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Fine mapping and conservation analysis of linear B-cell epitopes of peste des petits ruminants virus hemagglutinin protein

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ABSTRACT

Hemagglutinin protein (H), one of the two glycoproteins of peste des petits ruminants virus (PPRV), binds to its receptor on the host cell and acts as a major antigen that induces and confers highly protective immunity in the host. In order to delineate the epitopes on H protein, fine epitope mapping and conservation analysis of linear B-cell epitopes (BCEs) on PPRV H has been undertaken using biosynthetic peptides and rabbit anti-PPRV H sera. Thirteen linear BCEs were identified and their corresponding minimal motifs were located on the H protein of PPRV China/Tibet/Geg/07-30. Conservation analysis indicated that two of the 13 minimal motifs were conserved among 52 PPRV strains. Nine of the 13 peptides containing the minimal motifs were recognized using anti-PPRV serum from a goat immunized with PPRV vaccine strain Nigeria 75/1. Identified epitopes and their motifs improve our understanding of the antigenic characteristics of PPRV H and provide a basis for the development of epitope-based diagnostic assays and multiple epitopes vaccine.

1. Introduction

Peste des petits ruminants (PPR) is a highly infectious disease of domestic and wild small ruminants that is caused by PPR virus (PPRV), a member of the genus *Morbillivirus* that includes rinderpest virus (RPV), measles virus (MeV), canine distemper virus (CDV), phocine distemper virus (PDV), cetacean morbillivirus (CeMV), and feline morbillivirus (FeMV) (Torsson et al., 2016). Clinical symptoms associated with PPR include pyrexia, congestion of mucosal surfaces, ocular and nasal discharge, erosive stomatitis and diarrhea (Liang et al., 2016; Libeau, 2015). As a notifiable disease listed by the World Organization for Animal Health (OIE), PPR has significant socioeconomic impact on poverty alleviation in developing countries, and the OIE together with the Food and Agriculture Organization (FAO) has launched a control and eradication strategy aimed at the global elimination of the disease by 2030. PPRVs have been classified into four lineages based on partial sequences of the nucleocapsid (N) or fusion (F) genes even though the virus is serologically monotypic. Until recently, these lineages have been associated with the geographic distribution of the virus. Lineages I, II and III were common in Africa and lineage IV in the Middle East and Asia (Adombi et al., 2016; Santhamani et al., 2016). However, during the last decade, lineage IV viruses has been regularly reported in different African countries and appear to be spreading more efficiently within this continent (Adombi et al., 2016).

The non-segmented, single-stranded, negative-sense RNA genome of PPRV encodes six structural and two or three non-structural proteins in the order 3′-N-P/C/V/W-M-F-H-L-5′ (Baron, 2015). Among the three viral proteins (N, H, and F) able to induce an immune response, the two surface glycoproteins H and F stimulate neutralizing and protective antibody production in the host and have been targeted in the development of recombinant vaccines used to confer protection in goats. Both recombinant proteins are reported to confer immunity when expressed either together or separately (Libeau, 2015; Liu et al., 2014).

Lymphocytes play a fundamental role in protecting the host against invading pathogens by conferring both cell- and antibody-mediated immune responses. Following stimulation by the pathogen, B cell lymphocytes secrete neutralizing antibodies that are highly effective in clearing foreign invaders such as PPRV (Munir et al., 2013b). Therefore, mapping B cell epitopes (BCEs) on the viral protein is a prerequisite for designing efficient recombinant multi-epitope vaccines. Furthermore, identification of epitopic domains in the PPRV structural proteins will facilitate the development of epidemiological surveillance methods to

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monitor the immune status of animals. BCE mapping is also helpful for advancing DIVA (differentiation of infected versus vaccinated animals) strategies.

BCEs are categorized either as linear (composed of continuous amino acids [aa]) or conformational (discontinuous aa). In nature, the majority of BCEs are discontinuous but, due to difficulties in the mapping and design of such epitopes, more research has centered on linear BCEs (Han et al., 2013). Although further effort is required to identify the epitopes on viral proteins, there have been several reports describing the antigenic regions of the PPRV H and N proteins (Choi et al., 2005; Dechamma et al., 2006; Renukaradhya et al., 2002). Employing four different monoclonal antibodies and deletion mutants of PPRV H expressed in both *Escherichia coli* and mammalian cells (Renukaradhya et al., 2002), B cell epitopic regions having neutralizing antibody binding capacities have been mapped to two regions of the PPRV H protein from 263 to 368 aa and 538 to 609 aa. However, there are no reports of in-depth mapping and minimal motif identification of BCEs associated with PPRV H protein.

Another convenient and highly efficient method of epitope screening that has been adopted for fine mapping of epitopes on the coronavirus S protein (Hua et al., 2005; Sun et al., 2008) and IgG-epitome decoding of proteins from oncogenic human papillomavirus type 58 (Xu et al., 2016) involves the use of biosynthetic peptides. Biosynthetic peptides and polyclonal serum against PPRV N protein were employed in our previous study for fine epitope mapping and conservation analysis of linear B-cell epitopes on the PPRV N protein (Yu et al., 2015). This strategy has now been adopted to identify 13 linear epitopes and their minimal motifs on the PPRV H protein. Our data increase our knowledge of BCE distribution on the PPRV H protein and provide a more solid foundation for the rational design of multi-epitope based vaccines and diagnostic antigens.

