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Efficacy and immunogenicity of recombinant swinepox virus expressing the A epitope of the TGEV S protein

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ABSTRACT

To explore the possibility of developing a vaccine against transmissible gastroenteritis virus (TGEV) infection, a recombinant swinepox virus (rSPV-SA) expressing a TGEV protective antigen has been constructed. Immune responses and protection efficacy of the vaccination vector were assessed in both mice and pig models. An indirect ELISA assay suggested that when mice were vaccinated with rSPV-SA, the level of IgG against TGEV was enhanced dramatically. The cytokine assays were employed and the results indicated that both the Th1-type and Th2-type cytokine levels raised after vaccination with rSPV-SA in mice models. Results from the passive immunity protection test of new born piglets demonstrated that the recombinant live-vector vaccine, rSPV-SA, could 100% protect piglets from the SPV infection, and there was no significant clinical symptom in the rSPV-SA treatment group during this experiment. The data suggest that the novel recombinant swinepox virus is a potential vaccine against TGEV infection.

1. Introduction

Transmissible gastroenteritis virus (TGEV) is a member of Coronaviridae, which is the etiological agent of transmissible gastroenteritis. Although the virus is capable of infecting swine of all ages, suckling piglets are the most susceptible and have a mortality rate up to 100% [1]. TGEV is a pleomorphic enveloped virus containing a positive-stranded RNA genome and four structural proteins: the spike (S) protein, the integral membrane (M) protein, the minor envelope (E) protein, and the nucleocapsid (N) protein. Among which, the spike (s) protein, one of the key structural membrane proteins of coronaviruses, is an attractive target for generating neutralizing antibodies against the virus due to the critical role it plays in the host cell invasion [2,3]. Precisely, the S protein mediates the attachment of virus particles to targets via binding of itself to the specific receptors. At the N terminus of the S protein, there are four antigenic sites, A, B, C, and D, which have been shown to be involved in the stimulation of neutralizing antibodies (Fig. 1) [4]. Previous studies have determined that the A site (which is fully dependent on glycosylation for proper folding) is predominantly responsible for stimulating neutralizing host antibodies [5–10].

SPV is a natural mild attenuated virus and has been widely applied as a vaccine. Given that Poxvirus-vectors can prevent a great deal of important diseases in both humans and animals it is not surprising that many of these vectors been licensed and used extensively [11–13]. Additionally, SPV is a safe vaccine vector as there is no risk of cross-species infection[14]. Therefore, both for biological and clinical practicality, SPV is regarded as an appropriate and promising veterinary vaccine for swine, owing to its ability to effectively express foreign genes, its large packaging capacity for recombinant DNA, its low cost of delivery and its specific host restriction [15]. The potential value of SPV as a live vector vaccine is being studied extensively. Because SPV is able to pack large amounts of recombinant DNA and to induce appropriate immune responses in vivo, it is a promising candidate for the development of a recombinant vaccine [16,17]. As of yet, pigs are the only known hosts of swinepox virus and therefore may be useful in developing a safe vaccine for clinical application [18,19].

In this study, we constructed a recombinant swinepox virus expressing S-A (A epitope of the S protein) of TGEV and characterized recombinant virus replication and expression of the S protein in PK-15 cells. We further investigated the potential of this approach for use in the vaccination of pigs against TGE.

2. Materials and methods

2.1. Cells and viruses

Wild type swinepox virus (wtSPV, Kasza strain, ATCC: VR363), swine testicle cells (ST, ATCC: CRL174), and porcine kidney cells (PK-15, ATCC: CCL-33) were purchased from the American

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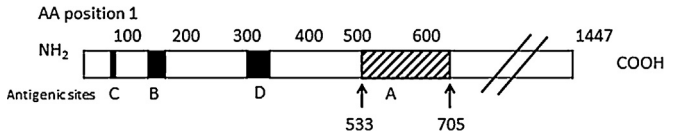

Fig. 1. Schematic representation of the four antigenic sites in the S protein of TGEV. The antigenic sites (C, B, D and A from left to right) of the TGEV S protein is shown using the Madrid nomenclature.

Type Culture Collection. Swine transmissible gastroenteritis virus (TGEV, China strain, SHXB) was purchased from the Jiangsu Academy of Agricultural Sciences, and the titer was determined as 1×10^8 PFU/ml ST cell. TGEV convalescent positive serum was purchased from the Jiangsu Academy of Agricultural Sciences, and the neutralizing antibodies were used at a dilution of 1:10,000.

