Arch Virol (2014) 159:3021–3028
DOI 10.1007/s00705-014-2174-4

ORIGINAL ARTICLE

Characterisation of novel linear antigen epitopes on North American-type porcine reproductive and respiratory syndrome virus M protein

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Received: 18 March 2014 / Accepted: 30 June 2014 / Published online: 19 July 2014

Abstract The M protein, encoded by the porcine reproductive and respiratory syndrome virus (PRRSV) ORF6 gene, is considered to be one of the most conserved PRRSV proteins. In recent decades, highly specific monoclonal antibodies (Mabs) have been exploited to provide reliable diagnoses for many diseases. In this study, two different Mab clones targeting the linear epitopes on the PRRSV M protein were generated and characterized. Both Mabs showed binding activity against the native PRRSV virion and recombinant M protein when analyzed by immunofluorescence assay (IFA) and Western blot. The targeted epitope of each Mab was mapped by serial truncation of the M protein to generate overlapping fragments. Fine epitope mapping was then performed using a panel of expressed polypeptides. The polypeptide sequences of the two epitopes recognized by Mabs 1C8 and 3F7 were ^{3}SSLD^{6} and ^{155}VLGGRKAVK^{163}, respectively, with the former being a newly identified epitope on the M protein. In both cases, these two epitopes were finely mapped for the first time. Alignments of Mab epitope sequences revealed that the two epitopes on the M protein were highly conserved between the North American-type strains. These Mabs, along with their mapped epitopes, are useful for the development of diagnostic and research tools, including immunofluorescence, ELISA and Western blot.

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Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped single-stranded positive-sense RNA virus belonging to the order *Nidovirales*, the family *Arteriviridae*, and the genus *Arterivirus*, together with equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV) of mice, and simian hemorrhagic fever virus (SHFV) [1, 2]. The isolates can be divided into two distinct genotypes, represented by the prototypical European and North American strains Lelystad virus (LV) [3] and ATCC VR-2332 [1, 4]. PRRSV is the causative agent of porcine reproductive and respiratory syndrome (PRRS), one of the most economically important infectious diseases for the pig industry worldwide [5–7]. The disease is characterized by reproductive failure in sows and respiratory disease in pigs [8–10]. In 2006, highly pathogenic (HP) strains of PRRSV emerged in Jiangxi province of China and quickly spread throughout China and other Southeast Asian countries, causing significant damage to the Asian swine-breeding industry [11]. Disease resulting from HP-PRRSV infection is more severe, characterized by high fever (41 °C), high illness rates (50–100 %) and high mortality (20–100 %) in pigs of all ages [12–14].

The M protein, an 18- to 19-kDa class III membrane protein, is encoded by the ORF6 gene. It is non-glycosylated and is the most conserved structural protein of PRRSV and arteriviruses in general [15–17]. In the virion, the GP5 and M proteins are found as a disulphide-linked

heterodimer, which is essential for the infectivity of arteriviruses [18–21]. It has been reported that the presence of the M protein increases the immune response against GP5 by increasing the cellular immune response and the production of neutralizing antibodies [22]. In addition, the Calmette-Guérin (BCG) vaccine strain of *Mycobacterium bovisbacille* expressing the M protein successfully induced the development of neutralizing antibodies in mice, indicating that the M protein contains neutralizing epitopes [23].

However, although B-cell epitopes on the M protein have been reported [24], serodiagnostic assays based on these epitopes have not yet been established. Here, we have precisely mapped novel B-cell-specific epitopes in addition to generating two monoclonal antibodies (Mabs) against HP-PRRSV. The epitope mapping reported in this paper may facilitate the development of diagnostic tests for the serological detection of this virus while providing valuable insight into the antigenic structure of HP-PRRSV.

