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Hypervariable antigenic region 1 of classical swine fever virus E2 protein impacts antibody neutralization

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ABSTRACT

Envelope glycoprotein E2 of classical swine fever virus (CSFV) is the major antigen that induces neutralizing antibodies and confers protection against CSFV infection. There are three hypervariable antigenic regions (HAR1, HAR2 and HAR3) of E2 that are different between the group 1 vaccine C-strain and group 2 clinical isolates. This study was aimed to characterize the antigenic epitope region recognized by monoclonal antibody 4F4 (mAb-4F4) that is present in the group 2 field isolate HZ1-08, but not in the C-strain, and examine its impact on neutralization titers when antisera from different recombinant viruses were cross-examined. Indirect ELISA with C-strain E2-based chimeric proteins carrying the three HAR regions showed that the mAb-4F4 bound to HAR1 from HZ1-08 E2, but not to HAR2 or HAR3, indicating that the specific epitope is located in the HAR1 region. Of the 6 major residues differences between C-strain and field isolates, Glu713 in the HAR1 region of strain HZ1-08 is critical for mAb-4F4 binding either at the recombinant protein level or using intact recombinant viruses carrying single mutations. C-strain-based recombinant viruses carrying the most antigenic part of E2 or HAR1 from strain HZ1-08 remained non-pathogenic to pigs and induced good antibody responses. By cross-neutralization assay, we observed that the anti-C-strain serum lost most of its neutralization capacity to RecC-HZ-E2 and QZ-14 (subgroup 2.1d field isolate in 2014), and *vice versa*. More importantly, the RecC-HAR1 virus remained competent in neutralizing ReC-HZ-E2 and QZ-14 strains without compromising the neutralization capability to the recombinant C-strain. Thus, we propose that chimeric C-strain carrying the HAR1 region of field isolates is a good vaccine candidate for classical swine fever.

1. Introduction

Classical swine fever (CSF) is a highly contagious disease of swine and wild boar caused by classical swine fever virus (CSFV), an enveloped RNA virus with a genome size of approximately 12.3 kb. A single ORF, flanked by 5′-terminal and 3′-terminal non-translated regions, encodes a polyprotein of 4000 amino acids (aa) which is processed by viral and cellular proteases into four structural (C, Erns, E1, and E2) and eight non-structural proteins (Npro, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [1].

The glycoprotein E2 plays multiple roles in viral life cycle, such as virus attachment, entry into target cells [2], cell tropism [3] and virulence [4]. It is the most immunogenic protein of the virus and induces neutralizing antibodies effective against lethal challenge [5]. CSFV consists of a single serotype. However, analysis of E2 nucleotide has revealed genetic diversity of CSFV strains that could be classified into three groups. Group 1 (with subgroups 1.1, 1.2 and 1.3) contains most of the historical strains, including C-strains. Group 2 (with subgroups 2.1, 2.2 and 2.3) comprises most of the current field strains. Group 3 (with subgroups 3.1, 3.2, 3.3 and 3.4) includes most of the strains distributed in some particular geographic regions [6].

Variations of E2 in field strains influence the reaction patterns and cross-neutralization with sera from heterologous strains and monoclonal antibodies (mAbs) [7,8]. Probing with different mAbs could reveal the antigenic structure and diversity of E2 from different CSFV isolates [9,10]. Four antigenic domains, A to D, are located within the N-terminal half of E2 and constitute two independent antigenic units, in the order of domains B/C and A/D (aa690-800 and aa766-865, respectively) [11,12]. Early studies reported several important antigenic regions involving both conformational and linear epitopes within E2. The two regions ^{772}LFDGSNP^{778} and ^{829}TAVSPTTLR^{837} were considered to be CSFV specific [13,14], while ^{994}YYEP^{997} is conserved among pestiviruses [15]. The regions ^{692}CKEDYRY^{698} and ^{753}RYLASLHKKALPTSV^{767} have not yet been

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tested for their specificity [16,17]. In addition, the residues E^{713} and D^{729} are responsible for antigenic specificity of field strains in Taiwan, while the D^{705} and K^{761} residues are specific for the vaccine strain [16].

Our previous report proposed three hypervariable antigenic regions of E2, HAR1 (aa702-731), HAR2 (aa774-799) and HAR3 (aa841-864) [18]. HAR1 and HAR2 fall within the B/C domain. Recent studies indicate that the virus populations in the field have switched from group 1 to group 2 [19–22]. There has been chronic or atypical form of the disease in the endemic areas with recovery of subgroup 2.1 isolates in vaccinated pig farms in China [19,20,23]. These findings suggest that classical group 1 lapinized C-strain vaccine is facing challenge with regard to its protective efficacy against the field strains. This study was aimed to identify the critical region of antigenic divergence of the E2 of field CSFV isolates recognized by a specific monoclonal antibody 4F4 that might impact viral neutralization. The results could be extended for future development of recombinant C-strain-based candidate vaccine strains containing E2 or its major antigenic region from prevalent clinical isolates for enhanced protection of classical swine fever in the field.

