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Sero-monitoring of Peste Des Petits Ruminants (PPR) Antibodies in Small and Large Ruminants in Bangladesh

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Abstract: Sero-monitoring of peste des petits ruminants (PPR) in 750 goats and 500 cattle were studied 15 different districts of Bangladesh. This study was undertaken with a view to evaluate the serum antibody level against PPR virus in goats, determination of level of PPR antibodies in large ruminants and finally to determine the risk of goat population to PPR based on age. The experiment was conducted at the Virology Laboratory of Bangladesh Livestock Research Institute (BLRI), Savar, Dhaka. In most districts, the antibody level against PPR Virus (PPRV) in goats varied between 4-98% with an average 49.33% and only 3-10% was found positive against rinderpest in C-ELISA The highest level of antibody against PPRV was found in Hill tracts (98%) and the lowest in Pabna (4%). In terms of Rinderpest (RP) antibody as being detected by C-ELISA, it was observed that the sera collected from Jessore, Faridpur and Tangail showed very low levels of antibody, which varied between 3-10%. The results from the examination of cattle sera from four selected areas of Bangladesh showed that antibody level against PPRV varied from 17.64-36.84% with an average 24.67% and 0-26.31% found positive against RP in C-ELISA. So the present findings are the indication of future problem in proper evaluation of rinderpest vaccination programme in Bangladesh particularly with low sero-conversion in vaccinating herds as they have already been sensitized by PPRV.

Key words: Sero-monitoring, Sero-conversion, C-ELISA

Introduction

Peste des petits ruminants (PPR) is the most contagious viral infection of small ruminants particularly in goats which emerged as an epidemic during 1993 in the western districts of Bangladesh (Sil *et al.,* 1995). Since then, the disease is being spreading as an epidemic and at present it has touched each and every administrative district of Bangladesh (Sil *et al.,* 1995*).* The current initiative of goat farming taken by Department of Livestock Service and different NGO's as well as private enterprises is seriously being discouraged because of devastating effect of PPR and is considered as a major constraint of an effort to develop goat and goat populations in this country. In particular, present epidemics of PPR not only killed a millions of goat but also crippled the noble approach of goat farming in Bangladesh. Outbreaks of PPR were first reported in 1993 at the western part of Bangladesh. Since then, it spreads all over the country and killed more than five millions of goats (Sil *et al.,* 1995).

The etiological agent of PPR virus is a member of the morbilli virus (Paul *et al.,* 1979) under the family Paramyxoviridae. PPR virus is a negative single stranded non-segmented, enveloped RNA pleomorphic virus particle. A thorough epidemiological study in different organized goat farms reported that out breaks of PPR has always been associated with introduction of new goats in farm which had a 100% morbidity with varying degrees of mortality (50-98%) depending on the management practices and environmental stresses (Sil *et al.,*1995). Studies conducted in a goat farm vacinated with live attenuated rinderpest vaccine have apparently failed to protect the vaccinated animals. Therefore, the present research was designed and carried out to evaluate the serum antibody level against PPR virus in goats, determine the level of PPR antibodies in large ruminants and to determine the risk of goat population to PPR virus based on age. These will help us for future control of PPR as well as rinderpest eradication campaign lead by South Asian Rinderpest Eradication Campaign (SAREC).

Materials and Methods

The serum survey of goat was conducted in 15 different districts of Bangladesh during 1997- '98. Fifty samples were collected from each district. In addition to this, the preserved samples of BLRI (collected in 1993 PPR outbreaks) were also used in this study. The analyses of samples were carried out at the Virology Laboratory, Bangladesh Livestock Research Institute (BLRI). Savar, Dhaka. FAO/IAEA joint Division, Vienna, supplied all the materials, chemicals etc for the detection of PPR antibody.

Computerized Immuno-scan micro plate reader (BDSL) photometer with an interference filter of 492 mm was used.

Reagent and Sample Preparation: Anderson *et al.*, 1991, developed the C-ELISA kit. All reagents and chemicals preparation were done exactly as details in the C-ELISA kit manual.