Materials and methods

Viruses, cells, plasmids, and sera

HP-PRRSV HuN4 (GenBank accession no. EF635006) was isolated in China, and its pathogenicity has been characterized [14, 25, 26]. The vaccine strain HuN4-F112 was obtained by culturing the parent strain HP-PRRSV HuN4 in Marc-145 cells for 112 passages [25]. TJM-F92 and JXA1-R are commercial vaccines available in China. The cell lines Marc-145, SP2/0 and 293T and the eukaryotic expression vectors pCAGGS-HuN4-F112-GP2, GP3, GP4, GP5, M, and N were maintained in our laboratory. PRRSV-positive serum was obtained from a piglet that was initially immunized with the vaccine strain HP-PRRSV HuN4-F112 and then inoculated three times with the virulent strain HP-PRRSV HuN4-F5 [27]. PRRSV-free serum was obtained from a specific-pathogen-free pig.

Virus purification

HuN4-F112 ($10^{7.0}$ $TCID_{50}$/0.1 mL) was used as the immunogen for production of PRRSV-specific Mabs. Marc-145 cell monolayers were inoculated with virus for 24–48 h at 37 °C. Supernatants containing infectious virus were obtained by subjecting infected Marc-145 cells to three freeze-thaw cycles, followed by centrifugation at 5000 × g for 30 min at 4 °C. The supernatants were then ultracentrifuged at 30,000 × g for 3 h at 4 °C (SW32Ti, Beckman, USA). The pellet was resuspended in PBS and stored at −20 °C.

Production and characterization of Mabs against HuN4-F112

Female BALB/c mice (Laboratory Animal Center of Harbin Veterinary Research Institute, CAAS), aged 4–6 weeks, were immunized intraperitoneally three times at 2-week intervals with HuN4-F112 ($10^{9.0}$ $TCID_{50}$). The first immunization was given using Freund's complete adjuvant (FCA; Sigma, St. Louis, MO, USA) and for the subsequent two, Freund's incomplete adjuvant (FICA; Sigma, St. Louis, MO, USA) was used. A final booster of virus alone was also given intraperitoneally. Three days after the final booster injection, spleen cells were fused with SP2/0 cells using 50 % (v/v) polyethylene glycol (Sigma). The fused cells were cultured successively in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL Co. Ltd., USA) containing HAT (Sigma) and HT (Sigma) and then in DMEM supplemented only with 20 % fetal bovine serum (Hyclone Laboratories Inc., South Logan, UT, USA).

The hybridomas were screened by immunofluorescence assay (IFA) for secretion of the desired antibodies. In brief, Marc-145 cell monolayers were infected with HuN4-F112 and incubated for 24–48 h at 37 °C. Cells were harvested by digestion and centrifugation and washed once with PBS. Eight-hole glass slides were coated with infected cells, air dried, and fixed with cold acetone. IFA was performed using the hybridoma supernatants and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Zsbio, Beijing, China) as primary and secondary antibody, respectively. The samples were analyzed using a fluorescence microscope (Nikon TS100, Japan). The selected clones were subcloned by limiting dilution. Ascitic fluids were produced in FICA-primed BALB/c mice.

Transient transfection was performed to identify the structural proteins that were bound by the generated Mabs. 293T cells were transiently transfected with the eukaryotic expression constructs pCAGGS-HuN4-F112-GP2, -GP3, -GP4, -GP5, -M and -N using X-tremeGENE HP DNA Transfection Reagent (Roche, Basel, Switzerland) according to the manufacturer's protocol. pCAGGS-transfected cells were used as a negative control. Marc-145 cell monolayers were infected with HuN4, TJM-F92 and JXA1-R, cells were harvested, and IFA was performed as described above. The isotypes of the Mabs produced were determined using a Pierce® Rapid ELISA Mouse Mab Isotyping Kit (Thermo Scientific, MA, USA) according to the manufacturer's instructions.

