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Glyceraldehyde-3-Phosphate Dehydrogenase, an Immunogenic *Streptococcus equi* ssp. *zooepidemicus* Adhesion Protein and Protective Antigen Ⓢ

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Streptococcus equi **ssp.** ***zooepidemicus*** **(*Streptococcus zooepidemicus*, SEZ) is an important pathogen associated with opportunistic infections of a wide range of species, including pigs and humans. The absence of a suitable vaccine makes it difficult to control SEZ infection. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been previously identified as an immunogenic protein using immunoproteomic techniques. In the present study, we confirmed that the sequence of GAPDH was highly conserved with other *Streptococcus* spp. The purified recombinant GAPDH could elicit a significant humoral antibody response in mice and confer significant protection against challenge with a lethal dose of SEZ. GAPDH could adhere to the Hep-2 cells, confirmed by flow cytometry, and inhibit adherence of SEZ to Hep-2 cells in an adherence inhibition assay. In addition, real-time PCR demonstrated that GAPDH was induced *in vivo* following infection of mice with SEZ. These suggest that GAPDH could play an important role in the pathogenesis of SEZ infection and could be a target for vaccination against SEZ.**

Key words: *Streptococcus equi* ssp. *zooepidemicus*, vaccine candidate, glyceraldehyde-3-phosphate dehydrogenase

Streptococcus equi ssp. *zooepidemicus* (*Streptococcus zooepidemicus*, SEZ) is a Lancefield group C beta-hemolytic *Streptococcus* responsible for septicemia, meningitis, purulent arthritis, endocarditis, and mastitis in a wide range of species, including horses, pigs, sheep, cows, and several other mammalian species [4, 17, 20]. The infections lead to significant welfare and economic costs [11], most notably to the pig industry in China [8, 9]. However, attempts to control the diseases due to SEZ are hampered by a lack of knowledge of protective antigens and pathogenesis of SEZ.

In previous attempts to develop vaccines against SEZ using acid extracts or live attenuated strains of SEZ, there have been the disadvantage that the presence of some complicated components probably induce a dominant but non-protective response and sometimes could cause serious side effects [13]. Recombinant subunit vaccines using M-like protein (SzP), a virulence-associated factor of SEZ, induced protection against homologous challenge [6, 12, 16]. However, SzP contains a variable region that has been used to differentiate groups of SEZ [23, 25], so it is unclear what level of cross-protection will be conferred by this antigen.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a glycolytic enzyme that catalyzes the conversion of glyceraldehyde-3-phosphate to 1,3-biphosphoglycerate, is a bacterial surface protein that, among other functions, contributes to adherence in pathogenic streptococci [3, 5]. Using immunoproteomics, GAPDH has been identified as a major extracellular antigen of SEZ [15]. In this study, the potential of GAPDH developed as a novel vaccine antigen against SEZ infection and the adherence of GAPDH to Hep-2 cells were examined.

MATERIALS AND METHODS

Bacterial Strain and Growth Conditions

SEZ strain C55138 (China Institute of Veterinary Drug Control) was originally recovered from a diseased pig with septicemia in Sichuan, China [8]. It was grown on tryptone soya broth (TSB) (Oxoid, Wesel, Germany) or tryptone soya agar (TSA) (Difco Laboratories, Detroit, MI, USA) plus 5% newborn calf serum at 37°C under aerobic conditions.

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Table 1. Primer sequences used for clone and real-time PCR.

