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Prevalence of Peste Des Petits Ruminants Virus (PPRV) in Mardan, Hangu and Kohat District of Pakistan; Comparative Analysis of PPRV Suspected serum samples using Competitive ELISA (cELISA) and Agar Gel Immunodiffusion (AGID)

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Abstract

Peste des petits Ruminants (PPR) is an acute, febrile, highly contagious and economically important viral disease of small ruminants. However PPR is more prevalent in sheep and goat. Competitive ELISA, Virus neutrilization test, and RT-PCR are the available techniques for diagnosis of PPR which give rapid detection where as Agar gel immunodiffusion and Counter immunoelectrophoresis were previously used for PPR detection. In this study two serological techniques were compared for PPR diagnosis. The main aim of this study was to evaluate the comparative sensitivity of both techniques for PPR detection. For this purpose one hundred and sixty PPR suspected serum samples collected from goats and sheep flocks (unvaccinated) from three Districts of NWFP including Mardan, Hangu and Kohat were analyzed in National Veterinary Laboratories, Islamabad. Out of these 160 samples, fifty (50) were found positive for PPR antibodies with cELISA (Prevalence = 31.25%). The cELISA positive serum samples however gave negative results when tested with AGID although the control well was always positive. Thus it was concluded that cELISA technique is more sensitive and specific than AGID for PPR antibody detection.

Keywords: Prevalence, PPR, cELISA and AGID

Introduction

Morbilli-viruses are highly contagious pathogens that cause some of the most devastating viral diseases of humans and animals worldwide. They include measles virus (MV), canine distemper virus (CDV), rinderpest virus (RPV), and peste des petits ruminants virus (PPRV). They cause fever, coryza, conjunctivitis, gastroenteritis, and pneumonia in their respective host species. All members produce both cytoplasmic and intranuclear inclusion bodies. The major sites of viral propagation are lymphoid tissues.

The acute diseases are usually accompanied by profound lymphopenia and immunosuppression, leading to secondary and opportunistic infections (Murphy *et al.*, 1999).

Morbilliviruses are enveloped, non-segmented negative strand RNA viruses and constitute a genus within the family *Paramyxoviridae* and the order Mononegavirales. Morbilliviruses are a pleomorphic particle with a lipid envelope which encloses a helical nucleocapsid (Gibbs *et al.*, 1979). Nucleocapsids are usually filamentous with a herring-bone appearance; 600-800(-1000) nm long; 18 nm in diameter. The total genome length is 15200-15900 nucleotide. Full length genome sequences of Morbilliviruses are available. The genome is divided into six transcriptional units encoding two non structural (V and C protein) and six structural proteins: the nucleocapsid protein (Np), which encapsulates the virus genomic RNA, the phosphoprotein (P), which associates with the polymerase (L for large protein), the matrix (M) protein, the fusion (F) and the haemagglutinin (H) (Barrett, 1999).

Peste des petits ruminants (PPR) is characterized by high fever, ocular and nasal discharge, pneumonia, necrosis and ulceration of the mucous membrane and inflammation of the gastro-intestinal tract which leads to severe diarrhea and high mortality. It affects small ruminants, especially goats, which are highly susceptible, and occasionally wild animals. In 1942 in West Africa, it was first described as psuedo rinderpest, pneumoenteritis complex and

stomatitis-pneumenteritis syndrome (Braide, 1981).

Tears, nasal discharge, coughed secretions, and all secretions and excretions of incubating and sick animals are the source of PPRV. PPR virus, like other morbilliviruses, is lymphotropic and epitheliotropic (Scott, 1981). Consequently, it induces the most severe lesions in organ systems rich in lymphoid and epithelial tissues. The respiratory route is the likely portal to entry. After the entry of the virus through the respiratory tract system, it localizes and then start replicates in the pharyngeal and mandibular lymph nodes as well as tonsil. Viremia may develop 2-3 days after infection and 1-2 days before the first clinical sign appears. Subsequently viremia results in dissemination of the virus to spleen, bone marrow and mucosa of the gastro-intestinal tract and the respiratory system.

This study used two techniques i.e. AGID and cELISA for the detection of antibodies in PPR suspected serum samples in order to achieved the objective to screen out the number of PPR suspected samples from the given population of three districts of NWFP (160 serum samples), also to perform the comparative analysis of both these techniques against PPR antibodies detection.

Materials and Methods

Source of samples: A total of one hundred and sixty (160) serum samples were collected from goats and sheep flocks (unvaccinated) from three Districts of NWFP including Mardan, Hangu and Kohat were analyzed in National Veterinary Laboratories, Islamabad for PPR detection using competitive enzyme linked immunossorbant assay and agar gel immunodiffusion test. cELISA was used as a screening test for the presence of antibodies against PPRV. The positive samples from cELISA were then tested with AGID for their comparative sensitivity.

