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Characterization of a serologic marker candidate for development of a live-attenuated DIVA vaccine against porcine reproductive and respiratory syndrome virus

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Abstract

DIVA (differentiating infected from vaccinated animals) vaccines have proven extremely useful for control and eradication of infectious diseases in livestock. We describe here the characterization of a serologic marker epitope, so-called epitope-M201, which can be a potential target for development of a live-attenuated DIVA vaccine against porcine reproductive and respiratory syndrome virus (PRRSV). Epitope-M201 is located at the carboxyl terminus (residues 161-174) of the viral M protein. The epitope is highly immunodominant and well-conserved among type-II PRRSV isolates. Rabbit polyclonal antibodies prepared against this epitope are non-neutralizing; thus, the epitope does not seem to contribute to the protective immunity against PRRSV infection. Importantly, the immunogenicity of epitope-M201 can be disrupted through the introduction of a single amino acid mutation which does not adversely affect the viral replication. All together, our results provide an important starting point for the development of a live-attenuated DIVA vaccine against type-II PRRSV.

Keywords

PRRSV; M protein; Serological marker epitope; DIVA vaccines

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) was first reported in the U.S. in late 1980s [1]. Since then, the disease has been widespread in most swine-producing countries worldwide, causing substantial economic losses to the swine producers. Typical clinical signs associated with the disease include late-term reproductive failure in pregnant sows and...
respiratory distress in young pigs [2]. Recently, a highly pathogenic form of PRRS (HP-PRRS) has emerged in Asia, causing death in pigs of all ages with the mortality up to 100% [3]. The etiologic agent of PRRS is an RNA virus that belongs to the family Arteriviridae, the order Nidovirales, and is referred to as PRRS virus (PRRSV) [4–6]. The PRRSV genome is a linear, positive sense and single stranded RNA molecule of about 15 kb in length, which is flanked by a methyl-cap and a poly (A) tail at its 5’ and 3’ end, respectively [7–9]. It encodes a total of 14 non-structural and 8 structural proteins [7–9], none of which are known to be dispensable for the viral replication cycle.

Vaccines for protection against PRRSV infection have been commercially available since 1994. Two types of PRRSV vaccines are licensed for clinical applications including inactivated and live-attenuated vaccines. No subunit PRRSV vaccines are available at the present. It has been demonstrated that live-attenuated vaccines are much more effective than inactivated vaccines [10,11]. Under field conditions, the use of live-attenuated PRRSV vaccines improves pigs’ performance but co-circulation of field virus and vaccine virus has been reported to occur in the vaccinated herds [12,13]. Under experimental conditions, current live-attenuated vaccines protect the vaccinated pigs from development of clinical disease but they often fail to prevent infection and shedding of the challenge viruses [11,14–16]. One major limitation of the current live-attenuated PRRSV vaccines is that they do not allow serological discrimination between naturally infected and vaccinated animals, a property designated as DIVA (differentiating infected from vaccinated animals).

DIVA vaccines are vaccines that lack at least one antigenic component (so-called serologic marker antigen) when compared to the corresponding wild-type viruses [17]. Therefore, only wild-type virus infected, but not vaccinated, animals will develop antibodies to the marker antigen. Consequently, serological assays that detect antibodies specific to the marker antigen can be used to identify wild-type virus infected animals in the vaccinated population. The use of DIVA vaccines is preferred or even mandatory in animal health campaigns aiming toward control and eradication of important animal diseases [17–19]. Classically, live-attenuated DIVA vaccines are generated through deletion of an entire gene encoding an immunogenic, non-essential protein [17]. While technically straightforward in the case of some double-stranded DNA viruses like Pseudorabies Virus (Suid Herpesvirus 1), it is very difficult to delete an entire gene of a smaller RNA genome virus like PRRSV, whose genes are all essential for the productive viral infection [20–23]. An alternative approach to develop live-attenuated DIVA vaccines for RNA viruses is to selectively eliminate a small protein fragment or an epitope, instead of deleting the whole protein [24–26]. Previously, we identified several immunodominant B-cell epitopes located in non-structural protein 2 (nsp2) and in different structural proteins of a type-II PRRSV strain FL12 [27]. In the present study, we provide detailed characterization of a conserved and immunodominant epitope located at the carboxyl-terminus of PRRSV M protein and propose to use this epitope as a serologic marker for development of live-attenuated DIVA vaccines against type-II PRRSV.
2. Materials and methods