Viruses: Peste des petits ruminants viruses was obtained from the Virology Laboratory, BLRI, Savar, Dhaka. Original samples were collected during 1993 outbreaks from Meharpur, primarily was identified as PPR by agar gel immuno-diffusion test and susceptible host inoculation and confirmed as PPR virus by World Reference Laboratory, UK using genome analysis.

PPR hyper-immune Serum: Peste des petits ruminants specific antiserum was raised in a group of 5 PPR susceptible goats. Goats were infected by PPR virus using 5 GID_{50} ml% (obtained form the Virology Laboratory, BLRI) per animal at mid cervical region sub-cutaneously. At the onset of disease, animals were given 10 ml of PPR hyper-immune serum and LA oxytetracycline at a does of 1ml kg^{-1} body weight per animal. Serum neutralizing antibody titer were measured form each animals after 30 days of recovery. For the production of hyperimmune serum, each recovered animals was boostered by 1 ml of virulent PPR virus ($10GID_{50}$ ml^{-1}) and serum was collected after 20 days. Titer were further measured by TCID 50 test in Vero cell culture and stored at -20°C until use (Ekue *et al.*, 1992).

Growth Medium: Eagle's Minimum Medium (EMEM, Sigma Company Ltd.) to which 5% fetal calf serum (FCS, Sigma), 1% penicillin/streptomycin (5000 IU/ml, 5000mg ml^{-1}), L-glutamiZZnd 2% sodium bicarbonatZZre added and used as ceZZulture growth medium. MZZenance medium was prepaZZby adding 1% FCS in EMEZZouble strength medium was prepared containing all components in double amount was needed for cell growth medium.

Cell Culture: Vero cell (African Green monkey kidney cell line) obtained from Gonoshasthay Vaccine Research Laboratory (GVRL), Savar, Dhaka and was used for the propagation of field isolates of PPR virus. Vero cell line was used for virus isolation and propagation (Chandran *et al.*, 1995).

Rinderpest Virus Antigen Stock: Freeze dried cell culture derived rinderpest virus antigen was supplied in glass vials at 0.5 ml volume. Freeze dried antigen rinderpest virus antigen was dissolved in 1 ml of sterile water (supplied in ELISA kit) and mixed gently until completely dissolved. The antigen was stored in a 100 µl volume in cryo-vials at −20°C. At the time of test cryo-vials containing antigen was thawed at 37°C (Water bath and used at 1:100 dilution for the C-ELISA test.

Anti Rinderpest Monoclonal Antibody Stock: Freeze dried hybridoma cell culture supernatant (mouse anti RPV monoclonal antibody) was supplied in glass vials at 0.5 ml volume. Freeze dried antibody was dissolved in 1 ml of sterile water (supplied in ELISA kit) and mixed gently until completely dissolved. The antibody was stored in a 100 µl volume in cryo-vials at-20°C. At the time of test, Cryo-vials containing antibody was thawed at 37°C (Water bath) ad used at 1:100 dilution for the C-ELISA test.

Anti-species Conjugate Stock: Freeze-dried horse radish peroxides (HRPO) conjugate, rabbit anti-mouse immuno-globulin (supplied by IAEA) were used. HRPO conjugate stock was further sub-divided in to 500 µl aliquots in 1 ml cryo-preservation vials (supplied in ELISA kit) and used at 1:1000 dilution for the test.

Control Serum Stocks: Freeze dried C^{++}anti RPV antibody positive (strong), C^{+} anti RPV antibody positive (Moderate) and C^{-} anti RPV negative antibodies were supplied in glass vials at 0.5 ml volumes. Freeze dried antibodies were then dissolved in 1 ml of the sterile water supplied in ELISA kit and mixed gently until completely dissolved. The antibody stock was stored in a 100 ul volume in cryo-vial at-20°C. At the time of the test cryo-vial containing antibody were thawed at 37°C (water bath) and used at 1:1000 dilution for the ELISA test procedure.