Competitive ELISA (cELISA): Micro-titre plates (NUNC, Denmark) were coated with 1:100 dilutions of the PPRV antigens (working volume = 50ul/ well) in PBS (PH 7.4) and incubated at 37°C for 1hour on an Orbital shaker (IKA-SCHUTTL 2r MITS4) (250 Shaking/ min). At the end of the incubation period, the antigen was discarded from the plate by inverting the plate over the sink and tapping or jerking it down with a single motion of the hand. The plate was washed three times by filling up the wells with the washing buffer (PBS diluted four times with distilled water) and then discarding the buffer by inverting the plate over the sink and tapping it over a towel (washer, Lab system, Finland).

Following three washings with PBS and blot drying the plates on towel, 40 µl of blocking buffer (PBS, 0.5% Tween-20 and negative serum) were distributed to all (96) wells and 60 ìl of additional blocking buffer was added to each of the conjugate control (Cc) wells (A1 &A2) and 10 µl of additional blocking buffer was added to monoclonal antibody control (Cm) wells (F1, F2, G1 and G2). After that 10µl of control sera were added to the control wells i.e. strong positive serum control (C++) to each of the four designated wells in the plate: B1, B2, C1& C2, weak positive serum control (C+) to each of the four designated wells in the plate: D1, D2, E1 & E2 and negative serum control (C-) to each of the two designated wells in the plate: H1 & H2.

10 µl of the test sera were added in a set of two wells using a separate tip for each sample (vertical duplicates) duplicate starting from A3, B3 and so on. After that 50 µl of MAb diluted 1/100 in blocking buffer was added to all the wells except A1 and A2 and then put the plate in shaking incubation at 37°C for 1 hour. After that the plates were washed three times with PBS and blot dried over a towel, 50 µl of anti-mouse conjugate diluted 1/1000 were added in all the wells and then incubate at 37°C for 1 hour in shaking condition. Then again the plates were washed three times with PBS and blot dried over a towel. After that Orthro-phenyldiamine solution was prepared in hydrogen peroxide and 50 µl of that substrate/conjugate mixture were added to all the (96) wells. The plate was kept at room temperature for 10 minutes and the reaction was stopped by adding 1M Sulphuric acid to all the (96) wells. Finally the plate was read on an ELISA reader (BDSL Immunoskan Model No.355, Finland) at 492 nm. The absorbance was converted to percentage inhibition (PI) using the formula:

PI = 100 - (absorbance of the test wells) x 100
(Absorbance of the MAb control wells)

Sera samples with PI greater than 50% were considered to be positive.

Agar gel immuno-diffusion test: 1% Noble agar (Oxoid, England) was dispensed using micropipette (Eppendorf, Germany) in normal saline, containing thiomersal (Sigma) (0.4 g/liter) as a bacterio-static agent, into Petri dishes (6 ml/ 5 cm dish). Then the gel was allowed to solidify. After that the wells were punched in the agar following a hexagonal pattern with a central well. The wells were 5 mm in diameter and 5 mm apart. The central well was filled with 50 µl of control PPR antigen, the one peripheral well was filled with 50 µl of hyper immune serum, and the remaining peripheral wells were filled with 50 µl of test sera. Then the plate was placed in humidity chamber at 37°C for 18-24 hours. The precipitin line developed between the serum and antigens within 18-24 hours was considered as a positive result while no precipitin line or line appeared after the specified time, was considered to be a negative result.

Table-1: Serological screening of PPR suspected sera samples from different districts of NWFP using cELISA.

Location	Species		Samples tested for cELISA	cELISA positive samples	% Prevalence
	Goat	Sheep			
Mardan	-	80	80	14	17.5
Kohat	30	10	40	15	37.5
Hangu	30	10	40	21	52.5
Total	160		160	50	31.25

Result Interpretation: These PPR suspected sera samples were first tested by cELISA and samples showing PI value greater than 50% were considered positive. The PI values of different samples were measured with filter having wave length of 492nm. ELISA data interchange (EDI) software, provided by International atomic energy agency (IAEA) and FAO (UN) was used to get and analyze the results.

Results

Out of one hundred and sixty (160) sera samples tested at National Veterinary Laboratories for PPR, fifty (50) were found positive for cELISA (Table 1). These PPR suspected sera samples were brought from three different areas of NWFP to the National Veterinary laboratory, Islamabad for testing. These PPR suspected sera samples were first tested by cELISA and the sera samples showing OD value greater than 50% were considered to be positive. The OD values of different samples were measured at 492nm. The microtitre plates were read by software ELISA data interchange (EDI) prepared and provided by International atomic energy (IAEA) and FAO (UN).

