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Design and immunogenicity analysis of the combined vaccine against zoonotic hepatitis E and foot-and-mouth disease

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ABSTRACT

Aim: Design and immunogenicity assessment of the combined vaccine candidate against zoonotic hepatitis E virus (HEV) and foot-and-mouth disease virus (FMDV).
Methods: Using the molecular cloning technology, we produced and purified 9 HEV ORF2-truncated proteins (HEV genotype 4). Then, we compared their thermal stability, antigenicity, and immunogenicity to select the best HEV immunogen. Next, we used the adjuvant Montanide ISA-206 to prepare different formulations of HEV vaccine alone, FMDV vaccine alone and HEV-FMDV combined vaccine. The formulations were injected into mice and the induced humoral immune responses were monitored up 12 weeks post-immunization.
Results: The HEV p222 protein could self-assemble into VLPs (~34 nm) and showed higher stability and better antigenicity/immunogenicity than the other HEV antigens, thus it was selected as the best HEV immunogen. Mice immunization with the FMDV vaccine alone induced high FMDV-specific antibody titers in a dose-dependent manner; the HEV p222 protein also induced high levels of anti-HEV antibodies but in a dose-independent manner. The HEV-FMDV combination induced anti-FMDV antibody titers 7–16-fold higher than the titers induced by the FMDV vaccine alone, and HEV-specific antibody titers 2.4-fold higher than those induced by the HEV p222 antigen alone.
Conclusion: Herein, we proposed a new approach for the control of zoonotic HEV infection through its control in its main host (pig). We also designed the first HEV-FMDV combined vaccine and the preliminary analyses revealed a synergistic effect on the immunogenicity of both HEV and FMDV antigens.

1. Introduction

Hepatitis E virus (HEV) causes acute, fulminant and chronic viral hepatitis, accounting for about 20 million infections annually worldwide and leading to an estimated 70,000 deaths [1]. Traditionally, only a single serotype of HEV has been recognized, and four well-known genotypes that infect humans, namely genotypes 1, 2, 3 and 4 [2]. Genotypes 1 and 2 are pathogenic only for humans, whereas, genotypes 3 and 4 infect both human populations and several animal species such as swine, deer, wild boars, and rabbits [3]. It has been reported that there is an antigenic cross-reactivity among HEV strains, and previous HEV infection conferred a cross-genotype and cross-host-species protection against human HEV genotypes 1 and 4 in rhesus macaques [4]. Likewise, Sanford et al. [5] found that the prior infection with a genotype 3 swine HEV protected pigs from subsequent challenges with homologous and heterologous genotypes 3 and 4 human HEV. Additionally, pigs immunized with truncated recombinant capsid antigens produced strong anti-HEV IgG responses and protected the animals against a genotype 3 mammalian HEV [6]. HEV is difficult to propagate in primary hepatocyte culture as well as in various other cell lines. The viral replication usually progresses extremely slowly, and the infection with low virion counts results in a non-productive HEV replication.

In recent years, a continuously growing number of reports described zoonotic hepatitis E as a major challenge for public health in the industrialized countries [7–9]. In China, early cases of hepatitis E were mainly caused by genotype 1 strains, but in the last two decades, there was the emergence of genotype 4 infections [10,11]. All the isolated strains in a study of hepatitis E patients from 2001 to 2011 in eastern China belonged to the geno-

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type 4, very similar to swine HEV strains [10] and zoonotic HEV strains were also detected in several other provinces [10,12].

There are three effective approaches to control an infection such as HEV: eliminate the pathogen source (reservoir), block the transmission route and/or immunize susceptible populations. This latter has been the most extensively studied approach [13,14]. However, given the fact that hepatitis E in China is mainly caused by the genotype 4 and these genotype 4 strains comes mainly from pigs [15], the development and use of pig vaccines emerge as a promising approach for the control of HEV infection. Such vaccine would significantly decrease the source of HEV genotype 4, and therefore would reduce or even eliminate the genotype 4 infection in the human populations. One of the major obstacles in developing a swine HEV vaccine is the low benefit/cost ratio given that the HEV infection in swine is usually asymptomatic. To solve this problem and increase the benefit/cost ratio, we developed a combined vaccine candidate against HEV and against one of the most serious swine diseases: foot-and-mouth disease (FMD).

Foot-and-mouth disease is a highly infectious disease of cloven-hoofed animals that spreads rapidly and can lead to large-scale epidemics and cause a significant socioeconomic impact. The causative agent, foot-and-mouth disease virus (FMDV), belongs to genus Aphthovirus of the family *Picornaviridae*. Seven FMDV serotypes (A, O, C, Asia 1, SAT 1, SAT 2 and SAT 3) have been identified so far with the serotype O as the most prevalent in swine in China [16]. In recent years, two strains caused the major FMD outbreaks in China and its neighboring countries: the O/Mya/98 strain of the Southeast Asia (SEA) topotype and the porcinophilic strain (Cathay topotype) [16,17]. Vaccination is still the most common method of controlling FMD and it is mandatory in government disease control programs in China. Therefore, a swine combined vaccine against HEV and FMDV would be interesting for the swine breeding industry: it could prevent one the most devastating swine diseases (FMDV infection) on one hand, and reduce the source of HEV genotype 4 causing infections in human populations on the other hand.

It has been previously reported that mice could be used as immune models for the evaluation of pig vaccines. Cao et al. reported that a multi-epitope peptide vaccine greatly increased the neutralising antibody response against FMDV in mice, and then they showed that the same multi-epitope protein vaccine completely protected pigs against a virulent FMDV challenge [18,19]. An other report showed that FMDV types O, A, and Asia1 were well adapted for mice and the pathogenesis of each virus was evaluated in the mouse model [20].

In the present study, we conducted an investigation on HEV-FMDV combined vaccine candidates. First, we produced several HEV proteins that contain the most immunogenic region of the HEV capsid protein (ORF2) and comprising the dominant neutralizing epitopes [21], namely p179 (439-617aa), p188 (aa430-617), p189 (aa439-927), p196 (aa422-617), p199 (aa439-637), p209 (aa439-647), p216 (aa422-637), p222 (aa439-660) and p231 (aa430-660). Next, we compared the immunogenicity/antigenicity and stability of these proteins to select the best candidate to be combined with the FMDV vaccine. After the selection of the best HEV immunogen, we prepared different HEV-FMDV combined vaccine formulations and investigated their immunogenicity in mice.

2. Materials and methods

2.1. Animals

All animal immunizations were conducted on female BALB/c mice (6–8 weeks-old) purchased from Animal Resource Centre (Yangzhou, China), and kept in a pathogen-free environment. All animal experiments were performed according to the guidelines for animal experimentation of the Institutional Animal Care and Use Committee of Southeast University.