Gene	Primer sequence used for clone and real-time PCR	
gapdh	Clone	Forward: 5'-TCTTGGATCCCGTCTTATCC-3' (*Bam*HI)
		Reverse: 5'-CATTTCGTTGAATTCCCAT-3' (*Eco*RI)
gapdh	Real-time PCR	Forward: 5'-GCTCCTGGTGGAAACG-3'
		Reverse: 5'-GCCATTGGTGCAAGACA-3'
16S rRNA	Real-time PCR	Forward: 5'-ATCCGAACTGAGATTGGC-3'
		Reverse: 5'-CCCTTATGACCTGGGCTA-3'

Cloning, Expression, and Purification of GAPDH

DNA fragments encoding GAPDH were obtained by PCR amplification using the SEZ genome sequence as a source of DNA. Restriction sites used for the cloning were included in the forward and reverse primers (Table 1). The PCR product was ligated into the expression vector pET-28a (Novagen, Madison, WI, USA) in frame with the his6 tag sequence at the N terminus to produce pGAPDH. pGAPDH was transformed into *Escherichia coli* DH5α, and then transferred into *E. coli* BL21 (DE3) for expression. The recombinant protein was puried by nickel-nitriloacetic acid (Ni-NTA) affinity chromatography following induction with isopropyl-β-D-1-thiogalactopyranoside (Sigma, St. Louis, MO, USA). Recombinant protein fractions were filtered through a 0.22 μm membrane (Millipore, Bedford, MA, USA) and stored at −80°C.

SDS-PAGE and Western Blotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analyses were performed as previously described [16]. Convalescent porcine sera against SEZ prepared earlier [26] (1:200) and goat anti-porcine IgG (H+L)-HRP (1:5,000) (Southern Biotech, Cambridge, UK) were applied as the first and the second antibodies, respectively. The membrane was developed with the ECL Plus Western Blotting Detection System (GE Healthcare, Piscataway, NJ, USA) and imaged on a Kodak 2000MM Image Station. All experiments were performed in triplicate.

Immunization and Challenge

All the experimental protocols were approved by the Animal Care and Use Committee of Guangdong Province and performed accordingly. The approval ID or permit numbers were SCXK (Guangdong) 2009-0011 and SYXK (Guangdong) 2011-0112. Thirty BALB/c mice (4-week-old female) were randomly assigned to three groups of 10 mice each. Purified recombinant GAPDH (50 μg), dissolved in 50 μl of phosphate-buffered saline (PBS, pH 7.4) and absorbed to an equal volume of complete Freund's adjuvant (Sigma, St. Louis, MO, USA), was applied to immunize the mice in group 1. Subsequent booster injections of the same antigen emulsified in 50 μl of incomplete Freund's adjuvant (Sigma, St. Louis, MO, USA) were given on day 14.

Mice in group 2 served as a positive control and were immunized with inactivated vaccine in which mice were first injected with 0.5 ml of an emulsified mixture of formalin (final concentration 0.8%), inactivated SEZ C55138 strain (2×10^8 CFU/ml), and complete Freund's adjuvant, according to a previous study [9]. Subsequent booster injections were given on the 14th day with the same inactivated strain emulsified in incomplete Freund's adjuvant.

Mice in group 3 were inoculated with PBS emulsified in the same adjuvant and served as a negative control. Mice in all groups were immunized by intraperitoneal injection. On day 14 after booster immunization, sera were obtained from each group by tail vein bleeding and then all mice in each group were challenged by intraperitoneal injection with a lethal dose of 2×10^5 CFU of SEZ C55138 in 0.5 ml of PBS.

Determination of Antibody Titers

The presence of antibodies in sera were determined by ELISA using microtiter plates (Nunc, Roskilde, Denmark) coated with purified recombinant GAPDH (200 ng/100 μl) as described previously [1]. After saturation of the plates with 5% skim milk solution for 2 h at 37°C, serially diluted mice sera were added and incubated for 30 min at 37°C. Bound antibodies against immunoglobulin (Ig) subtypes were detected with rabbit anti-mouse IgG-HRP (Southern Biotech, Cambridge, UK), IgG1-HRP (Southern Biotech, Cambridge, UK), or IgG2a-HRP (Southern Biotech, Cambridge, UK). The plates were read with a microplate ELISA reader at an optical density (OD) of 630 nm. End-point titers were calculated as the reciprocal of the last serum dilution yielding 50% of the maximum OD value above the background with a value of 0.08.