2.2. Construction and identification of the rSPV-SA plasmid

The pUSZ11 swinepox virus vector was generated previously [20]. Two primers, SA1 (5′-GCGTCGACATGGGTCTTGGTATGA-AGCGTAG-3′) and SA2 (5′-CGGGATCCTTA TAGCGTCCTGT-TAGTTTGTC-3′) were used to amplify the S-A gene (516 bp, KM980444) from the TGEV genome, which was inserted into the pUSZ11 plasmid to construct pUSZ11-SA subsequently. The recombinant swinepox virus, rSPV-SA, was generated by homologous recombination of wtSPV with pUSZ11-S-A as previously described [21]. PCR and indirect immunofluorescence were employed to analyze the S-A gene expression and the expression of S protein. The replication capacity and genetic stability of rSPV-SA were also evaluated by.

2.3. Generation and screening of rSPV-SA

The generation and screening of the recombinant swinepox virus assays were performed as described previously [20]. A subconfluent culture of PK-15 cells was infected with wtSPV (0.02 moi) for 2 h, and subsequently transfected with 10 μg of the pUSZ11-SA plasmid using Exfect™ Transfection Reagent (Vazyme Biotech Co., Ltd). After 72 h, PK-15 cells were harvested and lysed by five rounds of freezing and thawing. Subsequently, the lysate was used to infect PK-15 cells grown in a 12-well plate for further purification of recombinant viruses. 1.5 ml of medium with 1% LMP agarose (DingGuo, beijing, china) was added to each well and incubation was continued for five days until plaques became visible under a light microscope. After 1–2 days, a second overlay medium containing X-gal was added. The plaques were resuspended in 0.3 ml of medium with 2% FBS. Plaque isolation was repeated for 5–6 rounds until all plaques in a given well were stained blue. The recombinant SPV bearing S-A of TGEV was designated as rSPV-SA.

2.4. PCR analysis of the recombinant swinepox virus

The rSPV-SA genomic DNA from the PK-15 cells infected with rSPV-SA was extracted by SDS-protease K-phenol. We utilized wtSPV genomic DNA from PK15 cells infected with wtSPV as a negative control. PCR was performed for 5 min at 94 °C; followed by 32 cycles of 1 min at 94 °C, 1 min at 50 °C, and 1 min at 72 °C. Amplifications were performed with DNA polymerase (Promega, Shanghai, China) using primers SA1 (5′-GCGTCGACATGGGTCTTGGTATGAAGCGTAG-3′) and SA2 (5′-CGGGATCCTTATAGCGTCCTGTTAGTTTGTC-3′).

2.5. Immunofluorescence assays

Indirect immunofluorescence assays (IFA) were performed as described previously [21]. PK-15 cells were grown on a 24-well plate and infected with the wtSPV and rSPV-SA at 1×10^8 PFU/ml per well. PBS-treated cells were used as a negative control. At 60 h post-infection, cells were washed three times in PBST and fixed with cold methanol for 10 min at −20 °C. Cells were then washed three times with PBST and blocked by PBST with 10% BSA. Preparations were incubated for 1 h at 37 °C with TGEV convalescent positive serum (1:1000 in dilution buffer, PBST with 1% BSA). After three washes with PBST, cells were treated with the rhodamine-conjugated secondary antibody (Staphylococcal protein A-Rhod, Boshide, Wuhan, China) at a 1:5000 dilution (diluted in PBS) for 30 min at 37 °C. After a final wash with PBS, all wells were examined by fluorescence microscopy (Zeiss, Germany).

2.6. Immunization of mice and swine

Nine six-week-old BALB/C mice were randomly divided into three groups (3 mice per group), and immunized three times at 0, 14, and 28 days with rSPV-SA (1×10^8 PFU/ml in 0.2 ml of PBS) or wtSPV (1×10^8 PFU/ml in 0.2 ml of PBS), the control group injected with PBS.

