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Recombinant plasmids containing CpG with porcine host defense peptides (PR-39/pBD-1) modulates the innate and adaptive intestinal immune responses (including maternal-derived) in piglets

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ABSTRACT

CpG oligodeoxynucleotides (CpG-ODN) is an immunoenhancer, which is composed of unmethylated cytosine and guanine. Host Defense Peptides (HDPs) are small molecule polypeptides with various immunological activities that have been shown to induce a stronger innate immune response in piglets with synthetic CpG-ODN. Therefore, combination of CpG-ODN and HDPs was expected to be a novel immunoadjuvant with high efficiency, low toxicity and great potential. However, cost of synthetic HDPs or CpG-ODN is too high to be advantageous for animal farming. In this study, in order to improve the immune function of vaccine and reduce cost, a series of recombinant plasmids (containing HDPs gene (PR-39/pBD-1) and different numbers of CpG motifs) were constructed. In vitro, porcine lymphocytes were stimulated by recombinant plasmids to verify the immunostimulatory function of recombinant plasmids. In vivo, recombinant plasmids were used to immunize piglets with Enterotoxigenic *Escherichia coli* (ETEC) vaccine to analyze effects of recombinant plasmids on the mucosal immune responses. In addition, dosage screening and capability of maternal antibody responses were also investigated. Our results showed that recombinant plasmids had strong adjuvant effects especially the plasmid pVAX49-PR-39 and pVAX49-pBD-1. Moreover, there was no diarrhea in piglets using pVAX49-PR-39 or pVAX49-pBD-1 as adjuvants. These findings suggested that recombinant plasmids (containing PR-39/pBD-1 and CpG) as adjuvants of vaccines could enhance immune stimulation better than HDPs or CpG alone. It has a good protective effect on maintaining health of newborn piglets. Among them, both plasmids pVAX49-PR-39 and pVAX49-pBD-1 could be used as effective vaccine adjuvants for piglets.

1. Introduction

CpG motif is a specific nucleotide sequence structure consisting of unmethylated cytosine and guanine dinucleotides as core sequence [1]. Oligodeoxynucleotide (ODN) containing CpG motif is called CpG oligodeoxynucleotides (CpG-ODN) [2]. CpG-ODN is a toll-like receptor agonist that activates B- and/or T-lymphocytes and improves immunoprotective responses in combination with vaccines in many studies [3]. A. Manuja et al. co-cultured CpG-ODN with *E. veneri* antigen, then they found that CpG-ODN could induce lymphocyte proliferation and produce a synergistic effect of eliciting an immune response [4]. CpG-ODN had been shown to stimulate many mammals, including humans, mice, rabbits, horses, cattle, buffalo, sheep, pigs, rats, cats, dogs and non-human primates [5–9]. Repeated use of vaccines caused weak immunogenicity, a highly effective vaccine adjuvant could solve this problem [10]. CpG-ODN was found to be a vaccine adjuvant. Such as recombinant gp85 protein and CpG-ODN adjuvant could enhance antibody response and cellular immune response [11]. The impact of CpG motifs on piglets had been extensively studied in our laboratory. Our previous findings showed that CpG-ODN could interact with defense peptides to affect the innate immunity of piglets [12]. In addition, CpG-ODN was used as an adjuvant for various vaccines (respiratory syndrome virus, Streptococcus suis septicemia, multi-killing Pasteurella vaccine, pseudorabies attenuated vaccine, etc.) to study its immune-

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enhancing effects in piglets [13–16]. It was demonstrated in our laboratory that the potential synergy of CpG-ODN together with vaccine against Enterotoxigenic *Escherichia coli* (ETEC) (with which neonatal piglets were susceptible to infection in our lab) in neonatal and weaning piglets [17].

Host defense peptides (HDPs) are a large group of innate immune effectors that are also termed antimicrobial peptides. HDPs play an important role in the body's innate immunity [2,18]. Moreover, they were widely found in organisms and had innate defense against small molecules that resist various pathogenic bacteria [19]. Similar to CpG-ODN, HDPs could also be used as a vaccine adjuvant to enhance the vaccine's immune effects [20]. Our findings showed that synthetic CpG-ODN and synthetic HDPs could induce stronger immune stimulatory responses than CpG-ODN alone or chemically synthesized HDPs alone [21]. Similar findings had been reported that the combined action of HDPs and CpG motif could increase the adjuvant effect of CpG on vaccines [22–24]. The combination of CpG-ODN and chemically synthesized HDPs as a vaccine adjuvant in pigs showed that the combined action of CpG-ODN and chemically synthesized HDPs could significantly enhance the ability of vaccine to produce specific antibodies. We found that CpG-ODN had synergistic effects with HDPs, and the combined action could produce a stronger immune stimulatory response [25]. However, the cost of synthetic CpG-ODN and HDPs is high, which is not conducive in farming. To address this deficiency, scientists used plasmids directly as vaccine adjuvants [26]. Mukesh Kumar et al. (2001) constructed a recombinant plasmid containing the IFN-γ and IL-12 genes, which proved to be effective as a vaccine adjuvant to improve human allergen immunity [27]. A similar finding was reported by Krieg AM et al. that the presence of a CpG motif provided the necessary immunoadjuvant signal for DNA vaccination [28]. In conclusion, the use of plasmids directly as vaccine adjuvants had proven to be a useful and low cost method.

Weaning piglet diarrhea which is caused by enterotoxigenic *Escherichia coli* (ETEC) vaccine is a disease often encountered in piglets. Diarrhea can be the result in a time of depressed feed intake and growth performance, and of further increased diarrhea and other diseases, bacterial overgrowth, villus atrophy and mortality. CpG ODN and HDPs had been confirmed to confer good protection against ETEC in weaning piglets.

Therefore, we aimed to explore the immunostimulatory effects of combination of CpG motif and HDPs on ETEC vaccine in piglets. A series of recombinant plasmids (containing HDPs (PR-39/pBD-1) and different numbers of CpG motifs) were constructed. The immune enhancing effects of recombinant plasmids were tested in vivo and in vitro. Since piglets are susceptible to stress, in order to reduce the harm to piglets, we considered not directly vaccinating piglets instead of immunizing sows and detecting maternal antibodies obtained in piglets. In addition, the clinical morbidity statistics were performed in piglets. The immune effect of the vaccine adjuvant was directly demonstrated by observing the diarrhea of the piglets. Our results suggested that recombinant plasmids had strong adjuvant effects. Moreover, administration of recombinant plasmids could induce much stronger mucosal immune responses to piglets.

