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Mapping of B-cell epitopic sites and delineation of functional domains on the hemagglutinin–neuraminidase protein of peste des petits ruminants virus

G.J. Renukaradhya [a], G. Sinnathamby [b,c], Shaguna Seth [b,d], M. Rajasekhar [a], M.S. Shaila [b,*]

[a] *Project Directorate on Animal Disease Monitoring and Surveillance (PD-ADMAS), Hebbal, Bangalore 560024, India*
[b] *Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore 560012, India*
[c] *Department of Microbiology and Immunology, Room 726, Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, PA 19107, USA*
[d] *Department of Microbiology and Immunology, Emory University School of Medicine, 3086 Rollins Research Center, 1510 Clifton Road, Atlanta, GA 30322, USA*

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Abstract

A recombinant baculovirus expressing membrane bound form of hemagglutinin–neuraminidase (HN) protein of peste des petits ruminants virus (PPRV) was employed to generate monoclonal antibodies (mAbs) against PPRV-HN protein. Four different mAbs were employed for mapping of regions on HN carrying B-cell epitopes using deletion mutants of PPRV-HN and RPV-H proteins expressed in *Escherichia coli* as well as PPRV-HN deletion proteins expressed transiently in mammalian cells. The immuno-reactivity pattern indicated that all mAbs bind to two discontinuous regions of amino acid sequence 263–368 and 538–609 and hence the epitopes identified are conformation-dependent. The binding regions for three mAbs were shown to be immunodominant employing competitive ELISA with vaccinated sheep sera. Delineation of functional domains on PPRV-HN was carried out by assessing the ability of these mAbs to inhibit neuramindase activity and hemagglutination activity. Two mAbs inhibited NA activity by more than 63% with substrate *N*-acetyl neuraminolactose, while with Fetuin one mAb showed inhibition of NA activity (95%). Of the three antigenic sites identified based on competitive inhibition assay, site 2 could be antigenically separated into 2a and 2b based on inhibition properties. All the four mAbs have virus neutralizing and recognized PPRV-HN in immunofluorescence assay.

Keywords: Peste des petits ruminants virus; Hemagglutinin–neuraminidase protein; Monoclonal antibodies; Conformational epitopes; Functional domains

1. Introduction

Peste des petits ruminants (PPR), also known as "goat plague", is an acute, highly contagious viral

* Corresponding author. Tel.: +91-80-360-0139/394-2702/309-2702; fax: +91-80-360-2697/46-872-72000
E-mail address: shaila@mcbl.iisc.ernet.in (M.S. Shaila).

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disease of goat and sheep, caused by peste des petits ruminants virus (PPRV) which is a member of the genus *Morbillivirus* under the family Paramyxoviridae. PPRV is widespread across sub-Saharan Africa and the Arabian Peninsula (Taylor, 1984). Epizootics are more frequent in western Africa and enzootics occur mainly in arid and semi-arid areas of Africa. When the first outbreak was diagnosed to be due to PPRV (Shaila et al., 1989), the disease was not known to exist in India till 1988 and since then has been reported in different parts of India (Kulkarni et al., 1996; Nanda et al., 1996; Govindarajan et al., 1997). PPR introduces severe economic losses by causing high mortality and severe morbidity in infected caprines and ovines.

PPRV has two surface glycoproteins—the hemagglutinin–neuraminidase (HN) and fusion (F) proteins—whose biological activities have been studied in isolation by transiently expressing the two genes in mammalian cells (Seth and Shaila, 2001). HN recognizes the host cells receptors while F protein mediates the fusion of the viral envelope with the host cell membrane. Monoclonal antibodies (mAbs) have been widely employed in determining the antigenic sites and to identify the functional domains important for biological activities on viral surface glycoproteins HN and F of paramyxo and morbilliviruses (Rydbeck et al., 1986; Portner et al., 1987; Komada et al., 1989). Though mAbs have been produced against PPRV-HN and nucleocapsid proteins (Libeau and Lefevre, 1990; Anderson and McKay, 1994), antigenic determinants on these proteins have not been mapped so far.

Langedijk et al. (1997) predicted that two morbilliviruses—PPRV and rinderpest virus (RPV)—possess neuraminidase (NA) activity based on extensive comparison of secondary structures of all known NAs with the predicted secondary structures of HN and H proteins. PPRV has been shown to possess hemagglutination activity (Ramachandran et al., 1995). The NA activity of PPRV H protein was demonstrated after purifying the protein from infected cells or from purified virus, which also showed HA activity (Shyam and Shaila, unpublished results). The NA activity was conclusively demonstrated using the cloned HN gene to transiently express the HN protein transfected in mammalian cells (Seth and Shaila, 2001). The host cell receptor for PPRV was shown to be an NA sensitive structure containing sialic acid linked in α2–3 linkage (Shyam and Shaila, unpublished results).

In paramyxoviruses, functional inhibition studies have been carried out using mAbs to locate the antigenic sites relative to the hemagglutinin and NA activities (Iorio and Bratt, 1984). Four distinct antigenic sites on HN of Sendai virus were identified and the biological functions of HN were related to its antigenic structure using anti-HN antibodies in inhibition assays (Portner et al., 1987).

Recently, neutralizing immunodominant epitopes on RPV-H have been mapped using selected mAb-resistant mutants of lapinized strain of RPV (Sugiyama et al., 2002). However, there is no information concerning the major antigenic region(s) on the PPRV-HN protein. We have generated a recombinant baculovirus expressing HN protein of PPRV (Ind. AP94/1 strain) and shown that the expressed protein gets incorporated into extracellular baculovirus particles (rECV) (Chattopadhyay et al., unpublished results). Further, we demonstrated that rECV induces the generation of virus neutralizing antibodies and HN-specific lymphoproliferative responses in goats (Sinnathamby et al., 2001a).

In this work, we report the development of mAbs against PPRV-HN using rECV and characterization of the antigenic determinants recognized by these mAbs on PPRV-HN and RPV-H proteins by employing overlapping deletion proteins of PPRV-HN and RPV-H expressed in *Escherichia coli* as well as eucaryotic cells. We identified discontinuous regions on these proteins recognized by mAbs and present indirect evidence that the epitopes are conformation-dependent. We have also attempted to delineate the functional domains on PPRV-HN by evaluating the ability of these mAbs to inhibit NA activity and hemagglutination. Based on the mAb reactivities, the regions on HN protein contributing to NA activity have been identified.

