Cross-Protective Efficacy of Recombinant Transferrin-Binding Protein A of Haemophilus parasuis in Guinea Pigs

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The causative agent of Glasser’s disease in swine is Haemophilus parasuis. Commercial bacterins are widely used for protection of the swine population. However, cross protection is limited because H. parasuis has more than 15 serovars. Transferrin-binding protein A has shown potential as a broad-spectrum vaccine candidate against homologous and heterologous strains. Here we amplified the full-length tbpA gene from an H. parasuis serovar 13 isolate and cloned it into a pET-SUMO expression vector. We then expressed and purified the TbpA protein by Ni affinity chromatography. First, the immunogenicity and protective efficacy of the protein were evaluated in guinea pigs by two subcutaneous immunizations with different doses of Montanide IMS 206 VG adjuvant. The immunized guinea pigs were, respectively, challenged on week 3 after a booster immunization with homologous strain LJ3 (serovar 13) and heterologous strain FX1 (serovar 4), and vaccine-inoculated groups were compared with nonvaccinated controls. All immunized groups showed serum antibody titers higher than those of negative-control groups. Furthermore, the cytokine and chemokine levels were evaluated at the transcriptional level by the real-time PCR analysis of six cytokines and chemokines. Gamma interferon and interleukin-5 in groups immunized with 100 μg were elevated more than 15-fold over those in negative-control groups. The protection rates were 80 and 60% after a challenge with strains LJ3 and FX1, respectively, in the groups vaccinated with 100 μg of recombinant TbpA protein. Subsequently, the data showed that guinea pigs immunized with a single dose (100 μg) were protected at levels of 80, 80, and 60% against LJ3, FX1, and another heterologous strain, SZ (serovar 14), respectively. The results indicate for the first time that TbpA protein cross protects guinea pigs against serovars 13, 4, and 14 of H. parasuis. Taken together, these results suggest that the recombinant TbpA protein is a promising vaccine candidate that needs to be confirmed in a swine population.

Haemophilus parasuis, a nonhemolytic, Gram-negative, NAD-dependent, rod-shaped bacterium, belongs to the family Pasteurellaceae (1). H. parasuis is a commensal of the upper respiratory tract of healthy pigs. However, it is considered an important pathogen and the etiological agent of Glasser’s disease, which is characterized by fibrinous polyserositis, polyarthritis, and meningitis. This disease is one of the main causes of death in the swine industry, resulting in huge economic losses worldwide (2). Kielstein et al. have identified 15 serovars of H. parasuis that range from highly virulent to nonvirulent, but a high percentage of the field isolates obtained have not been serotyped thus far (3, 4). Different serovars of H. parasuis range from highly virulent to nonvirulent (5).

Control of Glasser’s disease has traditionally been carried out by means of commercial or autogenous bacterins (6). These vaccines generally offer strong protection against a challenge with the homologous serovar, but the development of cross protection depends on strains and serovars of H. parasuis (7–9). The lack of effective vaccines against a broad spectrum of strains has limited disease control. With the development of modern vaccines based on molecular techniques, subunit vaccines have attracted more attention, with particular interest in the identification of protein-based vaccine candidates (10–12). It has been shown that recombinant vaccines based on outer membrane proteins (OMPs) provided partial protection against a challenge with H. parasuis (13). Fifteen novel immunogenic OMPs have been identified, and four of them, OMP2, D15, PalA, and HPS06257, have proven to have strong potential as vaccine candidates (14). Few studies of the virulence factors or other immunogens involved in the pathogenicity or immunity of H. parasuis have been reported to date. Therefore, it is necessary to identify novel and more efficient immunoprotective antigens contributing to the development of a vaccine that can protect pigs against H. parasuis infection.