Quantitative Real-Time PCR to Measure Expression of *gapdh* *In Vivo* and *In Vitro*

Bacteria harvested from three SEZ-infected mice, and total RNA from *in vitro* and *in vivo* harvested bacteria, were prepared according to a previous study [18]. cDNAs were synthesised using the Reverse Transcription System (Promega, Madison, WI, USA). Each cDNA sample was used as a template for a real-time PCR in an amplification mixture containing SYBR Green (TaKaRa, Dalian, China). All reactions were performed in triplicate on the LightCycler 480 (Roche, Indianapolis, IN, USA). For each run, the Ct value of the endogenous control *16S rRNA* gene was subtracted from the Ct value of each gene (ΔCt = Ct test gene - Ct *16S rRNA*) to normalize the amount of sample cDNA added to each reaction. For a comparison of the expression of each gene *in vitro* and *in vivo*, the ΔCt value of the gene *in vitro* was subtracted from the ΔCt value of the gene *in vivo* (ΔΔCt = ΔCt *in vivo* - ΔCt *in vitro*). Relative changes were calculated using the formula $2^{-\Delta\Delta Ct}$ [14]. Data are presented as means ± standard deviation (SD) of triplicate reactions for each gene transcript. Each of the primers used for real-time PCR are provided in Table 1.

Hep-2 Cell Adherence Assay

The adherence of the GAPDH protein to Hep-2 cells was carried out as previously described [21], with some modification. Hep-2 cells were incubated with 2 μg of purified recombinant GAPDH or bovine serum albumin (BSA) in 200 μl of PBS containing 1% BSA (PBS-BSA) for 45 min on ice, washed twice in ice-cold PBS-BSA and incubated for another 45 min on ice with mouse antibodies against GAPDH (100 µl). After washing, the cells were incubated with goat anti-mouse IgG-FITC (Santa Cruz, CA, USA) for 45 min on ice. The cells were then washed once in PBS-BSA and analyzed by flow cytometry using a Beckton-Dickinson FACS-Calibur (Becton Dickinson, San Jose, CA, USA).

Adherence Inhibition Assay

To determine whether GAPDH was involved in the adherence of SEZ to host cells, an inhibition assay was performed as described previously [21]. Hep-2 cells were cultured in 24-well cell plates (approximately 5×10^5 cells/well) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum at 37°C in a humidified incubator. The cells were washed with PBS three times and then incubated with 10 µg/ml purified recombinant GAPDH for 2 h at 37°C. Cells treated with 10 µg/ml BSA served as a control. All cells were washed with PBS three times, and then 1 ml of SEZ C55138 suspended in DMEM at a concentration of 5×10^6 CFU/ml was added to each well and incubated for 2 h at 37°C in 5% CO_2. The number of CFU associated with the monolayer was determined by viable counts after being washed with PBS to remove non-adherent bacteria. The percent amount of inhibition of GAPDH to adherence was calculated as [1 − (number of CFU recovered in the GAPDH-treated cells/number of CFU recovered in the BSA-treated cells)] × 100.

Bioinformatics and Statistical Analysis

Multiple sequence alignment and homology analysis was carried out using DNAMAN Biosoftware (version 5.2.10). Data were analyzed using Student's t-test and were shown as means ± SD. For *in vivo* virulence experiments, survival was analyzed using the log rank test. For all tests, statistical significance was defined at $p < 0.05$.

RESULTS

Sequence Alignment and Production of Recombinant GAPDH

Sequence alignment demonstrated that the GAPDH protein was highly conserved with other *Streptococcus* spp. and that SEZ isolates had nearly identical sequences (Supplementary Table). The *gapdh* gene of SEZ C55138 had 100% identity with SEZ MGCS10565 and encoded a 306 amino acid fusion protein with a predicted molecular mass of 32.4 kDa. Expression of the GAPDH protein was demonstrated by comparing band sizes of induced and non-induced cultures of the producer strain with their predicted size by SDS-PAGE. Purified recombinant GAPDH protein was recovered by Ni-NTA affinity chromatography and the protein exhibited immunoreactivity to convalescent sera against SEZ by Western blot analysis (Fig. 1).