Eight one-month-old swine (Large White) were randomly divided into four groups (2 pigs per group) and were immunized twice at 0 and 28 days with infectious rSPV-SA (1×10^8 PFU/ml in 2 ml of PBS), inactivated-TGEV (1×10^8 PFU/ml in 2 ml of PBS), wtSPV (1×10^8 PFU/ml in 2 ml of PBS) or PBS, each time via three routes: oral, nasal, and intraperitoneal. Serum was collected 14 days after the last immunization.

Twelve one-day-old pigs were randomly divided into four groups for passive immunization experiments (3 pigs per group). High titers of antibodies were collected from piglets following the first immunization. Mice and swine serum were incubated at 56 °C 30 min to complement inactivated.

All experimental protocols involving mice or swine were approved by the Laboratory Animal Monitoring Committee of Jiangsu Province.

2.7. Western blot assays

PK-15 cell monolayers were infected with wtSPV and rSPV-SA (moi of 5) and incubated for 72 h at 37 °C. Extracts, representing approximately 1×10^5 cells, were electrophoresed through an SDS-12% polyacrylamide gel and the separated proteins were transferred onto a PVDF membrane. After a 2 h transfer, the membrane was blocked with 5% skim milk in phosphate buffered saline with 0.05% Tween-20 (PBST) overnight at 4 °C. The membrane was incubated with swine convalescent serum (1:1000 dilution) containing TGEV for 2 h at 37 °C and washed three times with PBST. Immunodetection was performed with staphy-lococcal protein A-HRP at 37 °C. Following the secondary antibody probing, the membrane was washed four times with PBST. The membrane was then developed with 3,3′-diaminoben-zidine substrate until optimal color development was observed.

2.8. Indirect ELISA

Serum was collected from mice and pigs, and detected the TGEV-specific antibodies by indirect ELISA. The purified TGEV was resuspended in 100 μl PBS (pH 7.2), and used the best titer of virus for coating 96-well plates, which was determined by titration. Samples were then incubated overnight at 4 °C. This incubation was followed by three PBST washes, and blocking with 5% skim milk (in PBST) at 37 °C for 2 h. Serum samples were serially diluted and

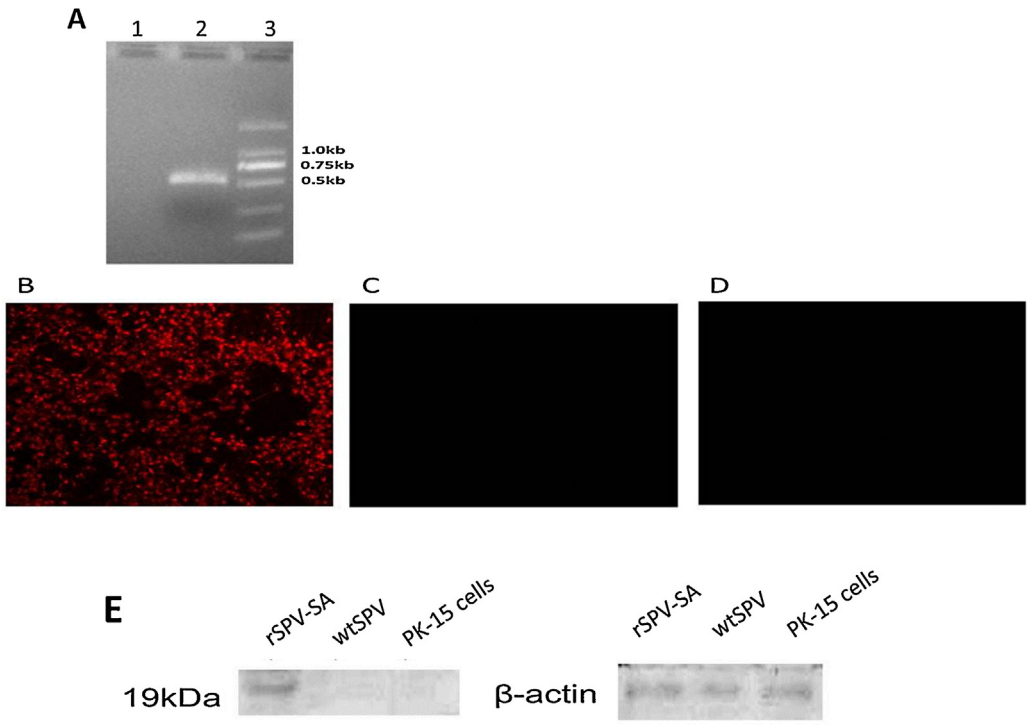

Fig. 2. Characterization of recombinant swinepox virus. (A) PCR analysis of rSPV-SA. Lane (1) wtSPV; Lane (2) rSPV-SA; Lane (3) DL2000 DNA marker. Identification of SA by IFA on PK-15 cells infected with (B) rSPV-SA, (C) wt SPV, and (D) PBS treated cells.(E)Western blot analyses of rSPV-SA.