2.1. Antibodies and viruses

A hybridoma cell line expressing monoclonal antibody (MAb) specific to the peptide-M201 (\(161\)AVKQGVVRLVKYAK\(174\)) [27] was produced through a contract with the Immunological Resource Center (Urbana, IL). The peptide was conjugated with Keyhole Lipet Hemocyanin (KLH) and used to immunized mice. After the hybridoma cell line was achieved, ascites fluid production and antibody purification and HRP-conjugation were done through a contract with GenScript USA Inc. (Piscataway, NJ). MAb SDOW-17 specific for PRRSV nucleocapsid protein was purchased from National Veterinary Services Laboratories (Ames, IA). PRRSV negative and positive control serum samples were collected from different experiments previously conducted in our laboratory [28,29]. These included sera from 36 pigs that had been infected with PRRSV strain FL12 or its derivative mutants and 28 pigs that had been infected with PRRSV strain PRRSV-01 or its derivative mutants. These mutant viruses carry different N-linked glycosylation sites in their GP3 and GP5 but they all have intact M protein sequence [28,29]. The PRRSV vaccine strain Prime Pac has been obtained from a commercial batch of that vaccine received in our laboratory in 1997 and maintained through several passages in MARC-145 cells [30]. The collection of 82 type-II PRRSV field-isolates used in this study were collected from Midwestern states (NE, IA and IL) of the U.S.

2.2. Plasmid construction

For alanine-scanning mutagenesis, PRRSV ORF6 carrying alanine substitutions within epitope-M201 region was prepared by site-directed mutagenesis using synthesized mutagenic primers (Table 1). The resulting PCR products were cloned into the pIHA plasmid [31] at the XhoI and NotI restriction enzyme sites. The megaprimmer PCR method [32] was used to prepare mutant pFL12 plasmids that contain mutations in the epitope-M201 region of the PRRSV strain FL12 [33].

2.3. Recovery and in vitro characterization of mutant viruses

Recovery of mutant viruses from the full-genome cDNA clones was done as previously described [33,34]. Multiple step growth kinetics were performed in MARC-145 [35]. Reactivity of the mutant viruses with different MAbs was analyzed by indirect immunofluorescent assay [30].

2.4. Blocking ELISA

Details of the bELISA protocol are described in the supplemental section.

2.5. Animal experiments

All pigs used in this research were housed and handled following the protocols approved by the Institutional Animal Care Committee at University of Nebraska-Lincoln. The pigs were purchased from a swine herd with certified records of absence of PRRSV infection and were accommodated in an isolated bio-safety level-2 facility. The pigs were about 3–4 weeks of age at the beginning of the experiment. The pigs were separately infected with one of the
following PRRSV strains: PP-virus, Q164R and N168R. In all cases, the pigs were inoculated intramuscularly with $10^{5.0}$ TCID$_{50}$ of virus diluted in 2 ml of cell culture medium. Serum samples were collected from all animals before infection and periodically after infection and stored in small aliquots at −80 °C for serological and virological analysis.