2.2. Selection of HEV fragment and FMDV vaccine

We have previously produced 9 different HEV ORF2-truncated proteins: p179 (439-617aa), p188 (aa430-617), p189 (aa439-927), p196 (aa422-617), p199 (aa439-637), p209 (aa439-647), p216 (aa422-637), p222 (aa439-660) and p231 (aa430-660), which were all derived from a Chinese strain belonging to HEV genotype 4 (GenBank: AY789228). All HEV proteins were expressed in E. coli and. Briefly, all the target gene fragments were inserted into the plasmid pET28a(+). The various recombinant constructs were transformed into *E. coli* competent BL21 (DE3) cells. Then, the target recombinant proteins were expressed by isopropyl-b-D-thiogalactoside (IPTG) induction in Luria Broth/kanamycin medium. Next, the cells were pelleted by centrifugation, washed, resuspended, lyzed, and the soluble fractions were purified by Ni-NTA affinity chromatography, as previously described [22–24]. The proteins were analyzed by SDS-PAGE, aliquoted and stored at −70 °C until further use.

To select the best HEV protein to be combined with the FMDV vaccine, we first analyzed the thermal stability of the produced HEV ORF2 proteins as follows: the proteins were stored at 37 °C for 7 days and the degradation rate was observed by SDS-PAGE at 1, 3 and 7 days of storage. Next, we analyzed the immunoreactivity of the most stable HEV fragments against the neutralizing monoclonal antibodies (mAbs) 5G5 by indirect ELISA. Further, the selected HEV protein was diluted in the oil-adjuvant Montanide ISA-206 (SEPPIC, France) and then injected intra-muscularly into mice at day 0 and day 15 (booster dose); the serum samples were taken 2 weeks after the second immunization for determination of HEV IgG antibody by indirect ELISA and compare the immunogenicity of the HEV immunogens. The selected proteins were diluted to 0.1 mg/mL, applied to a carbon-coated grid, stained with 2% uranyl acetate for 15 min, and the samples were observed with a transmission electron microscope (H-7650, HITACHI, Japan) to characterize the morphology of the formed virus-like particles.

Concerning the FMDV vaccine, a commercial inactivated FMDV type O vaccines (Type O, Strain O/Mya98/XJ/2010 + Strain O/GX/09-7) was provided by Jinyu Baoling Biological Medicine Company (Huhehaote, China).

2.3. Vaccine formulations

The monovalent inactivated FMDV vaccine containing approximately 2.0 μg/ml of whole virus antigen [19] was adjusted to three doses of 0.5, 1, 2 μg/ml. The concentration of HEVp222 was adjusted to 25, 50, 100 μg/ml, and then mixed with the oil-adjuvant Montanide ISA-206 in a 50:50 (w/w) ratio. The antigens and adjuvant were mixed and stirred at a low shear rate (300 rpm) for 10 min at 30 °C in an auto-mix blender to form double emulsions (water-in-oil-in-water). Likewise, different formulations of FMDV-HEV combined vaccine were prepared: F1 + E25, F1 + E50, F1 + E100, F2 + E25 and F0.5 + E25 (μg/ml), where F refers to inactivated FMDV vaccine and E refers to HEVp222; the numbers following the F and E refers to the concentration of the corresponding antigen in μg/ml.

2.4. Vaccination and experimental design

Balb/c mice were randomly separated into 12 vaccine groups and 3 control groups (n = 10 for each group). The animals were immunized intra-muscularly by single injection of 0.2 ml of the corresponding vaccine formulation as summarized in Table 1.

Table 1
The monovalent inactivated FMDV vaccine, HEVp222 vaccine and different formulations of FMDV-HEV combined vaccine.

	GROUPS	Vaccine formulations	
		FMDV Ag (μg/ml)	HEV Ag (μg/ml)
FMDV vaccine	F0.5	0.5	–
	F1	1	–
	F2	2	–
HEV vaccine	E25	–	25
	E50	–	50
	E100	–	100
HEV- FMDV combined vaccine	F1 + E25	1	25
	F1 + E50	1	50
	F1 + E100	1	100
	E25 + F0.5	0.5	25
	E25 + F1	1	25
	E25 + F2	2	25
Control groups	C-1	–	–
	C-F1	0.5	–
	C-E25	–	25

The control group C-1 received only the Montanide ISA-206 adjuvant.

2.5. Serum collection

Baseline serum samples were collected from the mice prior to immunization. After vaccination, the serum samples were collected from all animals at 2, 4, 6, 8, 10 and 12 weeks post immunization. The Sera were stored at −70 °C until testing.

2.6. Anti-HEV IgG antibodies detection

Anti-HEV IgG antibodies in the collected mice sera were tested by an indirect-ELISA), as described previously [25,26]. Briefly, micro-well plates were coated with the HEV p166 antigen (HEV ORF2 aa452-617) at a concentration of 1 μg/ml. After overnight incubation at 4 °C, the wells were washed twice with PBS, containing 0.5% Tween-20 (PBST). The serial two-fold dilutions (starting at 1:100) of the serum samples were added to the wells (100 μl/well) and the plates were incubated at 37 °C for 1 h. Next, the plates were washed three times with PBST and 100 μl of 0.1 μg/ml HRP-conjugated goat anti-mouse IgG was added to each well, followed by a 60 min incubation at 37 °C. The unbound conjugate was removed by washing the plates three times with PBST. The TMB liquid substrate was added for color development and the plates were incubated at 37 °C for 10 min. The reactions were terminated with the addition of 50 μl/well of 2 M sulfuric acid. Finally, the OD values were read at 450 nm, with a 630 nm reference wavelength. Results were reported as geometric mean titers (GMTs) and the end-point titers were defined as the maximum dilution with a positive reaction.

2.7. Anti-FMDV antibody IgG detection

To detect produced anti-FMDV antibodies in the immunized mice, we designed a recombinant antigen that contains the G–H loop of the FMDV VP1 structural protein to be used in the indirect ELISA. This loop has been previously reported to contain the dominant neutralization epitope [19,27]. Therefore, the entire G–H loop domains of VP1 protein of O/GX/09-7 strain and O/Mya/98 were linked together by two glycines (G) residues, and then produced in E. coli. The individual G-H loops and the combined antigen were designated as L_{GX}, L_{MYA} and $L_{MYA\text{-}GX}$, respectively. The coding DNA sequence of the designed multiepitope $L_{MYA\text{-}GX}$ antigen was synthesized by Applied Biological Materials Inc. (www.abmgood.com) according to the most commonly occurring codons in E. coli. Then, the $L_{MYA\text{-}GX}$ gene was amplified by PCR, purified and inserted into the pGEX-4T-2 vector at the BamHI and XhoI restriction sites. The expression construct was transformed into *E. coli* (BL21) competent cells. The $L_{MYA\text{-}GX}$ protein was expressed and purified as a GST-tagged protein. The indirect ELISA conditions were optimized by checkerboard titration using serial dilutions of $L_{MYA\text{-}GX}$ protein tested against serial dilutions of known positive and negative mice sera. Dilutions that resulted in the maximum difference in absorbance at 450 nm for the positive and negative sera (P/N) were determined as the optimal working conditions for the detection of anti-FMDV IgGs.