Iron is an essential element for nearly all living organisms and participates in redox reactions, oxygen transport, and iron detoxification processes (15, 16). A low concentration of free iron, not enough to support the growth of bacteria in the host, represents major stress for bacterial pathogens. In order to adapt to the iron-restricted environment of the host, H. parasuis, like other members of the families Pasteurellaceae and Neisseriaceae, has iron acquisition pathways mediated by surface receptors that specifically bind transferrin from the host (17–19). The receptor is composed of two transferrin-binding proteins, TbpA (approximately 110 kDa) and TbpB (approximately 60 kDa), which makes the organism utilize porcine transferrin as the sole source of iron (20). Damage of iron uptake systems is likely to reduce virulence, so transferrin-binding proteins are known to be important for bacterial virulence. For this reason, these two proteins can be useful as candidate targets for H. parasuis. A number of studies have demonstrated that the transferrin-binding proteins from Neisseria...
meningitidis and Actinobacillus pleuropneumoniae showed good protection (21–23). In H. parasuis, TbpB has already been expressed and characterized, but recombinant TbpB (rTbpB) could not stimulate an immune response and gave no protection (6, 24, 25). However, the full-length tbpA gene has not been expressed and characterized to date. There is little information regarding the immunogenicity and protection of TbpA in this species.

The aim of this study was to evaluate the immunoprotective efficacy against H. parasuis induced by double or single immunization with TbpA protein. The tbpA gene of virulent H. parasuis strain LJ3 was successfully cloned and expressed. The immunogenicity of the recombinant protein and the capability of inducing inflammatory cytokine production were evaluated in the guinea pig model. Meanwhile, cross protection was assessed to see whether guinea pigs were protected against challenges with the homologous LJ3 (serovar 13) and heterologous FX1 (serovar 4) and SZ (serovar 14) strains. 

MATERIALS AND METHODS

Bacterial strains and growth conditions. H. parasuis strains LJ3, FX1, and SZ were isolated from separate pig farms located in Lujiang County, Feixi County, and Suzhou County in Anhui Province, where the highly pathogenic porcine reproductive and respiratory syndrome broke out in 2006 accompanied by Glasser’s disease. These three strains were cultured in tryptic soy broth or on tryptic soy agar supplemented with 0.01% NAD and 5% fetal calf serum at 37°C.

Construction of recombinant plasmids. The primers used to amplify the full-length tbpA gene were designed by referring to the sequence of H. parasuis SH0165 and were as follows: forward, 5′-GGCCATGAAAAATAAATTTAATTTTATAGCC-3′; reverse, 5′-CCGCTCCTAGTATGAGCCTCATCTCTACTGAAAGGA-3′. The underlined sequence in the forward primer was introduced to form an SfoI restriction enzyme site, and the one in the reverse primer was introduced to create an XhoI site. The DNA of strain LJ3 was extracted according the instructions in the TaKaRa MiniBEST bacterial genomic DNA extraction kit (TaKaRa, Dalian, China). The PCR conditions were as follows: initial denaturation at 98°C for 30 s, followed by 30 cycles of denaturation for 10 s at 98°C, annealing at 58°C for 30 s, and extension for 90 s at 72°C. Amplification was ended at 72°C for 10 min. The purified PCR product was digested with the restriction endonuclease Xhol (New England BioLabs, Boston, MA). A PET-SUMO plasmid that was modified to insert a SUMO protease cleavage site between a His tag and the target gene in order to easily purify the natural protein (26) was digested with SfoI and XhoI, and then the processed fragment was ligated with T4 DNA ligase at 16°C overnight. The ligation mixture was later used to transform E. coli DH5α. The recombinant positive clones were identified by PCR and enzyme digestion with SfoI and XhoI. The DNA primer was introduced to form an SfoI restriction enzyme site, and the one in the reverse primer was introduced to create an XhoI site. The DNA sequence of the recombinant fragment was confirmed by sequencing.