Through this EDI V2.3.1 software percentage inhibition (PI) values were obtained. Each serum sample was tested in duplicate and the PI value of each well was obtained through EDI which then gave the average PI value of that sample. The percentage inhibition was actually the amount of test sera antibodies to compete with the monoclonal antibodies against PPR to bind to the same PPR viral antigen epitope. The PI values in different ranges were obtained (Table 1 and 2).

The PPR positive sera samples obtained by cELISA were then tested for Agar gel immuno-diffusion test for their comparative sensitivity. As AGID is a qualitative test, it don't show the antibody titre in the test sera although it only detect the PPR antibodies against standard PPR antigen for AGID test. All the cELISA positive sera samples were shown negative result against AGID test but the control hyper immune sera show positive result with standard PPR antigen for AGID test. It concluded that cELISA is more sensitive and specific test for PPR diagnosis than AGID.

Discussion

Peste des petits ruminant (PPR) is an acute viral disease of goats and sheep characterized by fever, erosive stomatitis, conjunctivitis, gastroenteritis and pneumonia. Goats are usually more severely affected than sheep. PPR is caused by Peste des petits ruminants virus (PPRV). The PPRV is an enveloped negative sense single strand RNA virus, which belongs to *Morbillivirus* genus in the family *Paramyxoviridae* (Gibbs *et al.,* 1979).

Because PPR is clinically indistinguishable from Rinderpest, also there is need of differential diagnosis from other related diseases so a laboratory confirmation of PPR is of utmost importance, especially in regions where both the viruses are prevalent. Conventional serological tests like Agar gel immuno-diffusion (AGID) and Counter-immunoelectrophoresis (CIEP) can be used for the diagnosis of PPR, but these techniques are not sensitive enough so as to be used as reliable diagnostic tools. Though these tests are easy to perform, they are not sensitive and but specific enough making them less reliable techniques.

Table-2. Differnt PI values and frequency of samples collected from Kohat, Murdan and Hangu District

District	PI values range	Frequency
Kohat	50- 60	1
	61-70	0
	71-80	2
	81-90	10
	91-100	2
Murdan	50- 60	0
	61-70	0
	71-80	1
	81-90	9
	91-100	4
Hangu	50- 60	1
	61-70	0
	71-80	1
	81-90	9
	91-100	10

AGID is not sensitive enough to detect low quantities of the PPR antibodies, as may be the case

with mild forms of the disease (Diallo *et al*., 1995). Therefore, these tests have become obsolete with the availability of PPRV and RP specific monoclonal antibody based ELISAs. These assays are more sensitive, and also allow processing of a large number of samples within a short time.

Specific diagnosis of PPR can be made by virus neutralization test (VNT), c-ELISA and immunocapture ELISA (Libeau *et al.,* 1994). More sensitive tests such as polymerase chain reaction (PCR) has been developed and widely used for detection of PPRV.

During the present study PPRV was detected in suspected serum samples of sheep and goat collected from three different District of NWFP. Two techniques cELISA and AGID were used for this purpose. A total of 160 sera samples were tested by cELISA, out of which 50 were found positive for PPR.For cELISA, the microtitre cELISA kit (NUNC, Denmark). The same cELISA kit (NUNC, Denmark) was used by Ogansanmi *et al.,* (2003) for PPR detection. The results of their study suggest a need for continuous serological and clinical surveillance of PPR in wild ruminants in order to determine the prevalence of PPR, its effects on wildlife conservation and the possible role of these species in the transmission cycle of PPRV (Ogunsanmi *et al.*., 2003). Choi *et al.,* 2005 reported that the procedure of c-ELISA consists of four reaction steps: adsorption of the antigen onto a solid phase, competition between the serum and MAb, detection of the MAb bound to the antigen, and a substrate reaction. The presence of antibodies in serum samples will block reactivity of monoclonal antibody resulting in reduction of expected coloration enzyme labeled anti mouse conjugate and substrate- chromogen solution. The percentage inhibition of a given MAb (monoclonal antibody) was calculated from optical densities (OD) of the sample.

The OD of the sample was the OD in the presence of inhibitor and OD of the control was the OD without inhibitor. An inhibition of more than 50 percent was considered positive. The same procedure was used in this study for PPR detection. The capability of the c-ELISA to deal with a large number of samples at a time and its short turnaround time may better serve the needs of surveillance and control programs (Choi *et al*., 2005).

The positive results from cELISA was then analyzed by AGID in order to evaluate their comparative sensitivity .All the cELISA positive serum samples were shown negative result with AGID but the controls shown positive result in AGID. As AGID is a qualitative test it donot show the antibody titre in the test sera although it only detect presence of the PPR antibodies against standard PPR antigen for AGID test. It concluded that cELISA is more sensitive and specific test for PPR diagnosis than AGID but the control hyper immune sera show positive result with standard PPR antigen for AGID test. It thus concluded that cELISA is more sensitive and specific test for PPR diagnosis than AGID.

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