2. Materials and methods

2.1. Cells and viruses

PK-15 cells were grown at 37 °C and 5% CO_2 in Dulbecco's minimal essential medium (DMEM, Hyclone, South Logan, UT, USA) with 10% fetal calf serum (Hyclone). Four CSFV strains were used: C-strain and subgroup 2.1 clinical isolates (strain HZ1-08 [18], as well as two recent isolates QZ-14 and JH-14). The virus strains were propagated and titrated in PK-15 cells. Virus stocks were stored at −80 °C. Recombinant baculoviruses were grown and passaged in Sf9 cells with Sf-900™III SFM (Invitrogen, Eugene, Oregon, USA).

2.2. Construction of eukaryotic expression plasmids

The 1212-bp cDNA fragment encoding the signal sequence and full-length E2 of C-strain and three subgroup 2.1 strains (HZ1-08, QZ-14 and JH-14) as well as six mutant C-strain E2 proteins carrying single substitution ($^{D}705^{N}$, $^{L}709^{P}$, $^{G}713^{E}$, $^{N}723^{S}$, $^{D}725^{G}$ and $^{N}729^{D}$) in the HAR1 region of subgroup 2.1 strains were cloned into pcDNA3.1 according to the method previously described [24].

2.3. Expression of recombinant E2 proteins in baculoviruses

The vector pFastBac™HT B was used to construct recombinant plasmids containing the following target sequences according to Zhang [25]: truncated E2 without the transmembrane region from C-strain (C-tE2) and field isolate HZ1-08 (HZ-tE2) as well as C-strain E2-based mutant proteins containing HAR1 (aa702-731, C-HAR1), HAR2 (aa774-799, C-HAR2) or HAR3 (aa841-864, C-HAR3) regions from the field isolates. The plasmids were transfected into Sf9 cells by Cellfectin®II Reagent (Invitrogen). Recombinant baculoviruses were passaged at 28 °C three times, 72 h each to obtain viral stocks used for batch culture. Target proteins were purified from batch culture supernatants according to the manufacturer's instructions (Invitrogen).

2.4. Construction of C-strain-based recombinant viruses

Recombinant CSF viruses RecC-HZ-E2 and RecC-HAR1 were generated from the C-strain-based infectious clone pA-FL22 by exchanging the 870-bp and 90-bp antigenic regions, respectively, with the equivalent regions of the field isolate HZ1-08. The HAR1 deletion mutant was constructed and named as RecC-ΔHAR1. Each of the six different residues ($^{D}705^{N}$, $^{L}709^{P}$, $^{G}713^{E}$, $^{N}723^{S}$, $^{D}725^{G}$ and $^{N}729^{D}$) was mutated and introduced into pA-FL22 using the Quick Change XL Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). All substitutions were verified by sequencing. Each of the infectious clones was linearized by *Xho*I and transcribed *in vitro* using the T7 Megascript system (Ambion, Austin, TX, USA). After TURBO DNase I (Ambion) digestion, the RNA transcripts were precipitated with LiCl and quantified by spectrophotometer ND-1000 (Nano Drop Technologies, Marina, CA, USA). Each of the RNA transcripts was transfected into PK-15 cells by electroporation at 150 V with a BTX 630 electroporator (BTX, San Diego, CA, USA). Cells were grown in 25-cm^2 flasks and incubated for 4 days at 37 °C and 5% CO_2. Generation of virus particles was detected by immunofluorescence using the anti-E2 monoclonal antibody (mAb) as described below. Stocks of rescued viruses were stored at −80 °C.

2.5. Generation of monoclonal and polyclonal antibodies specific for CSFV or its E2

The murine mAbs 4F4 was generated against the recombinant HZ-E2-AD protein covering the two antigenic units (B/C + A/D) of the strain HZ1-08, four 5-week-old female specific-pathogen-free BALB/c mice were immunized subcutaneously with 0.1 mg of the purified protein emulsified in the complete Freund's adjuvant and intraperitoneally boosted twice with the protein emulsified in incomplete Freund's adjuvant at 2-week intervals. The mice were euthanized 2 weeks after the last boosting. Spleen cells were harvested and fused with SP2/0 myeloma cells using 50% (v/v) polyethylene glycol (PEG, Sigma–Aldrich, St. Louis, MO, USA). The resulting hybridomas secreting antibodies against the whole virus HZ1-08 were selected by immunofluorescence assay (IFA). Ascites were produced in pristine-primed BALB/c mice.

The rabbit antiserum to C-strain was generated by intravenous injection of 10^3 $TCID_{50}$ of virus into New Zealand white rabbits with boosting after 21 days. The pig immune sera against CSFVs: C-strain (RecC, 3 pigs), field isolate QZ-14 (2 pigs) as well as recombinant viruses RecC-HZ-E2 (3 pigs) and RecC-HAR1 (3 pigs) were produced by intramuscular injection in the neck region of 30-day-old CSFV-free pigs with about 10^4 $TCID_{50}$ in a biosafety level III facility, respectively. 3 pigs inoculated with 1 ml DMEM were set as mock. Each group was housed in separate rooms. Rectal temperature and clinical signs were recorded daily. Serum samples were collected at day 0, 3, 7, 10, 14, 21 and 28 post-immunization or post-infection (dpi) and stored at −80 °C until use. The sera collected at 28 days dpi from pigs of the same group were pooled for indirect ELISA and viral neutralization assays. The animal experiments were approved by the Laboratory Animal Management Committee of Zhejiang University (Approval No. 20141116).