Chromogen Stock: Constituted 3.7 mm ortho-phenyldiamine (OPD) in tablet form (supplied in ELISA kit) was used as chromogen.

Substrate Stock: Constituted 3%(W/V)/ 882 mM H_2O_2. The prepared substrate was stored at 4°C for future use.

Coating Buffer: Constituted 0.01 M phosphate buffered saline, pH 7.4 +/ 0.20. One tablet dissolved in 1 liter of locally produced distilled water and checked pH. Then prepared coating buffer was labeled and stored at 4°C for the period of two weeks.

Blocking Buffer: Constituted 0.01 M phosphate buffer saline, pH 7.4 +/ -0.20 plus 0.1%(v/v). Tween 20 plus o.3%

(v/v) normal bovine serum (C⁻control serum). Blocking buffer was prepared in 100 ml volume of PBS containing 100 µl between 20 µl and 300µl normal bovine serum (C⁻control serum). Prepared blocking buffer was labeled and stored at 4°C for the period of two weeks.

Washing Buffer: Constituents 0.002 M phosphate buffered saline, pH 7.4+/-0.20 and prepared in 1 liter of locally produced deionized water and used at 1: 4 dilution in distilled water.

Stopping Solution: One M sulfuric acid was used and was prepared in 1liter volume containing 55 ml of concentrated sulfuric acid in 945 ml of locally produced deionized water.

Serum Survey: In order to get information on the prevalence and distribution of PPR virus antibody in Bangladesh, a nation wide serum survey was organized. A total of 1250 from cattle and goat sera were tested against both PPRV and RPV. Seven hundred fifty goat sera were collected from the 15 different districts of Bangladesh. Five hundred randomized cattle sera were also collected from four different selected regions of the country. All sera samples were preserved at -20°C(Taylor, 1979).

Sampling Approach: Blood was collected by jugular vein puncture using venoject needles and vacutainer tubes. The blood was left to clot overnight in clot boxes serum was decanted into sterile tubes and kept in icebox for transportation to the laboratory. In the laboratory, the serum was centrifuged (at rpm for 10 minutes) to remove the remaining red cells before being transferred to 2 ml cryo vials and stored at -20°C until use.

Sera Testing: Rinderpest competitive ELISA was used for the analysis of the serum samples and 100 TCID neutralization test was done to screening with PPRV and RPV at the Virology Laboratory in BLRI. The test protocol was performed exactly as detailed in the C-ELISA kit manual. The C-ELISA results were read by the computer controlled immuno-skan plus analyzed using EDI version 2.11 software package supplied b the FAO/IAEA Division, Vienna. The results further analyzed using lotus 1 2 3 graphic (Release 5).

Competitive Elisa (c-elisa test): Rinderpest specific monoclonal antibody (Mab) C-ELISA was used for the detection of antibody against PPR and rinderpest.

Test Procedure: The test procedures consisting of several steps are given in Table 1(Anderson and Mckey, 1994).

Coating of Micro Plates: An aliquot of reconstituted RPV antigen stock was gently mixed to ensure uniform dispersion and diluted at 1:100 dilution in working solution. For each plate, 6 ml solution was prepared gently and then 50 µl of solution was transferred to each well of 96 wells plate. The plate gently agitated to ensure distribution of the antigen. Then micro plates were covered and incubated at 37°C for 1 h.

Table 1: Steps followed in C-ELISA for anti-RPV antibody

	Assay condition			
Assay steps	Incubation time	Incubation temperature	Plate shaking	Wash step
1 Coating of RPV antigen	1 hour	37°C	Yes	3X
2 Addition of test sera and anti RPV Mab	1 hour	37°C	Yes	3X
3 Addition of conjugate	1 hour	37°C	Yes	3X
4 Addition of substrate and chromogen	10 min	Room temp.	No	-
5 Addition of stopper solution	None	Room temp.	No	-
6 Reading of reaction	None	Room temp.	Tap to mix	-

The details of these procedures are described below: Addition of Test Sera, Control Sera and Monoclonal Antibody: Test sera and all 3 control sera (C++, C+ and C-) were agitated gently to ensure homogeneity. By inverting the micro plate and using an abrupt downward hand motion, the contents of all antigen coated micro plate in to a sink or other reservoir and slaved the inverted micro plate on to a tissue paper to remove all residual contents.