Epitope mapping with overlapping M protein peptide fragments

For epitope mapping of HuN4-F112-M, the ORF6 gene was divided into four overlapping segments for expression,

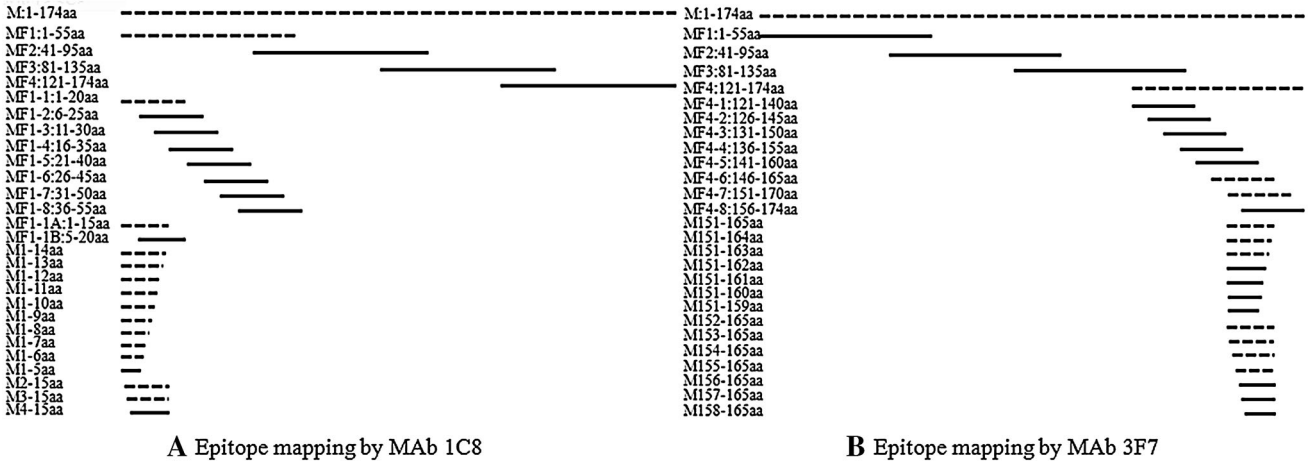

Fig. 1 Schematic diagram of the M protein, showing the expression constructs of the M fragments. **A** Strategy for mapping recombinant M protein by Mab 1C8. **B** Strategy for mapping recombinant M protein Mab 3F7. Peptides binding with corresponding Mabs are indicated by *dotted lines*

designated MF1–MF4. MF1 and MF4 were then divided into eight overlapping segments. Upon identification of bound polypeptide, we synthesized complementary oligonucleotide pairs, annealed them together, and cloned them into the *BamH*I and *Xho*I sites of the pET32a (+) and pGEX-6p-1 expression vectors, respectively, before introducing them into *E. coli* BL21 (DE3) cells by transformation (Fig. 1).

The Trx and GST fusion proteins were subjected to 12 % SDS-PAGE and then transferred to nitrocellulose membranes (PALL, NY, USA) for Western blot. After blocking, the membranes were incubated with the Mabs of interest at 37 °C for 1 h. After washing three times with PBS containing 0.5 % Tween-20 (PBST), the membranes were incubated with IRDye-700-conjugated goat anti-mouse IgG (Rockland, Gilbertsville, PA, USA) as the secondary antibody. Proteins were visualized by scanning the membranes with the LI-COR Odyssey infrared image system (LI-COR Biotechnology, USA).

Sequence alignments of the epitopes recognized by Mabs 1C8 and 3F7

Nucleotide sequences of geographically distinct PRRSV strains were retrieved from GenBank, followed by deduction and alignment of the amino acid sequences using the DNASTAR MegAlign software (DNASTAR Inc., Madison, WI, USA). The representative PRRSV strains are listed in Table 1.