2. Material and methods

2.1. Preparation of recombinant PPRV H protein

Recombinant H protein of PPRV China/Tibet/Geg/07-30 (ACQ44671.1) was employed as the immunization antigen and was prepared as described previously (Xu et al., 2009; Zhang et al., 2012). PPRV China/Tibet/Geg/07-30 was the most prevalent strain in China when this research was initiated, and the first PPR virus genome to be sequenced in China. Briefly, the codon optimized H gene of PPRV was synthesized, cloned into the prokaryotic expression vector pET-32a, and expressed in *E. coli* BL21. After separation of the gene product using SDS-PAGE and purification by electro-elution of the excised gel band, the protein was concentrated with PEG 8000 and stored at −70 °C prior to use as the immunogen.

2.2. Immunization of animals

Four female New Zealand white rabbits (~2 kg body weight) were purchased from the Shanghai SLAC Laboratory Animal Co. Ltd (Shanghai, China) and maintained according to the standard laboratory animal care protocols approved by the Institutional Animal Care Committee of the SAAS. Three rabbits were injected intramuscularly with 0.5 mg of purified recombinant H protein emulsified in complete Freund's adjuvant (CFA) (Sigma Aldrich, San Francisco, USA) at multiple sites on the back of each animal. Three booster injections of 0.25 mg of the same antigen per injection in incomplete Freund's adjuvant were administered at two-week intervals. Animals were bled 7 days after the third booster and the serum was separated and stored at −70 °C until use. The antibody titer of the combined sera from the three immunized rabbits (designated RAH01) was determined by ELISA as previously described except the coating antigen was replaced by PPRV H protein (Zhang et al., 2012). Non-immune serum from the remaining one rabbit that received only CFA was used as the negative control.

2.3. Biosynthesis of 8-mer and 16-mer peptides

Using truncated glutathione S-transferase (the initial 188 aa, designated GST188) as carrier, 74 16-mer peptides and one 17-mer peptide with an overlap of 8 aa residues covering the entire H protein of PPRV China/Tibet/Geg/07-30 (ACQ44671.1) were expressed as fusion proteins as described earlier (Yu et al., 2015). In addition, a series of overlapping 8-mer peptides (7 aa residues overlap) for each of the positive 16-mers identified later (i.e. P1, P9–P11, P13, P28, P45–48, P58, P60, P63, and P74) were similarly expressed. Briefly, the synthesized, then annealed, DNA fragments encoding each short peptide, incorporated with *Bam*H I and TAA-*Sal* I cohesive ends on their 5′- and 3′-terminals respectively, were inserted into the *Bam*H I and *Sal* I sites of plasmid pXXGST-1. *E. coli* BL21 (DE3) (*pLysS*) was employed to express the fusion peptides. Positive clones were selected by SDS-PAGE and confirmed by DNA sequencing. Cell pellets containing each fusion peptide were stored at −20 °C until BCE mapping by western blot.

2.4. SDS-PAGE and western blots

SDS-PAGE and western blots were performed as previously described (Yu et al., 2015) with minor modifications. Briefly, cell pellets were resolved by SDS-PAGE using 15% gels. Gels were either stained with Coomassie brilliant blue G-250 for confirming the bands of fusion proteins or processed for western blotting by electro-transferring the proteins onto 0.2 μm nitrocellulose membrane (Whatman GmbH, Dossel, Germany). Membranes were then blocked with 5% (w/v) skimmed milk powder in phosphate-buffered saline (PBS), incubated sequentially with RAH01 (dilution, 1:4000) and goat anti-rabbit IgG conjugated to horseradish peroxidase (dilution, 1:80,000) (Protein Tech Group, Inc., Chicago, USA), and visualized by enhanced chemiluminescence using ECL plus western blot detection reagents (GE Healthcare, Chalfont St Giles, UK) according to the manufacturer's instructions.

2.5. 3D modelling of PPRV H protein

A search of the Protein Databank Archive (RCSB PDB) (http://www.rcsb.org/pdb/home/home.do) revealed no 3D structures of PPRV H protein. The 3D structures of the N-terminal cytoplasmic tail (aa 1–39), the stalk region (aa 60-155), and the C-terminal receptor-binding head domain (aa 155–609) of PPRV H protein were predicted using the Protein Homology/analogY Recognition Engine V 2.0 (Phyre2) (Kelley et al., 2015) server (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) in intensive modeling mode.

2.6. Conservation analysis of the epitope motifs among PPRV strains

Full-length aa sequences of the H proteins from 52 PPRV strains available in GenBank were aligned using ClustalX2 software. Analysis of the BCE-containing sequences of PPRV H was performed with MEGA software (version 6.0).

3. Results

3.1. Identification of 16-mers containing linear BCEs

Initially, antigenic regions containing linear BCEs were broadly delineated using a series of longer peptide fragments covering the entire PPRV H protein and the rabbit polyclonal anti-serum RAH01. Seventy-four overlapping 16-aa-long fragments and a single 17-mer fragment were fusion expressed with GST188 in *E. coli*, and the fusion proteins were observed as approximately 22 kDa bands by SDS-PAGE (Fig. 1). Among the 75 expression products, the following 14 peptides were

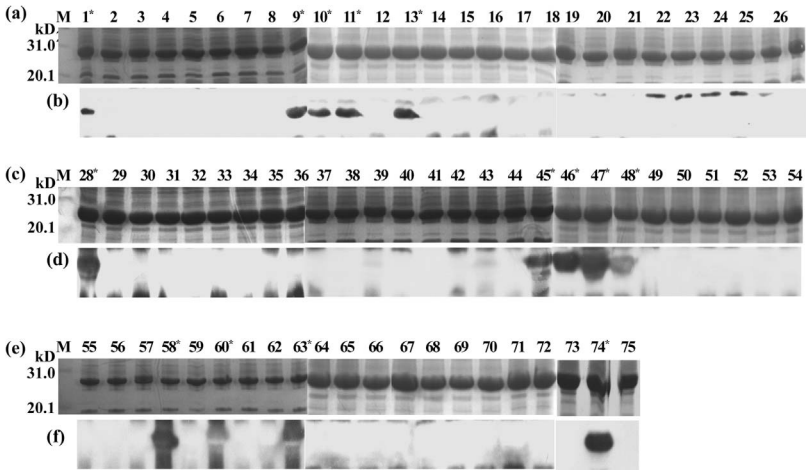


Fig. 1. SDS-PAGE and western blotting analysis of 16-mer and 17-mer PPRV H protein-derived peptides.

