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Short communication

Intragastric administration of attenuated *Salmonella typhimurium* harbouring transmissible gastroenteritis virus (TGEV) DNA vaccine induced specific antibody production

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ABSTRACT

Attenuated *Salmonella typhimurium* was selected as a transgenic vehicle for the development of live mucosal vaccines against transmissible gastroenteritis virus (TGEV). A 2.2 kb DNA fragment, encoding for N-terminal domain glycoprotein S of TGEV, was amplified by RT-PCR and cloned into eukaryotic expression vector pVAX1. The recombinant plasmid pVAX-S was transformed by electroporation into attenuated *S. typhimurium* SL7207, the expression and translation of the pVAX-S delivered by recombinant *S. typhimurium* SL7207 (pVAX-S) was detected in vitro and in vivo respectively. BALB/c mice were inoculated orally with SL7207 (pVAX-S) at different dosages, the bacterium was safe to mice at dosage of 2×10^9 CFU and eventually eliminated from the spleen and liver at week 4 post-immunization. Mice immunized with different dosages of SL7207 (pVAX-S) elicited specific anti-TGEV local mucosal and humoral responses as measured by indirect ELISA assay. Moreover, the immunogenicity of the DNA vaccine was highly dependent on the dosage of the attenuated bacteria used for oral administration, 10^9 CFU dosage group showed higher antibody response than 10^8 CFU and 10^7 CFU dosages groups during week 4–8 post-immunization. The results indicated that attenuated *S. typhimurium* could be used as a delivery vector for oral immunization of TGEV DNA vaccine.

1. Introduction

Transmissible gastroenteritis (TGE) causes serious intestinal infectious diseases of swine, which especially severe in newborn pigs less than two weeks old, resulting in nearly 100% mortality. Its causative agent, transmissible gastroenteritis virus (TGEV), belonging to the coronavirus, has a positive single-stranded RNA about 28.6 kb and consists of three major structural proteins: a phosphorylated nucleoprotein (N) and two glycoproteins, the membrane (M) and the spike (S) proteins [1]. The envelope S protein is highly glycosylated and mediates the binding of TGEV to its cellular receptor, porcine aminopeptidase N (pAPN) [2,3]. As the major inducer of TGEV-neutralizing antibodies, the S protein has been used mainly for the induction of protective immunity to TGEV and four major antigenic sites (A, B, C and D) have been defined on the amino-terminal domain [4,5]. Earlier studies have shown that the intact globular N-terminal half of the S protein is sufficient to achieve a protective immune response equivalent to that induced by the full S protein [6].

The TGEV infects the villous enterocytes, which causes localized infections to the intestinal tracts, resulting in villous atrophy and consequently a malabsorptive diarrhea [1]. Protection of the suckling piglets from TGEV infection requires the induction of secretory immunoglobulin A (sIgA) in milk, especially in colostrums. Typically, IgA-producing plasma cells in the mammary gland of pregnant sows originate from the gut-associated lymphoid tissues (GALT) where they were activated by different antigenic components [7,8]. Thus, new developments have focused on the production and delivery of the S protein through oral immunization. Several systems have been used to express the full-length or truncated TGEV S proteins such as *Salmonella* [9–11], *Lactobacillus casei* [12] and plants [13–17]. However, the expression level of antigen protein in transgenic plants is generally low and will lose its bioactivity due to the degradation of stomach acid [13,18]. Prokaryotic expression antigen protein in bacterial will greatly affect its immunogenicity for lacking of glycosylation and post-translational modification [1,19]. As fore-mentioned, an easy and effective oral vaccine suitable for protecting against TGEV infection is highly needed in livestock farming.

In recent years, attenuated Gram-positive and Gram-negative intracellular bacteria (such as *Salmonella*, *Shigella* and *Listeria*) have been used as carriers for efficient delivery of either DNA vaccine or vaccine antigens for oral immunization. This strategy allows

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administration of DNA vaccines via mucosal surfaces as well as delivery of the plasmid DNA directly to professional antigen presenting cells (APCs), which can elicit strong humoral and cellular responses against the pathogens [20–22]. In particular, attenuated *Salmonella typhimurium* (*S. typhimurium*) has been used to deliver DNA vaccines encoding immunogens of pathogenic microorganisms including HIV, HBV, HSV, NDV, IBDV, PRRSV and *Toxoplasma gondii* [23–29]. However, the use of *S. typhimurium* as a DNA vaccine vector in TGEV has almost not been reported.