Addition of Conjugate: A working dilution of the conjugate was prepared immediately before the end of the serum/monoclonal antibody incubation in a volume sufficient for all the micro plates (6 ml of working dilution per micro plate). Both the conjugate stock and its working dilution was handled with care, agitation was gentle but

thorough. After one hour of serum incubation, micro plates were removed from the incubator and were washed by wash buffer. Immediately after washing, 50 µl volumes of the working dilution of conjugate was added to all 96 wells of the micro plates. Then micro plates was covered and incubated for 1 h at 37°C.

Addition of Substrate / chromogen and Stopping Solution: Immediately before the end of the conjugate incubated a working dilution of the substrate/chromogen solution was prepared in a volume sufficient for the number of microplates (e.g. for 6 ml/ plate, dilution of 30 µl of substrate stock (H_2O_2) in 6 ml of chromogen stock (OPD) solution, this represented 3.7 OPD and 3.5 mM H_2O_2) and 1:200 dilution was used. After one of conjugate incubation, micro plate was washed. A clean micro plate (not coated with Ag) was used as the blanking plate for the photometric reading. Immediately after washing, 50 µl volumes of the substrate chromogen solution was added to the wells of microphates, in the test run. Immediately begin timing of the substrate/chromogen development and incubated at room temperature for 10 min without plate shaking. After 10 min of substrate/ chromogen incubation 50 µl volumes of stopping solution (1M H2SO4) was immediately added to the wells of the micro plate, starting with the first column of the "blanking plate" followed by all 96 wells of the micro plates in the test run. Briefly shaked the micro plate to ensure through mixing. All wells contained 50µl of substrate/chromogen solution plus 50 µl of stopping solution.

Measurement of Substrate Development: The micro plate reader should be turned on and allowed to warm up for at least 15 min before reading the first micro plate. This warm up period is necessary to ensure uniformity of reading for all micro plates. OPD was read at 942 nm. "Blanking plate" was placed in the carriage of the micro plate reader and initiated the blanking sequence. Micro plate of the test was placed in the carriage of blanked reader and initiated reader sequences. It was repeated for each microplate.

Tissue Culture Infective $Dose_{50}$ ($Tcid_{50}$) Test:
$TCID_{50}$ Test: PPR field virus and all Vero cell passaged virus titration were preformed in Vero cell monolayers prepared in 96 wells tissue culture plate (Sigma). The medium was removed, the cells was washed with PBS and then 100 µl of ten fold dilution of the test virus added in 4 wells (10^{-1}-10^{-8}) with one raw being kept as control. Following incubation, the cells were given 250 µl of virus maintenance media and incubated at 37°C and observed daily to detect CPE such as syncitia formation, rounding of the cells etc. Following incubation the supernatant was removed and stained by 2 crystal violet (w/v in 98% methanol).

100 Tcid Neutralization Test: Sera were screened using their neutralizing activity against reference PPRV by 100 $TCID_{50}$ neutralization test.

Screening of Serum by Neutralization Test: The test was performed in 96 well plates (CORNING, UK) containing monolayers of Vero cells. The infectivity titer of the virus was previously determined by $TCID_{50}$ in PBS and mixed with an equal volume of 1:10 dilution of specific PPR anti-serum and test serum (depending upon test) and incubated at room temperature for 45 min. Following incubation, 200 µl of the virus serum was pipetted into the monolayer of each 96 well plates, which had been washed with PBS, controls were kept in each plate. Following incubation for 30 min at room temperature, 200 ul virus maintenance medium was added and incubated at 30°. After incubation at 37°C for the requisite number of days, CPE were visualized by 2% crystal violet following removing of supernatant medium.