The indirect ELISA was performed as described above (detection of anti-HEV antibodies), except that the micro-wells were coated with $L_{MYA\text{-}GX}$ antigen at a concentration of 2 μg/ml.

2.8. HEV neutralization assay in vitro

Neutralizing activity of the elicited anti-HEV antibodies was tested by an in vitro PCR based neutralization assay [28,29]. The mice serum samples were inactivated by heating at 60 °C for 30 min and diluted in Hanks' solution. Then, approximately 100 times the cell culture infectious dose of HEV of genotype 4 strain NJ703 [GenBank: AY789228] diluted in 100 ul of Hanks' solution, was mixed with 100 μl of the immune serum serially diluted at 1:40, 1:60,1:80,1:100,1:120 and 1:140. After incubation at 37 ℃ for 1 h, the mixture was inoculated onto a cell monolayer of A549 and incubated at 37 for 2 h. After 5–6 days culture at 37 ℃ in 5% CO2, the cells were washed three times with Hanks' solution, followed by RNA extraction with TRIzol reagent (GibcoBRL). Detection of the presence of HEV was determined by One-step RT-PCR (QIAGEN, Germany) using universal HEV PCR primers, JM-2 (5′-CCG ACA GAA TTG ATT TCG TCG GC) and JM-4 (5′-TCG GCG GCG GTG AGA GAG AGC CA) that would amplify a 287 bp fragment from the HEV genome. All the RT-PCR products were separated by 1% (w/v) agarose gel electrophoresis, stained with ethidium bromide and photographed under UV light. HEV neutralization was determined by the absence of detectable HEV RNA in the inoculated cell culture.

2.9. Statistical methods

Statistical analysis was performed using GraphPadPrizm6.02 Software. Two-way ANOVA followed by Tukey post-hoc test was used to assess the differences in antibody responses between the different vaccinated groups. P-value ≤ 0.05 was considered statistically significant; not significant (NS) indicates $P > 0.05$.

3. Results

3.1. Selection of the HEV immunogen

Nine purified HEV ORF2-truncated proteins were stored at 37 °C for 7 days for an accelerated stability test. As shown in Fig. 1, after 7 days, a small amount of the p179 and p222 proteins were degraded while the other proteins were completely degraded into smaller fragments of different sizes (Fig. 1A). Therefore, p179 and p222 were selected as stable HEV proteins for further analyses.

Next, we sought to compare the immunoreactivity of p179 and p222 against the HEV mAb 5G5. The results revealed that both proteins had a good antigenicity, with slightly stronger reactivity with p222 (Fig. 1B). However, when injected into mice, p222 elicited a significantly stronger humoral response and induced production of higher anti-HEV antibody titers than p179 (Fig. 1C). The visual-

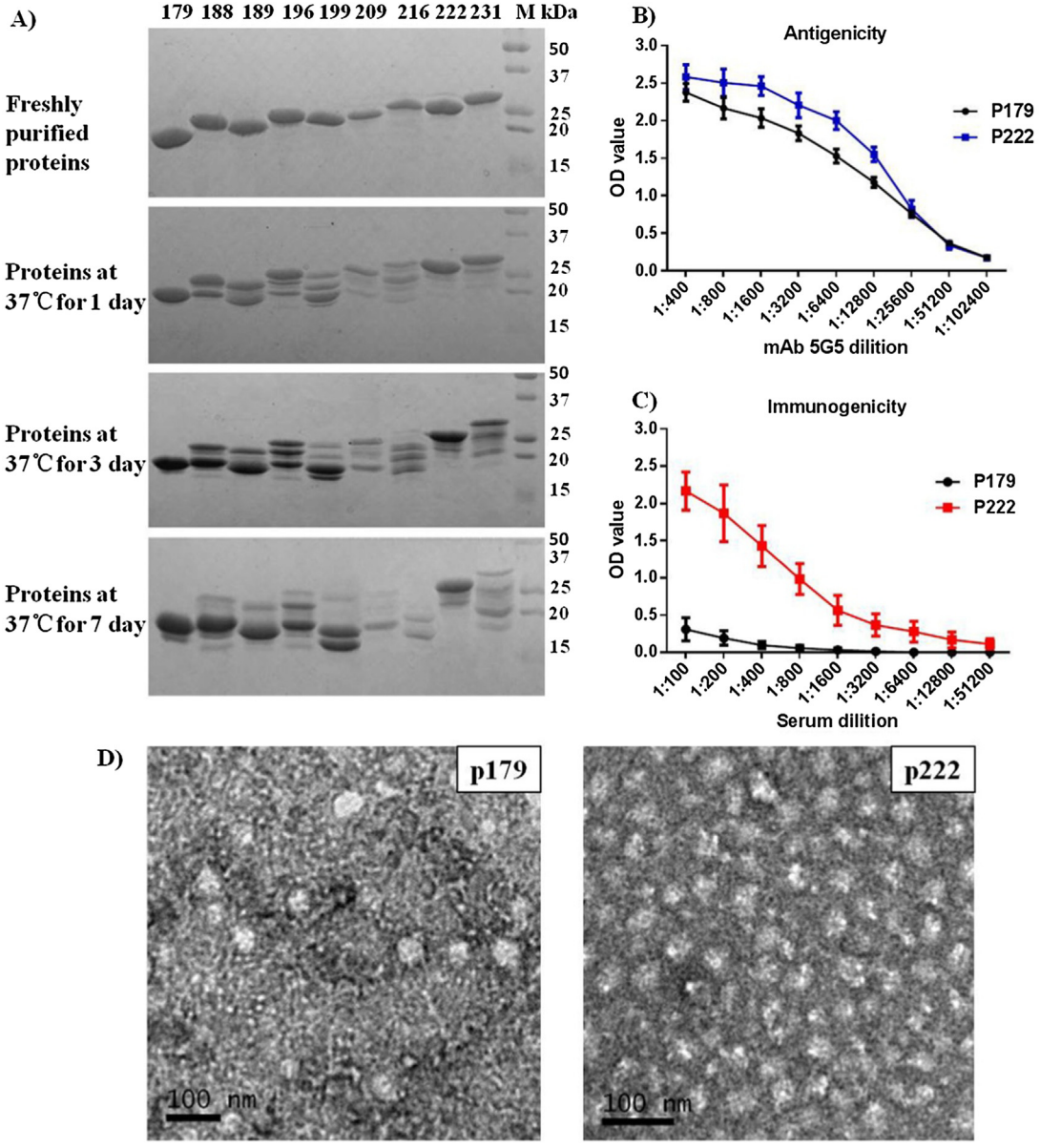

Fig. 1. Selection of the best HEV immunogen. (A) Stability of the different HEV ORF2-truncated protein stored at 37 °C for 7 days as analyzed by SDS-PAGE at 0, 1, 3 and 7 days of storage. (B) Comparison of the antigenicity of p179 and p222 proteins by evaluating their immunoreactivity against the HEV neutralizing monoclonal antibody 5G5. (C) Comparison of the immunogenicity of p179 and p222 proteins by evaluating the humoral immune response upon injection into mice. (D) Electron micrographs of negatively-stained p179 and p222 virus-like particles.

ization of the p179 and p222 particles under an electron microscope revealed that p222 self-assembled into virus-like particles (VLP) with similar morphology than the native virus, while p179 formed fewer particles with distinct morphologies (Fig. 1D). Taken altogether, p222 was selected as the best HEV immunogen to be considered as a second-generation recombinant HEV vaccine on one hand, and as the best candidate to be combined with the FMDV vaccine for the development of the HEV-FMDV combined vaccine on the other hand.