3. Results

3.1. Epitope-M201 is conserved and immunodominant

Epitope-M201, spanning the residues 161–174 of M protein (Fig. 1A) is well conserved among type-II PRRSV isolates. Analysis of 100 type-II PRRSV M protein sequences collected from GenBank revealed that all of the amino acid residues of epitope-M201 were identical except the residue at position 164, where 16% of the analyzed sequences had a substitution from glutamine to arginine (Fig. 1B). The monoclonal antibody specific to epitope-M201 (MAb-201) recognized 92% ($n = 82$) PRRSV field-isolates originating from the Midwestern states of the U.S. (Fig. 1C), confirming the conservation of this epitope. Through pepscan analysis, we reported previously that 100% ($n = 15$) of pigs infected with the highly virulent PRRSV strain FL12 developed antibodies against epitope-M201 [27]. To further confirm the immunogenicity of this epitope, we developed a bELISA that allowed us to measure antibodies to epitope-M201 more specifically. Using this bELISA we showed that 63 out of 64 pigs (98.4%) infected pigs had antibodies specific to epitope-M201 at 35 days p.i. (Fig. 1D). Detailed analysis revealed that antibodies to epitope-M201 appeared at about 14 days p.i. (Fig. 1E). Together, the results presented here confirm that epitope-M201 is highly immunodominant as previously anticipated [27].

3.2. Core amino acid sequence of epitope-M201

To determine the core amino acid residues of the epitope-M201, we constructed an expression vector encoding the M protein of PRRSV strain FL12. Using this vector as a backbone, we performed alanine-scanning mutagenesis where amino acids of the epitope-M201 were replaced by alanine (Table 2). Cells expressing the Mut-4 construct carrying alanine substitutions at the last 4 amino acids of epitope-M201 were still recognized by MAb-201. By contrast, cells transfected with mutant plasmids carrying substitutions within the first 10 amino acids were not recognized by MAb-201. The results indicated that the core amino acid sequence of epitope-M201 resided within its first 10 amino acids (residue 161–170).

3.3. Generation and characterization of N168R mutant virus

Mutations that disrupt the antigenicity of epitope-M201, as determined by transient expression, were incorporated into the pFL12 plasmid [33]. At 48 h post-electroporation with the mutant RNA transcripts, cells were immunostained with MAb SDOW-17 specific to viral N protein. Specific signal was detected from cells electroporated with all mutant RNA transcripts (data not shown), suggesting that mutations within epitope-M201 did not affect viral sub-genomic mRNA transcription. However, we could not recover viable mutant viruses from any of those constructs (Table 2), indicating that amino acids within the epitope-M201 region were essential for productive infection.
By comparison between Mut-4 and Mut-6 constructs (Table 2), it appeared that a single amino acid substitution from asparagine to alanine at position 168 was sufficient to abolish the antigenicity of epitope-M201. Further analysis revealed that 5 out of 16 amino acids at the carboxyl-terminus of the FL12 M protein, including 3 residues inside epitope-M201 and 2 residues immediately upstream of epitope-M201, were positively charged. Consequently, a mutant virus N168R was generated by mutating asparagine 168 to arginine, a positive charge amino acid, instead of alanine. The N168R mutant virus replicated as efficiently as the parental wild-type FL12 in cell culture (Fig. 2A). More importantly, the N168R mutant did not react with MAb-201 (Fig. 2B), indicating that antigenicity of epitope-M201 was successfully disrupted.

To study the stability and immunogenicity of the N168R mutation 4 recently weaned pigs were infected with N168R mutant virus. Analysis of the consensus sequences of the viruses from serum samples collected at 7 days p.i. revealed that two viruses exhibited arginine to asparagine mutation; one virus displayed arginine to serine mutation and one had an ambiguous sequence (Fig. 2C). Detailed analysis of the sequencing chromatograms indicated that all the serum-derived viruses carried a mix of nucleotide sequences at codon 168 (Fig. 2D). All pigs infected with N168R mutant were tested positive by the bELISA at 32 days p.i. (Fig. 2E). The results further confirmed that reversion mutation had occurred in all 4 infected pigs. As expected, all infected pigs were tested positive by the IDEXX ELISA (Fig. 2F).