Results

Serum samples from 750 goats and 500 cattle were collected from 15 different districts of Bangladesh. They were tested for detection of antibody against PPR and rinderpest. The experiment was conducted by using 100 $TCID_{50}$ neutralization against PPRV and monoclonal antibody based rinderpest C-ELISA test. Comparative levels of rinderpest and PPR antibodies were studied in goats and cattle. Serum samples of goats were first tested by rinderpest C-ELISA test then by 100 $TCID_{50}$ neutralization test against PPRV in Vero cells. Serum samples which were found negative in C-ELISA but neutralize PPRV at 100 $TCID_{50}$ level were but had a lower level of neutralization index against PPRV (1:10) indicated that the antibody was specific to rinderpest. A total of 750 goat and 500 cattle sera were tested against both peste des petits ruminants virus (PPRV) and rinerpest virus(RPV) in C-ELISA.
In Table 2, it was revealed that in most districts the antibody level against PPRV varied between 40-60% while only 3-10% was found positive RP in C-ELISA. The highest level of antibody against PPRV was foud in Hill tracts (98%) while lowest in Pabna (4%). In terms of RP antibody as being detected by C-ELISA, it was observed that sera collected from Jessore, Jhinaidah, Faridpur and Tangail showed very low level of antibody which varied between 3-10% and found negative in rest of the districts.

In another study (Table 3), the comparative levels of serum antibodies of PPR and RP in cattle serum were evaluated in cattle sera collected from four different areas of Bangladesh. A total of 500 cattle sera were tested against both PPRV and RP. The results from the examination of cattle sera showed 17.64% positive against RP. In contrast, 36.84% of the cattle from Jessore district were sero-positive against PPR and 26.31% were sero-positive against rinderpest virus.

In Rajshahi district, 27.53% of the cattle sera were found positive against PPR and 11.59% sero-positive against RP. Twenty per cent of the cattle in Mymensingh district were found sero-positive against PPR virus and only 6.67% were sero-postitive against rinderpest virus.

Levels of serum antibody against PPR based in age were also examined and shown in Table-4. During the outbreak of PPR in 1998, the serum antibody of PPR in goats of different age groups varied from 0-4.74% and goats under six month of age groups was found absolutely negative. The serum antibody titer against

PPRV were found absolutely negative under 12 months of age, while goats above one year had a antibody range 3.39%-4.74%. But sera collected during 1997/1998 showed that the antibody against PPRV had markedly increased. Kids under 6 months of age had higher PPR antibody (61.8%) and only 2.19% sera were found positive in goats of 12 months of age. On the other hand, goats of 1-2 years and above 2 years of age had a PPR antibody 41.32% and 49.47% respectively.

Discussion

Sero-survey of goats and cattle was conducted using a total of 1250 ruminants sera (small and large) from 15 different districts of Bangladesh. Serum antibody level against PPRV and RPV were also compared between the sera collected in 1993 and in 1997-98. Absence of rinderpest virus during the first incidence of PPRV in Bangladesh. However, the presence of PPRV neutralizing antibody tested by 100 $TCID_{50}$ in Vero cells specifically

Table 2: Comparative level of PPR. and Rinderpest (RP) antibodies in goats in different districts of Bangladesh

Name of Districts	No. of Goat sera collected	No. positive for PPR	% positive for PPR	No. positive for RP	% positive for RP
Chittagong Hill tracts	50	48	98	0	0
Noakhali	50	15	30	0	0
Khulna	50	16	32	0	0
Jessore	50	21	42	0	0
Jhinaidah	50	24	48	5	10
Rajshahi	50	40	80	3	5
Pabna	50	2	4	0	0
Noagoan	50	25	50	0	0
Rangpur	50	24	48	0	0
Kurigram	50	23	46	0	0
Sylhet	50	26	52	0	0
Dhaka	50	25	50	0	0
Faridpur	50	24	48	2	3
Tangail	50	26	51	2	4
Feni	50	31	62	0	0
Total	750	370	49.33	12	1.6