Expression and purification of the recombinant protein. The recombinant plasmid PET-SUMO-tbpA was transformed into E. coli Rosetta(DE3) competent cells. An overnight cell culture was grown at 37°C under agitation (120 rpm) in the presence of 50 μg/ml ampicillin. When the concentration of the cell suspension reached an optical density at 600 nm (OD600) of 0.5 to 0.6, 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce protein expression at 20°C (120 rpm) overnight. The cells were centrifuged at 10,000 X g for 15 min, and the pellets were resuspended in lysis buffer (30 mM Tris-HCl, 100 mM NaCl, pH 8.0) and then lysed by sonication. The lysate was centrifuged at 10,000 X g for 10 min, and the pellets were resuspended in lysis buffer (20 mMol/liter Tris-HCl, 150 mmol/liter NaCl, 2 mol/liter urea, 0.5% Tween 20, 20 mmol/liter imidazole, pH 8.0) and sonicated for 20 min. The target protein was purified from the suspension over a His-Ni resin affinity chromatography column (Novagen). Finally, the target proteins were eluted in 500 mM imidazole buffer.

Immunoblotting test. The expressed and purified TbpA protein obtained as described above was analyzed by 10% SDS-PAGE. For Western blotting, purified TbpA was examined by 10% SDS-PAGE and electro-transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk in PBST (phosphate-buffered saline [PBS] containing 0.05% Tween 20) at 4°C overnight and then incubated for 1 h at room temperature (RT) with rabbit anti-H. parasuis polyclonal serum that was produced by immunizing a rabbit with the whole inactivated LJ3 strain according to the hyperimmune preparation protocol (diluted 1:500 with 5% milk in PBST). Horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma; 1:5,000) was used as the secondary antibody. After incubation for 1 h at RT, the membranes were washed three times with PBST and then developed with ECL liquids A and B by following the manufacturer’s instructions. Immunobinding bands were visualized by exposure on the film.

Protective efficacy against H. parasuis of two subcutaneous immunizations of guinea pigs with TbpA. (i) Immunization, sample collection, and challenge. One hundred or 50 μg of rTbpA protein was combined with Montanide IMS 206 VG adjuvant (Seppic, Inc., Paris, France) at a 1:3 ratio and stored at 4°C until use. A total of 30 guinea pigs were purchased from Beijing Vital River Ltd. (Beijing, China) and housed in a biosafety level 2 animal facility. The animal experiment was approved by the biosafety and animal welfare committee of the institute and local government (SYXK (G)2010-0003). In the present experiment, guinea pigs were randomly allocated to six groups of five animals each and given two subcutaneous vaccinations 3 weeks apart. Two groups (A and B) were immunized with 200-μl emulsion volumes containing 100 μg of purified TbpA per guinea pig. The other two groups (C and D) were immunized with 200-μl emulsion volumes containing 50 μg of this protein. As controls, groups E and F were vaccinated subcutaneously with 200-μl emulsion volumes only. The immunization procedure is shown in Table 1.

<table>
<thead>
<tr>
<th>Group</th>
<th>Antigen</th>
<th>Dosage/μl</th>
<th>Adjuvant</th>
<th>Route</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>TbpA</td>
<td>50 μg/200 μl</td>
<td>206.4</td>
<td>s.c.</td>
</tr>
<tr>
<td>B</td>
<td>TbpA</td>
<td>50 μg/200 μl</td>
<td>206</td>
<td>s.c.</td>
</tr>
<tr>
<td>C</td>
<td>TbpA</td>
<td>100 μg/200 μl</td>
<td>206</td>
<td>s.c.</td>
</tr>
<tr>
<td>D</td>
<td>TbpA</td>
<td>100 μg/200 μl</td>
<td>206</td>
<td>s.c.</td>
</tr>
<tr>
<td>E</td>
<td>None (PBS)</td>
<td>200 μl</td>
<td>206</td>
<td>s.c.</td>
</tr>
<tr>
<td>F</td>
<td>None (PBS)</td>
<td>200 μl</td>
<td>206</td>
<td>s.c.</td>
</tr>
</tbody>
</table>

a There were five guinea pigs per group.

b Schedule: priming-boosting with a 3-wk interval.
c 206, Montanide IMS 206 VG.
d s.c., subcutaneous.