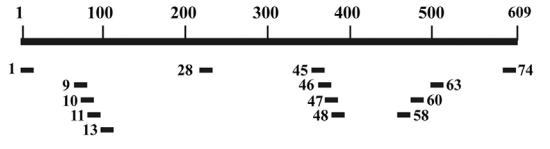
Seventy-four 16-mer peptides and one 17-mer peptide covering the entire H protein of PPRV China/Tibet/Geg/07-30 were expressed in *E. coli* strain BL21. After induction, cells were harvested by centrifugation and cell pellets were resolved by SDS-PAGE using 15% gels. Gels were either stained or processed for western blots. Nitrocellulose membranes were first blocked with 5% (w/v) skimmed milk, incubated sequentially with RAH01 (dilution, 1:4000) and goat anti-rabbit IgG (1:80,000 dilution), and then visualized by enhanced chemiluminescence.

a. c. e: SDS-PAGE analysis of 74 GST fusion expressed 16-mers and one 17-mer.

b. d. f: Western blot analysis of 74 GST fusion expressed 16-mers and one 17-mer.

1–75: GST fusion expressed 16-mer (P1-P74) and 17-mer (P75) peptides.

*: denotes the positive GST fusion expressed 16-mer.



No.	Position	No.	Position	No.	Position	No.	Position
1	1-16	13	97-112	47	369-384	63	497-512
9	65-80	28	217-232	48	377-392	74	585-600
10	73-88	45	353-368	58	457-472		
11	81-96	46	361-376	60	473-488		

Fig. 2. Distribution of positive 16-mer PPRV H protein-derived peptides.

The short segments represent the 16-mer PPRV H protein-derived peptides. Numbers assigned to the 16-mer peptides are the same as in Fig. 1. Numbers shown above the long fragment denote the initial and terminal amino acids of the PPRV China/Tibet/Geg/07-30H protein sequence (ACQ44671.1).

identified as positive by western blotting: P1, P9–P11, P13, P28, P45–P48, P58, P60, P63 and P74 (Fig. 1). Of these, six 16-mer sequences (P1, P9–P11, P13 and P28) were located in the amino-terminus half of the H protein (aa 1–232, Figs. 1 and 2), while the remaining eight positive 16-mer sequences were located closer to the carboxyl terminus (aa 353–609, Figs. 1 and 2).

3.2. Identification of the minimal motifs of the detected BCEs

Fourteen sets of overlapping 8-mer peptides fusion expressed with GST188 in *E. coli* and RAH01 anti-serum were used in a second round of fine epitope characterization of the 14 positive 16-mer peptides identified in the first round. Within every set (except P13), one to six of the 8-mers stained with the antiserum and shared 3 aa (P1), 5 aa (P11 and P47), 6 aa (P46 and P63) or 7 aa (P45, P48, P58, P60 and P74) (Fig. 3). These shared amino acids were tentatively designated as minimal epitope motifs. Since the P13 8-mer sets yielded no positives, two sets of nested polypeptides larger than 8-mer were synthesized and one minimal motif of 10-aa was subsequently identified (see below). For easy description, where two neighboring 16-mers shared the same motifs (as in the case of P9 and P10, P10 and P11, and P46 and P47), the motifs were assigned to the latter peptide. Consequently, the 13 minimal binding motifs of the PPRV H protein were ascribed to residues ^{8}INA^{10} (in P1), ^{73}RLNTNIKL^{80} (in P10), ^{84}IDHQT^{88} (in P11), ^{98}IIGDEVGIRI^{107} (in P13), ^{224}EEGLFGRT^{231} (in P28), ^{359}KDDEANW^{365} (in P45), ^{367}VPSTDV^{372} (in P46), ^{372}VRDLQ^{376} (in P47), ^{383}VEACKTR^{389} (in P48), ^{463}FLGMINT^{470} (in P58), ^{478}VMPHILT^{484} (in P60), ^{504}VDDDIK^{509} (in P63) and ^{590}TDEEVHT^{596} (in P74) (Figs. 2–4).

3.3. Identification of minimal motifs on peptide P13

Since RAH01 failed to react with any of the P13 8-mer sets, two serial 9-15-mer peptides with one amino acid subtracted progressively from either the C- or N-terminus of P13 were fusion expressed with GST118 in *E. coli* and used to identify the minimal binding motifs in this 16-mer peptide. As shown in Fig. 3, RAH01 reacted with the fusion expressed 15- to 11-mer serial peptides (13–10 to 13–14, Fig. 3) of P13 in which 1 to 5 amino acid residue(s), respectively were deleted from the C-terminal end, and with the 15-mer peptide P13-10′ which had one amino acid residue subtracted from the N-terminal end. Therefore, it was concluded that one 10-aa minimal epitope motif, ^{98}IIGDEVGIRI^{107}, existed in the 16-mer P13 peptide (Figs. 3 and 4).