3.2. Expression and purification of the FMDV $L_{MYA\text{-}GX}$ antigen

The GST-tagged $L_{MYA\text{-}GX}$ protein was successfully expressed in E. coli after IPTG induction as revealed by SDS-PAGE, where a specific band of approximately 32.6 kDa was visualized at the expected position (Fig. 2B). The purified protein was also analyzed by SDS–PAGE and the results showed that the purity of the $L_{MYA\text{-}GX}$ protein was above 90% (Fig. 2C).

3.3. Monitoring of HEV- and FMDV-specific antibodies in the mice immunized with HEV or FMDV vaccines separately

Total anti-FMDV IgG antibodies were monitored by indirect ELISA in the serum samples from mice immunized with three different concentrations of the inactivated FMDV vaccine (0.5, 1, 2 μg/ml) (Fig. 3A and Table 2A). Anti-FMDV antibodies were detected in all the immunized mice at week 2 but the levels were very low. As expected, the antibody titers increased overtime to reach the highest level at week 6 for the group F0.5 and at week 10 for the groups F1 and F2. The antibody titers in the group F0.5 were lower than in the two other groups all throughout the experiment, which was expected given that these latter groups received a higher dose of

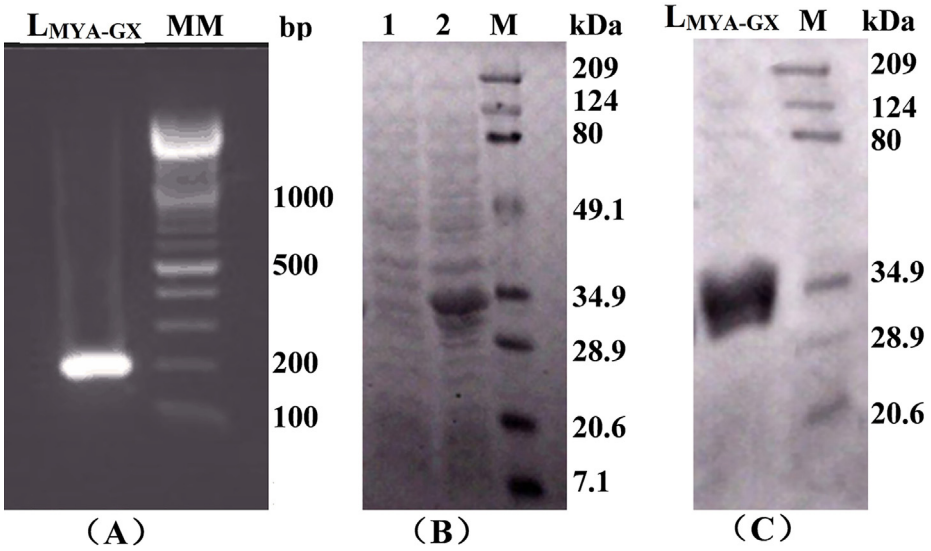

Fig. 2. Expression of FMDV L_{MYA-GX} antigen in E. coli. (A) Visualization of the amplified L_{MYA-GX} gene on 1.5% agarose gel. (B) SDS-PAGE analysis of the expression of L_{MYA-GX} antigen in E. coli before (1) and after (2) IPTG induction. (C) SDS-PAGE analysis of the purified L_{MYA-GX} antigen. MM: 100 bp DNA ladder. M: molecular weight makers in kiloDaltons (Fermentas USA).

the FMDV vaccine. However, the results were not dose-dependent in the first four weeks in the F1 and F2 groups: the levels of the anti-FMDV antibodies were higher in the F1 group at weeks 2 and 4; then they reached similar levels at week 10 in both groups. The responses were negative in the control group 1-C that received only the adjuvant during the whole experiment. Overall, during the 12 weeks of the experiment and taking into account the four groups, there was a positive correlation between the dose and titers of the induced FMDV-specific antibodies.

Likewise, the sera collected from the mice immunized with different dosages of the HEV recombinant vaccine (25, 50, 100 μg/ml) were analyzed for the presence of anti-HEV antibodies by indirect ELISA (Fig. 3B and Table 2B). The anti-HEV antibodies were not detectable at week 2 in all groups. Starting from week 4, the HEV-specific IgGs started increasing in a dose-independent manner in the vaccine groups and reached the highest levels at week 10 in the E25 and E100 groups, and at week 12 in the E50 group. At week 4, the antibody titers in the E25 and E100 were 4-fold higher than in the E50 groups. However, at week 10, the levels of the anti-HEV antibodies in the E25 group were 5-fold higher than in the E50 group and 2-fold higher than in the E100 group.

Therefore, according to these results, the concentrations of 25 μg/ml of the HEV immunogen and 1 μg/ml of the FMDV immunogen were selected for the preparation HEV-FMDV combined vaccine formulations.

3.4. Monitoring of HEV- and FMDV-specific antibodies in the mice immunized with different HEV-FMDV combined vaccine formulations

In order to determine whether combining the HEV and FMDV immunogens together could affect their respective immunogenicity we set the following experiments:

Experiment 1: The FMDV immunogen (1 μg/ml) was combined with three different doses of the HEV p222 protein (25, 50 and 100 μg/ml). After injection into mice, we monitored the production of FMDV-specific antibodies. The results (Fig. 3C and Table 3A) revealed that all three combined vaccine formulations induced the production of anti-FMDV IgGs detectable at week 2 post-immunization. Interestingly, the levels of anti-FMDV antibodies in the three combined vaccine groups were significantly higher than the control group F1 that received only the inactivated FMDV vaccine. In addition, anti-FMDV IgG titers were increasing as the combined HEV immunogen dose was increasing. The antibody levels reached a peak at week 12 in the F1 + E25 and F1 + E50 groups, and at week 10 in the F1 + E100 group. At the end of the experiment (week 12), the antibody titers remained as high as at week 10 in the F1 + E100 group. These results indicated that the addition of the HEV p222 immunogen enhanced the immunogenicity of the FMDV vaccine.