2.6. Binding of recombinant proteins with monoclonal antibody by indirect ELISA

Indirect ELISA was performed in triplicate under stringent conditions to avoid nonspecific binding [18]. Binding to mAb-4F4 was compared among five proteins (C-tE2, HZ-tE2, C-HAR1, C-HAR2 and C-HAR3) expressed in the baculovirus system. Wells of plates were coated with 5 μg/mL (0.5 μg/well) of proteins and the antibodies were above saturation levels to ensure that antibody concentration was not limiting. All OD_{450} values were normalized to anti-His-tag binding and presented as mean ± SD of three independent experiments.

2.7. Indirect immunofluorescence assay

Cells infected with viruses on 24-well plates (Costar; Corning, NY, USA) were fixed with ethanol–acetone (1:1 by volume) for 20 min at −20 °C, washed twice in PBS (10 mM), and blocked in PBS containing 1% BSA. Different primary antibodies diluted in PBS containing 0.5% non-fat milk powder were incubated for 2 h at 37 °C. The plate was washed three times with PBS and incubated with Alexa-Fluor-labeled anti-mouse or anti-rabbit secondary antibodies (488 nm or 568 nm; Invitrogen) at 37 °C for 1 h. The cells were washed and examined under a fluorescence microscope (X81, Olympus, Japan).

2.8. Quantification of viral RNA by real-time RT-PCR

The viral RNA was extracted from the cell culture supernatants of the infected cells or the whole blood of infected pigs using an RNA extraction kit (Invitrogen) Viral RNA level was quantified with the primers CSFV-F (5′-GAACTGGGCTAGC CATG-3′) and CSFV-R (5′-ACTGTCCTGTACTCAGGAC-3′) via real-time RT-PCR in qPCR machine (BIO-RAD iQ™5, Berkeley, CA, USA). Viral RNA copy numbers were interpolated from a reference curve established by detecting the CT values of the serially diluted recombinant pUC18 vector carrying the 5-UTR sequence of CSFV. The results were expressed as the mean of the logarithmic viral RNA copies per ml blood following a previously described method [26].

2.9. Single-step growth curve

Growth characteristics of rescued mutant viruses RecC-HZ-E2 and RecC-HAR1 were evaluated relative to the wild-type recombinant C-strain virus in a single-step curve. Monolayer PK-15 cells in 24-well plates were infected with 150 $TCID_{50}$ (MOI = 0.001). After absorption for 1 h at 37 °C, the supernatant samples were removed and replaced with a volume of 0.7 ml of fresh medium. Cell samples were collected at 12, 24, 48, 72, and 96 h post-infection (hpi) for virus titration and quantification of viral RNA [24].

2.10. Virus neutralization in vitro

Virus neutralization was assessed as described previously [20]. Antibody titers of pig sera were determined by indirect ELISA using prokaryotic protein HZ-E2-AD and C-E2-AD as the coating antigen. Briefly, each of the stock viruses (C-strain, RecC-HZ-E2 and QZ-14, all at about 200 $TCID_{50}$) was mixed with an equal volume of 2-fold serially diluted pig immune sera and incubated at 37 °C for 1 h before being inoculated onto the pre-seeded PK-15 cells in 96-well plates. The starting dilution factor of each serum was 1:50. At 72 hpi, cells were fixed and stained for glycoprotein E2 by IFA. The protective dose 50 (PD50) is calculated by Trimmed Spearman–Karber method with formula: lgPD50 = L + d (S-0.5). L logarithmic lowest serum dilution, d is the dilution factor, S is the sum of protection ratio.

3. Results

3.1. Reaction patterns of monoclonal antibody 4F4 with CSFV Strains

Indirect immunofluorescence assay showed that mAb-4F4 reacted only to PK-15 cells infected with group 2 strains but not those with vaccine C-strain (Fig. 1A). Cells transiently expressing the E2 also revealed the same pattern of reaction (Fig. 1B). Thus, we speculated that mAb-4F4 recognizes an epitope located in E2 proteins of subgroup 2.1 strains.