3.4. 3D structures of the minimal motifs of the identified BCEs

To clarify the spatial locations of the 13 minimal motifs of the BCEs identified on the PPRV H protein, 3-dimensional structural models of the N-terminal cytoplasmic tail (aa 1–39), the stalk region (aa 60–155), and the C-terminal head domain (aa 155–609) of the protein were generated using the Phyre2 server. Our data predicted that 412 of 471 residues (89%) constituting the head domain were modeled at > 90% confidence while the remaining 59 residues were modeled *ab initio*. However, none of the residues comprising the cytoplamic tail and stalk regions were modeled at this confidence level. Positioning of the mapped 13 BCE motifs on the 3D structure revealed distribution to be at regions of predicted loops, β-sheets and helixes (Fig. 5). Eleven of the 13 motifs were exposed or partially exposed on the surface of the predicted 3D structure (Fig. 5), suggesting that these were all antibody-accessible, whereas the remaining two motifs (epitope motifs 5 and 10 in Fig. 5) were almost entirely buried inside the PPRV H protein.

3.5. Conservation of BCE motifs in PPRV strains

Comparison of 52 available PPRV H protein sequences indicated that two of the 13 BCE motifs were fully conserved; i.e. ^{8}INA^{10} and ^{372}VRDLQ^{376}. The remaining eleven non-conserved BCE motifs (^{73}RLNTNIKL^{80}, ^{84}IDHQT^{88}, ^{98}IIGDEVGIRI^{107}, ^{224}EEGLFGRT^{231}, ^{359}KDDEANW^{365}, ^{367}VPSTDV^{372}, ^{383}VEACKTR^{389}, ^{463}FLGMINT^{470}, ^{478}VMPHILT^{484}, ^{504}VDDDIK^{509} and ^{590}TDEEVHT^{596}) exhibited at least one amino acid difference when compared with their counterparts (Table 1). For example, motif ^{98}IIGDEVGIRI^{107}, was conserved in all PPRV strains except those assigned to lineage I (ICV89 and E32/1969) (ABX75304.1 and AJT59441.1), in which I^{98} was substituted with V^{98}. None of the PPRV motifs identified were identical to the H proteins of RPV, MeV, CDV, PDV, CeMV, and FeMV, all of which belong to the genus *Morbillivirus* (data not shown).