Experiment 2: The 25 μg/ml HEV dosage was selected to be combined with three different concentration of FMDV vaccine (0.5, 1 and 2 μg/ml), and injected into mice. The results of the detection of the HEV-specific IgG showed that the HEV-FMDV formulations induced a stronger humoral response than the control group that received only 25 μg/ml of HEV p222 (Fig. 3D and Table 3B). The anti-HEV antibodies were detectable at week 4 post-immunization and their levels were increased to reach a peak at week 10 in all the groups. During the 12 weeks of experimentation, the E25 + F0.5 group registered the highest anti-HEV antibody titers followed by E25 + F2 and E25 + F1 groups. Although the addition of the FMDV vaccine seems to enhance the immunogenicity of the HEV immunogen, the increase in the anti-HEV antibodies was not dependent on the increase of the FMDV vaccine dose.

A negative reaction for the presence of both anti-HEV and anti-FMDV antibodies was registered all throughout the experiment in the negative control groups that received only the adjuvant.

Overall, the results of both experiments indicated that the HEV-FMDV combined vaccine formulations exhibited a synergistic effect on the immunogenicity of both immunogens.

3.5. Detection of anti-HEV neutralizing antibodies

Ability of the immune sera to neutralize HEV was determined using the PCR-based neutralization assay in vitro. The sera prepared from blood collected at 10 wpi from the animals immunized with Group C-E25 and Group E25 + F0.5 were used in the neutralization experiments. Corresponding pools of pre-inoculation sera were used as negative controls. As shown in Fig. 4, anti-HEV neutralizing titers of the Group C-E25 and Group E25 + F0.5 reached titers of 1:80 and 1:100, respectively. No neutralization was observed using pre-inoculation sera. Although the dosage of HEVp222 antigen was the same (25 μg/ml), the combined

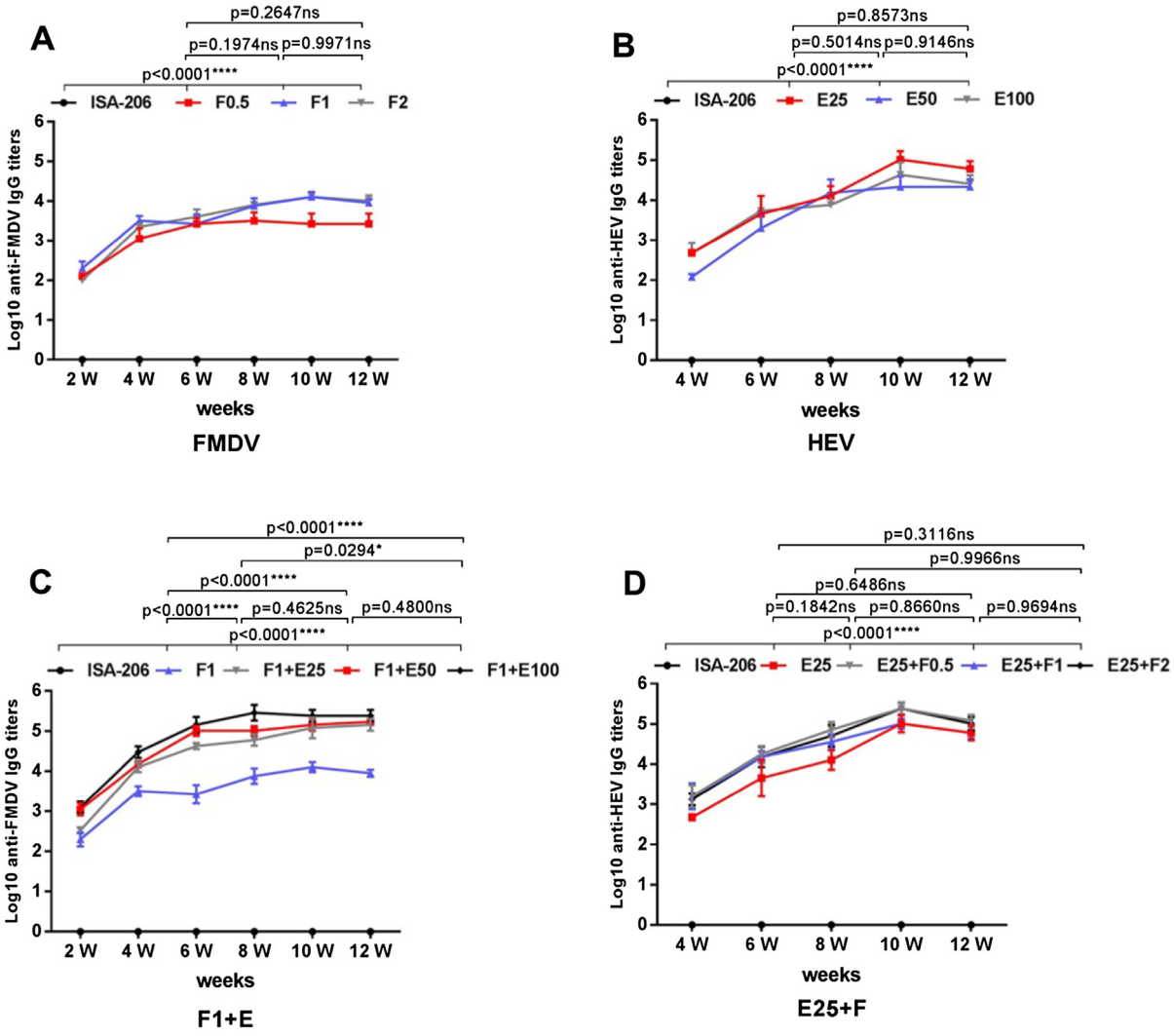

Fig. 3. Total IgG antibodies to HEV and FMDV of the immunized mice at the different time points of the experiment. Sera were pooled on weeks 2, 4, 6, 8, 10 and 12 post-vaccination and detected by ELISA. The different groups were analyzed by two-way ANOVA followed by Tukey test. (A) FMDV-specific IgGs antibodies of FMDV vaccine alone; (B) HEV-specific IgGs antibodies of HEV vaccine alone; (C) FMDV-specific IgGs antibodies of F1 + E combined vaccine; D) HEV-specific IgGs antibodies of E25 + F combined vaccine; *P < 0.05; ****P < 0.0001 (all the groups vs ISA206); ns: no significance P > 0.05.

Table 2
Monitoring of the anti-FMDV and anti-HEV antibodies in different groups vaccinated with different dosages of the FMDV or HEV vaccines.

A. Monitoring of the anti-FMDV antibodies titers							
	Week post-immunization						
Groups	0	2	4	6	8	10	12
C-1	–	–	–	–	–	–	–
F0.5	–	126	1131	3200	3200	2691	2691
F1	–	200	3200	4032	7611	12,800	9051
F2	–	100	2263	4032	8063	12,800	10,159
B. Monitoring of the anti-HEV antibodies titers							
	Week post-immunization						
Groups	0	2	4	6	8	10	12
C-1	–	–	–	–	–	–	–
E25	–	–	476	4525	12,800	102,400	60,887
E50	–	–	119	2016	15,222	18,102	21,527
E100	–	–	476	5382	7611	43,054	25,600

–: negative reaction when the serum was added at 1:100 dilution.