In this study, we used the attenuated intracellular bacteria, attenuated *S. typhimurium* SL7207, as a carrier for delivery of DNA vaccines encoding the N-terminal half of TGEV glycoprotein S. Our data indicated that orogastric intubation of the recombinant *S. typhimurium* could induce a specific immune response against TGEV.

2. Materials and methods

2.1. Bacterial strains, plasmid, virus and cell lines

The attenuated *S. typhimurium aro*A$^-$ strain SL7207 (*S. typhimurium* 2337-65 derivative hisG46, DEL407 [*aro*A::Tn10 (Tc^s)]) was kindly provided by Professor Kai Schulze of Helmholtz Centre for Infection Research, Germany. Eukaryotic expression vector, pVAX1 contains cytomegalovirus (CMV) immediate-early promoter for efficient expression and bovine growth hormone (BGH) poly A signal for mRNA stability, purchased from Invitrogen. The virulent TGEV strain SC-H was isolated from Sichuan, P.R. China in 2005. ST cells and COS-7 cells were purchased from China Center for Type Culture Collection (Wuhan, China).

2.2. Experiment mice

Female BALB/c mice (six weeks old, 17–22 g), purchased from Chengdu Institute of Biological Products (Chengdu, China), were maintained in animal holding laboratory under controlled condition with temperature of 25 ± 10 °C, humidity of 40 ± 10% and had free access to standard mouse diet and water.

2.3. Construction of expression plasmid

Viral RNA was extracted from TGEV-infected ST cells using Mini BEST Viral RNA/DNA Extraction Kit (TaKaRa). A 2.1 kb DNA fragment, encoding for the N-terminal of glycoprotein S was amplified from virus genomic RNA by RT-PCR, using the primers S1: 5′-CGGAAGCTTACCATGGAAAAACTATTTGTG-3′ (forward) and S2: 5′-TCGGATCCTTAGCCACTAAGTAGCGTCCT-3′ (reverse), which respectively introduce the HindIII and BamHI restriction sites at the 5′ ends of the primers. The amplified DNA fragment of TGEV S gene was cloned into a pMD19-T vector (TaKaRa) and sequenced. Then, the S gene fragment was released from the pMD19-T plasmid by HindIII and BamHI digestion and ligated into the pVAX1 vector cut by the same enzymes, the resulting plasmid was named pVAX-S.

2.4. Transient expression of the recombinant plasmid

Six-well tissue culture plates (Costar) were seeded with COS-7 cells. Monolayer of 70–80% confluent cells were transiently transfected with the plasmid pVAX-S and empty plasmid pVAX1 using Lipofectamine 2000 (Invitrogen). Thirty-two hours after transfection, cells were washed with phosphate-buffered-saline (PBS), then fixed with ice-cold methanol/acetone (1:1) at 4 °C for 30 min and washed once again with PBS. Diluted primary and secondary antibodies were incubated at 37 °C for 1 h, respectively. Primary antibodies were antiserum of pig to TGEV, which were kindly provided by researcher Li Feng (Harbin Veterinary Research Institute, China) and secondary antibodies were FITC-conjugated-rabbit-anti-pig IgG (Sigma).

2.5. Transformation of attenuated S. typhimurium

The purified plasmid pVAX-S or control vector pVAX1 was transformed into *S. typhimurium* competent cells by electroporation at 2.5 kV, 25 μF and 200–400 Ω. The positive transformants were selected on LB agar containing 50 μg/mL kanamycin, and then were verified by PCR amplification and digestion with restriction enzymes. The *Salmonella* strains containing plasmid pVAX-S or pVAX1 were named strain SL7207 (pVAX-S) and strain SL7207 (pVAX) respectively.