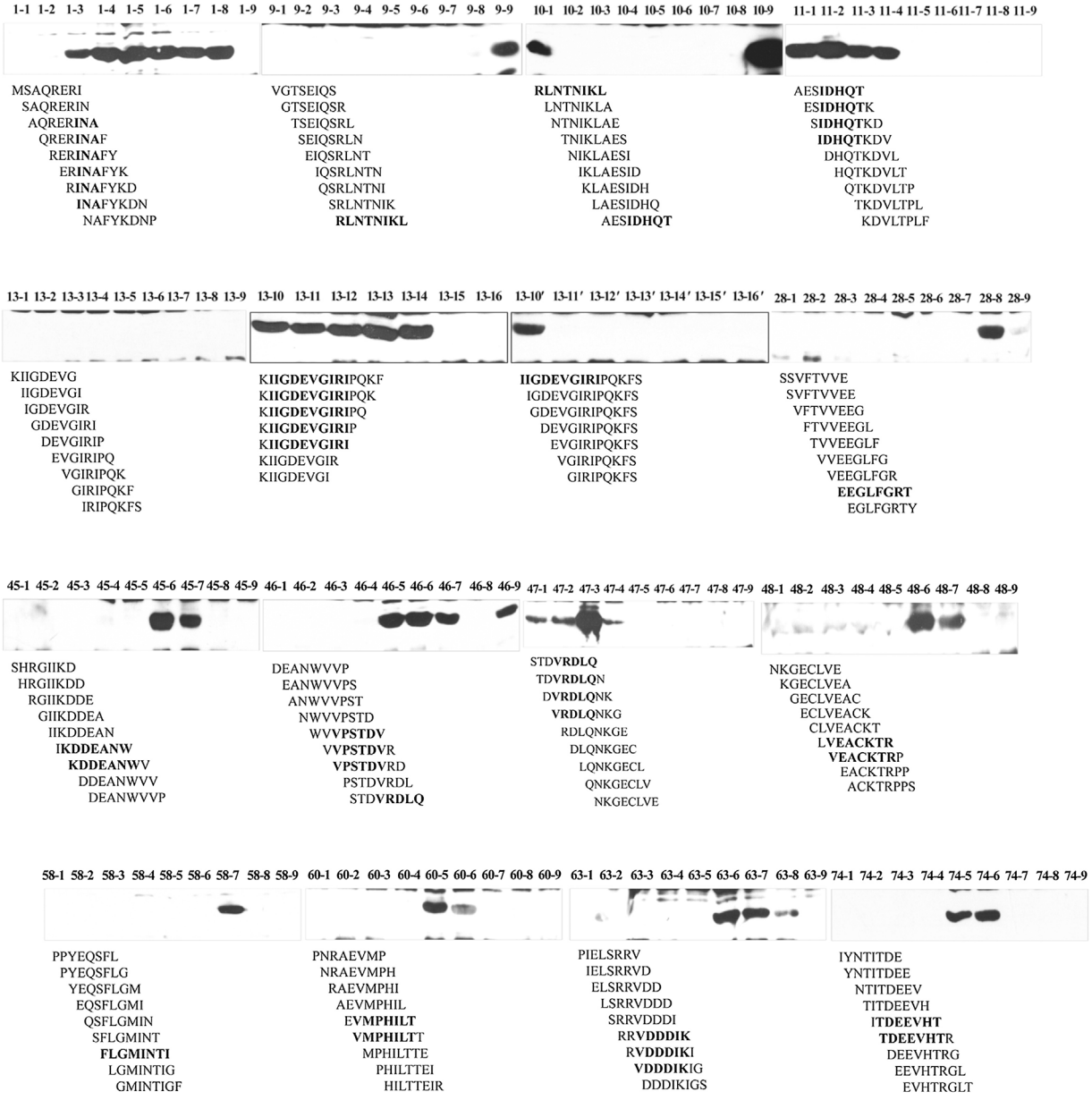


Fig. 3. Determination of minimal motifs of the identified BCEs by western blotting.
All 8-mers or similar short peptides of PPRV H protein were expressed in *E. coli* strain BL21. After induction, cells were harvested by centrifugation and cell pellets were resolved by SDS-PAGE using 15% gels. Gels were either stained or processed for western blotting. Nitrocellulose membranes was first blocked with 5% (w/v) skimmed milk, incubated sequentially with RAH01 (dilution, 1:4000) and goat anti-rabbit IgG (1:80,000 dilution), and then visualized by enhanced chemiluminescence. Hyphenated numbers represent the sequential numbers of the expressed peptides used to screen the minimal motifs of the positive 16-mers.

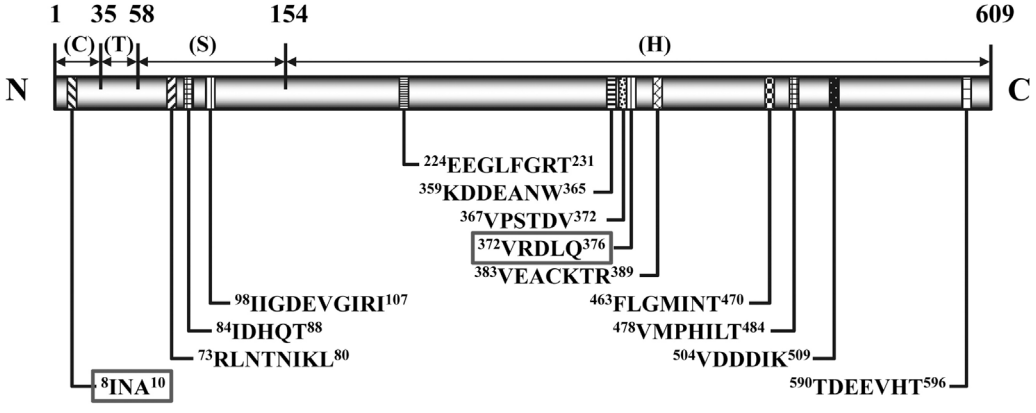


Fig. 4. Distribution of 13 BCEs minimal motifs on the PPRV H protein.
The horizontal bar represents the primary structure of the H protein (aa 1–609). Capital letters in brackets: C, cytoplasmic tail; T, transmembrane region; S, stalk region; H, head domain. The positions of each BCE minimal motifs are indicated with superscripts. Conserved BCE motifs are boxed.

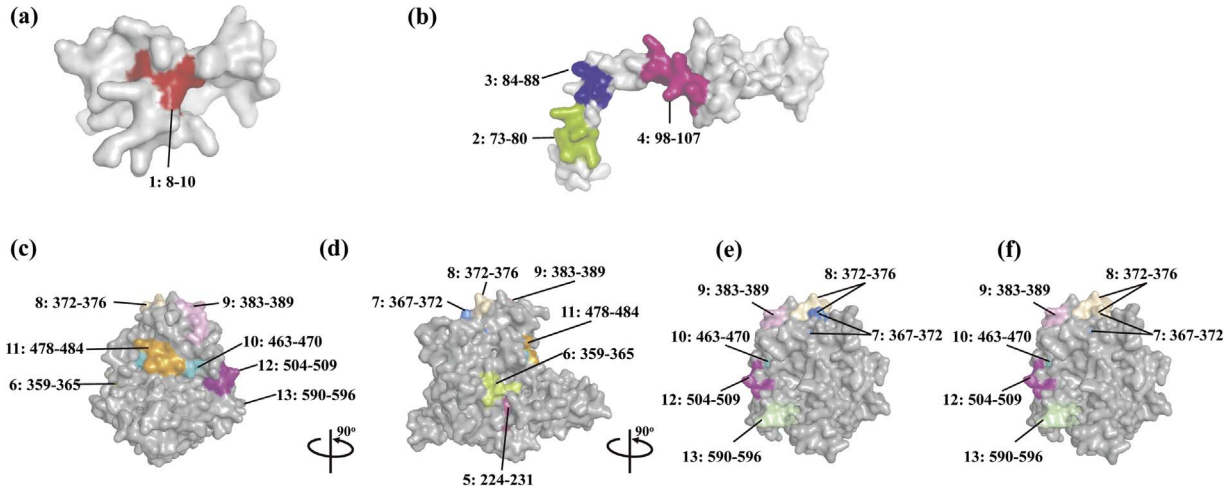


Fig. 5. Positioning of the mapped 13 BCEs minimal motifs (Nos 1–13) on the predicted 3D structure of H protein.
The 3D structure models of the cytoplasmic tail region (a), the stalk region (b) and the head domain (c–f) of the PPRV H protein were predicted with Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) using intensive modeling, and visualized using the PyMOL molecular graphics and modeling system. (f) is the same as (e) except the color of the shared residue V^{372} which is in marine (the color of BCE 7) in (e), while in wheat (the color of BCE 8) in (f). The BCEs minimal motifs identified in this study are highlighted in different colors (1, red; 2, split pea; 3, blue; 4, hot pink; 5, warm pink; 6, lemon; 7, marine; 8, wheat; 9, pink; 10, cyan; 11, orange; 12, magenta; 13, pale green).