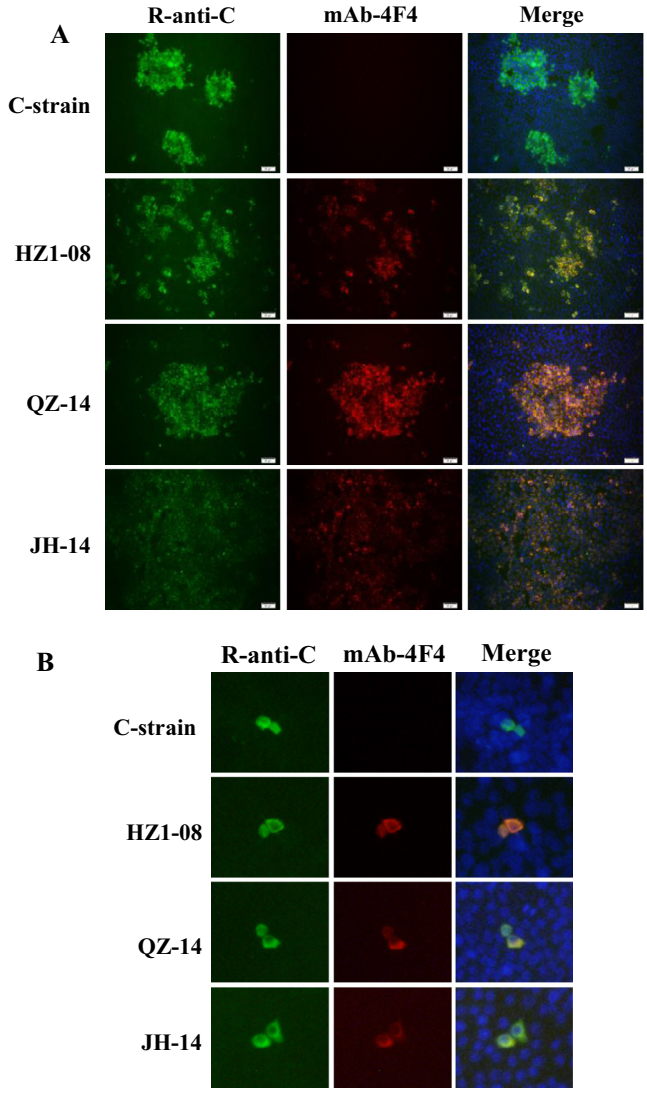

Fig. 1. Reaction patterns of the monoclonal antibody 4F4 to different classical swine fever virus strains (A) or to recombinant E2 expressed in PK-15 cells (B) Rabbit anti-C-strain polyclonal antibodies were used for comparison.

3.2. Neutralizing activity of monoclonal antibody against CSFV Strains

The specificity of mAb-4F4 to group 2 strains suggests that the epitope may be involved in viral neutralization. Fig. 2 shows that the mAb-4F4 had the neutralization titer of more than $10^{-3.8}$ to group 2 strains QZ-07, QZ-14 and JH14, but with no neutralization activity to the C-strain, indicating that the epitope of mAb-4F4 is involved in neutralization in group 2 strains.

3.3. Identification of the antigenic region associated with monoclonal antibody 4F4

We further characterized whether the specific antigenic region recognized by mAb-4F4 is located in three hypervariable antigenic regions [18]. Indirect ELISA was used to examine the binding of the mAb-4F4 (with anti-C-strain sera as reference) with the recombinant E2 proteins or C-strain E2-based mutants containing HAR1, HAR2 and HAR3 regions of group 2 viruses expressed in baculovirus system (Fig. 3A). MAb-4F4 bound to C-HAR1 and the HZ-tE2 protein, but not to other proteins. All recombinant proteins reacted well with the anti-C strain serum. This suggests that

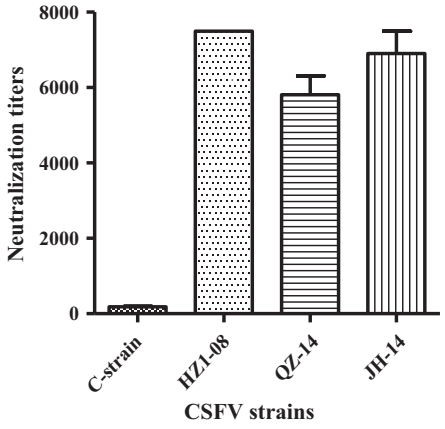

Fig. 2. Neutralization of different classical swine fever virus strains by the monoclonal antibody 4F4 in PK-15 cells. At 72 h post-infection, the cells were fixed and stained for immunofluorescence.

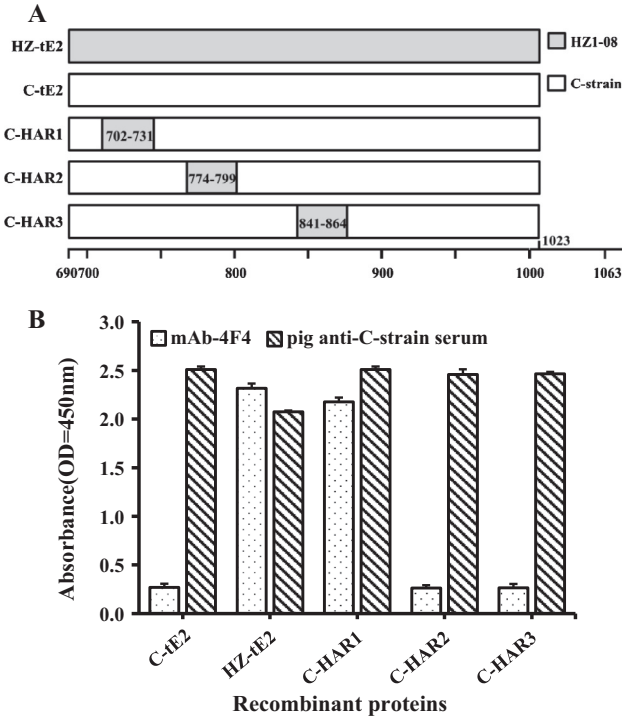

Fig. 3. Determination of the antigenic regions of classical swine fever virus E2 recognized by the monoclonal antibody 4F4. (A) Schematic presentation of the chimeric E2 proteins of classical swine fever virus using the C-strain E2 as backbone hybridized with HAR1 (aa702-731), HAR2 (aa774-799) and HAR3 (aa841-864) of a field isolate HZ1-08, named here at C-HAR1, C-HAR2 and C-HAR3, respectively. (B) The recombinant proteins expressed in baculoviruses were examined for binding with mAb-4F4 and pig anti-C-strain serum by indirect ELISA.

residues in HAR1, but not those of HAR2 or HAR3, contributed to the binding with mAb-4F4.