Table 3:Comparative levels of PPR and Rinderpest (RP) antibodies in cattle in four areas in Bangladesh

Survey areas	No. of cattle sera examined	No. positive for PPR	% positive for PPR	No. positive for PP	% positive for RP
Savar	100	17	17	0	0
Jessore	100	36	36	26	27
Rajshahi	250	68	27	28	11
Mymensingh	50	10	20	3	7
Total	500	131	25	35	9

indicated the involvement of PPR virus during 1993 outbreak. This finding was further strengthened by absence of PPRV antibody in kids below 6 months of age, which indicated that there is no circulatory antibody in the adults. From this study, it can also be concluded that PPR was not present in Bangladesh before 1993. On the other hand, the level of PPRV antibody was gradually increased (44.73%) due to the countrywide outbreak of PPR. At the same time the level of rinderpest antibody in goat has also increased (1.60%) due to mass rinderpest vaccination among goats (live vaccine) for the control of PPR in Bangladesh. Sudharshana *et al.* (1995) made serum survey over 663 sheep and goats and observed that 5.81% positive against PPRV and 3.5% against RP while 32.64% was positive against both the Indian observation. However, a varied level of PPRV antibody (4%-98%) in goats indicated

Table 4: Levels of Serum antibody against PPR based on age in goats

Age	No. of goat x % positive	% of PPR antibody ($100 TCID_{50}$ neutralization test)	
		1993-1994	1997-1998
0-6 Months	75	0	61.8
6 Months-1 Year	10	0	2.19
1 Year-2 Year	270	3.39	41.32
2 Years above	225	4.74	49.47

different stratum of immunity in herd. Animals above 2 years of age groups had acquired high level of PPRV antibody (41.32-49.47%) either due to PPRV due to PPRV infection or sero-conversion of RP vaccinated goats following exposure to PPR virus. This level reflected the higher level of maternal derived immunity in kids, which gradually declined by the 4-5 months of age; hence, kids over 6 months of age group remained highly susceptible to PPRV because of very low level of circulatory neutralizing antibody (2.19%). A similar result was observed by Taylor (1979), however, the author did not include kids below 6 months of age in his study, rater had classified goat into <1 year (16.10%), 2-2.5 years (16.80%) and > 3 years (62.50%). In another study, PPRV antibodies were found about 55% in Nigerian goats and sheep (Majiyagbe *et al.*, 1984) while 46.50% in sheep and goats were found sero-positive against PPRV in Cameroon (Ekue *et al.*, 1992). Similar results were observed by Ramesh and Rajasekher (1988) and showed the prevalence of 45.45% and 53.96% PPRV antibodies in sheep and goats, respectively. Sero-evaluation of 500 cattle sera from different areas of Bangladesh compared to 1993 and it might be due to the stimulation by the circulatory PPR virus in Bangladesh. On the other hand, very few animals had a rinderpest specific antibody which in an indication of absence of rinderpest virus in Bangladesh. Although the presence of certain percent of C-ELISA positive antiserum might be due to immigration of cattle from India where rinderpest mass vaccination has conducted under South Asian Rinderpest Eradication Campaign (SAREC) program. This finding is supported by the report of Anderson *et al.* (1994) in Nigeria and Ghana where cattle had a high titer of antibody against PPR virus, which interferes the rinderpest vaccination program. Present findings is an indication of future problem in evaluating rinderpest vaccination program in Bangladesh particularly with low sero-conversion in vaccinating herd as they have already sensitized by PPRV. So, this study also reflected the goat herd immunity against PPRV, which will be helpful for the designing of future control of PPR and using homologous vaccine. The present findings are the indication of future problem in proper evaluation of rinderpest vaccination programme in Bangladesh particularly with low sero-conversion in vaccinating herds as they have already been sensitized by PPRV.

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