Blocking ELISA

A blocking ELISA was designed to analyze the capability of PRRSV-positive sera to inhibit the binding of anti-PRRSV Mabs 1C8 and 3F7 to HuN4-F112. Known positive and negative sera were diluted serially in PBST (0.05 % Tween-20), beginning at 1:2. One hundred microliters of diluted serum was incubated for 1 h at 37 °C in an HuN4-F112-coated ELISA plate (Corning, NY, USA), blocked with 5 % skimmed milk, followed by addition of 100 μL of hybridoma supernatant. Binding of Mabs was detected using an HRP-conjugated anti-mouse immunoglobulin (Zsbio, Beijing, China). TMB substrate solution (Invitrogen, CA, USA) was then added. After

Table 1 PRRSV strains cited in this study

Isolate	Accession number	Type	Virulence
HUN4	EF635006	Type II	Highly virulent
HUB1	EF075945	Type II	Highly virulent
JX143	EU708726	Type II	Highly virulent
JXA1	EF112445	Type II	Highly virulent
JXwn06	EF641008	Type II	Highly virulent
Jiangxi-3	EU200961	Type II	Highly virulent
SX2009	FJ895329	Type II	Highly virulent
SY0608	EU144079	Type II	Highly virulent
WUH1	EU187484	Type II	Highly virulent
YN2008	EU880435	Type II	Highly virulent
CH-1a	AY032626	Type II	Virulent
CH2002	EU880438	Type II	Virulent
BJ-4	AF331831	Type II	Virulent
HN1	AY457635	Type II	Virulent
P129	AY585241	Type II	Virulent
VR2332	AY150564	Type II	Virulent
JXA1 P80	FJ548853	Type II	Vaccine
CH-1R	EU807840	Type II	Vaccine
MLV	AF066183	Type II	Vaccine
Lelystad virus	M96262	Type I	Virulent
NMEU09-1	GU047345	Type I	Virulent

10 min, the colorimetric reaction was stopped by adding 50 μL of 2 M sulfuric acid, and absorbance values were read at 450 nm using a microplate reader (Tecan, Männedorf, Switzerland). One hundred microliters of reagent were used per well; three washes with PBST were performed after each incubation.

Results

Production and identification of Mabs

Two Mabs, 1C8 and 3F7, were raised against HuN4-F112 and detected using IFA. The two Mabs specifically recognized the M protein expressed by transiently transfected cells, reacting positively with Marc-145 cells infected with the HP-PRRSV HuN4, and commercial vaccine strains (JXA1-R and TJM-F92) used in China. However, they did not bind to uninfected Marc-145 cells (data not shown). Mab 3F7, but not 1C8, recognized the European-type strain VP046 BIS. However, neither Mab recognized European-type strain DV (Fig. 2).

The isotypes of Mabs 1C8 and 3F7 were identified using a Pierce® Rapid ELISA Mouse Mab Isotyping Kit as IgG2a and IgG1, respectively. A neutralization assay was performed as described previously [28], and the results indicated that these Mabs did not neutralize PRRSV (data not shown). The supernatant IFA titer of both of these Mabs was