3.4. Fine mapping of residues responsible for mAb-4F4 binding

To further define the specific residues responsible for the recognition by mAb-4F4, the amino acid sequences of HAR1 between residues 690 and 720 derived from different subgroups of CSFVs were compared. We found that residues 705, 709, 713, 723, 725 and 729 in HAR1 region vary between group 2 strains and C-strain. Single substitutions of the C-strain-based E2 with these different residues were examined for their binding ability to mAb-4F4 by IFA. The mutant protein with $^{G}713^{E}$ had apparent reactivity to mAb-4F4, whereas other substitutions did not show significant changes (Fig. 4). It is clear that Glu713 in the field strains is the key residue for the mAb-4F4 reactivity.

3.5. Effects of different substitutions of E2 in C-strain-based recombinant viruses on reactivity to the monoclonal antibody 4F4

C-strain-based recombinant viruses either with HAR1 deletion (RecC-ΔHAR1) or with single substitutions (RecC-$^{D}705^{N}$, $^{L}709^{P}$, $^{G}713^{E}$, $^{N}723^{S}$, $^{D}725^{G}$ and $^{N}729^{D}$) and sequence replacement (RecC-HAR1 and RecC-HZ-E2), all based on the differences between C-strain and genotype 2.1 isolate HZ1-08, were constructed in order to verify the key residues of mAb-4F4 recognition in the whole virus. The recombinant virus RecC-ΔHAR1 failed to be rescued, suggesting that this region is essential for virus replication or release. The other viruses were successfully rescued. We further examined the reaction patterns of mAb-4F4 with the rescued viruses. Only single mutant virus RecC-$^{G}713^{E}$ showed reactivity

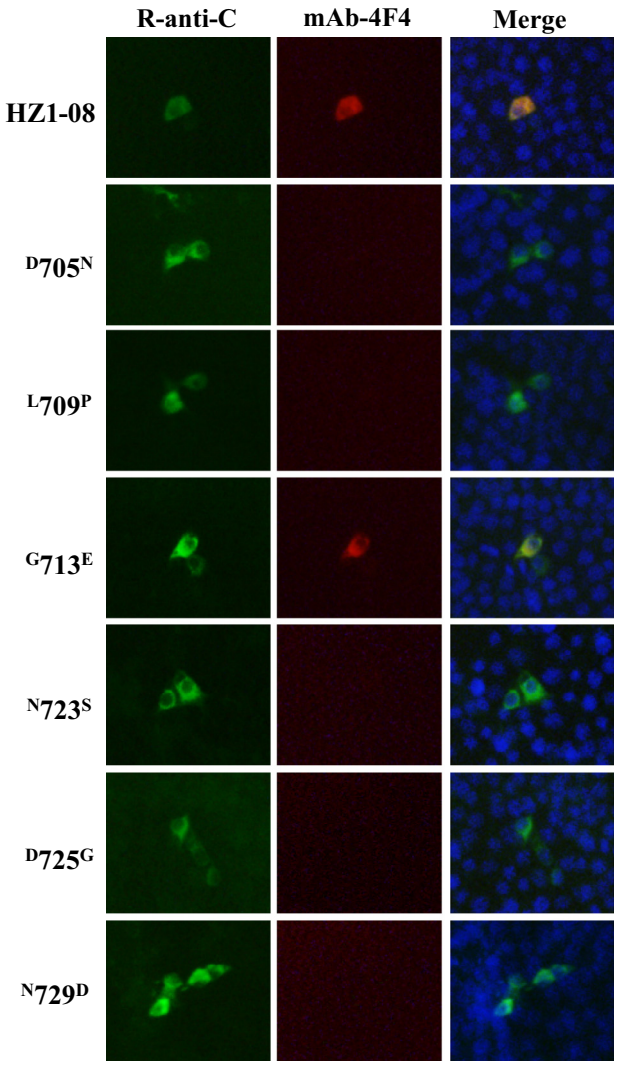

Fig. 4. Fine mapping of the E2 HAR1 epitope of classical swine fever virus HZ1-08 recognized by the monoclonal antibody 4F4. The C-strain-based E2 protein was mutated to substitute individual amino acids with those found at the same positions in subgroup 2.1 strains. Their expressions in PK-15 cells were probed with mAb-4F4 using the rabbit anti-C-strain serum (R-anti-C) as reference. The HZ1-08 E2 was used as control.

to mAb-4F4 and rabbit anti-C-strain serum, while other mutants exhibited binding to positive rabbit serum only. RecC-HAR1 and RecC-HZ-E2 also exhibited good reactivity to both antibodies (Fig. 5). These results indicate that Glu713 in the field strains was responsible for binding to mAb-4F4.