2. Materials and methods

2.1. Cells and viruses

Vero cells (originally obtained from National Centre for Cell Science, Pune, India) were cultured in Dulbecco's modified Eagle's medium (DMEM, Himedia, India) supplemented with 5% fetal calf serum (FCS, Gibco BRL). *Spodoptera frugiperda* (Sf-21) insect cells were cultured in TC-100 medium (Gibco BRL) supplemented with 10% fetal bovine serum (FBS, Gibco BRL). Sp2/0 myeloma cells and hybridoma cells were cultured in Iscove's modified Dulbecco's medium (IMDM, Gibco BRL), supplemented with 10% FBS. A recombinant baculovirus expressing PPRV-HN was propagated in Sf-21 cells as described earlier (Sinnathamby et al., 2001a). Vaccine strains of PPRV Nig75/1 and RPV (RBOK) were propagated in Vero cells. Parental clones of hybridoma were selected using IMDM supplemented with HAT (hypoxanthine, aminopterin and thymidine, Gibco BRL) and HT (hypoxanthine and thymidine, Gibco BRL, USA). Chicken (*Galus galus*) erythrocytes (RBCs) were collected by wing vein puncture from birds that were housed at the Institute of Animal Health and Veterinary Biologicals, Bangalore.

2.2. Production of recombinant PPRV-HN extracellular virus

Recombinant baculovirus expresses the PPRV-HN protein on the surface of assembled virus particles released from infected cells as extracellular virus (ECV) particles. The rECV was purified from the supernatant collected from Sf-21 cells infected with the virus at an moi of 5, employing sucrose density gradient centrifugation method described by Summers and Smith (1987). The protein content in the purified virus was estimated by the method of Lowry et al. (1951).

2.3. Titration of virus stocks by $TCID_{50}$ assay

Confluent Sf-21 cell monolayer was harvested and the cells were resuspended in TC-100 medium supplemented with 10% FBS at a density of 1×10^5 cells/ml. The virus stock was thawed in a 37 °C water bath. Tenfold dilutions of the virus were prepared in complete medium. Virus dilutions (100 μl) were added in replicates to the wells of a 96-well tissue culture plate. Cell suspension (100 μl) containing 10^4 cells/well was then added to the wells and the plate was incubated at 27 °C incubator. The plate was monitored every day for 4 days and the appearance of CPE was recorded. $TCID_{50}$ was calculated by employing Reed and Muench formula (Burleson et al., 1992).

2.4. Deletion mutants of PPRV-HN and RPV-H proteins

Generation of deletion mutants of RPV-H and PPRV-HN protein, expression as His-tagged fusions in *E. coli* strain BL21(DE3) and their purification have been described earlier (Sinnathamby et al., 2001a,b). The deletion mutants of PPRV-HN, NΔ241 and NΔ262CΔ101 and RPV-H deletions: NΔ448, NΔ511, NΔ359; CΔ41, NΔ112; Δ183–424; CΔ160, NΔ356; CΔ185 and NΔ112; CΔ427, employed in this study are schematically represented in Fig. 3A(i) and (iii).

Five deletion gene constructs of PPRV-HN (CΔ241; Δ385–537; NΔ508; Δ80–368 and CΔ412) are schematically represented in Fig. 3(ii) were generated in a eucaryotic expression vector pCMX under a cytomegalovirus promotor. The parental clone pSSHNCMX and pGEM. PPRV-HN was used to construct the deletion mutants used in this study. DNA digestions were carried out using appropriate amounts of enzymes and the insert fragments and dephosphorylated vectors were gel eluted from LMP agarose prior to ligation. Extracts prepared from CV1 cells transfected with the plasmids carrying different fragments of HN gene were used in ELISA. Briefly, CV1 cells were plated in DMEM containing 5% FCS, when the cells were 70% confluent washed the cells with 1 × PBS then lipofectamine (2 mg/ml) or polyethylene imine (PEI) (1 mg/ml, pH 7.0) and 7 μg of DNA were mixed in OPTI-MEM and after incubation for 30 min at room temperature added to the cells and incubated. The transfection mix was then removed completely, and DMEM was added and left for 24 h at 37 °C.

2.5. Recombinant baculovirus infected cell extract

Sf-21 cells infected with the recombinant baculovirus for 72 h with an moi of 5 were resuspended in PBS and sonicated in a Vibracell (USA) ultrasonic processor and then the lysed suspension was clarified at 10,000 × *g* for 10 min. The supernatant collected was used in indirect ELISA.

2.6. PPRV Nig 75/1, PPRV TN 87/1 and RPV (RBOK) infected cell extract

PPRV Nig 75/1, PPRV TN87/1 and RPV (RBOK) viruses were grown in Vero cells. At 60–70% confluency, Vero cells were infected at moi 10. After 48–72 h when 60–70% CPE was apparent, cell extract was prepared as described above.

2.7. Generation of hybridoma for PPRV-HN

Balb/c mice were immunized subcutaneously (s/c) with 500 μg of purified rECV (the HN protein content in rECV is estimated to be 8–10% of the total protein, Chattopadhyay et al., unpublished observation) in Freund's complete adjuvant (FCA). Mice were boosted s/c twice with 250 μg of rECV in Freund's incomplete adjuvant at 3 weeks interval. A week following the second booster injection, mice were bled and HN-specific ELISA titers were determined. A titer of 1:10,000 was considered optimum for proceeding with the fusion. Three weeks after the second booster, mice were further boosted with 500 μg of purified rECV intraperitonealy. Four days later, one of the immunized animals was sacrificed and spleen cells were fused with Sp2/0 myeloma cells by standard PEG-mediated fusion protocol (Bhavani et al., 1989).

2.8. Western blot analysis of the recombinant proteins

After electrophoresis of equal amounts of deletion proteins (protein content determined using Pierce Coomassie protein assay kit), the separated proteins were electroblotted onto nitrocellulose membrane, blocked in 3% gelatin in PBS, and probed with appropriately diluted mAbs. Blots were developed using H_2O_2 and diaminobenzidine (DAB) in PBS.

2.9. Indirect ELISA

ELISA plates were coated with PPRV Nig 75/1 or RPV-RBOK infected Vero cell extracts at a concentration of 1 μg/well for an hour at 37 °C or overnight at 4 °C. The plate was washed in PBS thrice and blocked with blocking buffer (3% bovine gelatin + 0.1% Tween-20 in PBS) for 1 h at 37 °C. Plates were then treated with serial dilutions of PPRV-HN-specific mouse hyperimmune sera/hybridoma culture supernatant/ascitic fluid in PBS at 37 °C for an hour. Plates were washed and treated with anti-mouse Ig-HRP or anti-mouse IgM-HRP conjugate at 37 °C for an hour. The reaction was developed using *O*-phenylenediamine dihydrochloride and H_2O_2 and terminated using 2 N H_2SO_4. Plates were then read in a microtiter plate reader at 490 nm. Supernatant collected from each established hybridoma clone was subjected to an isotyping ELISA using an isotyping kit (Boehringer Mannheim, Germany) according to the directions of the manufacturers.