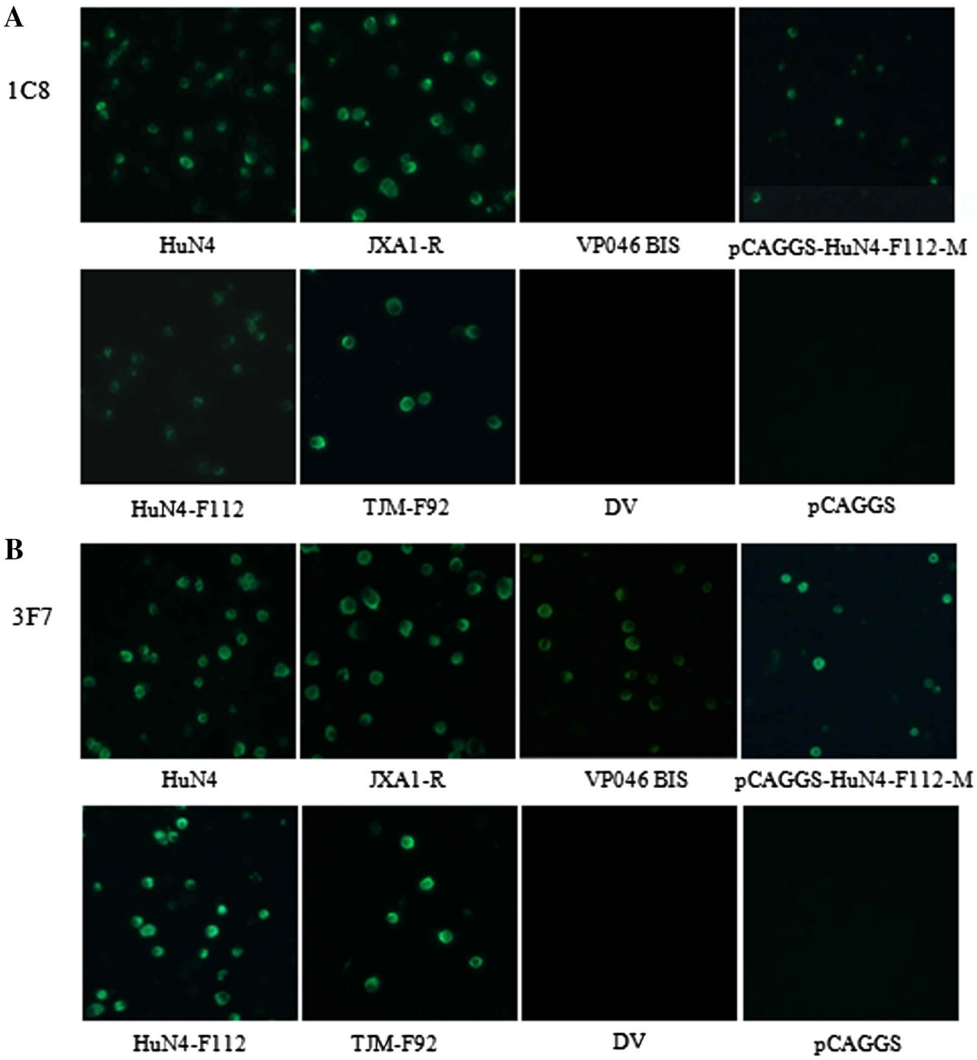

Fig. 2 Reactivity of Mab 1C8 (A) and Mab 3F7 (B) with HP-PRRSV HuN4 and vaccine strains in Marc-145 cells and transiently transfected 293T cells expressing M protein. HuN4-F112 (attenuated in our laboratory), JXA1-R, TJM-F92, VP046 BIS, and DV are commercial vaccines used in China. Among these vaccines, VP046 BIS and DV are European-type vaccine strains. Mab 1C8 recognized North-American-type strains but not European-type strains. However, Mab 3F7 recognized not only North-American-type strains but also the European-type strain VP046 BIS. The two Mabs specifically recognized M protein expressed in transiently transfected cells. pCAGGS-transfected 293T cells were used as a negative control