Table 3
Monitoring of the anti-FMDV and anti-HEV antibodies in different groups vaccinated with formulations of HEV-FMDV combined vaccine.

A. Monitoring of the anti-FMDV antibodies titers							
	Weeks post-immunization						
Groups	0	2	4	6	8	10	12
C-1	–	–	–	–	–	–	–
C-F1	–	200	3200	4032	7611	12,800	9051
F1 + E25	–	336	12,800	43,054	60,887	121,775	144,815
F1 + E50	–	1131	13,325	102,400	102,400	144,815	172,215
F1 + E100	–	1270	30,444	144,815	289,631	243,549	243,549
B. Monitoring of the anti-HEV antibodies titers							
	Weeks post-immunization						
Groups	0	2	4	6	8	10	12
C-1	–	–	–	–	–	–	–
C-E25	–	–	476	4525	12,800	102,400	60,887
E25 + F0.5	–	–	1600	18,102	72,408	243,549	113,650
E25 + F1	–	–	1600	15,222	36,204	102,400	60,887
E25 + F2	–	–	1345	15,222	51,200	243,549	102,400

–: negative reaction when the serum was added at 1:100 dilution.

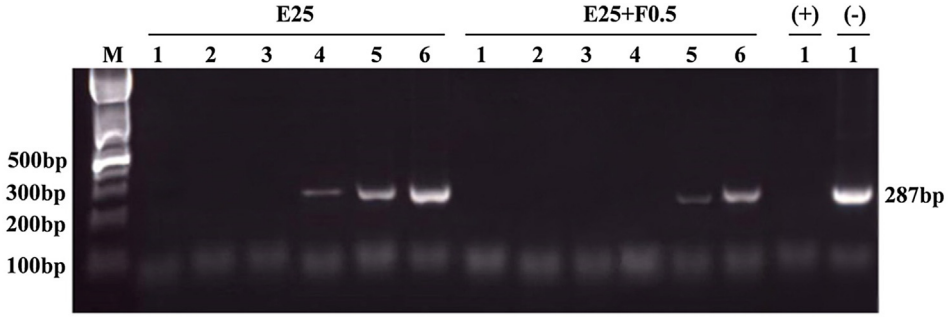

Fig. 4. HEV neutralization detected using an in vitro PCR-based neutralization assay. Comparison of anti-HEV neutralizing antibody titers between the mice immunized with the combined E25 + F0.5 vaccine or the monovalent E25 vaccine. 1 to 6 correspond to the serial dilutions of the sera, from1:40, 1:60, 1:80, 1:100, 1:120 and 1:140. (+): positive control, HEV inoculums were incubated with a known HEV neutralizing serum. (−): negative control, HEV inoculums were incubated with the pre-vaccination sera. M: DNA Molecular Weight Marker (Roche, Germany).

HEV-FMDV vaccine induced higher titers of neutralizing antibodies than the monovalent HEV vaccine, which was in accordance with the anti-HEV titers determined by ELISA. This detection implied that the inactivated FMDV vaccine in the HEV-FMDV combined vaccine candidate increased the immunogenicity of the HEV antigen. However, further protection experiments in vivo are needed to validate the efficacy of the this HEV-FMDV vaccine.

4. Discussion

To our knowledge, this is the first fundamental study on HEV-FMDV combined vaccine. We designed such a vaccine based on the following facts: (1) HEV infection is very common in China and mainly caused by genotype 4 strains [9]; (2) It has been widely recognized that pig is the main source of HEV genotype 4, and pig HEV strains can be transmitted to humans after the consumption of contaminated pork products [30] or even by having frequent contact with infected pigs [31]; (3) Therefore, vaccination of pigs against HEV can reduce the source of zoonotic infection in humans; (4) In order to achieve such a goal, the swine vaccine against hepatitis E should be widely used and thus should be interesting enough for the pig breeders in terms of benefit versus cost; (5) Therefore, a combined vaccine protecting against HEV and against a serious swine disease such as FMD would be an attractive vaccine for the swine industry as well as public health policy makers who could implement it in national programs for the control of zoonotic HEV (genotype 4) at its main source.

The dominant neutralizing epitopes of HEV have been localized on the ORF2 protein (region aa460-605) [32]. We have previously produced one of the two HEV vaccines that underwent clinical trials in China, the p179 protein (ORF2 aa439-617) [12]. In this study, besides the p179 protein, 8 other proteins were produced by C- and/or N-terminal truncation of the capsid protein, keeping the most immunogenic region intact (region aa460-605). For the selection of the best HEV immunogen to be combined with the FMDV vaccine, we analyzed four parameters: thermal stability, antigenicity, immunogenicity and the ability to selfassemble into VLPs. The results indicated that the p222 was the best HEV immunogen to be combined with the FMDV vaccine.

FMD is a serious and highly infectious disease of swine. In China, vaccination against FMDV is mandatory and have shown satisfactory results in regards to the control and prevention of the infection [33]. Herein, we selected a commercially available and widely used inactivated FMDV vaccine which is based on two epidemic FMDV type O strains: the O/Mya98/XJ/2010 and O/GX/09-7 strains. In order to compare the immunogenicity the of the FMDV and HEV vaccines as well as the different formulations of the HEV-FMDV combined vaccine, we adopted the indirect ELISA method to assess the humoral immune response they induced upon injection into mice. For the detection of the HEV-specific antibodies, we used the HEV p166 protein as a coating antigen (ORF2 aa452-617). This antigen has been used repeatedly for similar purposes [25,26]. Concerning the detection of the FMDV-specific antibodies, various ELISA formats have been developed,

including indirect ELISAs and competition-, blocking-, or sandwich-based assays. Recently, Cao et al. [34] produced a multi-epitope antigen based on the entire G–H loop domain of different FMDV strains and used it for the development of an indirect ELISA with high performance comparable to other existing methods. Similarly, we produced an antigen using the entire G–H loop domains of the FMDV O/Mya98/XJ/2010 and O/GX/09-7 strains (L_{MYA-GX}) and used it as the coating antigen in the indirect ELISA. Since the L_{MYA-GX} antigen is based on the same two strains than the selected FMDV vaccine, this increases the specificity of the detection method.