2.6. Plasmid transfer from attenuated S. typhimurium to mammalian host cells in vitro

Twelve-week-old BALB/c mice were sacrificed by cervical dislocation, and their peritoneal cavities were injected with 6 mL of RPMI 1640 medium. After gentle abdominal massage, the maximum amount of fluid was collected. The peritoneal exudates cells were separated by centrifugation and resuspended into 10 mL of RPMI 1640 medium. The isolated peritoneal macrophages were allowed to adhere for 3 h in a six-well tissue culture plates (Costar) in antibiotic-free medium, at which time the no-adherent cells were removed by gently washing plates two times with antibiotic-free medium. The adherent cells were infected with recombinant *S. typhimurium* strains SL7207 (pVAX) or SL7207 (pVAX-S) at a multiplicity of infection (MOI) of 50:1. After 20–30 min incubation at 37 °C, the infected cells were washed with PBS and incubated in fresh RPMI 1640 containing 10% fetal bovine serum (FBS) and 100 μg/mL gentamicin for 2 h. The medium was then removed and replaced with fresh RPMI 1640 containing 10% FBS and 10 μg/mL tetracycline. 42–60 h after infection, the expression of S gene was detected by indirect immunofluorescence assay (IFA).

2.7. RT-PCR detection the transcription of S gene in vivo

Six-week-old mice were inoculated intragastrically with 1×10^9 CFU SL7207 (pVAX-S), control mice were given with the same dosage of SL7207 (pVAX). Three days after the immunization, Payer's patches were removed from three mice and pooled. Cellular RNA was isolated from homogenized Payer's patches with Trizol (TaKaRa) according to the manufacturer's instructions. The transcripts of TGEV S gene in Payer's patches was analyzed by RT-PCR using specific primers, S3: 5′-AATTTTCCTTGTTCTAAATTGAC-3′ (forward) and S4: 5′-TTAATTTTCAAA ACTAATACGGTAAC-3′ (reverse), which contain the B, C site of S gene (549 bp in length). Mice β-actin specific primers, β1: 5′-CATGTGCCCATCTACGA-3′ (forward) and β2: 5′-ACAG GATTCCATACCCAAG-3′ (reverse), with its amplified fragment length 334 bp, was used as a quality control.

2.8. Safety and bacterial colonization in organs

S. typhimurium strains SL7207 (pVAX-S) were cultured in condition as described [30] previously. The bacterial cells were collected by centrifugation at 5000 × *g* for 10 min and resuspended in PBS containing 5% sodium bicarbonate (m/v) to the expected cell populations, as determined by plating serial dilution on LB agar plates. Three groups of six-week-old BALB/c mice, with eight mice in each group, were inoculated intragastrically using a gavage needle with SL7207 (pVAX-S) at dosage of 5×10^8, 1×10^9, 2×10^9 CFU and boosted with the same dosage two weeks later. The immunized mice were monitored daily for clinical changes. Two mice of each group were sacrificed every week post-immunization, spleens and livers were collected and homogenized in 2 mL PBS containing

0.1% Triton X-100 (v/v). The bacterial counts were determined by plating 100 μL of the homogenized spleens and livers samples on LB agar plates containing 50 μg/mL kanamycin. Bacteria colonies were picked randomly for PCR identification of TGEV S gene and digestion by restriction enzymes.

2.9. Mice immunization and sample collection

Six-week-old BALB/c mice were randomly divided into five groups, each of them consisting of twenty mice, and immunized three times with two weeks intervals. All mice were deprived of food and water for 4 h before oral immunization. Mice in groups A, B and C were inoculated intragastrically with SL7207 (pVAX-S) with different dosages of 10^7, 10^8 and 10^9 CFU per mouse, respectively. Mice in group D were inoculated intragastrically with the control strain SL7207 (pVAX) at 10^8 CFU per mouse. Group E mice received PBS as a negative control.

Three mice from each group were sacrificed for sera and intestinal lavage collection at weeks 0, 2, 4, 6 and 8 after the primary immunization. Blood were obtained from the submaxillary sinus of the mice, sera were prepared and stored at −20 °C prior to analysis. To prepare intestinal fluid, a 15 cm section of the small intestine was removed from mouse, cut into pieces and washed in 5 mL of PBS containing 1 mmol/L phenylmethylsulfonyl fluoride and 50 mmol/L EDTA (PBS-PE). Samples were centrifuged at 12,000 × *g* for 20 min to remove cellular debris. The supernatant were freeze–dried using freeze dryer and dissolved in 0.5 mL PBS-PE. After centrifuged at 12,000 × *g* for 10 min, the supernatant were collected as resultant intestinal lavage and stored at −20 °C prior to analysis.