3.6. Reactivity of the identified BCE motifs with anti-PPRV serum

Nine of the 13 selected 8- and 13-mers containing one of the identified minimal motif sequences were recognized by western blotting using anti-PPRV serum (1:200 dilution) from a goat immunized with PPRV vaccine strain Nigeria 75/1 as the primary antibody (Fig. 6). Nine of 11 BCE motifs predicted to be located or partially located on the surface of the H protein reacted with goat-anti-PPRV serum, albeit weakly in the case of epitopes 4, 7 and 12 (Fig. 6), and the remaining two epitopes (1 and 2 in Fig. 5) were situated within the cytoplasmic tail and stalk regions, respectively. Among the nine BCE motifs recognized by anti-PPRV serum, 8-mer epitopes “11-2” and “47-3” (Fig. 6, epitopes 3 and 8 in Fig. 5) should elicit a stronger antibody response.

4. Discussion

For infection to occur, enveloped viruses must first interact with membrane protein receptors on the surface of host cells and induce fusion of the viral membrane with the host cell membrane. PPRV H protein binds to its cognate receptor on the host cell during the first step of the viral infection process, and acts as a major antigen that stimulates a protective immune response in the host (Renukaradhya and Shaila, 2015). PPRV H protein has also been shown to be effective in inducing both humoral and cell-mediated immune responses. Recombinant vector viruses expressing H alone or combined with F, another PPRV glycoprotein, can induce high levels of antibodies and protect goats and sheep from experimental challenge with pathogenic PPRV (Caufour et al., 2014; Holzer et al., 2016). However, multi-epitope based recombinant vaccines offer numerous advantages compared to the complete protein including cost-effective production, multi-valency, and stability under different conditions. Moreover, the efficacy of epitope vaccines can be further improved by combining $CD4^+$ T helper and promiscuous epitopes, employing epitope enhancement techniques (such as immune-informatics or computational immunology), and adopting toll-like receptor (TLR) ligands as an adjuvant (Nezafat et al., 2014; Rosa et al., 2015). Consequently, in order to enable rational multi-epitope vaccine and diagnostic antigen design, it is imperative to delineate the BCEs on the target antigens. Although Shaila and coworkers have identified two discontinuous B cell epitopic regions (aa 263–368 and 538–609) and three regions that include T cell determinants (aa 123–137, 407–416, and 242–609) on the PPRV H protein (Renukaradhya et al., 2002; Sinnathamby et al., 2001, 2004), the total number of BCEs (or TCEs) and the precise nature and location of their minimal motifs still need to be determined.

As a continuation of fine epitope mapping of linear BCEs on the PPRV N protein (Yu et al., 2015), we carried out fine mapping analysis of PPRV H antigenic epitopes and revealed the existence of 13 such sequence segments and their minimal motifs. Based on sequence comparisons among different PPRV strains, two of these motifs are conserved. Primary amino acid sequence alignment of the hemagglutinin from members of the genus *Morbillivirus* indicates that, as a type II membrane glycoprotein, the H protein is comprised of an N-terminal cytoplasmic tail (aa 1–35), a transmembrane region (aa 36–58), a stalk region (aa 59–154), and a C-terminal receptor-binding head domain (aa 155 to the terminus) (Colf et al., 2007; Hashiguchi et al., 2011a). Accordingly, the two conserved motifs shown in Fig. 4 were located in the N-terminal cytoplasmic tail and head domain, respectively. Three of the eleven non-conserved motifs are located within the stalk region, and the remaining eight motifs are located within the C-terminal head domain. As expected, no antigenic motif is located within the transmembrane region.

Lack of reactivity between the 8-mer epitopes “28-8” and “58-7” (containing minimal epitopes 5 and 10, respectively) and anti-PPRV serum is consistent with their predicted deep-seated location within the H protein. However, the failure of goat anti-PPRV serum to react with the 8-mer epitopes “1-4” and “10-1” (containing minimal epitopes 1 and 2, respectively), which are predicted to be located on the surface of the H protein (Figs. 5 and 6), is unexpected. It is possible that the predicted surface location of epitopes 1 and 2 is incorrect since there are no crystal structure data relating to the cytoplasmic tail and stalk regions of other morbilliviruses that would allow Phyre2 to predict the 3D structure with high confidence. It is also possible that epitopes 1 and 2 fail to induce an immune response in the host since the former is enclosed in the intact virus particle while the latter may be buried in the tetrameric H protein complex during the formation of fusion machinery.

Although minimal B cell epitopes normally range from 5 to 20 amino acids long, improved methodology employed for fine mapping of linear B cell epitopes of PPRV N protein identified two 3-aa-long epitopes (^{462}ETP^{464} and ^{523}LLG^{525}) (Yu et al., 2015). Earlier, Xu et al. (2012) identified a 3-aa-long epitope, EGP$^{313–315}$, during the mapping of minimal motifs of B-cell epitopes on human zona pellucida glycoprotein-3. In a more recent study decoding the IgG-epitomes of E6, E7 and L1 proteins of oncogenic human papillomavirus type 58, Xu et al.

Table 1
Variance of nonconserved epitope motifs among PPRV strains.