2. Materials and methods

2.1. Animals

Landrace × Large White piglets (4–18 days old, 1–5 kg) and sows (1 year old, about 200 kg) were used in this study. All pigs were from the Swine Breeding Center of Guangzhou. The conventional pigs were kept under standard farming conditions.

2.2. Plasmid construction

The eukaryotic expression vector pVAX1 was stored in the laboratory. Recombinant plasmids (pVAX10 and pVAX49) were constructed and preserved by our lab. We inserted a 10/49 CpG motif (5′-TGCATCGATTTATCGATGCAG-3′) between the *Eco*RI and *Xho*I restriction sites of the vector pVAX1 to construct pVAX10 and pVAX49 [29,30].

The overlapped PCR technique was used to amplify the mature peptide genes of PR-39 and pBD-1. Specific primers were designed using primer design software Primer Premier 5.0. In addition, *Nhe*I and *Eco*RI restriction sites and protected bases, as well as Kozak sequences were added to mature peptide genes. All primers were listed in Tables S1 and S2 in Supplemental file. A set of eukaryotic expression vectors containing CpG motifs and PR-39/pBD-1 gene were obtained. The plasmids constructed in this study were listed in Table S3. The construction process of the recombinant plasmid is shown in the **Fig.S1.**

The plasmids without endotoxin were extracted using SanPrep endotoxin free plasmid extraction kit (*Sangon Biotech*, *Shanghai*, *China*) for stimulating cells.

2.3. Treatments

This study contained 5 individual experiments (2 cytology experiments and 3 animal experiments respectively). All animals were manipulated according to the procedures and consistent with the policies of the local animal care committee. In the year prior to the study, pigs were not immunized to prevent cross-reactivity with any vaccines that these animals may encounter. To evaluate the immune-enhancing effects of recombinant plasmids in piglets and its effect on maternal antibodies, we immunized piglets and pregnant sows by intramuscular injection (IM) with ETEC vaccine (ETEC vaccine was purchased from Shanghai Haili Biological Products Co., Ltd., containing *Escherichia coli* C600/PTK8899 strain at least 250 U/mL in the experiment). Every piglet was inoculated with 1 mL alone, or in combination with different doses of recombinant plasmids. All animals were kept for 1 month for examination or observation. Treatments for each experiment are as follows:

Experiment 1–2: Based on the perspective of in vitro cytology, the immunostimulatory functions of various plasmids were screened and verified.

Experiment 1: In order to determine the optimal stimulation time, lymphocytes were stimulated by recombinant plasmids at different times in vitro. The lymphocytes concentration was adjusted to 5.0×10^6 cells/mL in a complete RPMI-1640 medium, and the cell suspension was added to a 24-well cell culture plate at 1 mL per well. The recombinant plasmids were added to the cell suspension at a concentration of 10 μg/mL, respectively, and three replicates were made for each plasmid. For the control group, equal amounts of PBS were added and three replicates. The cell culture plates were placed in a 37 °C, 5% CO_2 incubator for 6 h, 18 h, 30 h, 36 h, 48 h, and 72 h. After the treatment, cells were collected by centrifugation at 2000 rpm for 3 min, and preserved at −80 °C for qPCR.

Experiment 2: The immunostimulatory function of recombinant plasmids was analyzed from the cytological point of view in vitro. Except that the stimulation time was the best time determined by experiment 1, the other experimental operations were the same as experiment 1.

Experiment 3–5: From the zoological perspective in vivo, screen and verify the immunostimulatory function of various plasmids.

Experiment 3: In order to make sure whether recombinant plasmids were effective for immune stimulation in piglets, seventy-two healthy 18-day-old piglets were randomly divided into 8 groups. Each group had 3 piglets, and about 5 kg of each pig. There were three repetitions in each group. Piglets were immunized intramuscular injection (IM). Based on our previous data, the dosage of plasmid pVAX10, pVAX10-PR-39, and

pVAX10-pBD-1 (1 μg/kg) and pVAX49, pVAX49-PR-39, and pVAX49-pBD-1 (10 μg/kg) were used. Grouping: (1) PBS; (2) PBS + vaccine; (3) 1 μg/kg pVAX10 + vaccine; (4) 1 μg/kg pVAX10-PR-39 + vaccine; (5) 1 μg/kg pVAX10-pBD-1 + vaccine; (6) 10 μg/kg pVAX49 + vaccine; (7) 10 μg/kg pVAX49-PR-39 + vaccine; (8) 10 μg/kg pVAX49-pBD-1 + vaccine. Samples (serum, saliva, nasal fluid, and stool) were collected from each group of piglets on days 3 and 7 post immunization. The natural incidence of piglets was observed.

Experiment 4: In order to screen for the optimal dosage of pVAX1-CpG-HDPs immunized piglets, piglets were randomly divided into 8 groups. Each group had 3 piglets, and 5 kg of each piglet. There were three repetitions in each group. Piglets were immunized IM. Grouping: (1) PBS; (2) PBS + vaccine; (3) 0.5 μg/kg pVAX49-PR-39 + vaccine; (4) 10 μg/kg pVAX49-PR-39 + vaccine; (5) 20 μg/kg pVAX49g-PR-39 + vaccine; (6) 0.5 μg/kg pVAX49-pBD-1 + vaccine; (7) 10 μg/kg pVAX49-pBD-1 + vaccine; (8) 20 μg/kg pVAX49-pBD- 1 + vaccine. Samples (serum, saliva, nasal fluid, and stool) were collected from each group of piglets on day 3 and 7 post immunization. The natural incidence of piglets was observed.

Experiment 5: To assess the effect of recombinant plasmids on maternal antibody level, plasmids and ETEC vaccine were inoculated on sows: 9 healthy pregnant sows were randomly divided into 3 groups, each with 3 sows, and each weighing about 200 kg. There were three repetitions in each group. Sows were immunized IM on day 14 before farrowing. Grouping: (1) PBS + vaccine; (2) 1 μg/kg pVAX49-PR-39 + vaccine; (3) 1 μg/kg pVAX49-pBD-1 + vaccine. On day 3 post-delivery, the sows in each group randomly selected three piglets from each sow for samples collection (serum, saliva, nasal fluid, and stool). The natural incidence of piglets was observed.