3.6. Evaluation of safety, immunogenicity cross-neutralization of the recombinant viruses RecC-HAR1 and RecC-HZ-E2 in pigs

RecC-HAR1 and RecC-HZ-E2 exhibited almost the same titers and replication ability as the wild-type RecC (Fig. 6), indicating that these replacements did not change the cell adaptation of virus. Then we attempted to assess their safety *in vivo*. The two recombinant viruses were able to induce fever in rabbits similar to the C-strain (data not shown). None of the pigs inoculated with Rec-C, RecC-HAR1 and RecC-HZ-E2 exhibited any clinical symptoms or fever throughout the experiment. Of the two pigs infected with field strain QZ-14, one showed intermittent elevation of temperature while the other showed persistent high fever (Fig. 7). Real-time RT-PCR shows that pigs inoculated with RecC, RecC-HZ-E2 and RecC-HAR1 had detectable, but low, levels of viral

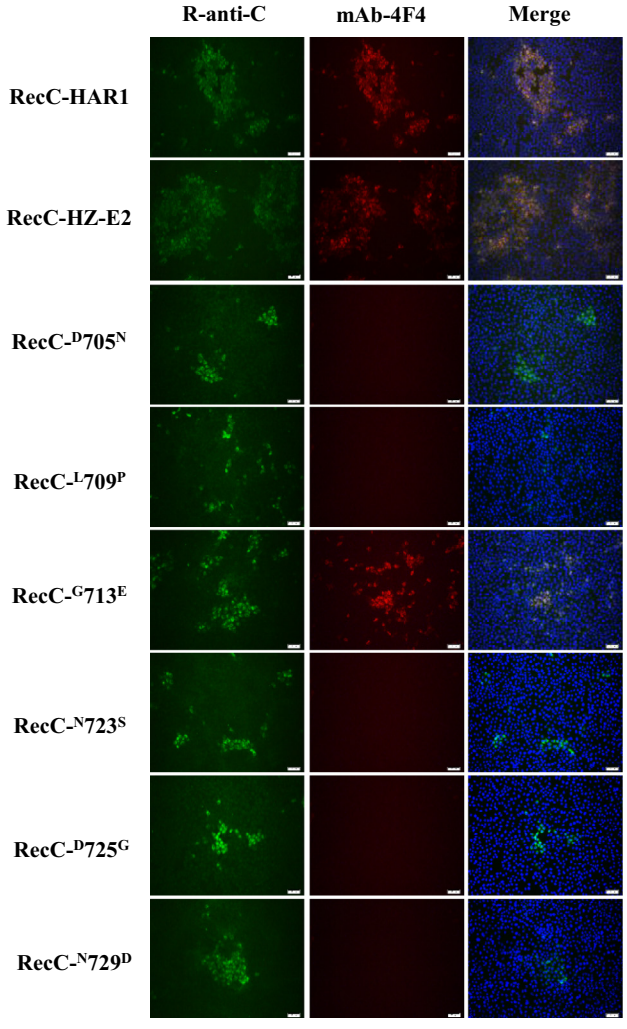

Fig. 5. Identification of a single amino acid substitution $^{G}713^{E}$ in the HAR1 region of E2 affected recognition of the recombinant classical swine fever viruses by the monoclonal antibody 4F4. PK-15 cell monolayers were infected with 200 $TCID_{50}$ of each virus and incubated at 37 °C for 72 h. The plates were fixed and differentially probed with mAb-4F4 and rabbit anti-C-strain serum. The RecC-HZ-E2 and RecC-HAR1 mutant viruses were used for comparison.

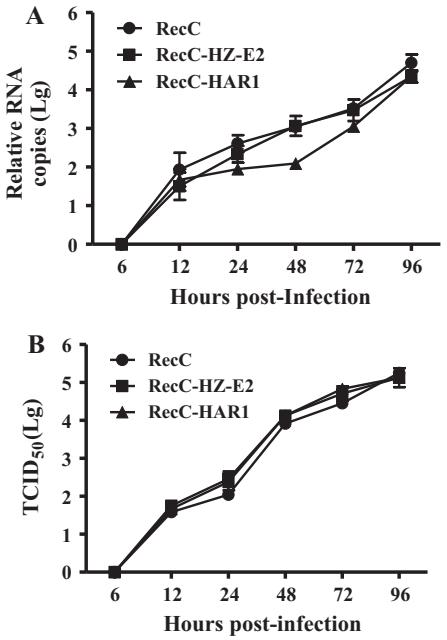

Fig. 6. Growth characteristics of the recombinant classical swine fever viruses RecC, RecC-HZ-E2 and RecC-HAR1 in PK-15 cells shown as virus titers (A) and RNA copies (B) Data represent mean ± SD from three independent experiments.

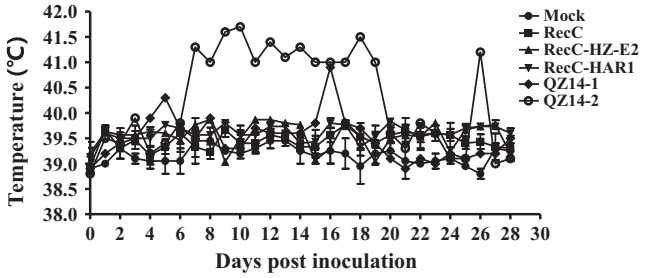

Fig. 7. Temperature profiles of pigs infected with recombinant classical swine fever viruses RecC, RecC-HZ-E2 and RecC-HAR1 as well as field isolate QZ-14. Rectal temperature was shown in average for recombinant viruses and individually for the two pigs infected with QZ-14.