2.10. Competition ELISA for HRP conjugated mAbs

Ascitic fluids from mice injected with three mAb clones (C10A1, D2E4 and F10E7) were subjected to ammonium sulphate precipitation at 50% saturation according to Harlow and Lane (1988). The concentration of precipitated protein was determined by Lowry method (Lowry et al., 1951). Concentrated mAb preparations were then coupled to HRP by standard procedures (Harlow and Lane, 1988). ELISA plates were coated with PPRV Nig 75/1 infected Vero cell extracts (1 μg/well) and blocked with blocking buffer. The reaction was developed and read as described for indirect ELISA. Percent inhibition (PI) of conjugated mAb by the unconjugated mAb is calculated using the following formula: $PI = 100 - [A - C]/[A - B] \times 100$, wherein, $A = OD_{490}$ in the absence of competitor (heterologous mAb), $B = OD_{490}$ in the presence of homologous mAb, C =

OD predetermined in the presence of competitor (heterologous mAb).

2.11. Competitive ELISA for identifying immunodominant epitopes

Competitive ELISA (C-ELISA) was performed essentially as described previously (Anderson and McKay, 1994). Briefly, the PPRV Nig 75/1 infected cell extract in PBS was used as an antigen for C-ELISA. Each well of a 96-well microtiter plate was coated with the antigen (5 μg/ml) at 4 °C overnight and treated with blocking buffer (PBS supplemented with 0.1% (v/v) Tween-20 and 0.3% (v/v) normal sheep serum seronegative for PPRV) for 1 h at 37 °C. After washing five times in phosphate buffer saline (0.2 × PBS), appropriate dilutions of test sera (10 negative and 50 positive sera) and anti-HN mAbs (A6E9, C10A1, D2E4 and F10E7) were added. Serum controls (strong positive, weak positive and negative sheep sera) and a mAb (0% competition) control were included. Following incubation at 37 °C for 1 h on an orbital shaker, plates were washed and anti-mouse HRPO conjugate (predetermined dilution) was added. After a final incubation, substrate/chromogen (OPD/H_2O_2) was added and the color allowed to develop for 10–15 min. Plates were read on ELISA reader at 490 nm, and analyzed using an enzyme immunoassay (EIA) software (Biologicals Diagnostic Supplies Ltd., UK) and the OD values were converted to percentage inhibition (PI) values using the following formula. PI = 100 − [OD in test well/OD in 0% control well] × 100.

Out of 60 test sera (10 negative and 50 positive for PPRV antibodies) used in C-ELISA were collected from five different vaccinated herds. From each herd, 10 positive and two negative were collected and subjected for C-ELISA with all the four PPRV-HN mAbs separately.

2.12. Immunofluorescence

Vero cells grown on cover slips were infected with RPV (RBOK) and PPRV (Nig 75/1) at 10 moi and when the cells showed 30–40% cytopathic effect (CPE), cover slips were washed gently in PBS. Acetone fixed cells (intra-cytoplasmic detection) or unfixed cells were used for immunofluorescence as described by Harlow and Lane (1988).

2.13. NA and NI assays

NA assay was performed according to Aymard-Henry et al. (1973). For neuraminidase inhibition (NI) assay, PPRV Nig 75/1 infected cell extract (dilution which gave the OD of 0.6 in NA activity) was mixed with double diluted ascites of different PPRV-HN mAbs with an initial dilution of 1 in 5 in PBS and the assay was performed. The OD values obtained for each dilution of the ascites was then expressed as percentage activity remaining using the following formula:

$$\text{NI activity (\%)} = \left(\frac{\text{OD of the test mAb after inhibition}}{\text{OD of the virus control}}\right) \times 100.$$

2.14. Hemagglutination and hemagglutination inhibition assays

Hemagglutination assay was performed according to Rosanff (1961). For hemagglutination inhibition (HI) assay, ascitic fluids were double diluted in PBS in a volume of 25 μl starting with an initial dilution of 1 in 5 in a round bottom 96-well plate. Twenty-five microliters of four HA units of PPRV antigen (PPRV Nig 75/1 infected cell extract) was added to the wells. Then the standard procedure of HI was followed (Norrby, 1962).

2.15. Virus neutralization assay

The ability of mAbs to neutralize virus infectivity was tested using the procedures described by Barrett et al. (1989). Briefly, equal volumes (25 μl) of twofold dilutions of heat-inactivated (56 °C, 1 h) ascitic fluid and PPRV (Nig 75/1 strain, 100 $TCID_{50}$) or RPV (RBOK strain, 100 $TCID_{50}$) were mixed in 96-well flat bottom tissue culture plates. 50 μl of trypsinized Vero cells resuspended at 10^5 cells/ml in DMEM containing 5% fetal calf serum

were added to each well and the plates were incubated at 37 °C for 3 days. Serum controls, cell controls, and virus controls (at 100, 10, and 1 $TCID_{50}$/well, respectively) were included on each plate. Development of CPE was monitored by light microscopy and the titers were expressed as the reciprocal of the highest dilution of ascites, which neutralized 50% of virus infectivity (Scott et al., 1986).

2.16. Hemolysis and hemolysis inhibition

Both hemolysis (HL) and hemolysis inhibition (HLI) assays were performed according to the method of Norrby and Gollmar (1975).

3. Results

3.1. Generation of hybridoma clones specific for PPRV-HN protein

From a single fusion, a total of 14 stably secreting parental clones were chosen. mAbs secreted by these clones were screened for their reactivities against PPRV-HN protein using extracts from baculo recombinant HN virus-infected Sf921 cells, PPRV Nig75/, PPRV TN87/1 and RPV (RBOK) infected Vero cells in indirect ELISA. Four parental clones were selected on the basis of stable and moderately high secretion. They were then subcloned by end point limiting dilution to achieve monoclonality. One subclone from each parental clone was selected based on the reactivity in indirect ELISA. The selected subclones were termed as A6EA9, C10A1, D2E4 and F10E7. Isotyping ELISA with the hybridoma culture supernatants indicated that three subclones secrete antibodies of IgM isotype and the other one IgG2b and all the four mAbs were found to possess k light chain (Table 1).

3.2. Characterization of PPRV-HN mAbs

Ascitic fluids of all the four mAbs were generated in Balb/c mice and assayed for their reactivity in ELISA. Three clones (C10A1, D2E4 and F10E7) were found to be cross-reactive with RPV-H while A6E9 was not. All the four mAbs recognized PPRV-HN from PPRV (Nig 75/1) infected Vero cells as detected by intracellular and cell surface immunofluorescence. Three of them recognized RPV (RBOK) infected Vero cells, while A6E9 did not react with H protein of RPV in infected cells (Table 1). The immunofluorescence pattern of reactivities is given in Fig. 1.