2.4. The expression of monocyte chemoattractant protein (MCP-1) in lymphocytes was detected by qPCR

Total RNA was isolated from lymphocyte cells, and reverse-transcribed into cDNA. cDNA was quantified with real-time PCR (Opticon-II, MJ, Germany) using a commercial reagent kit. A pair of oligonucleotide primers (Table S1) were designed by Primer Premier 5.0 software, based on the sequences registered in GenBank database (GenBank/EMBL/DDBJ accession number: MCP-1, NM 214214; β-actin, U07786). The reaction process was conducted in a Bio-Rad CFX Manager (Bio-Rad, Hercules, CA, USA). The Ct values of MCP-1 were normalized to those of β-actin to gain the ΔCt and the expression of MCP-1 in lymphocyte was represented as $2^{-\Delta\Delta Ct}$. Results were representative of three biological replicates.

2.5. Sample collection

2.5.1. Serum

A complete blood count was performed routinely to confirm normal hematological status. Blood samples were collected in evacuated test tubes with heparin (150 USP units) by venipuncture of jugular vein (pigs) using sterile equipment and procedure. All samples were stored at −20 °C until assayed by ELISA for IgG and IgA.

2.5.2. Saliva, nasal fluid, and stool

A cotton swab containing a small amount of PBS was used to extract saliva, nasal fluid and feces. Then, it was immersed in 0.5 mL of sterile PBS for 30 min, centrifuged at 5000 rpm for 5 min at room temperature. Then the soaking solution was collected at −20 °C for storage. Samples were stored at −20 °C until assayed by ELISA for IgG and IgA.

2.6. Cell preparation

Lymphocytes were prepared by density gradient centrifugation [31]. The porcine spleen was repeatedly ground with D-Hanks solution and an equal amount of Ficoll-Hypaque was added. The cells were centrifuged at 1500 rpm for 20 min to obtain a layer containing lymphocytes. Then, they were washed with a D-Hanks solution. Finally, they were diluted to 2.5–5.0 × 10^6 cells/mL with RPMI-1640 medium. Cells were incubated in a CO_2 incubator at 37 °C, 5% CO_2, saturated humidity.

2.7. Cellular proliferation assay

Untreated cells were cultured in RPMI-1640 medium with 10% fetal bovine serum and without the phenol red indicator, 0.15% sodium bicarbonate and 1% antibiotic/antimycotic. Purified peripheral blood mononuclear cells (PBMCs) were suspended in complete RPMI-1640 without the phenol red indicator to achieve 2.5 × 10^6 cells/mL. The cell suspension was added to the 96-well cell culture plate at 100 μl/well. PBS and Phytohemagglutinin (PHA) were added respectively as negative/positive control. Recombinant plasmids were added at a final concentration of 10 μg/mL, and three replications were performed in each group. The primary incubation was at 37 °C with 5% CO_2 for 24 h, followed by addition of 20 μl MTT (5 mg/mL) per well and a secondary incubation at 37 °C with 5% CO_2 for another 6 h. Centrifuge microplates and remove unreacted MTT, and then added 100 μl dimethyl sulfoxide (DMSO) per well to solubilize formazan, incubating 5 min with shaking. The absorbance plate was read using a 570 nm filter within minutes.

Lymphocyte proliferation was expressed as a stimulation index (SI), which was defined as mean OD of experimental wells/average of OD of the negative control wells.

2.8. Clinical observation

In the process of immunization, piglets were checked twice daily for diarrhea. Stool shedding of bacteria was monitored by daily stool sampling and the stool consistency was scored at sampling on a scale from 1 to 7. The score definitions were 1: hard, dry and cloddy; 2: firm; 3: soft but able to retain some shape; 4: soft and unable to retain any shape; 5: watery and dark; 6: watery and yellow; 7: foamy and yellow. A stool consistency score > 3 was defined as clinical signs of diarrhea. Clinical signs and morbidity were examined daily for 14 days post immunization.

2.9. Statistical analysis

All experiments were done at least three repetitions and results were expressed as mean ± standard deviation (SD). Data was analyzed using the statistical software program Systat 19.0 (SPSS). Differences in expression levels and ELISA titers were investigated using one-way analysis of variance (ANOVA). A p value of < 0.05 was considered significant. Differences among the treatment groups were tested using the post hoc Tukey–Kramer test.

3. Results

3.1. A quantitative real-time sequence detection system to measure porcine MCP-1 mRNA expression

Preliminary studies demonstrated that antibacterial peptides, were potent inducers of MCP-1, a chemokine that is chemotactic for monocytes/macrophages, T cells, NK cells, and neutrophils. Therefore, MCP-1 was chosen for screening porcine HDPs. Endo-free plasmid (use *endo*-free plasmid kit to extraction) pVAX49-PR-39 was used to stimulate lymphocytes cultured in vitro. In order to determine the optimal time, the expression level of MCP-1 at different stimulation times (6 h, 18 h,

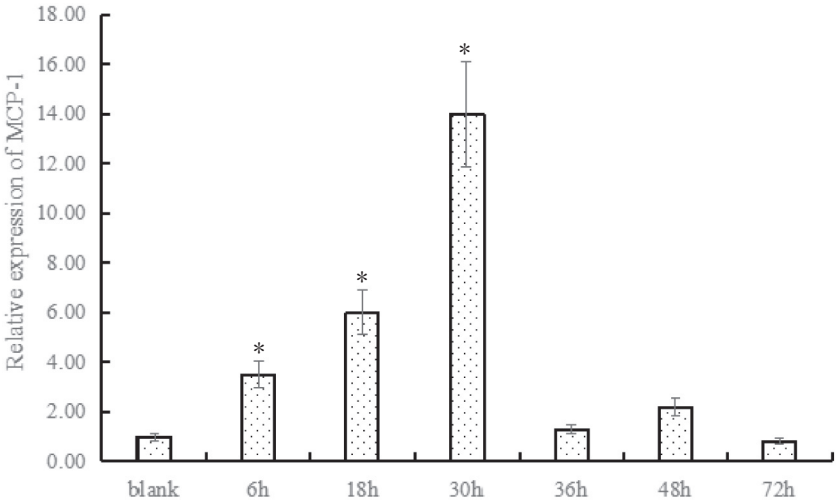

Fig. 1. Relative expression levels of MCP-1 in lymphocytes stimulated by pVAX49-PR39 at different times.
MCP-1 mRNA levels were determined by qPCR using actin gene as housekeeping gene. Mean relative quantity of mRNA ± standard deviation (SD) is shown. Results are representative of three biological replicates. *$p < 0.05$ indicated significant difference vs. blank group.