3.4. Generation and characterization of Q164R mutant virus

To seek a more stable mutant deprived of epitope-M201 immunogenicity, we investigated the M protein sequences of PRRSV strains/isolates that were naturally not recognized by MAb-201 (Fig. 1C) and found that they all carried a single amino acid substitution within the epitope-M201 region, from glutamine to arginine at position 164 (Q164R) (Fig. 3A). To test whether the Q164R substitution was sufficient to abolish the antibody response to epitope-M201, a group of 10 pigs were inoculated with the attenuated PRRSV vaccine strain Prime Pac (herein designated as PP-virus) [36], one of the PRRSV strains that were not recognized by MAb-201 (Fig. 3A). Serum samples collected from pigs infected with PP-virus were tested negative by the bELISA (Fig. 3B), indicating that the Q164R substitution eliminates the immunogenicity of epitope-M201.

Based on this finding, we constructed the Q164R mutant virus by mutating glutamine 164 to arginine. In addition, we also incorporated into the Q164R mutant virus two additional mutations (arginine 159 to lysine and lysine 160 to arginine) that are located immediately upstream of epitope-M201. We reasoned that the incorporation of these two additional mutations may help stabilize the Q164R mutation because they were commonly found in the epitope-M201 negative PRRSV isolates (Fig. 3A). The growth kinetics of Q164R mutant virus was slightly reduced as compared to the wild-type FL12 strain (Fig. 4A). Importantly, the Q164R mutant virus was no longer recognized by MAb-201 (Fig. 4B), indicating that antigenicity of epitope-M201 was successfully disrupted.

Two pigs were inoculated with Q164R mutant virus to study the stability and immunogenicity of the virus. ORF6 region of the virus in serum samples collected at 14
days p.i. was sequenced, which confirmed that all 3 mutations of the Q164R mutant virus were stably maintained at this time point (Fig. 4C and D). As expected, antibodies to epitope-M201 were absent in pigs infected with the Q164R mutant virus at all time-points analyzed (Fig. 4E). Conversely, all infected pigs were tested positive by the IDEXX ELISA (Fig. 4F). Together, the results confirm that the Q164R mutation eliminates the immunogenicity of epitope-M201.

4. Discussion

Since the initial application of DIVA vaccines to eradicate Pseudorabies Virus in pigs, epidemiological and regulatory considerations dictate that a DIVA vaccine should be designed based on a “negative” serologic marker antigen [17,19]. A serologic marker antigen is a viral protein or an epitope that is absent (thus “negative”) from the vaccine strains but consistently present in the corresponding wild-type viruses. Therefore, only animals that have been infected with wild-type virus will develop antibodies against the serologic marker antigen while the vaccinated animals will not. The most critical step in the development of a live-attenuated DIVA vaccine is the identification of a potential serologic marker antigen. Since a DIVA vaccine is only useful if it is used in conjunction with a companion diagnostic test, the marker antigen should be conserved and immunodominant to ensure that the companion diagnostic test based on this marker antigen will produce reliable diagnostic results. In addition, the marker antigen should be dispensable for the viral life cycle but should not contribute significantly to the overall immunogenicity of the vaccine. These two criteria are to ensure that elimination of the marker antigen from the viral genome will not adversely affect the viral replication and its ability to elicit protective immunity. The PRRSV genome encodes 14 non-structural and 8 structural proteins, several of which are well conserved and/or highly immunogenic [37–40]. Up to now, efforts to delete an entire gene of PRRSV have not been successful [20–23]. Therefore, the most feasible approach to develop a live-attenuated DIVA vaccine against PRRSV would be selectively eliminating a small protein fragment or an epitope, instead of deleting an entire protein. Thus far, efforts to develop a DIVA vaccine for PRRSV have been focused on deleting different immunogenic fragments of the viral nsp2 because this protein can tolerate large deletions [41–43]. However, the biggest shortcoming of the nsp2-mutant viruses, in the context of a DIVA vaccine, is that the differential peptide-based ELISA used in conjunction with the DIVA vaccine has very limited diagnostic sensitivity [42], mainly due to the substantial genetic variation of the nsp2 [44,45].