To determine whether these mAbs recognize distinct or overlapping antigenic sites, competitive binding assay was performed. F10E7 and A6E9 did not show competitive binding to PPRV-HN antigen in the presence of any of the four mAbs, while C10A1 and D2E4 competed with each other and prevented binding of the competing antibody to nearly 100% (Fig. 2).

3.3. Identification of regions on HN carrying B-cell epitopes using deletion mutants

Western blot analysis employing the *E. coli* expressed deletion mutants of RPV-H and PPRV-HN proteins revealed that all the four mAbs (A6E9, C10A1, D2E4 and F10E7) recognized PPRV-HN deletions NΔ241 and NΔ262CΔ101, while three mAbs (C10A1, D2E4 and F10E7) recognized three RPV-H deletions NΔ448, NΔ511 and NΔ359CΔ41 (Fig. 3). As expected, the RPV-H non-cross-reactive mAb A6E9 did not react with any of the RPV-H deletions tested (Fig. 3). From the immunoreactivity pattern using PPRV-HN deletions, the antigenic sites recognized by these four mAbs are localized to the region 263–508 aa and according to the immunoreactivity pattern with RPV-H deletions, the antigenic domain of three mAbs C10A1, D2E4 and F10E7 is in the region 512–568 aa. Extracts of CV1 cells transfected with five PPRV-HN deletion mutants were used for mAb reactivities in ELISA which suggested that all the four PPRV-HN mAbs reacted with four deletion mutants, namely CΔ241, Δ385–537, NΔ508, Δ80–368 and not with CΔ412 (Table 2) implying that the epitopes for these mAbs perhaps lie in two discontinuous regions 198–368 aa and 538–609 aa which are 171 aa apart.

Table 1
Properties of PPRV-HN protein mAbs

PPRV-HN mAbs	Antibody class	Light chain	Cross-reactivity with RPV	Immunofluorescence (IF)			
				RPV (RBOK)		PPRV (Nig 75/1)	
				Intracellular	Cell surface	Intracellular	Cell surface
A6E9	IgM	k	−	−	−	+++	++
C10A1	IgM	k	++	+	+++	++	+++
D2E4	IgG 2b	k	+++	+++	+++	+++	+++
F10E7	IgM	k	+	+	++	++	++

+: Low reactivity in ELISA or IF; ++: moderate reactivity in ELISA or IF; +++: high reactivity in ELISA or IF; −: negative reaction in ELISA or IF.

3.4. Identification of immunodominant B-cell epitopes on PPRV-HN

To determine if the B-cell epitopes recognized by the mAbs are immunodominant, we carried out a competitive ELISA in which sera from sheep vaccinated with tissue culture PPRV vaccine were used as competitors for mAbs for binding to the corresponding epitopic sites, mapped in the present work (Fig. 4). The ascites of all four mAbs had titers of over 1000 in indirect ELISA using rECV and PPRV Nig75/1 infected cell lysate. Binding of three mAbs to their epitopes was inhibited by immune sera suggesting that these epitopes/epitopic regions are immunodominant (263–368 aa and 538–609 aa). The immunodominant epitopic region as identified in the present work also contains sequences conserved in the neutralizing epitopes of H (587–592 aa) identified recently (Sugiyama et al., 2002) on the H protein of lapinized RPV.

3.5. Inhibition of biological functions of HN

The NA inhibition activity of various PPRV-HN mAbs were determined using two different substrates. The NI assay using substrate *N*-acetyl neuraminolactose indicated that two mAbs inhibited the NA with percent NA activity remaining at 17.6% (D2E4) and 36.9% (A6E9) (Fig. 5A). With Fetuin as substrate, only D2E4 exhibited strong inhibitory activity (95%) as shown in Fig. 5B.

All the four PPRV-HN mAbs showed HI activity with titers ranging from 100 to 400 (Table 3). Three PPRV-HN mAbs neutralized both PPR and RP viruses with titers ranging from 10 to 40 (Table 3) while, A6E9 neutralized the homologous (PPRV Nig 75/1) virus only.

To study whether binding of PPRV-HN mAbs to HN protein inhibits the fusion promotion activity, HLI assay was performed and the results indicated that none of the PPRV-HN mAbs inhibited the HL (Tables 3 and 4).

4. Discussion

Monoclonal antibodies are valuable tools in identifying antigenic determinants and functional domains of proteins. They are also useful in distinguishing closely related viruses and therefore are employed in diagnostic procedures for infectious diseases. We have reported the generation of mAbs to PPRV-HN protein in this study. As PPRV-HN and RPV-H are antigenically closely related, it is necessary to assess the cross-reactivity of mAbs generated against PPRV-HN with RPV-H and as expected, three of the four mAbs cross-reacted with RPV-H. Three of the four mAbs generated belong to IgM isotype and only one is IgG2b. The IgG2b (D2E4) antibody has high ELISA titer and was more stable than other three IgM mAbs upon repeated freeze-thaw cycles.

The present work is the first report wherein recombinant HN of PPRV produced in insect cells has been used successfully to generate mAbs. The immunoreactivity of three cross-reactive mAbs to PPRV-HN and RPV-H deletions has identified the