incubated at 37 °C for 1 h. The samples were set up at the same time and divided into three groups: the TGEV positive serum, the negative control serum (SPV positive serum) and the blank control (without serum). After three PBST washes, horseradish peroxidase (HRP)-conjugated goat anti-SPA IgG (1:10,000 diluted in PBST, Signalway Antibody) was added to each test well. The plates were then incubated at room temperature in the dark for 30 min and then washed three times with PBST. The TMB microwell peroxidase substrate system (TIANGEN) was used to develop the reaction. Samples were developed for 20 min and the reaction terminated with 2.0 M sulphuric acid. All assays were performed in duplicate. A microplate reader (Bio-Rad) was used to measure the reaction product at an absorbance of 450 nm [22].

2.9. Cytokine assay

Evaluation of cellular immunity was performed by detecting levels of IFN-γ and IL-4. Three mice were sacrificed at 30 days after the first inoculation with wtSPV (1×10^8 PFU/ml in 0.2 ml), PBS or rSPV-SA (1×10^8 PFU/ml in 0.2 ml). The mouse spleen was removed aseptically. Splenocytes were isolated, counted, and diluted to a density of 2×10^7 cells/100 μl. The evenly separated cells were aliquot into 96-well plates (100 μl/well). Then, 100 μl/well of DMEM with 200 $TCID_{50}$/100 μl TGEV was added to each well. After a 60 h incubation, the supernatants were collected and the mRNA of IFN-γ and IL-4 were probed by RT-qPCR relative to β-actin as described previously [23,24].

2.10. Assay for neutralizing antibodies

An assay for neutralizing antibodies was performed as described previously [18,19]. To explore whether mice or swine generated TGEV neutralizing antibodies, serum from the PBS, wtSPV, inactivated-TGEV and rSPV-SA treated mice and pig were collected at 0, 14, 21, 35, 42 days post-primary immunization (1:100–1:12,800 dilution in a 100 μl volume). And sera were mixed with equal volume of 100 $TCID_{50}$/ml TGEV and incubated at 37 °C. After 1.5 h incubation, we utilized sera treated viruses to infect ST cells in 96-well plates and overlaid cells with agar at 37 °C in a 5% CO_2 atmosphere. Cells were monitored daily for three days to detect TGEV-specific CPE.

2.11. Protection of swine by immune serum

The virulent TGEV strain SHXB (1×10^8 PFU/ml in 1 ml) was mixed with 3 ml of the porcine antiserum induced by recombinants rSPV-SA, inactivated TGEV or PBS, incubated at 37 °C for 60 min, and administered using a gastric tube to 2-day-old swine born from TGEV-seronegative sow. Inoculated animals were fed three times per day with formula for newborns that contained 3 ml of the antiserum. At three days following viral infection, small intestine tissue was collected from newborn pigs sectioned for histology. Microstructure characteristics were analyzed under the microscope (OLYMPUS, CX21FS1, Philippines) [25].

2.12. Statistical analysis

All data were analyzed using one-way ANOVA and values of $P < 0.05$ were considered significant.

3. Results

3.1. Characterization of the recombinant swinepox virus

To analyse the recombinant virus and confirm the presence of the SA gene, two specific primers were designed to amplify the inserted SA gene. The gene encoding the neutralizing antigen epitopes of TGEV is a 0.5 kb fragment and the specific fragments was detected in SPV-SA as shown in Fig. 2A. The recombinant SPV-SA was also confirmed by observing blue foci in plaque assays. rSPV-SA and wtSPV were determined to be approximately 1×10^8 PFU/ml in 1 ml for both. As shown in Fig. 2B, the IFA demonstrated that

the expression of the A epitope of the S protein was present in infected cells. Western blot analysis showed a specific band of target protein with a size of 19 kDa in the cell lysates infected with rSPV-SA (Fig. 2E). The 19 kDa molecular weight is consistent with the predicted size of the SA protein of TGEV. According to these data, we suggested that the A epitope of the S protein was expressed efficiently by the rSPV-SA virus (Fig. 2).