30 h, 36 h, 48 h, and 72 h) was detected by qRCR. As shown in Fig. 1, MCP-1 had the highest relative expression level after 30 h of treatment. Therefore, 30 h was selected as the stimulation time.

Different *endo*-free recombinant plasmids were used to stimulate lymphocytes. After 30 h of treatment, total RNA of lymphocytes was extracted, and the expression of the antimicrobial peptide gene in lymphocytes was detected by PCR amplification after reverse transcription. The results showed that antibacterial peptide genes were able to successfully transcribe cDNA in porcine lymphocytes. The expression level of MCP-1 in lymphocytes of different treatment groups was detected by qPCR (Fig. 2). In these 9 groups, pVAX49-PR-39 had the best stimulation effect. The plasmids containing 49 CpG motifs were more effective than plasmids containing 10 CpG motifs. Moreover, lymphocytes treated with plasmids containing HDPs gene and CpG motifs had higher relative expression level of MCP-1 than those treated with plasmids with CpG motifs alone.

3.2. Effects of recombinant plasmids on the proliferation of lymphocytes

After stimulation of lymphocytes with recombinant plasmids, pAVX10/49-HDPs had induced stronger proliferative responses than pVAX10/49 (Fig. 3) . The best effect was observed in pVAX49-PR-39 treatment group, which was significantly higher than PBS group ($p < 0.05$). pVAX10-pBD-1, pVAX49-PR-39 resulted in significantly high SI; SI values of pVAX1-PR-39, and pVAX1-pBD-1 were lower and insignificant ($p > 0.05$). Overall, the plasmid-treated group (49 CpG motifs and 10 CpG motifs) significantly stimulated the proliferation of lymphocytes.

3.3. Recombinant plasmids as a vaccine adjuvant for co-immunization of piglets with ETEC vaccine

As pVAX49-PR-39, pVAX49-pBD-1, pVAX10-PR-39, and pVAX10-pBD-1 could stimulate lymphocytes to express more MCP-1 and increase lymphocyte proliferation, these recombinant plasmids were selected for in vivo experiments. Recombinant plasmids were used as a vaccine adjuvant in piglets. Samples were collected from each group of piglets on days 3 and 7 post immunization.

The titer of antigen-specific IgA in serum was shown in Fig. 4(a). All treatment groups had higher antigen-specific IgA titers than vaccine group. The titer of IgA in pVAX10-HDPs and pVAX49-HDPs groups was more than pVAX10 and pVAX49 groups, respectively. Moreover, titers of IgA in pVAX49-HDPs group were more than pVAX10-HDPs group. IgA titers were lower on day 7 than day 3, but the trend was the same for both. The titer of each same group of IgG was lower than IgA (Fig. 4(b)). However, the trend was consistent with IgA titers.

Antigen-specific IgA titers were detected in oral mucus, nasal mucus, and stool of piglets after immunization with different reagents (Fig. 5). When pVAX49 was used in combination with the ETEC vaccine to immunize piglets, the IgA titer was significantly increased, significantly different from the vaccine group, and superior to the pVAX10 group. pVAX49-PR-39 and pVAX49-pBD-1 group had better IgA

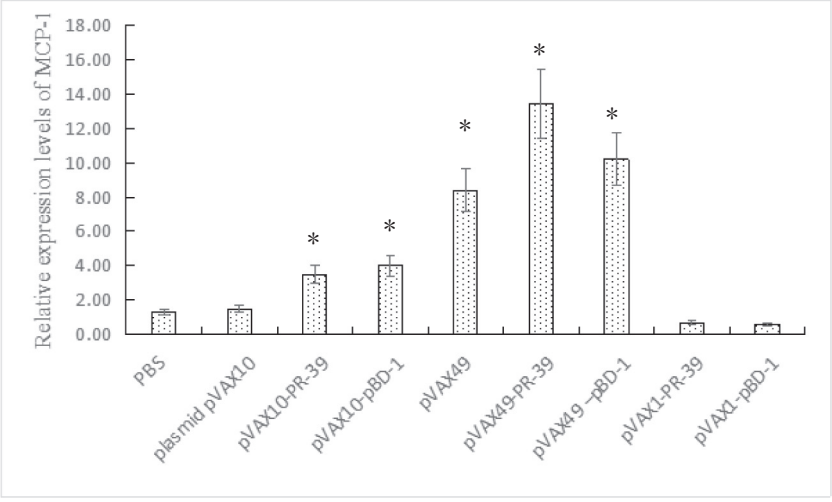

Fig. 2. Relative expression levels of MCP-1 in lymphocytes stimulated by different recombinant plasmids at the same time.
Relative expression levels of MCP-1 levels were determined by qPCR using actin gene as housekeeping gene. Mean relative quantity of mRNA ± SD is shown. 1–9 are respectively PBS, plasmid pVAX10, pVAX10-PR-39, pVAX10-pBD-1, pVAX49, pVAX49-PR-39, pVAX49–pBD-1, pVAX1-PR-39, and pVAX1-pBD-1. Results are representative of three biological replicates. *$p < 0.05$ indicated significant difference vs. PBS group.