Epitope-M201 can be a potential serologic marker candidate for development of a live-attenuated DIVA vaccine against PRRSV. The epitope is highly immunodominant and well-conserved among PRRSV isolates. In addition, the epitope does not seem to contribute significantly to the protective immunity against PRRSV infection because a polyclonal antibody specific to this epitope, as well as MAb-201, lacks PRRSV-neutralizing ability (unpublished results). Initially, we attempted to delete the whole or portions of epitope-M201 because this would minimize the chance of reversion to wild-type sequence. Unfortunately, we could not recover any mutant viruses carrying deletions in the epitope-M201 region (unpublished results). Similarly, we could not recover any viruses that carried multiple amino acid substitutions within epitope-M201 (Table 2). The results indicate the
important functions of this protein segment in the viral replication cycle. Epitope-M201 is located at the cytoplasmic tail of M protein, which has been suggested to interact with N protein [46]. It is possible that deletions or substitutions of multiple amino acids within epitope-M201 may interfere with the interaction between M and N proteins, thus, being detrimental to the virus. Through studying PRRSV isolates that were naturally not recognized by MAAb-201, we identified that a single amino acid substitution Q164R is sufficient to abolish the immunogenicity of epitope-M201. We demonstrated that the Q164R mutation can be introduced into the genome of the PRRSV strain FL12 without severely impairing the viral replication. Importantly, the Q164R mutant virus stably maintained its mutations and lost its capacity to elicit antibodies to epitope-M210 when it was inoculated into pigs. It has been previously reported that reversion to wild-type sequence occurred after the PRRSV mutant viruses were inoculated into pigs [47]. In the present study, we observed that the N168R mutant virus, but not the Q164R mutant virus, quickly reverted to wild-type sequence after the first week of inoculation into the pigs. We believe that the Q164R mutant virus was stable in vivo because it carries mutations mimicking the natural PRRSV sequences that have already been selected by nature.

Validation data based on a set of control serum samples collected from pigs experimentally infected with different PRRSV strains indicated that our bELISA could correctly detect 98.4% (n = 64) infected animals (Fig. 1D). However, before the definitive adoption of this bELISA as a companion serological test to be used with the epitope-M201-negative DIVA vaccine, it would be essential to validate the performance of this test with a large collection of clinical serum samples. Our results indicate that epitope-M201 is absent in approximately 10–15% of PRRSV field isolates (Fig. 1B and C). This will potentially affect the diagnostic sensitivity of our bELISA. In the scenario that our bELISA has significantly low diagnostic sensitivity when it is used with clinical serum samples, the test may need to be used for herd diagnosis instead of individual animal diagnosis. This way, the low diagnostic sensitivity of the test may be overcome by increasing the test samples collected from each herd.

Alternatively, we could consider the possibility of eliminating one additional marker epitope from the genome of the Q164R mutant virus to produce a double negative marker PRRSV strain that simultaneously lacks 2 immunodominant epitopes in its genome. This is based on the contention that the occurrence of PRRSV field strains that simultaneously lack two immunodominant marker epitopes would be negligible.

In summary, we report here the characterization of a potential serological marker epitope located in the M protein of PRRSV. We demonstrate that the immunogenicity of this epitope can be eliminated through the introduction of a single amino acid mutation which does not severely affect the viral replication. We believe that this mutation can be easily introduced into the genome of conventional live-attenuated type-II PRRSV vaccine strains in order to convert them into vaccine with DIVA capability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.
Acknowledgments