3.2. Humoral immune responses in mice model

To monitor the S-specific antibody titers of mice vaccinated with rSPV-SA, an ELISA was used. After an initial boost 7 days post-vaccination, the S-A-specific antibody titers increased gradually in mice (Fig. 3A). The antibody titers in rSPV-SA-immunized mice were higher at all-time points post-vaccination ($P < 0.01, n = 5$) compared to wtSPV or PBS-treated mice. The P/N value of the positive group was greater than 2.1. Persistent high levels of neutralizing antibodies (Fig. 4A) were detected in the rSPV-SA group with a mean titer of 1:1000 at 42 days post-inoculation.

3.3. Cellular immune responses in mice

In mice vaccinated with wtSPV and rSPV-SA at 30 days post-inoculation (Fig. 3B), the qPCR results showed a distinct variation of IL-4 and IFN-γ between wtSPV and rSPV-SA treatment groups. The relative quantity normalized by beta-actin of IL-4 mRNA in rSPV-SA was 1.47 times higher compared to wtSPV. The IFN-γ mRNA levels increased 1.45 fold in rSPV-SA when compared to wtSPV. The concentrations of IL-4 and IFN-γ in rSPV-SA-vaccinated mice were significantly higher than the control groups. These results indicate that rSPV-SA induces Th1-type and Th2-type cytokine responses during cellular immunity.

3.4. Safety of rSPV-SA in pigs

During the vaccination procedure, several poxes of 2 mmφ could be observed around the injection position at five days post-inoculation in both rSPV-SA and wtSPV treatment groups, respectively, which was not observed in the inactivated-TGEV and PBS treatment groups. This symptom could be disappeared spontaneously in the following 5 days. No pigs developed further symptoms such as fever or severe inflammation, and the spiritual condition and appetite of both treatment groups was considered as good. These two group pigs recovered in 14 days. All group pigs maintained rectal temperatures of 38.7–39.3 °C. From this experiment, we reveal that vaccination with rSPV-SA and wtSPV is well tolerated by pigs.

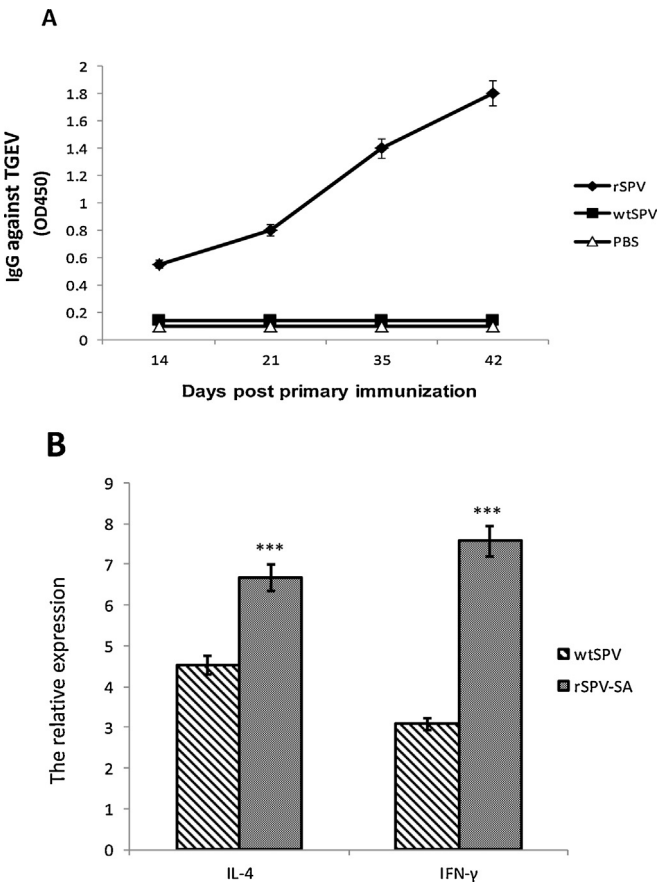

Fig. 3. rSPV-SA-induced immune responses in mice. (A) IgG antibodies (OD values) to TGEV were detected via ELISA using 1:1000 dilution. (B) Levels of secretion of cytokines in splenic lymphocytes in mice. Data are shown as mean ± S.D. The error bars represent standard deviations, and "*"indicates significant differences ($P < 0.05$) among the treatments for each kind of cytokine.