Lineage	H Protein_id[a]	Epitope motif sequence										
	ACQ44671.1	[73]RLNTNIKL[80]	[84]IDHQT[88]	[98]IIGDEVGIRI[107]	[224]EEGLFGRT[231]	[359]KDDEANW[365]	[367]VPSTDV[372]	[383]VEACKTR[389]	[463]FLGMINT[470]	[478]VMPHILT[484]	[504]VDDDIK[509]	[590]TDEEVHT[597]
I	ABX75304.1	RLNTNI***E***L	***V***DHQT	***V***IGDEVGIRI	EEGLFGRT	KD***N***EANW	VPSTDV	VEACKTR	FLGMINT	VMPHILT	***I***DDDIK	T***N***EEVH***K***
	AJT59441.1	RLNTNI***E***L	IDHQT	***V***IGDEVGIRI	EEGLFGRT	KD***N***EANW	VPSTDV	VEACKTR	***Y***LGMINT	VMPHILT	***I***DDDIK	T***N***EEVH***K***
II	ABX75312.1	RL***K***TNI***E***L	IDHQT	IIGDEVGIRI	EEGLFGRT	KD***N***EANW	VPSTDV	VEACKTR	FLGMINT	VMPHILT	***I***DDDIK	T***N***EEVH***M***
	AKT04315.1	RLNTNI***E***L	IDHQT	IIGDEVGIRI	EEGLFGRT	KD***N***EANW	VPSTDV	VEACKTR	FLGMINT	VMPHILT	***I***DDDIK	T***N***EEVHT
	AKT04323.1	RLNTNI***E***L	IDHQT	IIGDEVGIRI	EEGLFGRT	KD***N***EANW	VPSTDV	VEACKTR	FLGMINT	VMPHILT	***I***DDDIK	T***N***EEVR***T***
III	AIL54020.1	RLNTNI***E***L	IDHQT	IIGDEVGIRI	***G***EGLFGRT	KD***N***EANW	VPSTDV	VEACKTR	FLGMINT	VMPHILT	T***D***DDIK	T***N***EEVHT
	AIL54028.1	RLNTNI***E***L	IDHQT	IIGDEVGIRI	EEGLFGRT	KDDEANW	VPSTDV	VEACKTR	FLGMINT	V***T***PHILT	VDDDIK	T***N***EEVHT
IV	ABY61986.1	RLNTNI***E***L	IDHQT	IIGDEVGIRI	EEGLFGRT	KDDEANW	VPSTDV	VEACKTR	FLGMINT	VMPHILT	VDDDIK	TDEEVHT
	CAD54790.1	RLNTNI***E***L	IDHQT	IIGDEVGIRI	EEGLFGRT	KD***N***EANW	VPSTDV	VEACKTR	FLGMINT	VMPHILT	VDDDIK	TDEEVHT
	ANG60361.1	RLNTNI***E***L	IDHQT	IIGDEVGIRI	EEGLFGRT	KDDEA***S***W	VPSTDV	VEACKTR	FLGMINT	VMPHILT	VDDDIK	***S***GEEVHT
	AHF58488.1	RLNTNI***E***L	IDHQT	IIGDEVGIRI	EEGLFGRT	KDDEANW	VPST***E***V	VEACKTR	F***P***GMINT	VMPHILT	VDDD***L***K	TDEEVHT
	AIL29370.1	RLNTNI***E***L	IDHQT	IIGDEVGIRI	EEGLFGRT	KDDEANW	VPSTDV	***E***EACKTR	FLGMINT	VMPHILT	VDDDIK	TDEEVHT
	AIL54004.1	RLNTNI***E***L	IDHQT	IIGDEVGIRI	EEGLFGRT	KDDEANW	VPSTDV	VEACKTR	FLGMINT	VMPHILT	VDDDIK	TDEEVH***A***
	AJE30413.1	RLNTNIKL	IDHQT	IIGDEVGIRI	EEGLFGRT	KDDEANW	VPSTDV	VEACKTR	FLGMINT	VMPHILT	VDDDIK	T***G***EEVHT
	ALM55670.1	RLNTNIKL	IDHQT	IIGDEVGIRI	EEGLFGRT	KDDEANW	VPSTDV	VEACKTR	FLGMINT	VMPHILT	VDDDIK	T***N***EEVH***K***
	AKR81281.1	RLNTNIKL	IDHQT	IIGDEVGIRI	EEGLFGRT	KDDEANW	VPSTDV	VEACKTR	FLGMINT	VMPHILT	VDDDIK	TDEEVH***M***

[a] Number denotes the amino acid location in the PPRV China/Tibet/Geg/07-30H protein sequence (ACQ44671.1); Substituted aa shown in bold italics.
[a] Only one strain from each lineage for each variation is shown.

(2016) also identified a 3-aa-long epitope (SAP) on the HPV58-L1 protein.

Cell entry of enveloped viruses involves virus attachment followed by close apposition and fusion of the virion and cell membranes. Although less research work has focused on the precise mechanism of PPRV cell entry, studies on other members of *Morbillivirus* (especially MeV) indicate that the fusion machinery is composed of hetero-oligomeric complexes formed by tetrameric attachment (H) and trimeric fusion (F) glycoproteins (Plattet et al., 2016). While the head domains of the H protein recognize and bind multiple receptors, compelling functional and biochemical evidence indicate that the stalk region is predominantly involved in interaction with the F protein. A growing body of evidence suggests that engagement of H-heads with cognate receptors causes structural rearrangement within the H-stalk section, which is a strict requirement for triggering the F refolding cascade (F-activation) necessary to achieve membrane fusion (Corey and Iorio, 2007; Plattet et al., 2016). Should triggering of the fusion machinery turn out to be essential for initiating morbillivirus infection, novel antivirals could be developed that specifically target the receptor-binding sites and impede receptor interaction or micro-domains within the head and/or stalk domains, thereby preventing conformational changes that evoke F activation. In conceptual support of using the stalk domain of H protein as a potential drug target, Plattet and Plemper reported that a mAb that recognized a linear epitope in the CDV H stalk domain acted as a potent inhibitor of membrane fusion (Plattet and Plemper, 2013). Of the three BCE motifs, [73]RLNTNIKL[80], [84]IDHQT[88] and [98]IIGDEVGIRI[107] (corresponding to the 16-mers P10, P11 and P13, respectively) identified in the PPRV H-stalk region, the latter two were both predicted to be surface exposed and recognizable by goat anti-PPRV serum (Figs. 5b and 6). Theoretically, these BCE motifs should also be considered as candidates when designing PPRV antibody-based drugs or multi-epitope vaccines.