Blood samples were collected prior to the first immunization and 3 weeks after each immunization. All of the sera isolated from the blood samples were aliquoted and stored at −40°C until enzyme-linked immunosorbent assay (ELISA) analysis. Meanwhile, the fresh blood samples used to determine cytokine expression levels were also drawn 3 weeks after the second immunization.

At 3 weeks after second immunization, groups A, C, and E were intraperitoneally challenged with a lethal dose of H. parasuis strain LJ3 (1 X 1010 CFU) suspended in 0.2 ml of PBS. Groups B, D, and F were challenged with the same dose of H. parasuis strain FX1. All of the guinea pigs were monitored for morbidity and death for 5 days.

(ii) Antibody detection by ELISA. An indirect ELISA was used to determine the levels of anti-TbpA antibodies (IgG) in guinea pig serum. Nunc flat-bottom polystyrene 96-well plates were coated at 100 μl/well with purified TbpA protein at a concentration of 1 μg/ml in 0.02 M carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. Wells were

*TABLE 1 Experimental design for grouping, antigen dosage, and method of immunization.*

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were washed thrice with 300 μl of PBST and then blocked for 2 h at 37°C with 1% (wt/vol) bovine serum albumin in PBST at 200 μl/well. After three washings with PBST, serial 5-fold dilutions of each serum were made starting at 1:100 in blocking buffer, added to the wells in duplicates of 100 μl each, and adsorbed for 1 h at 37°C. Plates were washed three times with PBST, 100 μl of goat anti-guinea pig immunoglobulin G (IgG) conjugated with horseradish peroxidase (1:2,000 dilution; KPL) was added to every well, and the plates were incubated for 1 h at 37°C. This was followed by three washings. After the addition of 3,3′,5,5′-tetramethylbenzidine and 10 min of incubation in the dark at RT, the reaction was stopped with 2 M H2SO4. The absorbance at a wavelength of 450 nm of each well was read.

Statistical analysis. GraphPad PRISM 5.0 software was used to generate graphs of data for statistical analysis. Differences among all groups were processed by one-way analysis of variance, followed by the Tukey test, and a P value of <0.05 was considered statistically significant. Comparisons of animal survival were evaluated with the log-rank test.

RESULTS

tbpA was efficiently expressed in E. coli and had good immunogenicity. The full-length tbpA gene was highly efficiently expressed in E. coli Rosetta(DE3) cells. SDS-PAGE detection showed that the TbpA protein was expressed successfully at the predicted size (Fig. 1A). Meanwhile, the His-tagged TbpA protein after sonication was purified by Ni affinity chromatography (Fig. 1A). The molecular mass of TbpA was about 110 kDa, similar to the prediction. The result of Western blotting with a rabbit anti- H. parasuis polyclonal serum confirmed the band as we expected (Fig. 1B), which showed the protein had good immunogenicity to react with anti-TbpA antibody.

TbpA-specific antibody was induced in guinea pigs by two immunizations. To investigate whether the antibody was induced by the recombinant protein used to vaccinated guinea pigs, the antibody titers of the pooled sera from immunized groups and negative-control groups were measured by ELISA reader. Serum-