3.5. Neutralizing antibody production in swine

rSPV-SA induced a moderate level of TGEV-specific IgG as shown in Fig. 5. Persistent high levels of TGEV-specific neutralizing antibodies are shown in Fig. 4b. The second boost led to of the levels

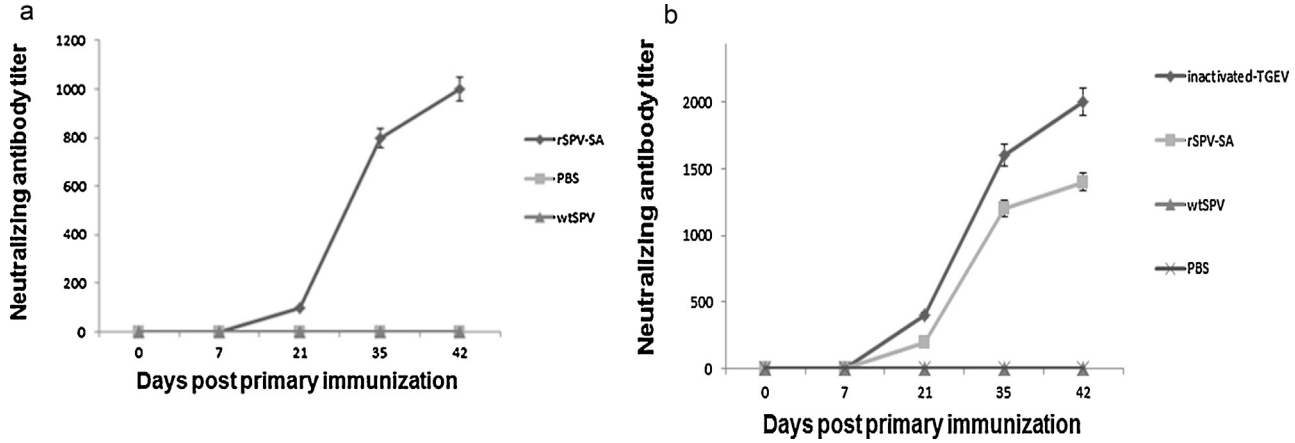

Fig. 4. TGEV-specific neutralizing antibodies were detected by a virus neutralizing assay using twofold serial dilutions. The titers of neutralizing antibodies were expressed as the reciprocal of the highest serum dilution in which no CPE was observed. (a) Response in mice. (b) Response in swine. Data are shown as mean ± S.D.

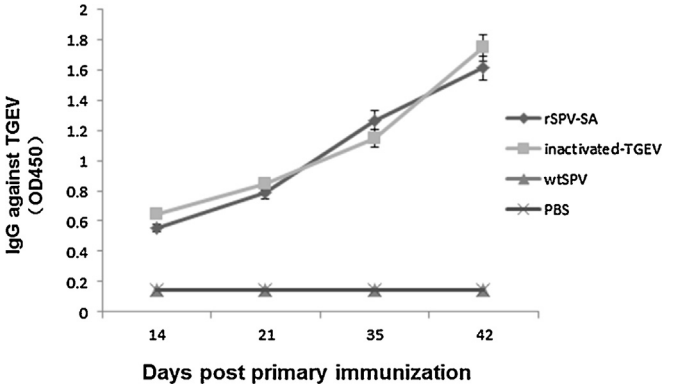

Fig. 5. rSPV-SA-induced immune response in swine. IgG antibodies (OD values) to TGEV were detected via ELISA using a 1:1000 dilution.

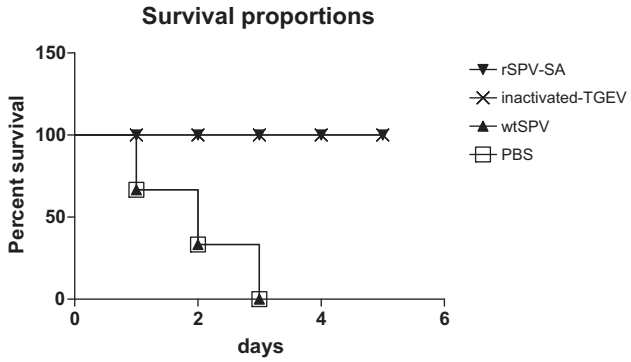

Fig. 6. Protection of newborn swine with porcine sera.

of TGEV-specific neutralizing antibodies. Persistent high levels of neutralizing antibodies were detected in the rSPV-SA group at a mean titer of 1:1400 in swine ($P < 0.05$, $n = 5$).