2.10. Measurement of antibody levels

All measurements of antibody levels in individual animal were determined in duplicate. For enzyme linked immunosorbent assays (ELISA) to determine serum IgG and intestinal IgA level of immunized mice, 96-well polystyrene microtitre plates (Costar) were coated overnight at 4 °C with 50 μL 5 μg/mL purified TGEV-antigen, in carbonate buffer (pH 9.6), and blocked for 1 h at 37 °C with PBS containing 1% (w/v) BSA. The coating antigen was prepared through sucrose density gradient centrifugation as described previously [4]. Plates were then washed three times with PBS containing 0.02% Tween 20 (PBST) and incubated with 100 μL of mice serum (1:40 diluted) or intestinal lavage samples for 1 h at 37 °C. After washed three times with PBST, HRP-conjugated goat anti-mice IgG or IgA (Sigma), diluted 1:2000 were used as the secondary antibody and incubated for 1 h at 37 °C. 3,3′,5,5′-tetramethyl benzidine (TMB) was employed as the substrate. After 20 min of incubation in the dark, the reaction was stopped by the addition of 50 μL of H_2SO_4 (2 mol/L), and the optical density at 450 nm was measured in an ELISA micro-plate reader. Total serum IgG and intestinal lavage IgA specific for TGEV were represented as the optical density. Data were analyzed using the one-sided Student's *t* test. Differences were considered statistically significant with $P < 0.05$.

3. Results

3.1. Construction and transient expression of pVAX-S in COS-7 cells

A 2.1 kb DNA fragment was amplified by RT-PCR from TGEV SC-H strain. Sequence analysis showed that the amplified TGEV SC-H strain S gene fragment was 2124 bp in length and encoded 707 amino acids. The result of sequence alignment showed that the predicted amino acids sequence of TGVE SC-H strain S gene fragment shared homology of 97.7%, 94.1%, 98.0%, 97.9%, 99.6%, 99.7%, 97.1%, 99.0% and 98.3% with the sequence of TS, 96-1933, HN2002, Miller, NEB72-RT, Purdue, TH-98, TFI and TO14 strains, indicating that the amino terminal half of the S glycoprotein is highly conserved among different TGEV strains. The S gene fragment was incorporated into pVAX1 vector and the recombination plasmid pVAX-S was detected by restriction analysis and PCR (Fig. 1). The expression of pVAX-S was demonstrated by indirect immunofluorescence assay. Cytoplasmic fluorescence was observed in COS-7 cells transfected with pVAX-S (Fig. 2A), but not with the parental vector pVAX1 (Fig. 2B).

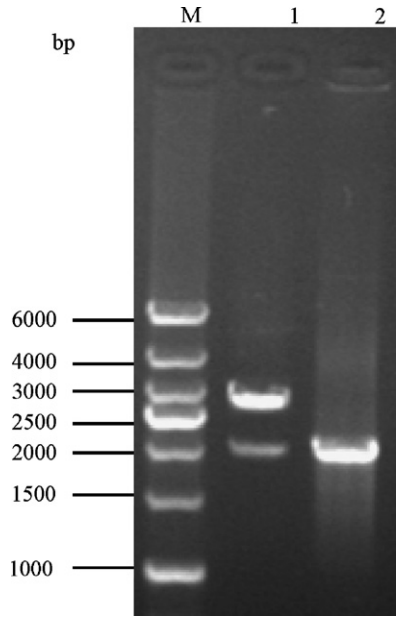

Fig. 1. PCR and restrictive digestion identification of recombination plasmid pVAX-S. Lane M, DL100–6000 marker; lane 1, pVAX-S digested by HindIII and BamHI; lane 2, S gene fragment amplified by PCR from pVAX-S.

3.2. Expression of TGEV S gene in vitro using attenuated S. typhimurium as a transgenic vehicle

To test whether the attenuated *S. typhimurium* has the potential to deliver TGEV DNA vaccine to APCs in vitro, mouse primary peritoneal macrophages were infected with attenuated *S. typhimurium* SL7207 (pVAX-S) harbouring TGEV S gene. Fifty hours after infection, the expression of S gene in macrophages was detected by IFA. Cytoplasm fluorescence was observed in cells infected with SL7207 (pVAX-S) (Fig. 3A), whereas no fluorescence was detected in the cells infected with attenuated *S. typhimurium* SL7207 (pVAX) which harbouring empty vector pVAX1 (Fig. 3B).

