; Values in parenthesis are shown in percentage; 95% CI average for goats 03.07-38.68 (p = 0.385); 95% CI average for sheep 09.09-98.45 (p = 0.016)

DISCUSSION

In present study, out of 2723 heads, 280 samples were collected randomly from three different farms, from about 10% of the herds and tested by cELISA. The results presented here are considered the first report of PPR antibodies in sheep and goats from governmental livestock farms in Pakistan. The overall prevalence of the PPR antibodies in sheep and goats was found to be 38.8 and 25.6%, respectively and seropositive animals were detected on all farms. These findings suggested that only those sheep capable of mounting a strong humoral antibody response to PPRV (high percent color inhibition) were able to survive infection. This should not be confused with the higher prevalence of antibodies to PPRV (proportion of sera samples with a % color inhibition of greater than 50%) observed in the sheep versus goat population. Sheep population showed a relatively higher level of serum antibodies against PPR than goats (Table 2). This may be attributed to a higher recovery rate and greater longevity of sheep verses goat which is in contrast to the serological profile reported by Viroji *et al*. (2001) and Abubakar *et al*. (2008). Although infected, sheep rarely suffer clinical disease (Roeder *et al*., 1994).

The prevalence of PPR in Pakistan was higher than that reported in some other countries. In Saudi Arabia, using a microtiter neutralization assay (known for its low sensitivity) the prevalence of PPR in sheep and goats was 3.1 and 0.6%, respectively (Al-Afaleq *et al*., 2004). In Yemen, the seroprevalence of PPR was 15% in sheep and 18% in goats (Taylor, 1997). Singh *et al*. (2004) reported a seroprevalence of 36.3% in sheep and 32.4% in goats. In Syria, the sheep flock prevalence was found

to be 96%, which is significantly higher than that in Pakistan (Taylor, 1997). A similar study in an outbreak with higher prevalence was also conducted in the Livestock Production and Research Institute, Bahadarnagar, Okara, Pakistan, showing 100% prevalence of PPR in thirty-five serum samples (Ahmed *et al*., 2005). This higher prevalence is due to the study conducted during a time when the highest seroprevalence was recorded in Pakistan. However, disease patterns indicate the disease is endemic throughout most of the year with incidence increasing in pre- and post-winter months. The greatest frequency of PPR outbreaks was reported during the period from January 2006 to April 2006 (Abubakar *et al*., 2008). These findings suggested the presence of this disease in the region and support the idea of initiating a surveillance network for this important disease.

The competitive ELISA used in the present investigation had high diagnostic specificity (99.8%) and sensitivity (90.5%) for the detection of PPRV antibody in convalescent sera when compared with the gold standard VNT (Anderson and McKay, 1994; Libeau *et al*., 1992).

All breeds available at all three forms were evaluated to determine breed predisposition. A mixed prevalence trend is seen in local (Thalli, Kachhi and Lohi) and imported (Karakal and Awassi) breeds of sheep. Of all the sheep breeds, lohi is found to be the most susceptible to PPR infection as well as developing a relatively higher level of serum antibodies against PPR. Lohi is a local breed found in southern Punjab in Pakistan. In the case of goats, local breeds (DDP, Nachhi, Beetal) showed higher prevalence than the imported breed (Angora) (Table 3).

The highest proportion of seropositive sheep came from the Okara District farm and the second highest was in sheep from the Layyah District Farm. This may be attributed to the proximity of these farms to many neighbouring villages, where PPR is considered endemic and to the unrestricted movement of relatively large numbers of cattle, sheep and goats from these 'endemic' regions into the surrounding districts. One of the factors that might contribute to these higher levels on farms could be the high concentration of animals in close proximity to each other, which would favor virus transmission. The lowest seroprevalence was observed in Sahiwal District Farm, whose geographical isolation makes the movement of animals difficult. The animals were not vaccinated against rinderpest or PPR. Therefore, the seroprevalence could only have resulted from field infection with PPR virus, either at common watering points or grazing areas. This variability in seroprevalence might be due to the nomadic grazing in eastern and northern part of Punjab. Climate conditions and seasonal forage availability dictate grazing patterns in the area of eastern and northern Punjab. The livestock spend April in subtropical and temperate forest grazing areas below 2,000 m. They graze the alpine areas from June to October, when low temperatures retard plant growth and then descend toward the plains or low valleys. During winter, livestock graze in abandoned cultivated lands and in valleys along water channels, roads and grazing grounds between agricultural fields (Dost, 1998). So, the nutritional status of the animals improves during the rainy season due to an increase in the availability of fodder that may lead to increased resistance.

The findings of this study suggest that animals younger than 6 months and older than 1 year have a better chance of seropositivity to PPR. These findings agree with previous reports (Ozkul *et al*., 2002; Singh *et al*., 2004). It is well documented that sheep and goats usually exposed to PPRV at a younger age may show positive sera for 1-2 years following exposure (Dhar *et al*., 2002; Ozkul *et al*., 2002; Singh *et al*., 2004). It is assessed that young animals are more susceptible than adults, which matches the serological profile reported by Viroji *et al*. (2001) and Roeder and Obi (1999). The assessment of seroprevalence of disease in diverse age groups of the goats and sheep gave the same trend at all three farms, with an insignificant deviation seen in this trend at layyah district farm in goats (Table 4). Most of the studies have shown that the prevalence of PPR antibodies in males is higher then in females (Rahman *et al*., 2004). However, in this study the only female goat sera found harbored more antibodies against PPR virus than males.

The study presented here indicated that a specific control program should be organized at these locations. Measures should be taken to ensure the eradication of the disease within the population and sound control measures should be adopted to avoid further spread of the disease to larger small

ruminants populations. In order to ensure a productive and healthy population of sheep and goats within the governmental livestock production scheme, regular PPR testing should be instituted. Proximity to other farm animals might serve as reservoirs for these herds. It would be helpful to also screen the prevalence of PPR in other farm animals and surrounding large rural small ruminants populations by some economical tests (Munir *et al*., 2008). Another test should be done to confirm positive animals and care should be taken in areas of high prevalence to determine the possible causes of the spread of the infection. Vaccination against PPR should occur every 3 years for the same animals, as the vaccine lasts for that period of time.

Infection with PPRV was demonstrated at three farms located in three different districts of Punjab that were considered pockets of PPR and animals were kept for productions. This study provided valuable data on the serologic status of PPR and suggested that PPR is endemic in all the governmental livestock farms in Pakistan. Given the high prevalence for PPR among all breeds (local and imported), it seems advisable to include PPR serology in the seromonitoring program to give a better indication of national herd immunity of sheep and goats against PPR. It also necessary for PPR experts to return and for appropriate PPR control measures in small ruminants to be instituted, especially at governmental livestock farms.

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