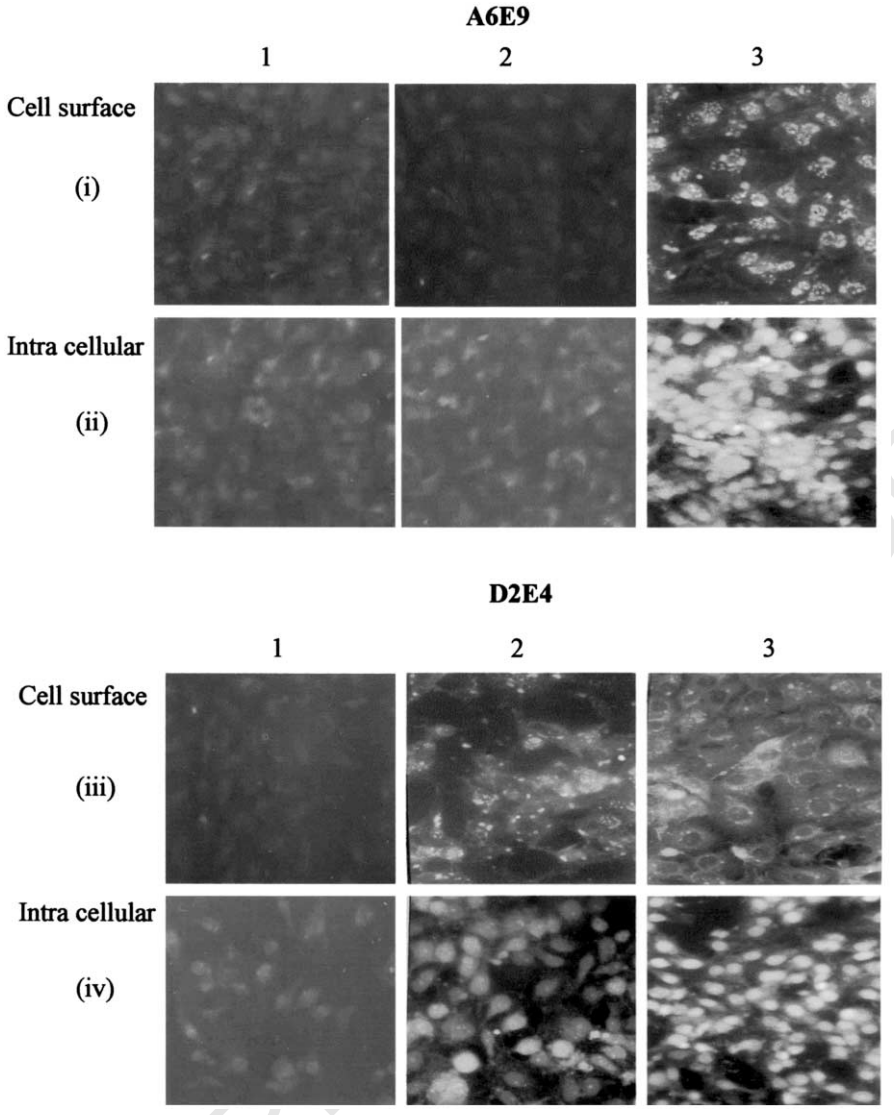

Fig. 1. Reactivity of PPRV-HN mAbs A6E9 and D2E4 by immunofluorescence staining of infected Vero cells: cells grown on coverslips were infected with RPV (RBOK) (column 2) and PPRV (Nig 75/1) (column 3), column 1—uninfected Vero cell control. At 48 h postinfection, only the coverslips intended for intracellular fluorescence study were fixed, rows (i) and (ii) coverslips were reacted with A6E9 (1 in 1500) and rows (iii) and (iv) coverslips with D2E4 (1 in 2500). After washing, all the coverslips were reacted with secondary antibody anti-mouse fluorescence isothiocyanate and the coverslips were examined under fluorescence microscope (magnification, 10 ×).

mAb binding region as a discontinuous region between aa 263–368 and 538–568 and for the cross-reactive mAb (A6E9) to aa 263–368 and 538–609 separated by 171 aa apart. This implies that all the four mAbs are binding to conformation-dependent epitopes. The three mAbs are also neutralizing, which suggests that the immunodominant B-cell epitope/epitopic domain identified in the present study are also of neutralizing nature. Earlier work on glycoprotein B of pseudorabies virus indicated that the discontinuous epitopes are only partially expressed in recombinant fragments

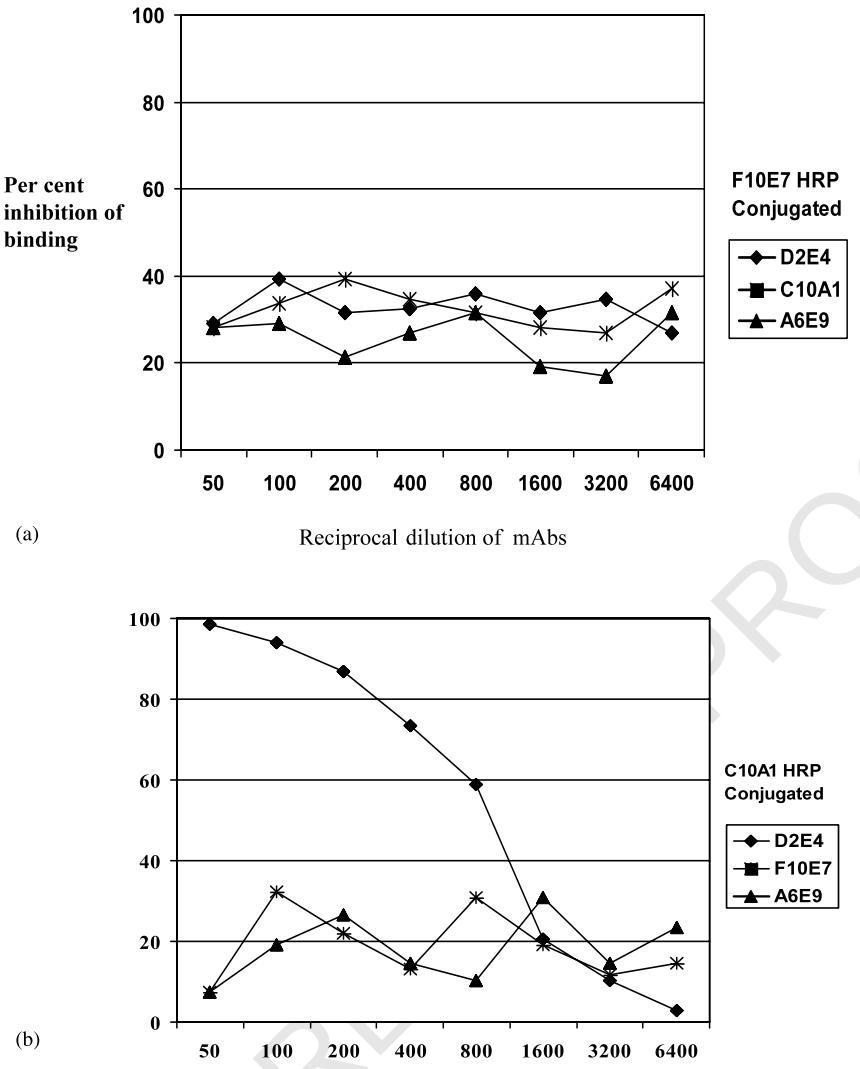

Fig. 2. Competition of horseradish peroxidase conjugated and unconjugated PPRV-HN mAbs by different combinations. The PI value is calculated by the formula, $PI = 100 - [A - C]/[A - B] \times 100$, wherein, $A = OD_{490}$ in the absence of competitor (heterologous mAb), $B = OD_{490}$ in the presence of homologous mAb, $C = OD$ pre-determined in the presence of competitor (heterologous mAb). The concentration of the conjugated mAb is fixed against varying twofold dilutions of different PPRV-HN unconjugated mAbs, to identify the overlapping and unique epitopes.