RNA in the blood, while QZ-14 infected pigs had elevated levels of viral RNA from 14 to 28 dpi (Fig 8). These findings indicate that the recombinant viruses remain safe as the vaccine C-strain.

Table 1 shows that the ELISA titers varied among the strains used for immunization or infection and between the two antigens used for coating. Immunization with RecC had higher titer than the chimeric viruses RecC-HZ-E2 and RecC-HAR1 in their corresponding coating antigens. However, the anti-RecC serum exhibited only 12.5% of its homologous titers when tested on the HZ-E2-AD as the antigen, indicating its lower reactivity to the heterologous group 2 antigen as a result of antigenic divergence between the vaccine C-strain and the HZ1-08 isolate. Surprisingly, the anti-QZ-14 sera had lowest titers, same with both antigen coatings although the virus had productive infection as shown by fever and high level of viral load in blood.

Similar scenarios were seen with the neutralization assay: higher titers with homologous strains for antisera from RecC and RecC-HZ-E2 (Table 1). However, the anti-C-strain serum had much lower neutralization capacity to RecC-HZ-E2 (26.6-fold or 96.3% less) or to QZ-14 (4.7-fold or 79% less) as compared with its homologous strain RecC. Correspondingly, the antisera from RecC-HZ-E2 and QZ-14 had lower neutralization titers to the RecC strain.

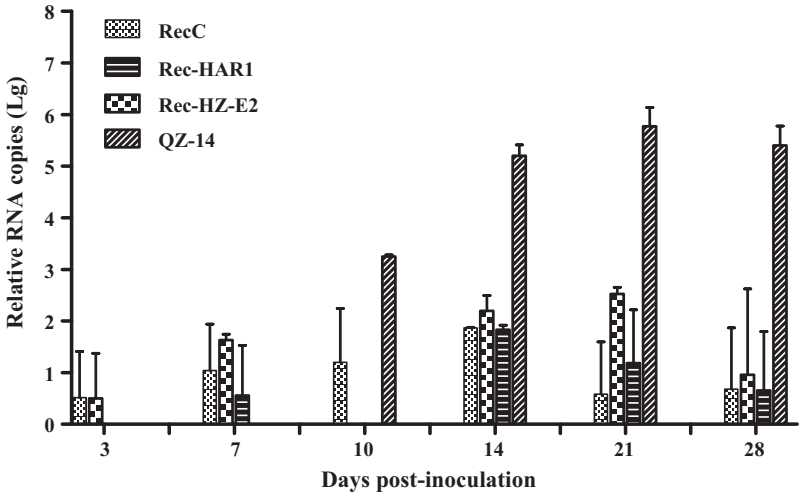

Fig. 8. Viral RNA copies in blood samples of pigs infected with recombinant classical swine fever viruses RecC, RecC-HZ-E2 and RecC-HAR1 as well as field isolate QZ-14. Data are shown as mean ± SD of each group of pigs (log_{10} copies/ml blood).

Table 1
Cross-reactivity or cross-neutralization of anti-CSFV antibodies to heterologous antigens by indirect ELISA or to heterologous strains by *in vitro* neutralization assay.

Pig antisera[a]	ELISA titer (1:x)[b]		Neutralization titer (1:x) [b]		
	C-E2-AD	HZ-E2-AD	RecC	RecC-HZ-E2	QZ-14
Anti-RecC					
%[c]	6400	800	6309	237	1333
	100	12.5	**100**	3.7	21
Anti-RecC-HZ-E2					
%	800	1600	562	6309	4467
	50	**100**	8.9	**100**	70.8
Anti-RecC-HAR1					
%	1600	3200	3758	3162	6309
	50	**100**	119	**100**	199
Anti-QZ-14					
%	800	400	199	2660	1585
	200	**100**	12.5	168	**100**

[a] Antisera were from blood samples of pigs infected with recombinant classical swine fever viruses RecC, RecC-HZ-E2 and RecC-HAR1 as well as field isolate QZ-14.
[b] (1) Antibody titers as measured by indirect ELISA using prokaryotic recombinant E2 proteins of C-strain and strain HZ1-08 as the coating antigens or by *in vitro* neutralization assay on the recombinant viruses and the field isolate QZ-14. (2) Neutralization titers were expressed as average 50% protective dose (PD_{50}) of three independent experiments.
[c] The titers to homologous antigen or homologous strain were normalized to 100% (bold and underlined) for comparison of cross-reactivity or cross-neutralization with heterologous antigens or strains across the rows. Anti-RecC-HAR1 and anti-QZ-14 sera are considered 'homologous' to HZ-E2-AD antigen or RecCHZ-E2 virus.

Importantly, the antisera from RecC-HZ-E2 and RecC-HAR1 had good neutralization capacity with the homologous strain RecC-HZ-E2 and the heterologous field strain QZ-14. Of particular note is the good neutralization to all three viruses with the introduction of the HAR1 region of HZ1-08 into the C-strain backbone.