3.3. Transcription of TGEV S gene in vivo using attenuated S. typhimurium as transgenic vehicle

Payer's patches is the mainly colonization site of attenuated *S. typhimurium* and important immunologically relevant site in the context of the mucosal responsiveness. To test the delivery of TGEV DNA vaccine in vivo using attenuated *Salmonella* as a transgenic vehicle, total cellular RNA was isolated from the mice payer's patches on day 3 after the inoculation with attenuated *S. typhimurium* SL7207 (pVAX-S) and the transcription of TGEV S gene was analyzed by RT-PCR. As shown in Fig. 4, a DNA fragment about 540 bp was only amplified from the RNA of mice immunized with SL7207 (pVAX-S). Meanwhile, there were no DNA fragments amplified from RNA prior to reverse transcription with the same primers, or from RNA of control mice immunized with SL7207 (pVAX). Murine β-actin DNA fragment (330 bp) was amplified from all samples.

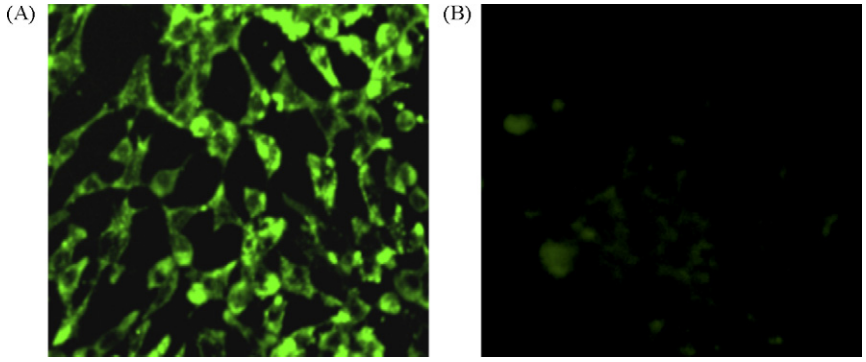

Fig. 2. Indirect immunofluorescence detection the expression of pVAX-S in COS-7 cells (×200). (A) Indirect immunofluorescence result of COS-7 cells transfected with pVAX-S; (B) indirect immunofluorescence result of COS-7 cells transfected with pVAX1.

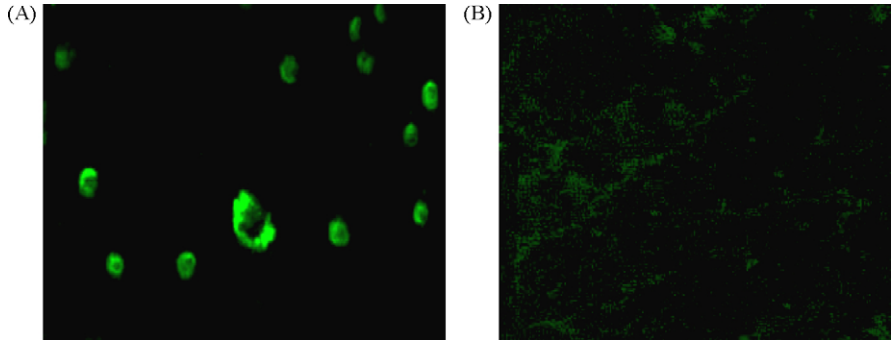

Fig. 3. Indirect immunofluorescence detection the expression of S gene after in vitro infection of mouse peritoneal macrophages with recombinant attenuated *S. typhimurium* (×200). (A) Indirect immunofluorescence result of mouse peritoneal macrophages infected with SL7207 (pVAX-S); (B) indirect immunofluorescence result of mouse peritoneal macrophages infected with SL7207 (pVAX)

3.4. Safety and colonization of recombinant *S. typhimurium* in organs

BALB/c mice were inoculated orally with SL7207 (pVAX-S) at different dosages for safety analysis. No clinical aberrations and visible lesions in liver and spleen were observed during the four weeks observation period. The kinetics of colonization and persistence of the bacteria in vivo was investigated. Bacteria could be isolated from in liver and spleen of different dosage group during weeks 1–3 post-inoculation and were eventually eliminated from liver and spleen at four weeks post-inoculation (Fig. 5). PCR and enzyme digestion revealed the constant presence of S gene in recovered bacterial isolates.