(Zaripov et al., 1999). Further, Harper et al. (1990) have shown that there would be a partial restoration of the discontinuous epitopes in recombinant fragments just before Western blotting which enables binding of these mAbs to the respective regions with less intensity. Despite the lack of authentic higher-order structure of recombinant proteins when expressed in *E. coli* (Cason, 1994), the mAbs bind to the linear constituents of the discontinuous regions in Western blot analysis. The epitopes on two discontinuous regions, which are 171 aa apart behave similar to the epitope identified by Zaripov et al. (1999) for the glycoprotein B of pseudorabies virus.

Comparison of deduced amino acid sequences of the HN protein from 13 isolates of PPRV (Shyam et al., unpublished observations) which include four Indian isolates revealed that the

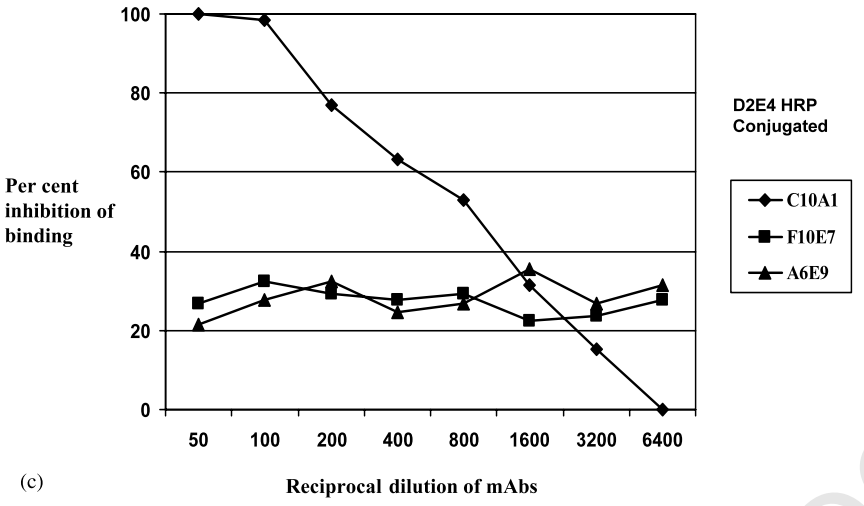

Fig. 2 (*Continued*)

sequence in the region 527–552 is highly conserved, indicating conservation of B-cell epitopic regions. The region 263–368 aa and 538–609 aa where PPRV-HN mAbs bind may be assembled together in the tertiary structure of the HN protein to represent an antigenically conserved region.

Using the four anti-HN mAbs, two distinct antigenic sites have been topographically mapped on the PPRV-HN molecule by competitive binding assay. A similar grouping based on C-ELISA has been reported for Sendai virus HN glycoprotein (Portner et al., 1987).

Functional inhibition assays by different PPRV-HN mAbs of biological activities like neutralization of PPRV Nig 75/1 and RPV (RBOK) in VNT, inhibition of the NA activity upon binding to NA site of HN molecule by mAbs, measured using Fetuin and *N*-acetyl neuraminolactose as substrates, hemagglutination inhibition assay and HI activity using PPRV Nig 75/1 infected cell extract, gave very useful insights about the HN molecule. The results of all these assays suggested that the HN protein presented by the baculovirus recombinant "resembles" the native protein of PPRV wild-type virus.

As reported earlier by Iorio and Bratt (1984), the mAbs binding to different sites on the HN protein can be grouped depending on the extent of inhibition of NA activity with different substrates, since the inhibition varies with the site of their binding. In this work, with Fetuin as the substrate for NA, only one mAb D2E4 showed NI activity (94.8% of activity being inhibited) and none of the other mAbs showed inhibition, while two mAbs (D2E4 and A6E9) showed inhibition with *N*-acetyl neuraminolactose, a low molecular weight substrate. Fetuin is a bulky molecule (m.w.: 43,000) and is expected to be acted upon by part of HN molecule responsible for NA activity to a greater extent than *N*-acetyl neuraminolactose (m.w.: 633) (Iorio and Bratt, 1984). In NI assay, the present results indicated that one mAb (D2E4) is able to inhibit NA activity with Fetuin, as against two mAbs (D2E4 and A6E9) which showed NI activity with *N*-acetyl neuraminolactose as the substrate; the reason could be that the mAb A6E9 is of IgM class, its affinity to the antigen is very weak as seen in ELISA, Western blot and in kinetic analysis (data not shown), the IgM mAb dissociates at a much faster rate than IgG mAb. Because of its huge size, Fetuin would have successfully competed out A6E9 mAb from binding to epitope on NA site, as compared with the smaller substrate, *N*-acetyl neuraminolactose with reduced steric hindrance of nearly 100-fold (Iorio and Bratt, 1984), increasing the likelihood of mAb A6E9 to directly block NA site without much competition by substrate *N*-acetyl neuraminolactose.

A significant observation is that D2E4 showed more inhibitory activity than A6E9 with both the