Immune Response

Antibodies against GAPDH were determined in sera obtained from mice on day 14 after the booster injection. The levels of specific IgG titers against GAPDH were significantly higher in the immunized group ($p < 0.001$) than in the negative control groups (Fig. 2A). To reveal the type of immune response, the subclass responses were assessed using ELISAs specific for mouse IgG1 and IgG2a. IgG1 was associated with Th2-like response, whereas IgG2a was associated with Th1-like response. Although the nature of these experiments did not allow an accurate quantification of different immunoglobulin subclasses, the test indicated that GAPDH could induce a high titer of IgG1 and IgG2a (Fig. 2B).

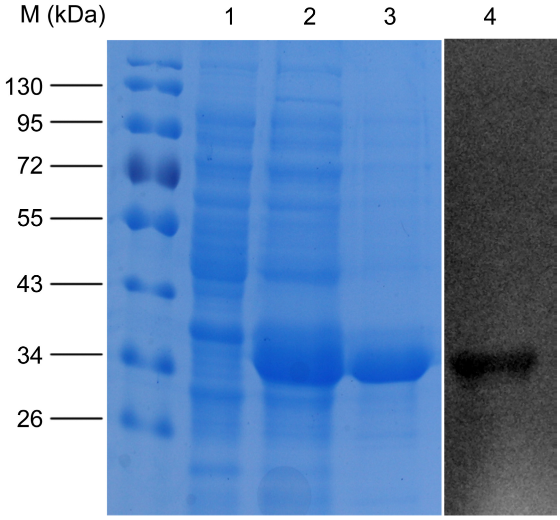

Fig. 1. Representative SDS-PAGE and Western blot analysis of recombinant GAPDH.

Lanes 1–3 show SDS-PAGE analysis of extracted proteins from the non-induced or induced GAPDH-producer *E. coli* strains and Ni-NTA purification of $(His)_6$ GAPDH, respectively. Lane 4 shows the Western blot analysis of recombinant GAPDH with convalescent sera against *Streptococcus equi* ssp. *zooepidemicus*. Molecular size markers are indicated (kDa).

Challenge of Mice Immunized with GAPDH

On day 14 after booster immunization, mice in all three groups were challenged with a lethal dose of 2×10^5 CFU log-phase SEZ C55138. Five mice in the negative control group died on day 3 post-challenge and all of the remaining mice showed significant clinical signs, including ruffled hair coats and a slow response to stimuli, and died successively within 6 days. In comparison, mice immunized with inactive SEZ had less severe clinical signs than mice in the negative control group and all of the mice recovered from day 3 post-challenge. Two out of 10 mice in the test group also showed severe clinical signs and died on day 4. The remaining mice showed mild clinical signs, such as depression and weakness, and one died on day 5. The mice gradually improved from day 6 and no mice died from day 6 to the end of the study (Fig. 3). Furthermore, bacteria could not be isolated from the surviving mice on day 14 post-challenge. These results indicated that GAPDH was able to protect mice against SEZ infection.