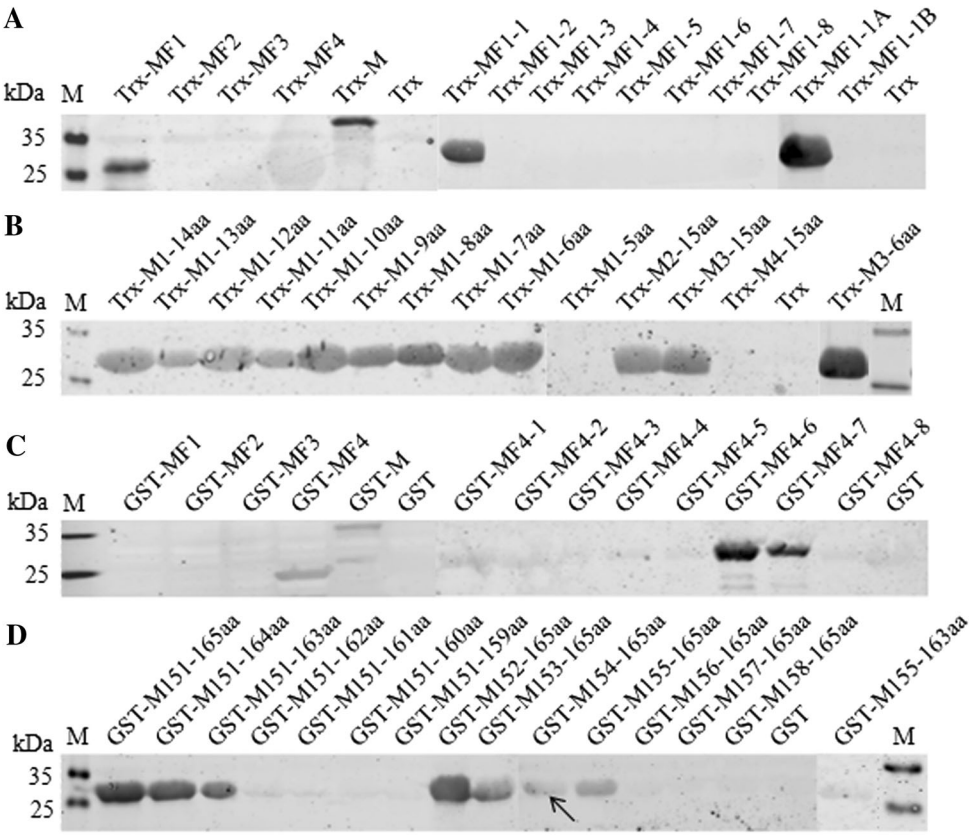

Fig. 3 Truncated M fragments identified by Western blot. A and B: Reactivity of Mab 1C8 with truncated segments. C and D: Reactivity of Mab 3F7 with truncated segments. The segments in A and C were amplified using PCR, while the segments in B and D were obtained by primer annealing. aa 3-6 and aa 155-163 in B and D were identified as the target epitopes

1:128, while Western blot titers were at least 1:512 (data not shown), suggesting that both were high-titer antibodies.

Identification of B-cell epitopes in the M protein recognized by Mabs 1C8 and 3F7

To locate linear antigenic epitopes within the M protein, four overlapping fragments (MF1–MF4) from ORF6 gene were prepared by PCR and cloned into the expression vectors pET32a (+) and pGEX-6p-1 for expression as Trx and GST fusion proteins. Epitope-containing fragments were then identified by Western blot with Mabs 1C8 and 3F7. The results showed that Trx-MF1 (aa 1-55) reacted with 1C8, while GST-MF4 (aa 121-174) reacted with 3F7. Trx-MF1 and GST-MF4 were then further divided into eight fragments (Trx-MF1-1–Trx-MF1-8 and GST-MF4-1–GST-MF4-8). When Mab 1C8 was probed with the Trx-MF1 recombinant polypeptides, only Trx-MF1-1 was recognized by 1C8. Similarly, Mab 3F7 was probed with the GST-MF4 recombinant polypeptides, with overlapping fragments GST-MF4-6 and GST-MF4-7 being recognized by 3F7. This indicated that the regions aa 1-15 and aa 151-165 were the epitope domains recognized by 1C8 and 3F7, respectively. To more precisely define the minimal epitopes recognized by the Mabs, the aa 1-15 and aa 151-165 fragments were further truncated from both ends. We found that 1C8 and 3F7 recognized the minimal epitopes aa 3-6 and aa 155-163, respectively (Fig. 3), indicating that the core sequences recognized by the Mabs 1C8 and Mab 3F7 were ^{3}SSLD^{6} and ^{155}VLGGRKAVK^{163}, respectively (Fig. 4).

Sequence alignments of the epitopes of different PRRSV strains

Amino acid sequence alignments revealed that the two epitopes (aa 3-6 and aa 155-163) on the M protein were both highly conserved between the HP-PRRSV strains and classical PRRSV. However, European strains demonstrated an amino acid deletion and S4→G4, R159→K159, and K160→R160 substitutions (Fig. 4).