One of the potential obstacles in the development of a combined vaccine is the possibility of interference between the combined immunogens. To evaluate this interference in the HEV-FMDV combined vaccine, we first sought to establish a baseline for the immunogenicity of HEV and FMDV immunogens separately. The injection of different doses of FMDV vaccine into mice elicited the production of specific antibodies in a dose-dependent manner. On the other hand, the mice immunization with different doses of HEV p222, produced HEV-specific antibodies in a dose-independent manner, with the highest antibody titers registered in the group E25 (receiving only 5 μg of p222). Similar results have been reported earlier: Mice vaccination with 5 μg of an HEV vaccine candidate has been reported to elicit a strong immune response that was estimated to persist for lifetime [35]; Likewise, administration of 0.4 μg of an HEV immunogen protected rhesus monkeys from hepatitis even after receiving intravenously as much as 300,000 times the monkey infectious dose of HEV [36].

A safe and efficient combined vaccine is always more attractive than the monovalent vaccine or association of monovalent vaccines, because of administration convenience, wide protection (multi-valence) and low cost of production. The first step towards a successful combined-vaccine is to demonstrate the compatibility of the antigens to combine and the absence of immunological interference between the vaccine components [37]. Several factors of both the combined vaccine and the vaccinee could lead to immunological interference and affect the immunogenicity of one or more of the valences of the combined vaccine [38]. Therefore, we sought to evaluate the immunological interference in the different HEV-FMDV combined vaccine formulations. The results indicated that the FMDV and HEV antigen were compatible and produced a synergistic effect on their immunogenicity. The HEV-FMDV combination induced anti-FMDV antibody titers 7–16-fold higher than the titers induced by the FMDV vaccine alone, and the combinations induced HEV-specific antibody titers 2.4-fold higher than those induced by the HEV p222 antigen alone. This demonstrates the absence of interference in the humoral responses induced by the HEV-FMDV combined vaccine.

Current methods for evaluating the neutralizing activity of HEV-specific antibodies include immunofluorescence focus assays (IFAs), real-time PCR, and high-throughput neutralization assay [39,40]. In this study, the basic neutralizing activity of the antibodies elicited by the HEV-FMDV combined vaccine candidate was determined by neutralization assay in vitro. The titers of HEV neutralizing antibodies in the vaccinated mice was 1:80 and 1:100 for HEV vaccine alone and HEV-FMDV combined vaccine, respectively. Even though the effects of neutralizing antibodies in vitro has not been identical with the protection levels in vivo, their similar protective functions were displayed in most of cases. However, the neutralization assay in vitro is just a preliminary step in the HEV-FMDV vaccine research, and the efficacy of such a vaccine in protecting vaccinated pigs would be conducted in future investigation. The inactivated FMDV vaccine used in the present work was approved by government departments and manufactured extensively, and its protective effect in pigs has been verified [41].

In summary, hepatitis E emerged as a serious health problem in industrialized countries, predominantly as porcine zoonosis associated with HEV genotypes 3 and 4 [6]. Numerous studies have also linked this zoonotic hepatitis E to the consumption of contaminated pork products or close contact with pig population (farmers and swine industry workers) [7,8,30,42,43]. Therefore, in this study, we presented a new approach for the control of zoonotic HEV infection through its control in its main host (pig). More interesting, this approach can be adapted to the needs of any given country or region based on the zoonotic HEV genotype (strains) circulating in the region, and according to the swine vaccines already implemented in the vaccination programs. Furthermore, herein, we designed the HEV-FMDV combined vaccine, according to the needs of mainland China, and the results of the preliminary assessment of its immunogenicity are very promising.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

Authors' contribution: J.H., Z.L. and N.B. conceived and planned the experiments. Z.L. N.B. S.B. and W.W. performed the experiments and summarized the results. Z.L., J.H., and N.B. contributed to the interpretation of the results. N.B. and Z.L. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the final version of the manuscript.

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References

[1] World Health Organization, Department of Immunization, Vaccines and Biologicals. The global prevalence of hepatitis E virus infection and susceptibility: a systematic review; 2010.
[2] Smith DB, Simmonds P, Jameel S, Emerson SU, Harrison TJ, Meng XJ, et al. Consensus proposals for classification of the family Hepeviridae. J Heneral Virol 2014;95:2223–32.
[3] Dalton HR, Izopet J. Transmission and epidemiology of Hepatitis E Virus genotype 3 and 4 infections. Cold Spring Harb Perspect Med 2018;8(11): a032144. https://doi.org/10.1101/cshperspect.a032144.
[4] Huang W, Zhang H, Harrison TJ, Lang S, Huang G, Wang Y. Cross-protection of hepatitis E virus genotypes 1 and 4 in rhesus macaques. J Med Virol 2008;80:824–32.
[5] Sanford BJ, Dryman BA, Huang YW, Feagins AR, Leroith T, Meng XJ. Prior infection of pigs with a genotype 3 swine hepatitis E virus (HEV) protects against subsequent challenges with homologous and heterologous genotypes 3 and 4 human HEV. Virus Res 2011;159:17–22.
[6] Sanford BJ, Opriessnig T, Kenney SP, Dryman BA, Córdoba L, Meng XJ. Assessment of the cross-protective capability of recombinant capsid proteins derived from pig, rat, and avian hepatitis E viruses (HEV) against challenge with a genotype 3 HEV in pigs. Vaccine 2012;30:6249–55.
[7] Hewitt PE, Ijaz S, Brailsford SR, Brett R, Dicks S, Haywood B, et al. Hepatitis E virus in blood components: a prevalence and transmission study in southeast England. Lancet (London, England) 2014;384:1766–73.
[8] Suin V, Klamer SE, Hutse V, Wautier M, Jacques M, Abady M, et al. Epidemiology and genotype 3 subtype dynamics of hepatitis E virus in Belgium, 2010 to 2017. Euro surveillance: bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin. 2019;24.
[9] Thom K, Gilhooly P, McGowan K, Malloy K, Jarvis LM, Crossan C, et al. Hepatitis E virus (HEV) in Scotland: evidence of recent increase in viral circulation in humans. Euro Surveillance: bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin. 2018;23.
[10] Dai X, Dong C, Zhou Z, Liang J, Dong M, Yang Y, et al. Hepatitis E virus genotype 4, Nanjing, China, 2001–2011. Emerg Infect Dis 2013;19:1528–30.
[11] Ma Z, Feng R, Zhao C, Harrison TJ, Li M, Qiao Z, et al. Seroprevalence and distribution of hepatitis E virus in various ethnic groups in Gansu province, China. Infect, Genet Evol: J Mol Epidemiol Evol Genet Infec Diseases 2010;10:614–9.