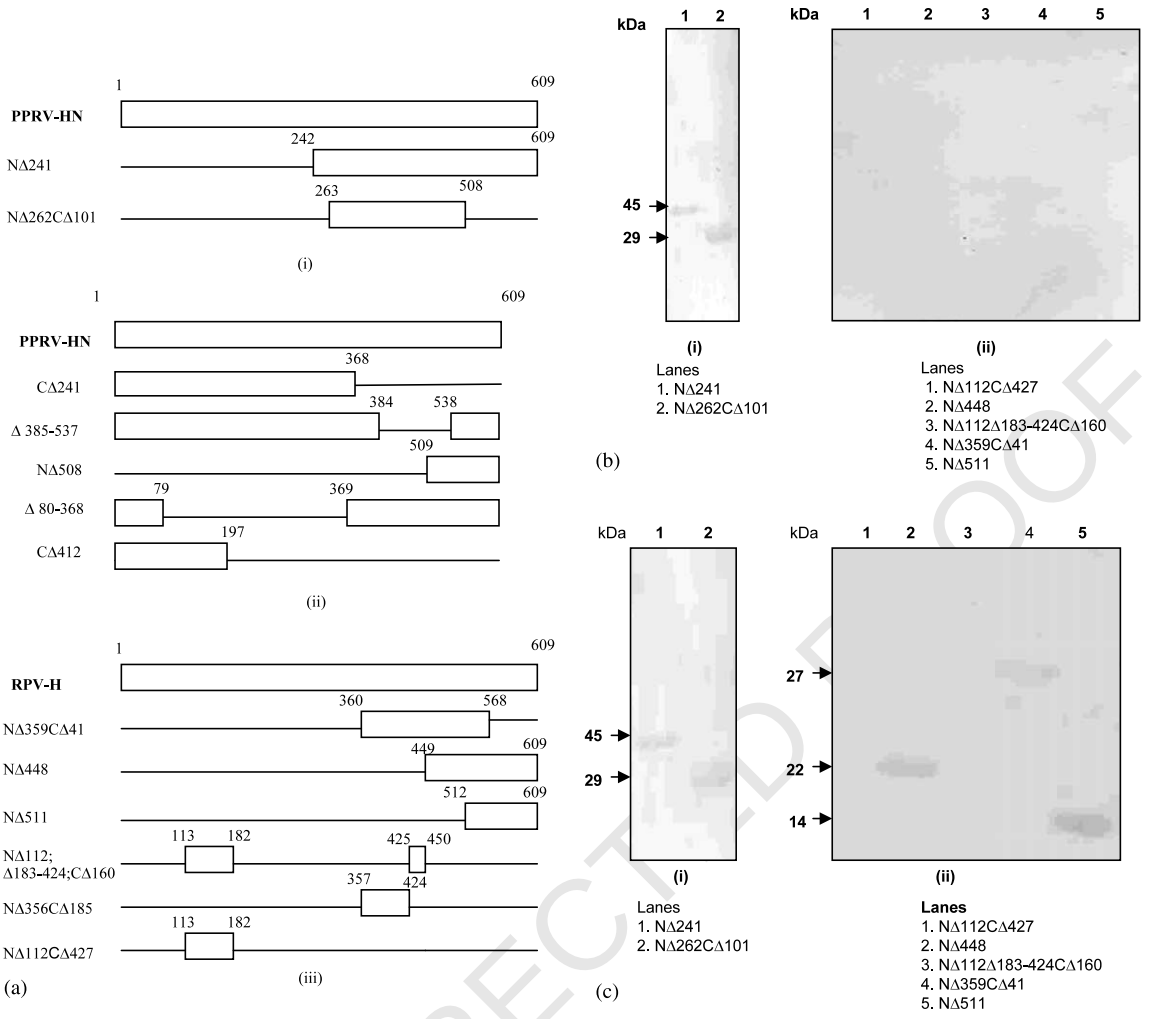

Fig. 3. (A) Schematic representation of overlapping deletion mutants: (i) fragments of PPRV-HN gene cloned in pRSET vectors and expressed as His-tagged recombinant fragments in *E. coli*, (ii) fragments of PPRV-HN gene cloned in PCMX vector and transfected in to CV1 cells and (iii) fragments of RPV-H gene cloned in pRSET vectors and expressed as His-tagged recombinant fragments in *E. coli.* Boxes represent the portions of the gene retained in the respective deletion mutant and the numbers indicate the amino acids. (B, C) Reactivity of PPRV-HN mAbs with PPRV-HN and RPV-H deletion proteins by Western immunoblot. Purified deletion proteins (1 μg) were electrophoressed on a 14% SDS polyacrylamide gel for panel (i), 12% for panel (ii), the blots were probed with mAbs (B) A6E9 (1 in 1500) and (C) C10A1, D2E4 and F10E7 (1 in 2500). The PPRV-HN deletion proteins. Panel (i): Lane 1, NΔ241; Lane 2, NΔ262CΔ101; and RPV-H deletion proteins; panel (ii): Lane 1, NΔ112CΔ427; Lane 2, NΔ448; Lane 3, NΔ112Δ183–424CΔ160; Lane 4, NΔ359CΔ41; and Lane 5 NΔ511 are expressed in *E. coli*. (C) is a representative Western blot with D2E4 mAb.

substrates. There may be different binding sites on the NA active site of the HN molecule for these two mAbs. This prediction is supported by the observation that mAb A6E9 is non-cross-reactive with RPV-H protein, whereas D2E4 cross-reacts and further, these two mAbs do not compete with each other in competitive binding assay, even though the binding site lies in the region 263–368 and 538–609 aa for A6E9 and 263–368 and 538–568 aa for D2E4. These results imply that these two mAbs are binding to two different HN sites on PPRV-HN.

Table 2
Reactivity of PPRV-HN mAbs with transfected cell extracts of HN deletion proteins in ELISA

PPRV-HN protein regions	Name of the deletion fragment	Reactivity in ELISA of PPRV-HN mAbs			
		A6E9	C10A1	D2E4	F10E7
1–368 aa	CΔ241	+	+	+	+
1–384 and 538–609 aa	Δ385–537	+	+	+	+
509–609 aa	NΔ508	+	+	+	+
1–79 and 369–609 aa	Δ80–368	+	+	+	+
1–197 aa	CΔ412	−	−	−	−

+: Signals with specific deletion protein fragment; −: negative with that specific deletion protein fragment. All the deletion fragments reacted with polyclonal hyperimmune serum against PPRV.

The functional inhibition assays using mAbs have helped to map the functional domains involved in the biological activity. Monoclonal antibody D2E4 competes with C10A1 in competitive binding assay and both the mAbs bind to two discontinuous regions on the PPRV-HN protein. In addition to this, D2E4 possesses NI activity while C10A1 does not, which suggests that these two mAbs are binding to two different HN sites on PPRV-HN within the mapped discontinuous region.

Out of four PPRV-HN mAbs, three bind to two discontinuous regions separated by 171 aa (263–368 and 538–568 aa sequence) and the fourth mAb (A6E9) also binds to two discontinuous regions separated by 171 aa (263–368 and 538–609 aa sequence). One of the two binding sites falls in the region beyond 538 aa (extreme carboxy terminal)