Recently, the complexes of the head domain of MeV H (highly homologous to PPRV H) with its receptors (SLAM, CD46 and nectin-4) were disclosed (Hashiguchi et al., 2011b; Santiago et al., 2010; Zhang et al., 2013). In combining the SLAM and nectin-4 receptors, the following residues of MeV H are involved: Pro[191]-Gly[196], Lys[387]-Gln[391], Pro[458]-Met[459], Leu[462]-Gly[465], Leu[482]-Phe[483], Val[485], Thr[498]-Leu[500], Glu[503], Asp[505]-Asp[507], Tyr[524], Leu[526], Thr[528], Asp[530]-Val[534], Tyr[541], and Tyr[543]-Arg[556]. Elucidation of the interaction between MeV H with its receptors provides an ideal template for homology modeling of other members of the genus *Morbillivirus*. Homology modeling and comparative interaction energy data indicated that the PPRV H-sheep SLAM binding interface and the MeV H-marmoset SLAM binding interface were consistent (Liang et al., 2016). Based on these observations, four linear BCE motifs identified herein, [383]VEACKTR[389], [463]FLGMINT[470], [478]VMPHILT[484], and [504]VDDDIK[509] in 16-mers P48, P58, P60 and P63, respectively, were located in the receptor binding pocket of PPRV H. Of these, [383]VEACKTR[389],[478]VMPHILT[484], and [504]VDDDIK[509] could be recognized by goat anti-PPRV serum (Figs. 5c and 6). Previous research on antigenic epitopes of MeV H showed that the majority of MeV H monoclonal antibodies with neutralizing capability were targeted to the receptor-binding area (Hashiguchi et al., 2011a; Tahara et al., 2016). Accordingly, it is tempting to anticipate that the three BCE motifs in the 16-mers P48, P60 and P63 have the potential to be used as candidates for PPRV multi-epitope vaccine development.

Serological cross-reactions among morbilliviruses, e.g. between PPRV and RPV, are one of the main constraints for achieving reliable diagnoses. Among the surface proteins, only H protein induces little cross-reaction between viruses of the genus (Libeau, 2015). Comparison of the amino acid sequences of the RPV and PPRV H proteins indicated only 50% similarity (Munir et al., 2013a). Hence, H protein (partial or intact) or mAb(s) against H could be used to develop a PPRV specific diagnosis kit. Methods relying on mAb with the capability of differentiating PPRV from RPV have been available (Anderson and McKay, 1994; Bruning-Richardson et al., 2011; Saliki et al., 1993; Singh et al.,

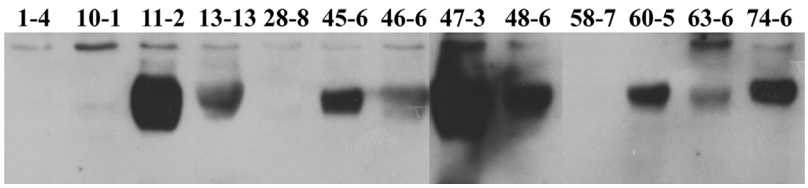


Fig. 6. Reactivity of the identified BCEs with goat anti-PPRV serum. Eight-mers and 12-mers containing the minimal BCE motifs were expressed in *E. coli* strain BL21. After induction, cells were harvested by centrifugation and cell pellets were resolved by SDS-PAGE using 15% gels. Gels were either stained or processed for western blots. Nitrocellulose membranes were first blocked with 5% (w/v) skimmed milk, incubated sequentially with anti-PPRV serum (1:200 dilution) from a goat immunized with PPRV vaccine strain Nigeria 75/1 and mouse anti-goat IgG (1:40,000 dilution), then visualized by enhanced chemiluminescence. Numbers above the lanes denote the 8-mers or 12-mers with BCE motifs included.

2004), but little is known about the location of the BCEs to which the mAbs combine. In our research, sequence alignment indicates that there is no sequence motif in the RPV H protein identical to the minimal motifs of epitopes found in PPRV H protein. Clearly, identification of 13 PPRV specific linear BCE motifs, including two conserved BCE motifs, on the H protein is of significance for establishing PPRV differential diagnoses although their specificities have yet to be validated experimentally.

To facilitate post-outbreak sero-surveillance of PPRV and its eradication, local veterinarians in endemic areas have made strong calls for a marker vaccine and a companion test to differentiate infected from vaccinated animals (DIVA project). Recently, Banyard's group have developed a novel strategy for constructing morbillivirus marker vaccines in which mutation of a specific mAb binding site on the RPV or PPRV H protein rendered the vaccine virus undetectable by the same mAb (Buczkowski et al., 2012; Muniraju et al., 2015). This, in tandem with the cH-ELISA, allows differentiation between vaccinated and naturally infected animals. Clearly, identification of the minimal motifs of 13 linear BCE motifs on the PPRV H provides additional candidate epitopes and antigenic data for the development of a PPRV DIVA vaccine.

In conclusion, biosynthetic peptides have been used to identify 13 linear BCE minimal motifs in the PPRV China/Tibet/Geg/07-30H protein, including two motifs conserved in 52 PPRV strains. Nine of the 13 peptides, each containing one of the identified BCE motifs, reacted with goat anti-PPRV serum. Identification of the BCE motifs further elucidates the antigenic characteristics of the H protein, and provides a solid foundation for understanding the nature of immunity against PPRV and for the development of epitope-based surveillance and vaccination.

Conflict of interest

The authors have no conflict of interest to declare.

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