Blocking ELISA

A blocking ELISA designed to detect the inhibition of specific Mab binding by incubation with positive sera further confirmed the specificity of the anti-HuN4-F112 Mabs. The OD_{450nm} value of PRRSV-positive serum was inversely proportional to serum concentration (Fig. 5). Therefore, the binding of the Mab 1C8 and Mab 3F7 to the antigen could be inhibited by positive serum. In other words, serum containing antibody to HuN4-F112 competed with the pretitrated Mabs for available epitope. These results showed that the two Mabs hold potential for the development of diagnostic and research tools.

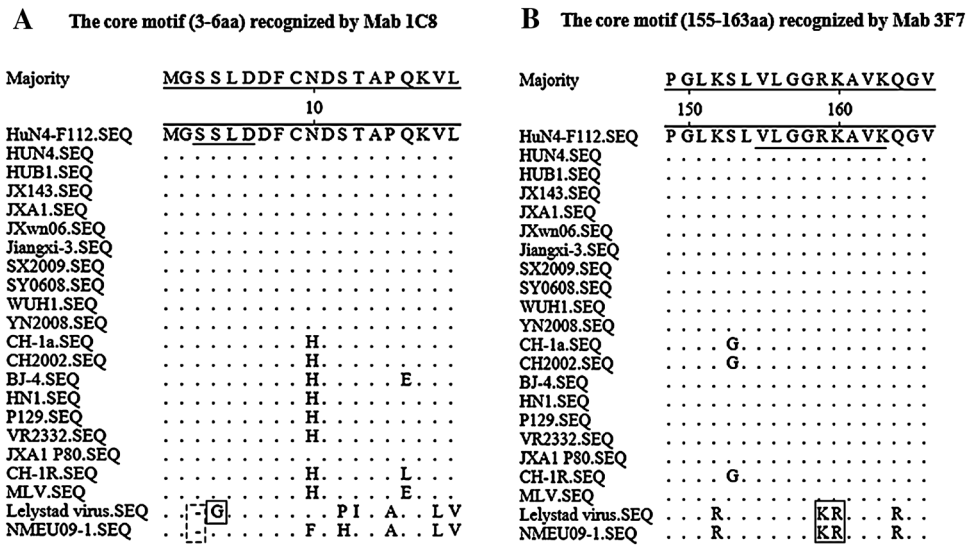

Fig. 4 Multiple sequence alignments of the M protein epitopes of HP-PRRSV, classical PRRSV and vaccine strains. The amino acid sequences of the epitopes identified are underlined. The two epitopes on the M protein were both highly conserved between the HP-PRRSV strains and classical PRRSV. Three amino acids varied between North American-type and European-type strains (black square frames). Hyphens (*dashed box*) represent amino acids deleted from the European PRRSV relative to the sequence of North American virus. Because of this deletion, the epitope M3-6aa does not exist in the European isolates. The amino acid sequences were aligned using the DNASTAR MegAlign software