TABLE 2 Primer sequences used for real-time PCR

<table>
<thead>
<tr>
<th>Gene product</th>
<th>Accession no.</th>
<th>Oligonucleotide*</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>U51572</td>
<td>F, CCCTTCCGCTGTAACCCACCT R, TGGCTGCTCACCCCTCCT</td>
<td>101</td>
</tr>
<tr>
<td>IL-2</td>
<td>AB010093</td>
<td>F, ACGCTACCTTTCTGTGGCTGTTG R, TGGCTGCTCACCCCTCCT</td>
<td>90</td>
</tr>
<tr>
<td>IL-5</td>
<td>U34588</td>
<td>F, AAGGGAAGACCTGCGAAGGTATG R, GGTGAAAGAAGTGCTGTTGGA</td>
<td>101</td>
</tr>
<tr>
<td>IL-8</td>
<td>NM_001173399.2</td>
<td>F, CAGTTGCTTTGCGGAGGTGTTG R, GTGGAAAGAAGTGCTGTTGGA</td>
<td>86</td>
</tr>
<tr>
<td>MCP-1</td>
<td>L04985</td>
<td>F, CTCAGCTCCTTCTGGCTCTGTT G, TTTTCTATACCCCTCACCCCT</td>
<td>143</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>NM_001172874.1</td>
<td>F, AGCAACAGGTGGCAGCTTTT G, TCAGTCCTCTGGCTCTGTT</td>
<td>134</td>
</tr>
<tr>
<td>TNF-α</td>
<td>NM_001173025</td>
<td>F, GCCCTTGAGTGAAGCCACCT G, GGAAATGGCAGAAGCTGCTGG</td>
<td>106</td>
</tr>
</tbody>
</table>

* F, forward; R, reverse.

live guinea pigs were collected on day 5 and fixed in 10% neutral formalin solution for a pathology assay. IgG and IgM levels were determined as described above.

TABLE 3 Experimental design for single immunization with LJ3 TbpA protein

<table>
<thead>
<tr>
<th>No. of guinea pigs/group</th>
<th>Antigen Dosage/vol</th>
<th>Adjuvant*</th>
<th>Route</th>
<th>Challenge strain (serovar)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>TbpA</td>
<td>100 μg/200 μl</td>
<td>206</td>
<td>s.c.* LJ3 (13)</td>
</tr>
<tr>
<td>5</td>
<td>TbpA</td>
<td>100 μg/200 μl</td>
<td>206</td>
<td>s.c. FX1 (4)</td>
</tr>
<tr>
<td>5</td>
<td>TbpA</td>
<td>100 μg/200 μl</td>
<td>206</td>
<td>s.c. SZ (14)</td>
</tr>
<tr>
<td>5</td>
<td>None (PBS)</td>
<td>200 μl</td>
<td>206</td>
<td>s.c. LJ3 (13)</td>
</tr>
<tr>
<td>5</td>
<td>None (PBS)</td>
<td>200 μl</td>
<td>206</td>
<td>s.c. FX1 (4)</td>
</tr>
<tr>
<td>5</td>
<td>None (PBS)</td>
<td>200 μl</td>
<td>206</td>
<td>s.c. SZ (14)</td>
</tr>
</tbody>
</table>

* 206, Montanide IMS 206 VG.  
  s.c., subcutaneous.

FIG 1 Expression and purification of rTbpA protein. (A) Expression and purification of TbpA in E. coli analyzed by10% SDS-PAGE and stained with Coomassie brilliant blue. Protein molecular size standard markers, lane M; expression product of noninduced recombinant bacterium, lane 1; expression product of induced recombinant bacterium, lane 2; supernatant of induced E. coli lysate, lane 3; precipitate of induced E. coli lysate, lane 4; purified protein after affinity chromatography, lane 5. Lanes M and 1 to 4 are from a single gel run, but four irrelevant lanes between lanes 2 and 3 have been removed; lane 5 is from a different gel in which all conditions were the same. (B) Purified TbpA was verified by immunoblotting with anti-HPS serum. Protein molecular size standard markers, lane M; purified TbpA protein, lane 1; negative control, lane 2.

TABLE 3 Gene product, Accession no. Oligonucleotide Product size (bp)