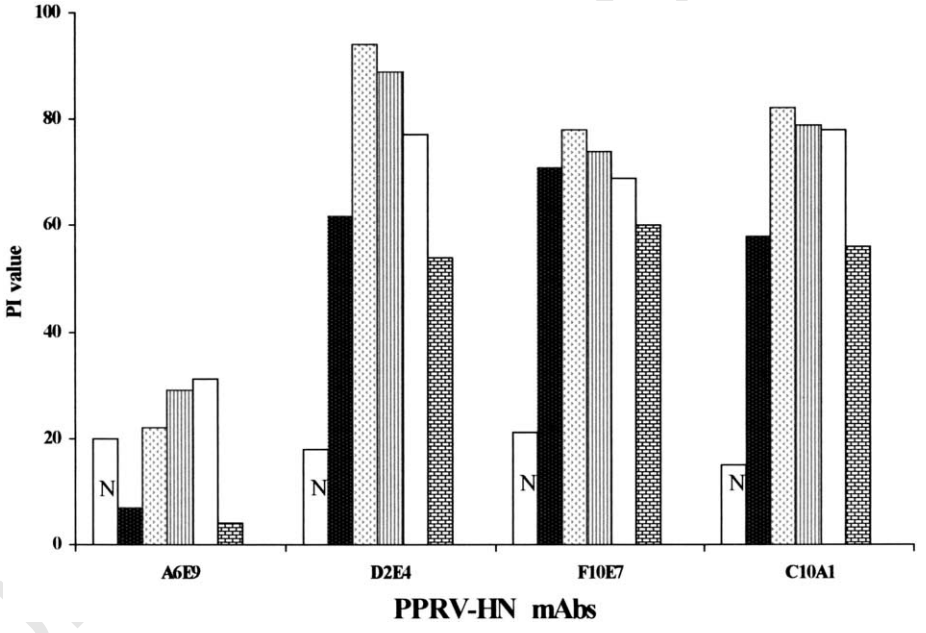

Fig. 4. Identification of the immunodominant B-cell epitope/epitopic domain on PPRV-HN protein using mAbs A6E9, C10A1, D2E4 and F10E7 as tested by C-ELISA using vaccinated sheep sera as competing antibody. Bars 2–6 under each mAb represent 10 positive sera from individual herds and bar 1 (N) represents average PI value of 10 negative sera from all the five herds (two samples per herd). The PI value is calculated by the formula, PI = 100−[OD in test well/OD in 0% control well] × 100. PI more than 50 was considered as positive for the presence of the PPRV-HN antibodies.

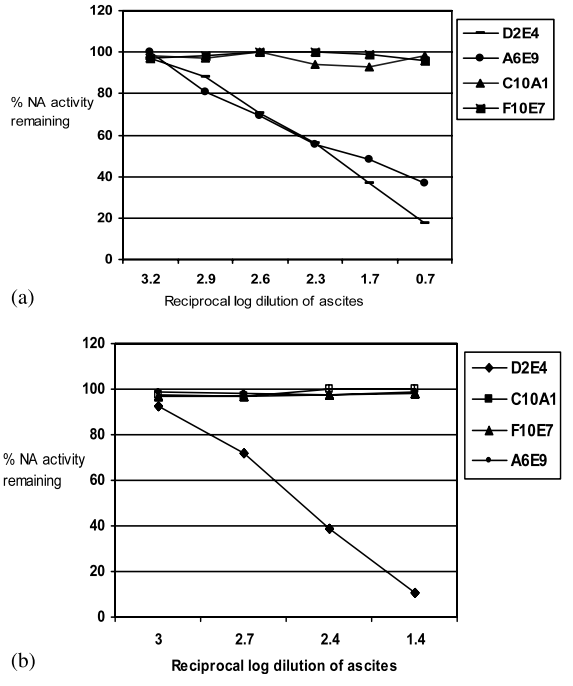

Fig. 5. NI activity of PPRV-HN mAbs using (A) *N*-acetyl neuraminolactose and (B) Fetuin as the substrate using PPPRV Nig 75/1 infected cell lysate as antigen. Percent NA activity inhibited = (OD of the test mAb after inhibition/OD of the virus control) × 100.

Table 3
Biological properties of PPRV-HN mAbs measured in terms of functional activities

mAb characterization	PPRV-HN mAbs			
	A6E9	C10A1	D2E4	F10E7
HI titer[a]	200	200	400	100
VNT[b]				
PPRV (Nig 75/1)	20	20	40	10
RPV (RBOK)	0	10	20	10
HLI titer[c]	–	–	–	–

–: No inhibition of HL by PPRV.
[a] Reciprocal dilutions of antibody which inhibits HA activity of PPRV.
[b] Reciprocal dilution of antibody which neutralizes 50% of the virus infectivity.
[c] Reciprocal dilution of antibody which inhibits HL of PPRV.

and two mAbs D2E4 and A6E9 possess NI activity. It has been shown recently that the residues DY at positions 283/284 contribute significantly to NA activity (Shaguna and Shaila, unpublished results). These two residues are analogous to DY at position 300–301 of human parainfluenza virus HN, which plays a role in the NA activity (Bando et al., 1990) and these two residues on PPRV-HN are within one of the two discontinuous region on the PPRV-HN mAb binding (263–368 aa) domain. Further, in other paramyxoviruses, homology modeling has revealed that R533 and Y551 residues may participate in substrate binding activity (Langedijk et al., 1997). In the region of 527–552 aa in the sequence of PPRV-HN, there is a 100% identity in 13 strains (includes four Indian isolates) (Shyam et al., unpublished results) and also in 270–276 aa sequence of PPRV-HN there is a 100% identity in 10 strains with only Y to H change in three African strains (Shyam et al., unpublished results). Therefore, it is likely that the two mAbs D2E4 and A6E9, which are binding to the highly conserved regions on HN protein recognize the aa involved in NA activity.

In accordance with the biological activities affected by PPRV-HN mAbs, they could be divided into three groups: group one comprising of an mAb D2E4 which has HI activity, neutralizing both homologous and heterologous viruses, cross-reacting with RPV in ELISA and possessing NA inhibition activity with both Fetuin and *N*-acetyl neuraminolactose substrates; the second group consists of the mAb A6E9 with low HI activity, neutralizing only homologous virus, non-cross-reacting with heterologous virus (RPV-RBOK) and possesses NI activity with substrate *N*-acetyl neuraminolactose only; and the third group showing all other activities except NI activity (F10E7 and C10A1). PPRV-HN mAbs can also be grouped based on their functional inhibition properties in to four groups (HN sites).

HN site 1 (F10E7) possessing neutralization, HI activities, does not compete with any mAb in C-ELISA and also without NI and HLI activities. HN site 2a (C10A1) possessing neutralization, HI activities, competes with one mAb (D2E4) in C-ELISA, but without NI and HLI activities. HN site 2b (D2E4) possessing neutralization, HI activities, competes with one mAb (C10A1) in C-ELISA, also has NI activity and not HLI activity.

Table 4
Summary of functional inhibition properties of PPRV-HN antigenic sites using PPRV-HN mAbs

PPRV-HN mAbs designation	HN site	Virus neutralization (VN)	NI		HI	HLI
			Fetuin	NANL		
F10E7	1	+	0	0	+	−
C10A1[a]	2a	++	0	0	++	−
D2E4	2b	+++	+++	+++	+++	−
A6E9	3	++	0	++	++	−

−: No activity; +: low activity; ++: moderate activity; +++: high activity.
[a] C10A1 subsite is involved in HI but not in NI activity.

HN site 3 (A6E9) possessing neutralization, HI activities, does not compete with other mAbs in C-ELISA, also has NI activity and no HLI activity.

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