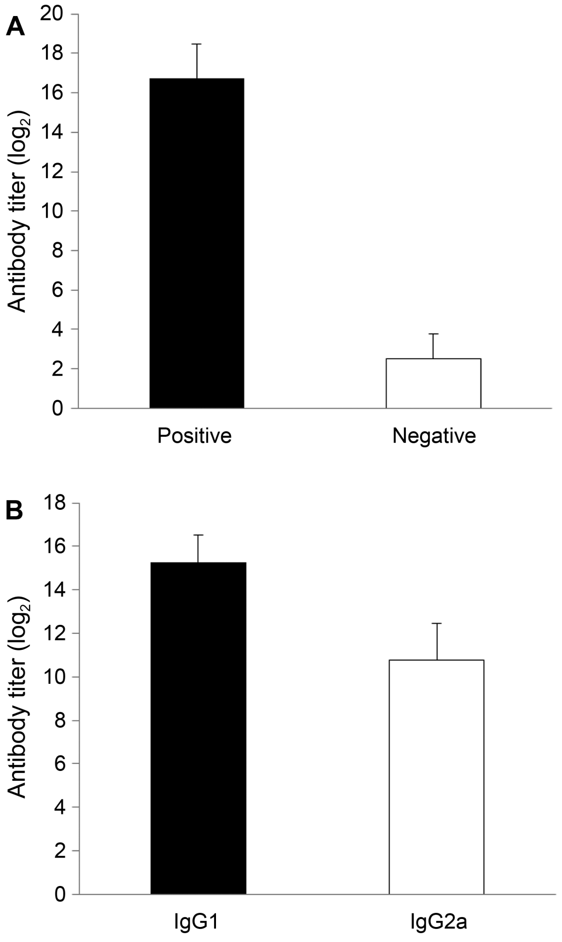

Fig. 2. Immune response induced by recombinant GAPDH in mice.

(**A**) Levels of specific GAPDH IgG were significantly higher in immunized mice than in the negative control group. (**B**) GAPDH induced a predominant IgG1 response compared with IgG2a.

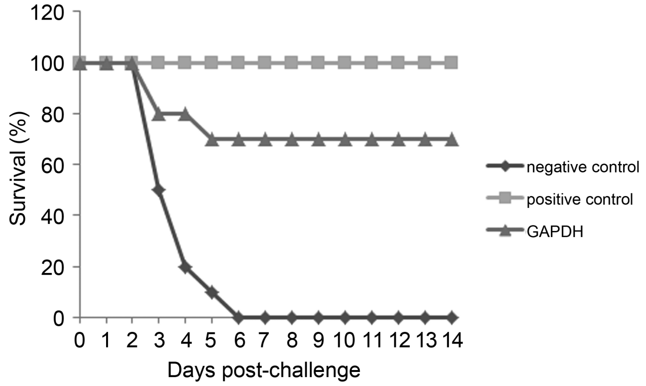

Fig. 3. Survival of mice immunized with GAPDH, inactive vaccine (positive control), or adjuvant (negative control) following challenge with *Streptococcus equi* ssp. *zooepidemicus*.

Each group consisted of 10 mice.

Quantification of the *In Vivo*-Induced Gene Transcripts by Real-Time PCR

Analysis of dissociation curves from infected samples and bacteria cultured *in vitro* revealed a single melting peak, and no specific fluorescence signal was detected from negative control samples, indicating a specific signal corresponding to *gapdh* and the endogenous control *16S rRNA*, respectively. Comparison of the calibration curves of *gapdh* and *16S rRNA* showed a similar primer efficiency of each gene, and the Ct value was in proportion to the copies of template (Fig. 4A). Further analysis of real-time PCR indicated that the level of expression of