3.6. Passive immunity

All newborn piglets in different groups were fed with the mixture of TGEV and the corresponding sera from pigs vaccinated with PBS, wtSPV, inactivated-TGEV, or rSPV-SA. Both PBS and wtSPV treatment groups developed a very severe diarrhea symptom, significantly losing of weight and appetite, and the mortality rate was up to 100% in 3 days. Meanwhile, in the inactivated-TGEV and rSPV-SA treatment groups, there was no obvious clinical symptoms and 0% morality rate observed (Fig. 6. and Table 1). After the sacrificing of all the piglets, the small intestine tissue from different groups were used for the pathological examination. Histological samples from PBS and wtSPV treatment groups showed prominent histopathological changes in the small intestine characterized by serious fracture of the small intestine mucosa, epithelial expansion, vacuolar degeneration, necrosis, shedding, and lamina propria congestive edema and hemorrhage. And such pathological changes were not observed during the examination of the samples from inactivated-TGEV and rSPV-SA treatment groups. All the results indicate that rSPV-SA inoculation provides complete protection passive immunity against TGEV challenge in pigs (Fig. 7).

4. Discussion

In the present study, we have engineered the swinepox virus to express the A epitope of the TGEV S protein. Our data showed that this recombinant virus was not only able to induce a strong immune response against the A antigen of TGEV in mice and pigs, but also had safety degree and protection efficacy against the virulent homologous TGEV infection in pigs. The traditional way to protect swine from TGEV infection is to immunize pregnant sows with inactivated or attenuated vaccine, resulting in the production of Secretory IgA in the colostrum. Despite the protection that Secretory IgA offers piglets, this method does not provide protection after the cessation of breastfeeding. Furthermore, inactivated or attenuated viral vaccines still maintain the ability of developing the viral toxicity [26]. Currently, there are various potential vaccines against TGEV but many present certain challenges, such as difficulties in the vaccination process, high production cost, low immunogenicity, biological risk and carcinogenicity.

The results from our experiments indicate that the rSPV-SA vaccine is a very promising candidate in the safety and immunogenicity respect. However, it should be noticed that, as an alive viral vector vaccine, it is possible that the SPV per se would interfere the vaccination. To evaluate this possibility, 2000 randomly sampled pig sera were tested via the agarose gel diffusion assay, and the results showed the SPV positive rate was around 1%. Moreover, there was no report of SPV epidemic in these recent years. Thus, together with gel diffusion assay, the interfering from SPV per se could be considered as minimum.

For the first time, we generated a recombinant SPV that expresses the neutralizing epitopes (A epitope in Protein S) of TGEV, and we verified that the S-A was expressed efficiently in our system. The antigen induced neutralizing antibodies against TGEV in ST cells, and potentiated strong TH1-type and TH2-type cytokine responses in our mouse model. These results suggest that rSPV-SA induces humoral and cellular immune responses effectively and efficiently. Given the Th1 cell secretion of IL-2, IFN-γ, IFN-α, and TFN-β, it is apparent that Th1 cells play an important role in anti-intracellular pathogenic infection. Because Th2 cells in our study secreted IL-4, IL-5, IL-6 and IL-10, it is likely that these cells were able to effectively stimulate B cell proliferation and hence, IgG and IgE antibody production (relevant to humoral immunity). We defined IFN-γ and IL-4 to be representative of the Th1-type and Th2-type cytokine secretory capacity, respectively. Our results indicate that in our hands, both Th1-type and Th2-type cytokine levels increased dramatically.

Due to the immature immune system, the maternal antibodies from breast milk account for the immune defensive ability of

Table 1
Diarrhea and virus shedding in piglets during virus challenge.