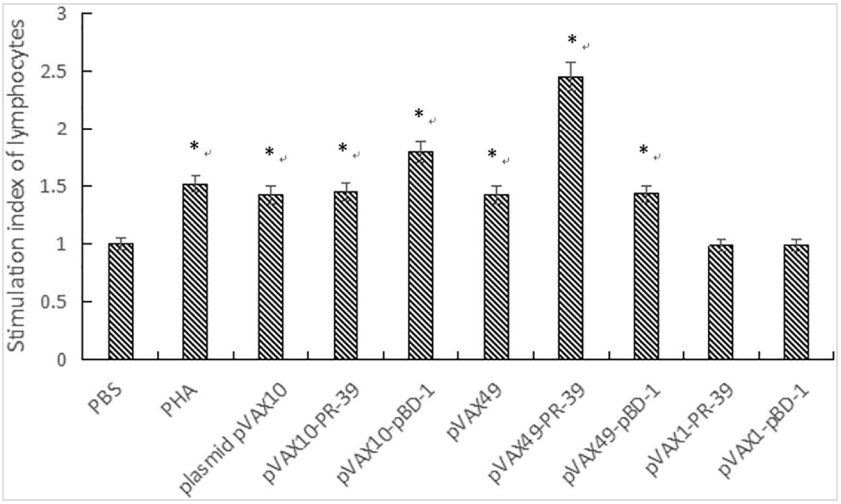

Fig. 3. Effect of recombinant plasmids on proliferation of porcine lymphocytes.
Different recombinant plasmids stimulate lymphocyte culture for 24 h and assay lymphocyte proliferation and transformation. Stimulation index (SI) was calculated: mean OD of experimental wells/average of OD of control wells. Mean SI ± SD is shown. Results are representative of three biological replicates. *$p < 0.05$ indicated significant difference vs. PBS group.

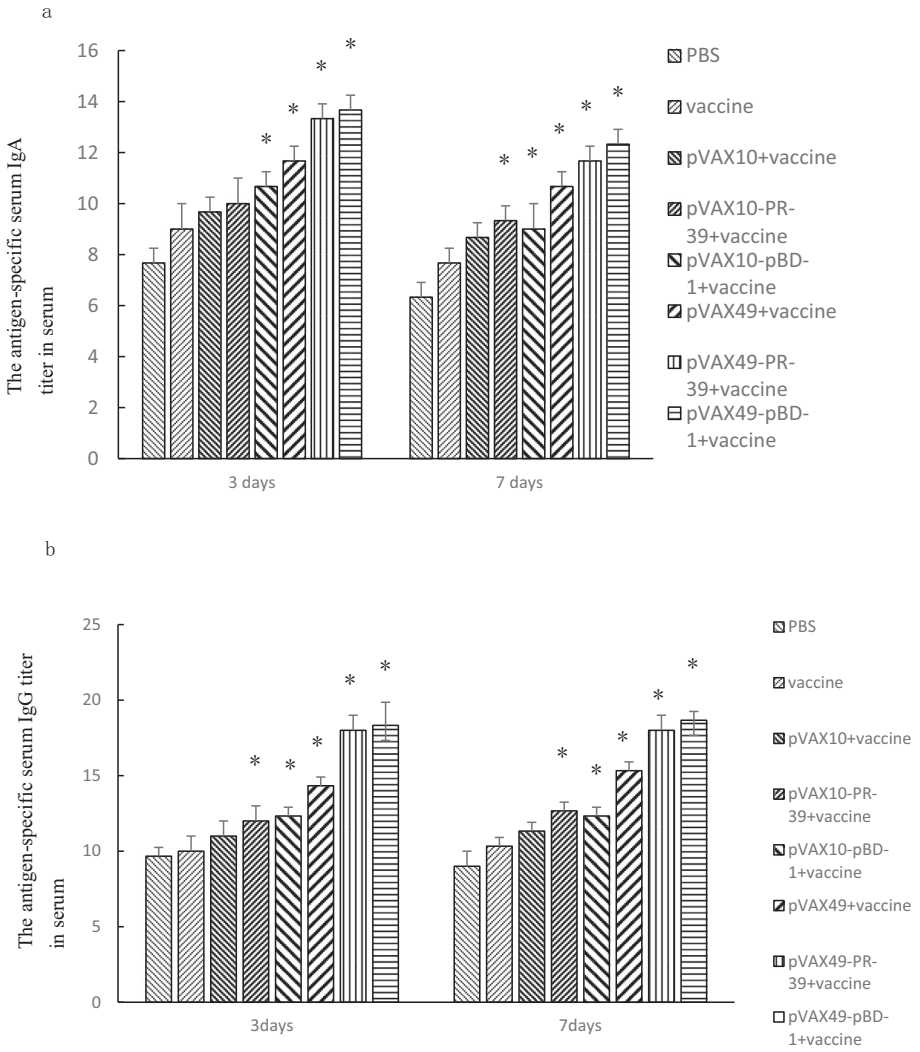

Fig. 4. The antigen-specific IgA (a) and IgG (b) titer in serum.
The antigen-specific serum IgA (a) and IgG (b) titer on day 3 and 7 post immunization.
Note: The titer values measured by ELISA were converted by the formula $\log_2 X$ (X = titer value).
The data in the figure are the mean values of the three replicates ± SD.*$p < 0.05$ indicated significant difference vs. Vaccine group.