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References


Fig. 1.
Epitope-M201 is highly conserved and immunodominant. (A) Prediction of transmembrane helices and topology of the PRRSV strain FL12 M protein. The figure was generated through the use of the web based application SOSUI engine ver. 1.11. Epitope-M201 is outlined in red at the carboxyl-terminus of the protein. (B) Sequence logo of epitope-M201 generated from 100 type-II PRRSV M protein sequences collected from GenBank. The relative positions of the amino acids are indicated in the X-axis. The height of symbols within the stack indicates the relative frequency of each amino at that position. The figure was generated through the use of the web based application WebLogo 3.3. (C) Reactivity of MAb-201 with 82 type-II PRRSV strains/isolates as determined by indirect immunofluorescence assay (IFA). (D) Validation of the bELISA. The positive and negative control sera were collected from 64 pigs before infection and at 35 days p.i. (details of the serum samples are described in Section 2). (E) Kinetics of seroconversion to epitope-M201 as determined by bELISA. The sera were collected from the same pigs mentioned in panel (D). The horizontal dotted line represents the cut-off of the assay. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)
Fig. 2.
Characterization of the N168R mutant virus. (A) Multiple step growth curves of the indicated viruses upon infection of MARC-145 cells. Viral titers are expressed as mean and standard error of mean (SEM) of data obtained from three independent experiments. (B) Reactivity of the indicated virus with different MAbs. (C) ORF6 region of viruses in the serum samples collected at 7 days p.i. was amplified by RT-PCR and subjected to DNA sequencing. The consensus sequences of the serum-derived viruses were aligned with that of input virus (before inoculation). Only the epitope-M201 region is shown. The conserved residues in the aligned sequences are indicated by (.). The X represents the ambiguous sequence. (D) Sequencing chromatograms of one representative serum-derived virus (from pig # 918) at 7 days p.i. Only region covering the mutation sites is shown. The triplet codon 168 is underlined. (E) Seroconversion to epitope-M201 of pigs infected with N168R mutant virus as measured by bELISA. Data are expressed as mean and SEM of 4 infected pigs. For the comparison purposes, kinetics of seroconversion to epitope-M201 of pigs infected with FL12 and PRRSV-01 (Fig. 1E) are shown. (F) Antibodies to N protein of pigs infected with N168R mutant virus as determined by the IDEXX ELISA. Data are expressed as mean and SEM of 4 infected pigs. The horizontal dotted lines in panels (E) and (F) indicate the cut-off of the assays.
Fig. 3.
A single amino acid substitution Q164R is sufficient to disrupt epitope-M201. (A) Multiple sequence alignment of the epitope-M201 region of the PRRSV isolates that were naturally not recognized by MAb-201. Conserved residues in the aligned sequences are indicated by (.). The gray box depicts epitope-M201 region. (B) Seroconversion to epitope-M201 of pigs infected with the attenuated PRRSV vaccine strain Prime Pac (PP-virus) as measured by bELISA. Data are expressed as mean and SEM of 10 infected pigs. Kinetics of seroconversion to epitope-M201 of pigs infected with FL12 and PRRSV-01 (Fig. 1E) are shown for the comparison purposes. The horizontal dotted line indicates the cut-off of the assay.
Fig. 4.
Characterization of the Q164R mutant virus. The experiments in this figure were done similar to those described in Fig. 3. (A) Multiple step growth curves. (B) Reactivity with different MAbs. (C) Multiple sequence alignment of the epitope-M201 regions of the viruses from serum samples collected at 14 days p.i. (D) Sequencing chromatograms of one representative serum-derived virus (from pig #860) at 14 days p.i. The triplet codons where amino acid mutations were introduced are underlined. (E) Seroconversion to epitope-M201 of pigs infected with Q164R mutant virus measured by bELISA. (F) Antibodies to N protein of pigs infected with Q164R mutant virus as determined by the commercial IDEXX ELISA.
### Table 1

**Primers used in this study**

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**For construction of full-length pFL12 plasmids containing different mutations in epitope-M201**

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Mutated nucleotides are underlined. Restriction enzyme sites incorporated into the primers for cloning purposes are shown in bold.
Table 2
Mutagenesis to disrupt the antigenicity of epitope-M201

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\(^a\)IFA: indirect immunofluorescence assay with the MAb-201.

\(^b\)ND, not done.