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</tr>
<tr>
<td>IL-2</td>
<td>AB010093</td>
<td>F, ACGCTACCTTTCTGTGGCTGTTG R, TGGCTGCTCACCCCTCCT 90</td>
</tr>
<tr>
<td>IL-5</td>
<td>U34588</td>
<td>F, AAGGGAAGACCTGCGAAGGTATG R, GGTGAAAGAAGTGCTGTTGGA 101</td>
</tr>
<tr>
<td>IL-8</td>
<td>NM_001173399.2</td>
<td>F, CAGTTGCTTTGCGGAGGTGTTG R, GTGGAAAGAAGTGCTGTTGGA 86</td>
</tr>
<tr>
<td>MCP-1</td>
<td>L04985</td>
<td>F, CTCAGCTCCTTCTGGCTCTGTT G, TTTTCTATACCCCTCACCCCT 143</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>NM_001172874.1</td>
<td>F, AGCAACAGGTGGCAGCTTTT G, TCAGTCCTCTGGCTCTGTT 134</td>
</tr>
<tr>
<td>TNF-α</td>
<td>NM_001173025</td>
<td>F, GCCCTTGAGTGAAGCCACCT G, GGAAATGGCAGAAGCTGCTGG 106</td>
</tr>
</tbody>
</table>

* F, forward; R, reverse.

were vaccinated with 100 μg of TbP protein with Montanide IMS 206 VG adjuvant, and the other three groups were treated with the emulsion only as negative controls. On day 14 postimmunization, blood samples were collected from each guinea pig and subsequently the guinea pigs were challenged with homologous strain L3J and heterologous strains FX1 and SZ according to the schedule shown in Table 3. All of the guinea pigs were monitored for morbidity and death for 5 days. The lungs of the dead or
ical analysis of vaccinated guinea pigs indicated that specific immune responses to the recombinant antigen were produced in the vaccinated animals, with significant increases in total IgG titers observed after primary and secondary immunizations (Fig. 2). High antibody levels were reached in the TbpA-immunized groups. The negative-control groups showed no detectable level of anti-TbpA antibody during the experimental period. Meanwhile, the TbpA-specific antibody level was only slightly higher in the groups immunized with 100 μg than in the groups immunized with 50 μg.

Cytokines and chemokines were stimulated at the transcriptional level after secondary immunization of guinea pigs. Cytokines and chemokines play big roles in the mediation of innate and adaptive immune responses, which cooperate to eliminate or combat pathogens. To evaluate whether the cytokines and chemokines induced by TbpA protein immunization might contribute to the protection of the guinea pigs in the present study, six cytokines or chemokines were tested at the transcriptional level by real-time reverse transcription-PCR. Significant increases in IFN-γ (P = 0.0246), IL-5 (P = 0.0071), and MCP-1 (P = 0.0426) mRNA expression were found to be at least 10-fold greater in the TbpA-immunized groups than in the control groups (Fig. 3). IFN-γ and IL-5 levels were 15-fold higher in the immunized groups than in the control groups. However, the IL-2, IL-8, and TNF-α levels in the TbpA-immunized groups were less than 10-fold higher than in the negative-control groups, a difference that was not statistically significant.

Protective efficacy of TbpA against homologous and heterologous serovars after two immunizations of guinea pigs. We next assessed whether the TbpA protein provides good protection or cross protection of guinea pigs against homologous strain LJ3 or heterologous strain FX1. At approximately 24 h after a challenge with lethal doses of strains LJ3 and FX1, 10 guinea pigs in the two negative-control groups inoculated with different strains of bacteria were all dead. However, the groups of guinea pigs immunized with TbpA protein survived in different ratios following the intraperitoneal inoculation of 1 × 10^10 CFU of H. parasuis, unlike the control group. A challenge of groups A, C, and E with strain LJ3 resulted in 80, 60, and 0% survival rates, respectively (Fig. 4A). One guinea pig each in groups A and C was dead on day 1 postchallenge. Another one in group C was dead on day 3 postchallenge. However, a challenge of groups B, D, and F with strain FX1 resulted in 60, 40, and 0% survival rates, respectively (Fig. 4B). One guinea pig in group B died on each of days 1 and 2 postchallenge, and another three guinea pigs in group D were dead on days 1 and 2 postchallenge.