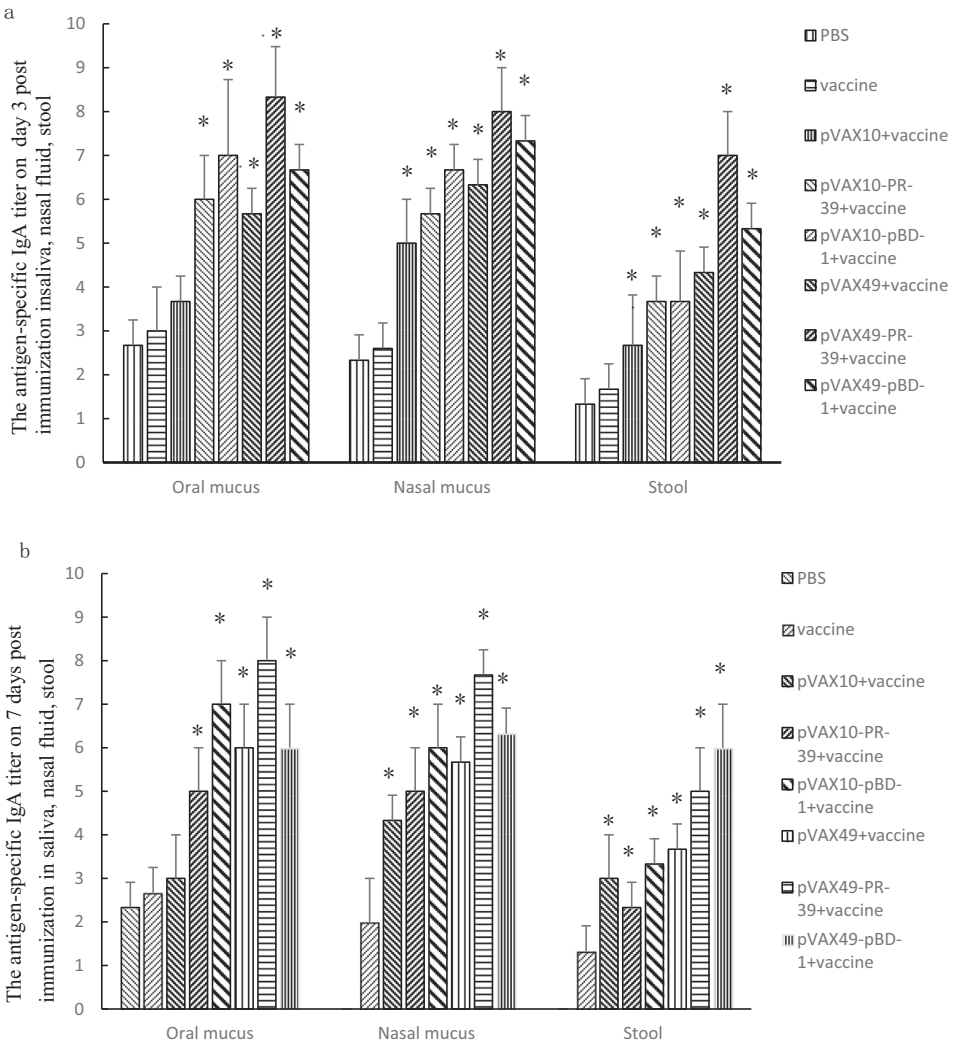

Fig. 5. The antigen-specific IgA titer on day 3 (a) and 7 (b) post immunization in saliva, nasal fluid, stool.
Note: The titer values measured by ELISA were converted by the formula $\log_2 X$ (X = titer value). The data in the figure are the mean values of the three replicates ± SD.*$p < 0.05$ indicated significant difference vs. Vaccine group. The nasal mucus and stool in PBS groups no detectable IgA antibodies.

induction than that of pVAX49 group. Among them, the pVAX49-PR-39 had the best immune-enhancing effect.

3.4. Recombinant plasmids for optimal dose screening

Recombinant plasmids pVAX49-PR-39 and pVAX49-pBD-1 with better immunological effects were used to immunize piglets with vaccines at different doses (0.5 μg/kg, 10 μg/kg, 20 μg/kg). Samples were collected from each group of piglets on days 3 and 7 post immunization.

Serum, saliva, nasal fluid, and stool as samples, antibody titers were detected by ELISA. As shown in Fig. 6, IgA titer in oral mucus, nasal mucus and stool were generally low, not as high in serum. However, antigen-specific antibodies were affected by the dose of pVAX49-HDPs. When the dose increased, the antibody responses were further boosted. pVAX49-PR-39 and pVAX49-pBD-1 had similar immunity-enhancing effects on ETEC vaccines. High doses of pVAX49-HDPs significantly increased the production of vaccine-specific IgA antibodies, and the 20 μg/kg dosage had the best effect.

3.5. Effects of recombinant plasmid on maternal antibody

Based on the results of immunizing piglets with different plasmids, pVAX49-PR-39 and pVAX49-pBD-1 were selected as adjuvant to co-immunize the sows. According to the dose experiment, we knew that dose of 20 μg/kg could achieve optimal immune enhancement. However, it was difficult to achieve so large dose of about 4000 μg per sow, 1 μg/kg was selected as the experimental dose. If the low dose was effective, then it meant that the higher dose would be more effective. Thereafter, it would need further dose screening investigation.

Serum, oral mucus, nasal mucus, and stool were collected. Maternal antibodies detected on piglets have antigen-specific IgA and IgG titers in the samples. Antigen-specific IgA and IgG titers were detected by ELISA. The results were shown in Fig. 7. Sows co-injected with pVAX49-PR-39 or pVAX49-pBD-1 and ETEC vaccine had significantly higher titers of maternal antibodies than ETEC vaccine alone ($p < 0.05$) in all body fluids (serum, oral mucus, nasal mucus, and stool) of piglets. In the vaccine group, specific antibodies could not be detected by ELISA in the nasal mucus and stool samples.

3.6. Piglet diarrhea statistics

The diarrhea of piglets was calculated throughout the immunization trial (Table 1), and no diarrhea was observed in the group co-injected with pVAX49-PR-39 or pVAX49-pBD-1 and ETEC vaccine. Diarrhea rate of piglets injected with ETEC vaccine was lower than that of PBS.