Sow group	Time after oral											
	12 h			24 h			36 h			48 h		
	a	*b*	*c* (%)	*a*	*b*	*c* (%)	*a*	*b*	*c* (%)	*a*	*b*	*c* (%)
rSPV-SA	–	–	0	–	–	0	–	–	0	–	–	0
Inactivated-TGEV	–	+	0	++	+	0	–	–	0	–	–	0
PBS	+	+	33	++	+	66	++	+	66	++	+	100
wtSPV	+		33			66	++	+	66	++	+	100

(a) –, no diarrhea; +mild diarrhea; ++, severe diarrhea. (b) TGEV in feces was detected using RT-PCR. (c) Mortality attributed to TGEV infection.

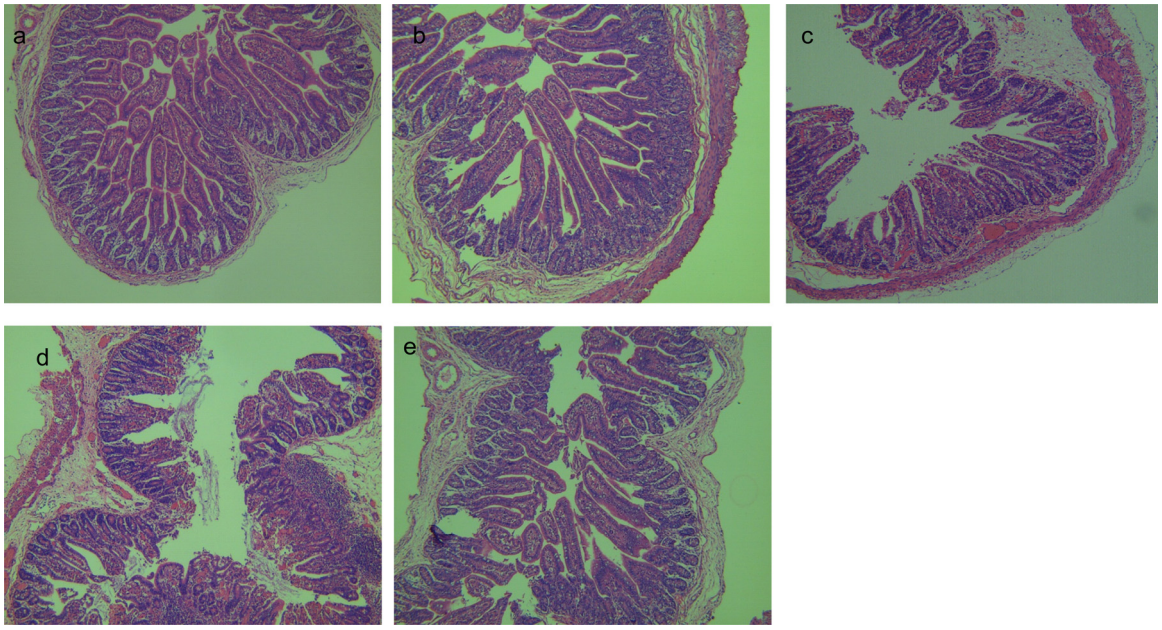

Fig. 7. Histopathological examination of pig small intestine in the rSPV-SA group (a), inactivated-TGEV group (b), wtSPV group (c), PBS group (d) and blank controls group (e) at 3 days post-infection. Hematoxylin and eosin staining (HE). Magnification, 100×.

piglets. Based on the feasibility and relevant published papers [25], we decided to mimic the breast milk from female pig by mixing the anti-serum generated by vaccinating the 30 days old piglets with SPV-SA and the normal cow milk, and use it for passive immunity protection test on the 1days old piglets. In the study, the recombinant SPV vectors were capable of protecting neonatal piglets against mortality and severe disease after a challenge with virus. The rSPV-SA vector induced high titers of antibodies in swine. However it is worth noting that rSPV-SA may enhance the anti-TGEV antibody titer in swine as well as the anti-SPV antibody, which will likely affect immune efficiency. With respect to clinical value, it is imperative to study this multivalent vaccine.

To summarize, we first report that SPV can be used as a live vector vaccine when it expresses the S-A protein of TGEV. We determined that not only B-cells, but also T-cells were induced successfully. Thus, rSPV-SA provides thorough protection against virulent TGEV challenge in swine. Lastly, our data indicate that rSPV-SA is a promising vaccine to prevent TGEV infection.

Conflict of interest statement

The authors of this paper have no financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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