A single TbpA immunization of guinea pigs provided good protection against homologous and heterologous serovars. To explore the efficacy of a single TbpA immunization of guinea pigs, we designed another experiment as described in Table 3. On day 14 postimmunization, the sera from each group were pooled and the antibody titers were detected by ELISA as described in Mate-
Materials and Methods. The IgG level in the TbpA-immunized groups was significantly higher than that in the negative-control groups, which was similar to the result of the first immunization in the two-immunization experiment (Fig. 5A). The IgM level in the TbpA-immunized group was much lower than the IgG level; however, in contrast to the negative-control group, the difference was statistically significant (Fig. 5B). The challenge data showed that the protective efficacies of even a single immunization of guinea pigs were 80, 80, and 60%, respectively, against a lethal dose of homologous and heterologous serovars (Fig. 6). The pathology assay result also matched that described above. In the Montanide IMS 206 VG adjuvant-immunized groups, the lungs of the guinea pigs generally had severe congestion, inflammatory cell infiltration, bronchopneumonia or interstitial pneumonia, and alveolar emphysema after a challenge with strains LJ3, FX1, and SZ on day 14 postimmunization. However, the lungs of guinea pigs in the TbpA-immunized groups had less severe pathologic lesions (data not shown).

DISCUSSION

Glasser’s disease is severe, with high morbidity and mortality rates in the swine population in China. Its etiological agent is *H. parasuis*, which is composed of more than 15 serovars. Therefore, prevention and control of Glasser’s disease are a big problem in China and in some European countries even though commercial bacterins are widely used in swine. Currently, protein-based vaccine is attractive as an alternative because it is efficient and readily prepared. Here, we used a serovar 13 strain prevalent in China as the template to clone and express its full-length *tbpA* gene. Protective efficacy was assessed in a guinea pig model. The results showed that TbpA had specific reactivity to *H. parasuis*-positive serum. Double or single immunization of guinea pigs with the recombinant protein in Montanide IMS VG 206 adjuvant elicited a strong immune response and provided good protection against a challenge with virulent strain LJ3 and the heterogenous serovars of mildly virulent strains FX1 and SZ. Therefore, the data show that TbpA might function as a new potent protective antigen, and it showed cross protection among serovars 4, 14, and 13 of *H. parasuis*.

Iron is an essential nutrient element for most organisms. Efficient strategies for its acquisition have been evolved. For access to these limited resources of iron, many pathogenic bacteria of the families *Neisseriaceae* and *Pasteurellaceae* encode two highly conserved transferrin-binding proteins that specifically bind the transferrin of their particular host organism. Indeed, their potential utility as vaccine immunogens has been demonstrated by studies carried out with some members of the *Pasteurellaceae* family, such as *Haemophilus influenzae* or *A. pleuropneumoniae* (27). The organization of high-affinity iron uptake systems composed of the *tonB*, *exbB*, *exbD*, *tbpB*, and *tbpA* genes in *H. parasuis* has been described previously (20). Transferrin-binding proteins have been associated with virulence in other members of this family. In *H. parasuis*, the expression of the *tbpB* gene has been reported. Unfortunately, the rTbpB protein gave no protection in piglets (10, 24, 25). Characterization of TbpA of *H. parasuis* and other Gram-negative organisms has shown that TbpA could be

![FIG 4](image1.jpg)

**FIG 4** Survival rates of TbpA-immunized guinea pigs following challenges with *H. parasuis* strains LJ3 (serovar 13, panel A) and FX1 (serovar 4, panel B). Groups were immunized subcutaneously with 50 or 100 µg of TbpA. Control groups were sham immunized with emulsions only. Each group consisted of five guinea pigs.

![FIG 5](image2.jpg)

**FIG 5** TbpA-specific IgG and IgM antibody levels in guinea pig sera on day 14 after single immunizations. Purified TbpA protein was used to coat a 96-well plate. (A) IgG titers in the sera of control guinea pigs and those immunized with 100 µg of antigen. (B) IgM titers in the sera of control guinea pigs and those immunized with 100 µg of antigen. *, P < 0.05.
The complete *tbpA* gene (2,700 bp) was successfully expressed by the SUMO fusion protein expression system. This new SUMO fusion protein expression vector allows the rapid cloning of any gene with two unique cloning sites by the sticky-end PCR method. This fusion protein technology enhanced the expression of recombinant proteins, protected proteins from degradation, improved the solubility of proteins, and simplified their purification and detection (26).