[12] Dong C, Dai X, Liang J, Dong M, Meng J. Seroprevalence of hepatitis e virus varies considerably among Chinese provinces. Hepatitis Month 2012;12:386–90.
[13] Cao YF, Tao H, Hu YM, Shi CB, Wu X, Liang Q, et al. A phase 1 randomized open-label clinical study to evaluate the safety and tolerability of a novel recombinant hepatitis E vaccine. Vaccine 2017;35:5073–80.
[14] Zhang J, Shih JW, Xia NS. Long-term efficacy of a hepatitis E vaccine. New Engl J Med 2015;372:2265–6.
[15] Sridhar S, Lo SK, Xing F, Yang J, Ye H, Chan JF, et al. Clinical characteristics and molecular epidemiology of hepatitis E in Shenzhen, China: a shift toward foodborne transmission of hepatitis E virus infection. Emerg Microbes Infect 2017;6:e115.
[16] Valdazo-Gonzalez B, Timina A, Scherbakov A, Abdul-Hamid NF, Knowles NJ, King DP. Multiple introductions of serotype O foot-and-mouth disease viruses into East Asia in 2010–2011. Vet Res 2013;44:76.
[17] Mahapatra M, Parida S. Foot and mouth disease vaccine strain selection: current approaches and future perspectives. Exp Rev Vacc 2018;17:577–91.
[18] Cao Y, Lu Z, Li P, Sun P, Fu Y, Bai X, et al. Improved neutralising antibody response against foot-and-mouth-disease virus in mice inoculated with a multi-epitope peptide vaccine using polyinosinic and poly-cytidylic acid as an adjuvant. J Virol Methods 2012;185:124–8.
[19] Cao Y, Lu Z, Li Y, Sun P, Li D, Li P, et al. Poly(I:C) combined with multi-epitope protein vaccine completely protects against virulent foot-and-mouth disease virus challenge in pigs. Antiviral Res 2013;97:145–53.
[20] Lee SY, Ko MK, Lee KN, Choi JH, You SH, Pyo HM, et al. Application of mouse model for effective evaluation of foot-and-mouth disease vaccine. Vaccine 2016;34:3731–7.
[21] Meng J, Dai X, Chang JC, Lopareva E, Pillot J, Fields HA, et al. Identification and characterization of the neutralization epitope(s) of the hepatitis E virus. Virology 2001;288:203–11.
[22] Dong C, Meng JH. Expression, purification and immunogenicity of a novel hepatitis E virus-like particle. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi 2006;22:339–42.
[23] Liu S, Tobias R, McClure S, Styba G, Shi Q, Jackowski G. Removal of endotoxin from recombinant protein preparations. Clin Biochem 1997;30:455–63.
[24] Reichelt P, Schwarz C, Donzeau M. Single step protocol to purify recombinant proteins with low endotoxin contents. Prot Expr Purif 2006;46:483–8.
[25] Wen J, Behloul N, Dai X, Dong C, Liang J, Zhang M, et al. Immunogenicity difference between two hepatitis E vaccines derived from genotype 1 and 4. Antiviral Res 2016;128:36–42.
[26] Behloul N, Wen J, Dai X, Dong C, Meng J. Antigenic composition and immunoreactivity differences between HEV recombinant capsid proteins generated from different genotypes. Infect, Genet Evol: J Mol Epidemiol Evol Genet Infec Diseases 2015;34:211–20.
[27] Xu H, Bao X, Lu Y, Liu Y, Deng B, Wang Y, et al. Immunogenicity of T7 bacteriophage nanoparticles displaying G-H loop of foot-and-mouth disease virus (FMDV). Vet Microbiol 2017;205:46–52.
[28] Meng J, Dubreuil P, Pillot J. A new PCR-based seroneutralization assay in cell culture for diagnosis of hepatitis E. J Clin Microbiol 1997;35:1373–7.
[29] Emerson SU, Clemente-Casares P, Moiduddin N, Arankalle VA, Torian U, Purcell RH. Putative neutralization epitopes and broad cross-genotype neutralization of Hepatitis E virus confirmed by a quantitative cell-culture assay. J Gen Virol 2006;87:697–704.
[30] Pavio N, Meng XJ, Doceul V. Zoonotic origin of hepatitis E. Curr Opin Virol 2015;10:34–41.
[31] Caruso C, Peletto S, Rosamilia A, Modesto P, Chiavacci L, Sona B, et al. Hepatitis E virus: a cross-sectional serological and virological study in pigs and Humans at Zoonotic Risk within a High-Density Pig Farming Area. Transbound Emerg Diseases 2017;64:1443–53.
[32] Wei W, Behloul N, Baha S, Liu Z, Aslam MS, Meng J. Dimerization: a structural feature for the protection of hepatitis E virus capsid protein against trypsinization. Sci Rep 2018;8:1738.
[33] Cai C, Li H, Edwards J, Hawkins C, Robertson ID. Meta-analysis on the efficacy of routine vaccination against foot and mouth disease (FMD) in China. Prevent Veter Med 2014;115:94–100.
[34] Cao Y, Zhou W, Xing X, Zhang J, Fu Y, Li K, et al. Indirect ELISA using a multi-epitope recombinant protein to detect antibodies against foot-and-mouth disease virus serotype O in pigs. J Virol Methods 2018;262:26–31.
[35] Kulkarni SP, Thanapati S, Arankalle VA, Tripathy AS. Specific memory B cell response and participation of CD4+ central and effector memory T cells in mice immunized with liposome encapsulated recombinant NE protein based Hepatitis E vaccine candidate. Vaccine 2016;34:5895–902.
[36] Tsarev SA, Tsareva TS, Emerson SU, Govindarajan S, Shapiro M, Gerin JL, et al. Recombinant vaccine against hepatitis E: dose response and protection against heterologous challenge. Vaccine 1997;15:1834–8.
[37] Bouvet J, Cariou C, Poulard A, Oberli F, Cupillard L, Guigal PM. Compatibility between a rabies vaccine and a combined vaccine against canine distemper, adenovirosis, parvovirosis, parainfluenza virus and leptospirosis. Vet Immunol Immunopathol 2018;205:93–6.
[38] Vidor E. The nature and consequences of intra- and inter-vaccine interference. J Comp Pathol 2007;137(Suppl 1):S62–6.
[39] Cai W, Tang ZM, Wen GP, Wang SL, Ji WF, Yang M, et al. A high-throughput neutralizing assay for antibodies and sera against hepatitis E virus. Sci Rep 2016;6:25141.
[40] Liu C, Cai W, Yin X, Tang Z, Wen G, Ambardekar C, et al. An optimized high-throughput neutralization assay for Hepatitis E Virus (HEV) involving detection of secreted Porf2. Viruses 2019;11.
[41] Li D, Lu ZJ, Xie BX, Sun P, Chen YL, Fu YF, et al. Alternative way to test the efficacy of swine FMD vaccines: measurement of pigs median infected dose (PID50) and regulation of live virus challenge dose. Virol J 2010;7:215.
[42] Shirazi R, Pozzi P, Wax M, Bar-Or I, Asulin E, Lustig Y, et al. Hepatitis E in pigs in Israel: seroprevalence, molecular characterisation and potential impact on humans. Euro surveillance: bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin 2018;23.
[43] Mughini-Gras L, Angeloni G, Salata C, Vonesch N, D'Amico W, Campagna G, et al. Hepatitis E virus infection in North Italy: high seroprevalence in swine herds and increased risk for swine workers. Epidemiol Infect 2017;145:3375–84.