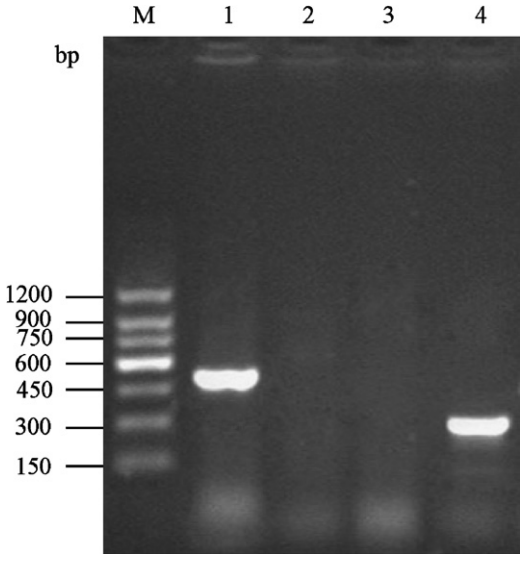

Fig. 4. RT-PCR detection the transcription of TGEV S gene in vivo. Mice were immunized orally with recombinant attenuated *S. typhimurium*, three days after immunization, transcription of S gene in Payer's patches was analyzed by RT-PCR using S gene specific primers. Lane M, DL100 bp marker; lane 1, mice were immunized orally with SL7207 (pVAX-S); lane 2, control mice were immunized orally with SL7207 (pVAX); lane 3, the RNA sample prior to reverse transcription served as controls for possible plasmid contamination; lane 4, amplification with β-actin specific primers served as a quality control for cellular RNA.

3.5. Mucosal antibody responses induced by attenuated *S. typhimurium* harbouring TGEV DNA vaccine

The ability of the recombinant *S. typhimurium* to induce a mucosal immune response was determined by measuring the level of IgA antibody to TGEV in intestinal lavage sample. As shown in Fig. 6, recombinant *S. typhimurium* induced detectable IgA antibodies to TGEV Ag in mice as rapidly as week 2 post-vaccination. Considerably enhanced antibodies titers could be observed in groups B and C, which received SL7207 (pVAX-S) at dosages of 10^8 and 10^9 CFU respectively, at week 4–6 post-immunization. In addition, the antibody level of group C was significantly higher ($P < 0.05$) than that of group B, at week 6 post-vaccination. However, only low-level antibody response could be detected from mice in group A, which received a low dose (10^7 CFU) of strain SL7207 (pVAX-S), throughout the whole experiment. No specific anti-TGEV antibodies were detected in intestinal lavage sample of the SL7207 (pVAX) group (group D) and PBS group (group E) during the experiment.

3.6. Humoral immune responses induced by attenuated *S. typhimurium* harbouring TGEV DNA vaccine

The ability of the recombinant *S. typhimurium* to induce a humoral immune response was determined by measuring the level

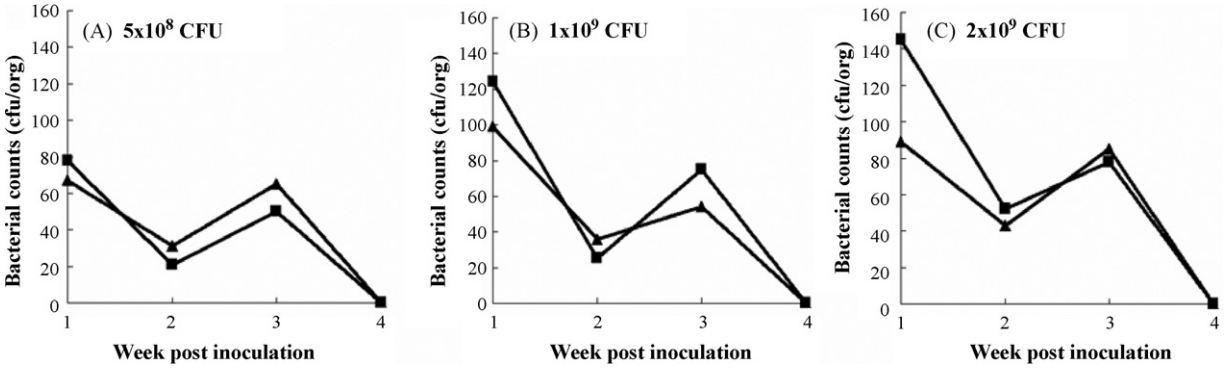

Fig. 5. Bacterial colonization in organs. Persistence of the SL7207 (pVAX-S) in spleen (▲) and liver (■) of mice inoculated orally with SL7207 (pVAX-S) at dosage of 5×10^8 CFU (A), 1×10^9 CFU (B), 2×10^9 CFU (C) and boosted with the same dosage two weeks later. Fresh spleens and livers were collected and homogenized in PBS for bacteria CFU determination on LB agar plates containing 50 μg/mL kanamycin every week.