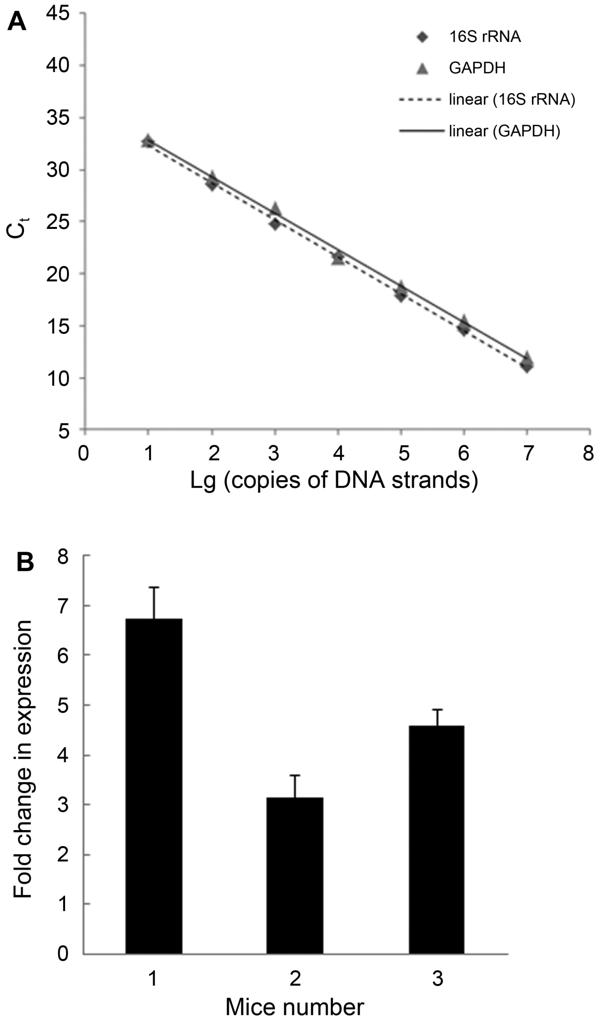

Fig. 4. Quantification of the *in vivo*-induced gene transcripts by real-time PCR.

(**A**) Calibration curves generated using the DNA standards for *gapdh* and *16S rRNA*. The target nucleic acid copies are plotted against the Ct values and the primers to these genes show a similar amplication efficiency. (**B**) Up-regulation of *gapdh* in spleens of three *Streptococcus equi* ssp. *zooepidemicus*-infected mice relative to SEZ cultured *in vitro*.

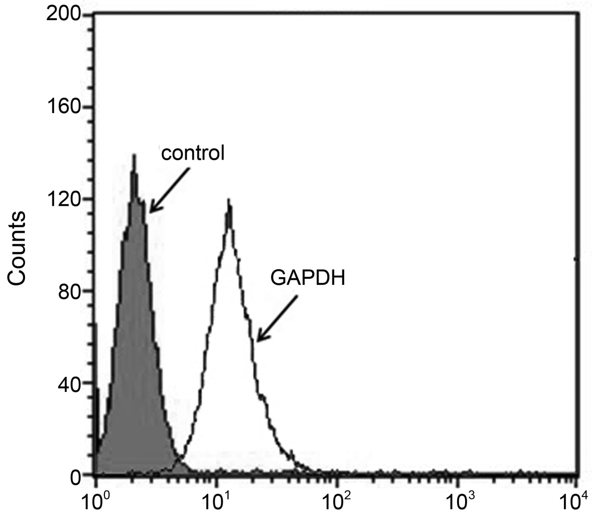

Fig. 5. Flow cytometric analysis of GAPDH binding to Hep-2 cells.
Mean fluorescence intensity (MFI) of cell after treatment with GAPDH (open histograms) or bovine serum albumin (shaded histograms). The MFI of the Hep-2 cells incubated with recombinant GAPDH was higher than control cells incubated with BSA.

gapdh in SEZ-infected animals was higher than that cultured *in vitro* (Fig. 4B). These indicated that the expression of *gapdh* is up-regulated *in vivo*.

Hep-2 Cells Binding by Recombinant GAPDH

To determine whether GAPDH is involved in bacterial adhesion to Hep-2 cells, a flow cytometric assay was applied. The cells were incubated with 2 μg of recombinant GAPDH, and then with mouse sera against GAPDH, stained with goat anti-mouse IgG-FITC before being examined with flow cytometry. The signicant MFI could be detected from the surface of the Hep-2 cells incubated with recombinant GAPDH, whereas no specific MFI could be observed from the negative control (Fig. 5). These indicated that the recombinant GAPDH could adhere to the Hep-2 cells.