In the immunized sow experiment (Experiment 5), the piglet

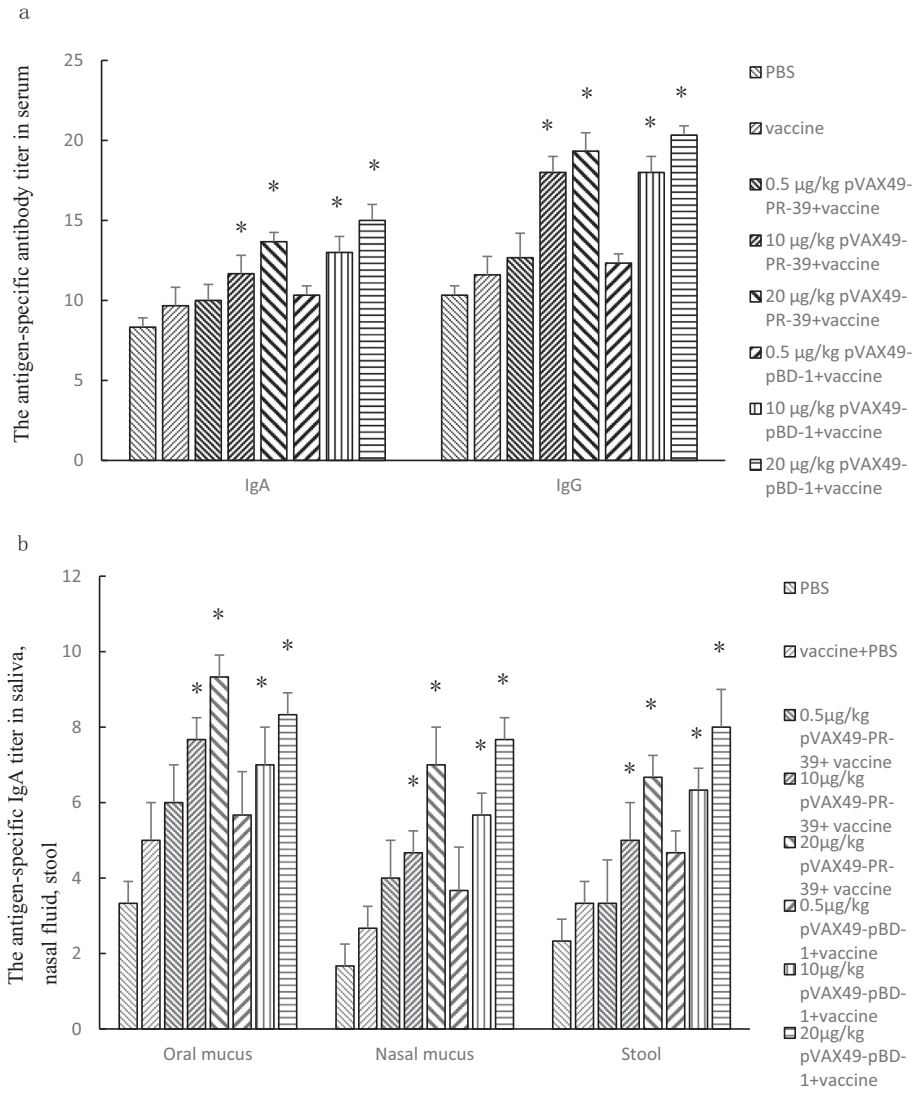

Fig. 6. Different dosage of recombinant plasmids for immunization of piglets.
a: The antigen-specific antibody titer in serum.
b: The antigen-specific IgA titer in saliva, nasal fluid, stool.
Note: The titer values measured by ELISA were converted by the formula $\log_2 X$ (X = titer value).
Data are the mean values of the three replicates ± SD. $^*p < 0.05$ indicated significant difference vs. Vaccine group.

diarrhea rate reached 25% for sows vaccinated with ETEC alone. No diarrhea was observed in groups co-injected with ETEC vaccine and pVAX49-PR-39 or pVAX49-pBD-1.

4. Discussion

Our laboratory had confirmed that CpG-ODN could be used as a vaccine adjuvant for piglet immune enhancement, and it could also be acted as a viral vaccine adjuvant together with innate defense-regulating peptide (IDR) to produce an immune response in newborn piglets [25]. To date, there have been proofs of HDPs as a vaccine adjuvant. Studies by Brogden et al. showed that human neutrophil peptide (HNP) defensins or human β-defensins (HBD), co-administered intranasally with the antigen ovalbumin (OVA), induced unique immune responses [32]. Our previous reports had demonstrated that synthetic peptides [33,34] showed good immune-enhancing activities with CpG-ODN in piglets [21]. However, the cost of synthetic HDPs or CpG-ODN is too high to be advantageous for animal farming. In order to improve the immune function of porcine vaccine and reduce cost, we constructed a series of recombinant plasmids containing CpG and HDPs.

Previous studies had shown that some plasmids could play an immunopotentiating role. Plasmids encoding cytokines such as IFN-γ and IL-12 were potential genetic adjuvants that increased the effectiveness of allergen vaccine [27]. Plasmid pcDNA3.1 and pCI stimulated porcine lymphocyte proliferation and significantly increased the level of IL-6 mRNA [35]. Different plasmids had different immune effects. For pcDNA1, there were no proliferative responses and the level of IL-6 mRNA was significantly reduced. This was because pcDNA1 only containing one CpG-motifs, however pcDNA3.1 and pCI containing two and three different motifs, respectively. pcDNA3.1 has a good immunostimulatory effect, pVAX1 which was used in this study is improved from pcDNA3.1. Moreover, pVAX1 is one of the eukaryotic expression vectors permitted by the U.S. Food and Drug Administration for the clinical use of vaccines. Our data further confirmed that pVAX1 has a good immune-stimulating function.

To verify whether the recombinant plasmids containing PR-39/pBD-1 gene and CpG motif had the function of immunoadjuvant in piglets, in vitro experiments were performed to screen the plasmid. Then recombinant plasmids with good performance in vitro and ETEC vaccines were co-injected to measure the titer of specific antibodies in body