of IgG antibody to TGEV in serum sample. As shown in Fig. 7, none of the groups had any detectable antibody response at week 2 post-vaccination. Mice in groups A, B and C, which received SL7207 (pVAX-S) at dosages of 10^7, 10^8 and 10^9 CFU respectively, showed different levels of antibody response during the experiment. Mice in groups B and C showed higher ($P < 0.05$) anti-TGEV ELISA antibody level than group A during week 4–8 post-immunization. At week 6 post-immunization, the antibodies titers generated in groups B and C reached their peak, meanwhile the antibodies level of group C was higher ($P < 0.05$) than group B. However, all through the experiment, mice in group A only induced a negligible antibody response at week 6 post-vaccination. There were no detectable specific anti-TGEV antibodies in the SL7207 (pVAX) group (group D) and PBS group (group E) during experiment.

4. Discussion

Most pathogenic microorganisms are either restricted to the mucosal membranes or in need of transit across the mucosal barrier during the early steps of infection. Thus, the elicitation of mucosal immune responses after vaccination is highly desired. A potential approach to achieve this aim is the use of attenuated *Salmonella* as carrier for heterologous antigens. In this report, we have demonstrated that a DNA vaccine encoding N-terminal of TGEV glycoprotein S, delivered by attenuated *S. typhimurium* is a simple and potent vaccine that elicits both serum IgG and mucosal IgA antibody response against TGEV. We have also demonstrated that the dosage of 10^9 CFU elicited a higher antibody response than that of 10^8 and 10^7 CFU.

It is generally assumed that after crossing the intestinal mucosal barrier (mainly via M cells) a large amount of attenuated *S. typhimurium* carrying the eukaryotic expression plasmid are taken up by APCs in local lymphoid tissues like Payer's patches. In these phagocytes, the bacteria will start to replicate and die possibly due to their metabolic attenuation. This should result in the release of their plasmid and the in vivo transfection of the infected cells which in turn will produce the antigen [31]. In this study, when using attenuated *Salmonella* as a vehicle for TGEV S gene eukaryotic expression plasmid, we detected the expression of S gene in mouse peritoneal macrophages by IFA in vitro and the transcription of S gene in Payer's patches by RT-PCR in vivo. The results indicated, when immunized orally, attenuated *S. typhimurium* SL7207 has the ability to deliver TGEV DNA vaccine for antigen expression by APCs.

Safety is a prerequisite when using live bacterial as vaccine carrier. Attenuated *S. typhimuriu* SL7207 with deletion mutations in *aro*A gene has impaired ability to grow in cultured macrophages and in mammalian tissues. In safety study, neither deaths nor side effects were found in mice post-inoculation with attenuated *S. typhimurium* SL7207 (pVAX-S) at different dosage (5×10^8 CFU, 1×10^9 CFU, 2×10^9 CFU). No bacterial were recovered in liver and

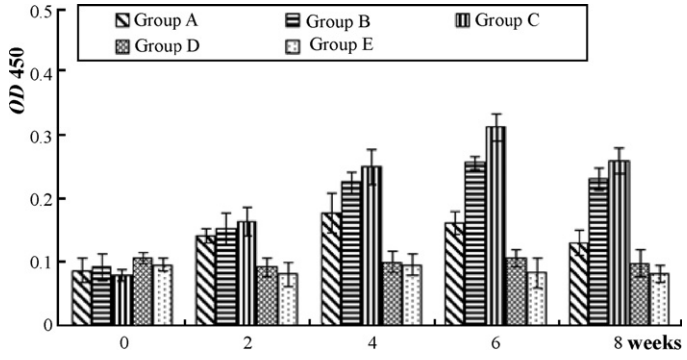

Fig. 6. End-point dilution ELISA analysis of the anti-TGEV IgA antibody in murine intestinal lavage after immunization. Six-week-old BALB/c mice were inoculated intragastrically with SL7207 (pVAX-S) three times with two weeks intervals at dosage of 10^7 (group A), 10^8 (group B) and 10^9 (group C) CFU per mouse. Control mice (group D) received 10^9 CFU of SL7207 (pVAX), while group E mice received PBS as negative control. Murine intestinal lavage, collected at week 0, 2, 4, 6, and 8 post-immunization were analyzed by end-point dilution ELISA assay using TGEV as coating antigen. Absorbance was measured at 450 nm in an automated plate reader. Results are expressed as means of the OD450 ±S.D. ($n = 3$). Numbers on *x*-axis indicate weeks post-immunization.