4. Discussion

E2 is an immune-dominant glycoprotein of CSFV and induces neutralizing antibodies that protect pigs from lethal infection [27,28]. Genetic analysis has revealed that the N-terminal half of E2 is more variable and may be responsible for its antigenic divergence among three groups of CSFV strains. It is of great importance to understand if such divergence from the vaccine strain would affect immunogenicity and neutralization titers [18,29]. We generated a mAb-4F4 that recognized the E2 of group 2 strains, but not the E2 of the group 1 vaccine strain. Fine mapping of the epitope narrowed down the region responsible for mAb-4F4 binding to the hypervariable antigenic region 1 of E2. We further proved that the anti-C-strain serum had significantly low level of neutralizing capacity to the field isolates. Replacement of the C-strain HAR1 region with that from the field isolate HZ1-08 markedly improved the neutralizing capacity to the field isolates while remaining competent in neutralizing the recombinant C-strain.

First, we determined the main regions of E2 that could be recognized by mAb-4F4 using the C-strain E2-based recombinant proteins in the baculovirus system with its HAR1, HAR2 or HAR3 region replaced with corresponding regions of strain HZ1-08 E2. Only the proteins containing HAR1 and E2 from HZ1-08 showed strong reactivity. Thus, binding to mAb-4F4 was defined to the HAR1 region of the group 2 field isolate. Alignment of HAR1 of E2 sequences from multiple CSFV isolates revealed that residues 705, 709, 713, 723, 725 and 729 in HAR1 of the group 2 strains differed from those of the C-strain. Immunostaining revealed that the residue Glu713 on the HAR1 region of strain HZ1-08 is critical for mAb-4F4 binding in PK-15 cells either expressing the recombinant peptides or being infected with recombinant viruses. This is in general agreement with an earlier report that Glu713 in the E2 epitope recognized by the mAb T33 is responsible for antigenic specificity for the field strain 94.4/IL/94/TWN (group 3) [30]. This also suggests that Glu713 in the B/C domain might share the same function in both group 2 and 3 strains. The variations of immuno-reactivity in specific epitopes between the vaccine C-strain and field isolates may have the potential for development of differential immunoassays.

Then, we determined if replacement of the C-strain E2 or HAR1 from the corresponding region of the clinical isolate HZ1-08 (RecC-HAR1 and RecC-HZ-E2) could alter virulence in pigs because E2 is a virulence determinant of CSFV [4]. The C-strain-based recombinant viruses did not cause any clinical symptoms (including fever) in pigs and viral RNA level was below the detection limit (10^2 copies/mL) in blood samples. This finding is in accordance with the C-strain vaccination in pigs where low level of viral RNA was detected in blood [31]. However, infection with the field isolate QZ14 led to high level of viral RNA starting from 10 dpi. These data suggest that the recombinant viruses remain to be

non-pathogenic irrespective of replacement of the most antigenic region of E2 of the C-strain with that from genotype 2.1 strains.

Further attempt was made to examine if replacement of the C-strain HAR1 or E2 with those of strain HZ1-08 would affect neutralizing antibody titers. We found that the anti-C-strain serum lost most of its neutralization capacity to RecC-HZ-E2 and QZ-14. This supports our early finding that the antibodies from the C-strain vaccine failed to neutralize the field isolates effectively [18]. Similarly, we observed good neutralization titers of RecC-HZ-E2 and field isolate QZ-14, but low titers to the recombinant C-strain when tested with the antisera from RecC-HZ-E2. These results suggest that cross-neutralization of the antisera between the group 1 and group 2 strains might be limited. A more important finding in this study is that RecC-HAR1 remained competent in neutralizing ReC-HZ-E2 and QZ-14 strains without compromising the neutralization capability to the recombinant C-strain, indicating that introduction of the HAR1 region, but not necessarily the major antigen region of E2, of field isolates into the C-strain backbone could produce 'balanced' neutralizing antibodies to both groups of CSFV strains. This also indicates that the epitope in HAR1 recognized by mAb-4F4 is one of the immune-dominant epitopes during natural infection. Earlier studies reported several neutralizing epitopes of CSFV E2 with two of them also located in the B/C domain [32–34]. However, none of the early studies ever examined cross-neutralization with the antisera from recombinant vaccine strains containing the epitope region from field isolates.

C-strain has been widely used in mainland China since 1957 [35]. However, CSFV may evolve in swine population under regular vaccination programs, leading to changes of genotypes and alterations in viral pathogenicity due to positive selection pressure [18,36]. Subgroup 2.1 strains, especially 2.1b and 2.1d, have recently appeared in some farms in China [19,22,23,37]. Because vaccination drives evolution of CSFV [38] and the anti-serum from C-strain vaccine has failed to have effective neutralization to the field isolates of group 2 as observed in this study, it is tempting to suggest that a chimeric C-strain containing the HAR1 region of epidemic strains could improve the vaccine efficacy. Since the HAR1 region recognized by mAb-4F4 is conserved in most group 2 CSFV strains, it may be utilized as a marker for differentiation between the vaccine C-strain and group 2 isolates.

Conflicts of interest

The authors declare no conflict of interest.

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