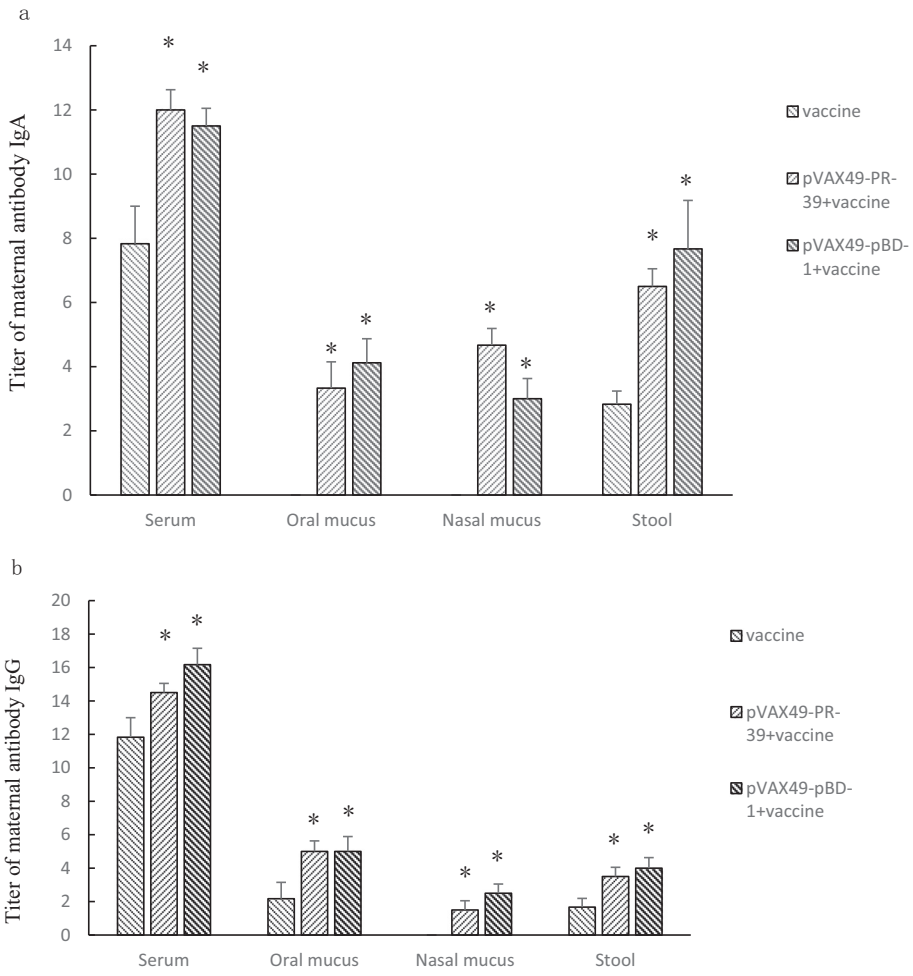

Fig. 7. Titer of maternal antibody IgA (a) and IgG (b).
The recombinant plasmids were separately mixed with vaccine and the sows were immunized by intramuscular injection of the neck. The immunization time of each group was 14 days before the birth. After the sows were born for 3 days, three piglets were randomly selected from each sow. and the serum, oral mucus, nasal mucus and stool of the piglets were taken aseptically. The antigen-specific IgA titer in piglets. The titer values measured by ELISA were converted by the formula $\log_2 X$ (X = titer value). The data in the figure are the mean values of the six replicates ± SD.*$p < 0.05$ indicated significant difference vs. Vaccine group. The oral mucus and nasal mucus in vaccine groups no detectable IgA/IgG antibodies.

fluids in piglets in vivo.

To assess immunoenhancing ability of recombinant plasmids, in vitro studies were performed using porcine spleen lymphocyte. Previous research showed that IDRs were potent inducers of MCP-1, a chemokine chemotactic for monocytes/macrophages cells, NK cells, and neutrophils [22]. Cao's research showed that CpG-ODN and HDPs could be used as immune potentiators to promote the expression of MCP-1 in piglet lymphocytes [21]. Therefore, MCP-1 was selected as an indicator for screening recombinant plasmids in this study. The relative expression level of MCP-1 in lymphocytes stimulated with plasmid containing CpG motifs and HDPs genes increased significantly ($p < 0.05$). Plasmids containing 49 CpG motifs had higher expression levels and SI than 10 CpG motifs. It indicated that plasmids encoding CpG motif had an immunostimulatory function, moreover number of CpG motif affected the immune stimulation effect. Plasmids encoding CpG motif and HDPs had better immunostimulatory effect than plasmids encoding CpG motif only.

Previous studies showed that CpG-ODN could form stable complexes with polycationic amino acids and peptides via electrostatic interactions [22]. This stable structure would be helpful to improve the immunostimulatory activity of CpG-ODN and enhance antigen-specific immune responses [36]. Our data clearly showed that there were synergistic effects between CpG and HDPs. Consequently we speculated that plasmid containing CpG motif binds to the expressed HDPs in the same manner as CpG-ODN, and exerts a stronger immunostimulatory function.

The immune system of piglets is immature, so they are highly sensitive to intestinal and respiratory infections [37]. Functional and quantitative deficiencies, involving both non-specific innate defenses and specific cellular and humoral defenses, have already been described in newborns [38]. Therefore, piglets had poor anti-infective ability and weak response to vaccines. The data in this study also illustrated this problem. Recombinant plasmids with better in vitro effects were used as adjuvants to immunize all piglets with the ETEC vaccine. The specific antibody IgG and IgA titers in body fluid samples of recombinant plasmids/vaccines groups were higher than those of vaccine-only. These results in vivo were consistent with that of in vitro experiments. It illustrated that the recombinant plasmid could be used as a vaccine adjuvant to enhance the immune effects. By a dosage screening using three adjuvant doses (0.5 μg/kg, 10 μg/kg, and 20 μg/kg), we observed that the higher the dose, the better the immune enhancement effects.

We also performed experiments on sows to test the maternal antibody responses in piglets, since the newborn piglets was prone to be stressed, and immunity of the piglets would be reduced and morbidity rate increase after vaccination. In order to reduce the damage to piglets, we chose to immunize sows to induce maternal antibodies. We found that the specific maternal antibody titers in body fluids of the piglets were significantly increased ($p < 0.05$). This indicated that pVAX49-PR-39/pBD-1 not only directly increased the antibody content of piglets, but also increased the maternal antibody level of piglets. Finally, based on observation of the clinical diarrhea of piglets during the experiment, we confirmed that pVAX49-PR-39/pBD-1 could be used as an adjuvant for ETEC vaccine to enhance immune activity and reduce the rate of diarrhea, which was more effective than direct co-immunized piglets.

In conclusion, our data demonstrated that plasmids encoding CpG motif and HDPs (PR-39/pBD-1) genes provided an effective adjuvant and increased the effectiveness of ETEC vaccine. By co-immunizing

Table 1
Piglets diarrhea statistics.

a

Group	D/T	Diarrhea ratio (%)
PBS	2/9	22.2
Vaccine	1/9	11.1
pVAX10-HDPs + vaccine	0/27	0
pVAX49-HDPs + vaccine	0/27	0

b

Group	D/T	Diarrhea ratio (%)
PBS	2/9	22.2
Vaccine	1/9	11.1
pVAX10-HDPs + vaccine	0/27	0
pVAX49-HDPs + vaccine	0/27	0

c

Groups	D/T	Diarrhea ratio (%)
Vaccine	5/20	25
pVAX49-PR-39 + vaccine	0/21	0
pVAX49-pBD-1 + vaccine	0/25	0

D/T: Number of diarrhea piglets/total piglets.
a: Data from experiment 3 (immunization enhancement effect of different plasmids on piglets).
b: experiment 4 (screening of different doses of recombinant plasmids) were counted.
c: Data from experiment 5 (recombinant plasmid to maternal antibody content) were counted.
Note: Each group was repeated 3 times, and the clinical observation was one month. Clinical signs and morbidity were examined daily for 14 days post immunization.

sows with recombinant plasmids and ETEC vaccine, piglets could obtain more maternal antibodies and reduce piglet diarrhea.

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Conflict of interest

The authors declare that they have no conflicts of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.03.007.

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