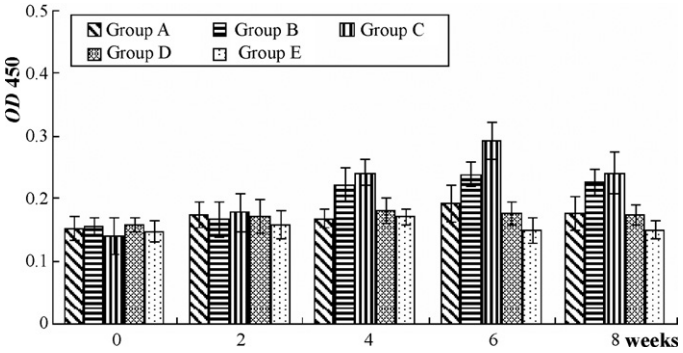

Fig. 7. End-point dilution ELISA analysis of the anti-TGEV IgG antibody in murine serum after immunization. Six-week-old BALB/c mice were intragastric immunization three times at two-week intervals with SL7207 (pVAX-S) at dosage of 10^7 (group A), 10^8 (group B) and 10^9 (group C) CFU per mice. Control mice (group D) received 10^9 CFU of SL7207 (pVAX) while group E mice received PBS as negative control. Murine serum, collected at weeks 0, 2, 4, 6, and 8 post-immunization were analyzed by end-point dilution ELISA assay using TGEV as coating antigen. Absorbance was measured at 450 nm in an automated plate reader. Results are expressed as means of the OD450 ±S.D. ($n = 3$). Numbers on *x*-axis indicate weeks post-primary immunization.

spleen in different dosage group after two weeks of boosting, indicated that attenuated *S. typhimurium* could be eliminated by immune system after completing the plasmid delivery. At the same time we noticed one week after boosting, bacterial separated from liver and spleen in different dosage groups were significantly lower than one week after first inoculation, which indicated the elimination of immune system to bacterial enhanced with the increasing of inoculation frequency. All the results above indicate that the use of attenuated *S. typhimurium* is an ideal choice of delivery vector for DNA vaccine orally, concerning safety for livestock.

Live bacterial vectors offer many potential clinical advantages. They are stable, easy and relatively inexpensive for mass-production. They are also able to deliver multiple antigens. Oral delivery increases the safety and ease of administration. In addition, bacteria naturally possess immunostimulatory molecules such as lipopolysaccharide (LPS) that can function as adjuvant to stimulate immune responses [32], furthermore, live bacterial vectors are able to induce systemic immune response, including humoral, cellular and mucosal immunity against pathogen infection [33]. When comparing the three different dosages of DNA vaccine delivered by the attenuated *S. typhimurium* SL7207 (pVAX-S), we found that immunogenicity of the TGEV DNA vaccine was highly dependent on the dosage of the attenuated bacteria used for oral administration. Delivery of the DNA vaccine at a low dosage (10^7 CFU) only elicits weak anti-TGEV IgG and IgA antibody through out the experiment. When the vaccination dosage increased to 10^8 CFU, the IgG and IgA antibody level was enhanced, meanwhile the highest antibody response was detected at week 6 post-vaccination when the vaccination dosage was increased to 10^9 CFU.

In summary, this study provided preliminary evidence that attenuated *S. typhimurium* strain SL7207could be utilized as the oral delivery vector for TGEV DNA vaccines. The target gene could be expressed not only in vitro but also in vivo to develop a specific humoral and mucosal immune response against TGEV. Although these data are preliminary and an ultimate TGEV vaccine may require incorporation of other TGEV antigens [34], or together with co-stimulatory molecules or immunomodulatory cytokines [35], the attenuated *S. typhimurium* may be an adequate delivery system to be tested in further experiments with the DNA vaccine of TGEV to obtain a maximum immune response.

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