An experimental animal model for the evaluation of *H. parasuis* pathogenesis and vaccination has been established. Studies have shown that the guinea pig is an especially suitable model of Glasser’s disease (30, 31). Here, we evaluated the protective potential of TbpA by using a guinea pig intraperitoneal challenge model. Our data prove that the guinea pig can be useful as a laboratory animal model for the examination of cellular factors associated with the virulence and immunogenicity of *H. parasuis*. Certainly, besides this experimental animal model, *H. parasuis* is an etiological agent in the swine population. Therefore, future work must assess protection in cesarean-derived, colostrum-deprived pigs, which have been proven to be suitable models for the study of *H. parasuis* pathogenesis (28, 32).

A humoral immune response is generally predominant in a subunit protein vaccination. In two-immunization experiment in the guinea pig model, IgG antibody was induced to a high level in the TbpA-immunized groups, no matter the vaccination dosage or times. The resultant data show that two TbpA-immunized groups were protected against the homologous and heterologous strains. This drove us to try to determine whether a single vaccination with 100 μg of rTbpA can provide similar or even better protection. Our subsequent experimental results indicate that the IgG antibody reached a high level on day 14 postimmunization. Moreover, in this experiment, IgM antibody, which is an early humoral immune response indicator, was tested on day 14 and its titer was much lower than the IgG titer in the TbpA-immunized groups. We did not analyze further to see if IgM played an important role against different *H. parasuis* infections in the early stage, but the final protection rates shown in Fig. 6 demonstrate that a single immunization provided similar or even better protection (against strain FX1) of guinea pigs challenged with the LJ3, FX1, and SZ strains on day 14. This implies that a single vaccination with 100 μg of rTbpA was sufficient to protect guinea pigs against the different serovars.

Besides the humoral immune response, measurement of cytokine levels in blood represents a systemic response to the recombinant protein and it is a reflection of the overall cytokine production in the organism for further investigations into the phenotype (Th1 and Th2) of the immune response. Th1 and Th2 cells secrete cytokines that enhance cell-mediated and humoral immunity, respectively. In this study, the levels of IFN-γ, IL-5, and MCP-1 transcriptionally stimulated in the immunized groups were significantly higher than those in the negative-control groups. Especially IFN-γ and IL-5 expression indicated that rTbpA might induce significant Th1 and Th2 immune responses. MCP-1 is known to be induced by IFN-γ and can be produced by a variety of cells, including macrophages, T cells, and granulocytes. However, the expression of all of these cytokines and chemokines at the protein level should be confirmed in a future vaccine evaluation. A previous study has highlighted the conclusion that the choice of adjuvant and the vehicle used for delivery will be critical for a Tbp-
based vaccine (33). In this study, cytokine expression further supported the use of Montanide IMS VG 206 adjuvant as a traditional Th1-type response enhancer in order to improve the capacity of TbpA to induce a strong Th1 immune response. In summary, TbpA with Montanide IMS VG 206 adjuvant induces humoral and cellular immune responses that might both contribute to protective immunity.

A previous study of Tbp proteins from the bovine pathogen Pasteurella haemolytica provided further evidence of the importance of TbpA (34). Studies with N. meningitidis and A. pleuropneumoniae have demonstrated the potential efficacy of TbpA as a vaccine antigen (21, 35). Our results provide an opportunity to assess whether the TbpA protein of H. parasuis can serve as an effective vaccine antigen to protect not only against serovar-specific H. parasuis but also against other serovars. Further preclinical studies with a swine population are needed to investigate whether the TbpA protein could be used as an effective vaccine to prevent Glasser’s disease. In conclusion, our study provides the basis for further identification of characteristics of the TbpA protein of H. parasuis and the development of new serological diagnostic reagents, subunit vaccines, and environmental detection systems.

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