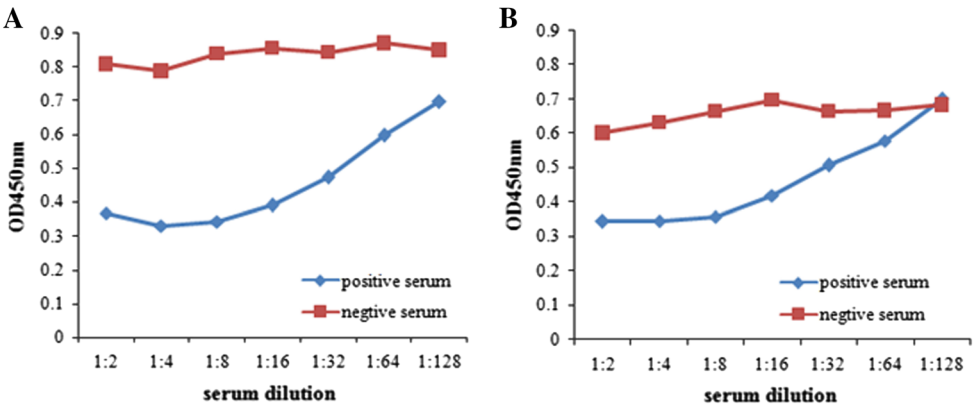

Fig. 5 PRRSV-positive serum blocks binding of Mabs 1C8 (A) and 3F7 (B). As the concentration of PRRSV-positive serum decreased, binding of Mabs to HuN4-F112 increased, as measured by ELISA. Conversely, both Mabs 1C8 and 3F7 could be outcompeted for available epitopes by PRRSV-positive serum. Negative serum failed to compete with Mabs for epitope binding

Discussion

PRRSV has been identified as the primary causative agent of PRRS, a disease resulting in significant economic losses to the global swine industry. However, antibody and antigen diagnostic methods are limited; no detection method has been approved for use to date in China except for the IDEXX HerdChek PRRS X3 antibody test kit. Therefore, there is an urgent need for the development of reliable diagnostic tools.

Previously, the M protein was identified as an important non-glycosylated protein. Precise mapping of epitopes within the M protein is important for understanding the antibody-mediated antiviral response and for developing epitope-based vaccines and diagnostic tools. Previous studies have identified many Mabs against PRRSV structural and nonstructural proteins, along with the target epitopes within these proteins [29–33]. However, diagnostic methods based on these Mabs have not yet been established. In our study, HuN4-F112 was ultracentrifuged and used as antigen to produce two Mabs, which reacted well with the M protein expressed in 293T cells, as determined by IFA. Western blot analysis indicated that the Mabs also reacted well with the recombinant M protein of HP-PRRSV.

Our data indicate that the two Mabs react with different epitopes on the M protein. At first, a series of prokaryotic expression vectors were constructed based on pGEX-6P-1, but poor definition of the positive band of the Mab 1C8:MF1 interaction led us to choose the pET32a (+) vector for subsequent experiments. Mab 1C8 recognizes a previously unidentified epitope, composed of residues 3–6, at the N-terminus of the M protein. Previously, residues 151–174 were identified as an immunoreactive peptide [24]. Consistent with this, the epitope recognized by Mab 3F7 is likely to comprise residues 155–163 at the C-terminal end of the M protein, and this, to the best of our knowledge, represents the first fine mapping of this epitope. Although residue 154 is not within the core sequence recognized by Mab 3F7, it may affect antibody affinity for this epitope (Fig. 3, arrow).

The two epitopes were aligned and compared with other PRRSV M protein sequences in the GenBank database (Fig. 4). The result shows that the two epitopes (aa 3-6 and aa 155-163) on the M protein are both highly conserved in all North American PRRSV strains, whereas European-type strains exhibit variability in these regions. In this study, IFA results indicated that Mab 1C8 could recognize North American-type PRRSV isolates but not European-type strains (Fig. 2). Therefore, Mab 1C8 has potential for use as a differential diagnostic tool for PRRSVs circulating in China.

It is known that PRRSV can induce antibodies in infected animals. In our study, binding of both Mabs to HuN4-F112 was blocked by positive serum of known specificity. The results suggest that the epitopes corresponding to these Mabs can also induce antibodies in pigs following natural infection.

Further research is required to clarify whether the Mabs identified in this study can be used in competitive or indirect ELISAs based on the M protein. This study is the first to describe finely mapped B-cell epitopes on the M protein of PRRSV. These Mabs will facilitate the development of serological diagnostic tests for this virus and also provide valuable insight into the antigenic structure of the M protein.

Acknowledgments This study was supported by the Heilongjiang Natural Science Funds for Distinguished Young Scholars (JC201314), the National Natural Science Foundation of China (grant no. 31001050) and the National High Technology Research and Development Program (863 plan) (Grant No. 2011AA10A208).

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