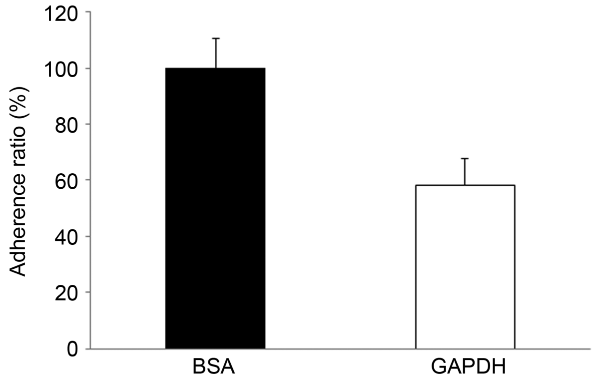

Fig. 6. Inhibition of *Streptococcus equi* ssp. *zooepidemicus* (SEZ) adhesion to Hep-2 cells by recombinant GAPDH.
Recombinant GAPDH was able to inhibit the adherence of SEZ to Hep-2 cells by 41.8% compared with BSA.

Inhibition of Adherence of SEZ to Hep-2 Cells by Recombinant GAPDH

To assess the contribution of GAPDH to the adherence of SEZ, an inhibition assay was applied. Hep-2 cells were treated with the purified GAPDH before adherence of SEZ to the cells, and BSA was served as the control. Recombinant GAPDH was able to inhibit the adherence of SEZ to Hep-2 cells by 41.8% compared with BSA (Fig. 6), which indicated that the GAPDH contributed to the adherence of SEZ to host cells ($p < 0.05$).

DISCUSSION

It is now apparent that many of the glycolytic enzymes are often localized to the surface of microbial pathogens, where they exhibit various functions, unrelated to their housekeeping roles [19, 24]. Currently, there is considerable interest in identifying the additional roles of these bacterial glycolytic enzymes. The GAPDHs of several bacterium species were found to be involved in host adhesion [7, 10, 24]. GAPDH of SEZ may play the same role in host adhesion as in *Streptococcus pyogenes*, because both proteins were homologous [2]. Besides this, the immunogenicity of GAPDH was confirmed in the previous study [15]. However, the potential of GAPDH developed as novel vaccine candidate remained to be tested.

In the present study, GAPDH protected mice against a lethal dose of SEZ. Although the protective effort of GAPDH was relatively weak compared with an inactivated SEZ vaccine, it still indicated that GAPDH was a good protective antigen. In addition, GAPDH is a housekeeping enzyme and is well conserved in many bacteria species [27]. This immunogenic protein shows little sequence variation among different SEZ strains and diverse clinical isolates, and could be a superior antigen for the development of broadly effective vaccines against SEZ.

In mice, serum IgG1 is associated with a Th2-type response, whereas serum IgG2a is associated with a Th1-type response, which is particularly effective at mediating bacterial opsonophagocytosis. In our study, GAPDH induced a high titer of IgG in mice, including IgG1, and subjectively less IgG2a. The humoral immune response against GAPDH may contribute to protection against lethal challenge.

That GAPDH could serve as a vaccine candidate was confirmed by the challenge study. Besides this, in the present study, we also explored the reasons by which GAPDH could confer a good protective efficiency. Adherence of GAPDH to the surface of Hep-2 cells was confirmed by flow cytometry and an adherence inhibition assay, which demonstrated that GAPDH could contribute to the adherence of SEZ to host epithelial cells. Undoubtedly, adherence to epithelial cells was important for SEZ to break through this first barrier of hosts [22]. We have confirmed that the expression of GAPDH was significantly up-regulated *in vivo*, by real-time PCR, suggesting that GAPDH might play an important role in the pathogenicity of SEZ. The roles of adherence and pathogenicity that GAPDH played could be the reasons for which it conferred a high protection efficacy.

In summary, we could draw a conclusion that purified recombinant GAPDH could confer a good protection efficacy against SEZ infection. Therefore, GAPDH has the potential to be developed as a novel and an effective vaccine candidate for SEZ. Further research is required to explore the effectiveness of GAPDH as a vaccine candidate to protect pigs against SEZ infection.

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