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Oral Immunization of Mice with Attenuated *Salmonella* Typhimurium Vaccine Expressing Transferrin-Binding Protein A (TbpA) of *Haemophilus Parasuis*

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ABSTRACT

***Background*:** *Haemophilus parasuis* is the etiological agent responsible for causing Glässer's disease in pigs, which are major respiratory pathogens that cause large economic losses in the pig industry worldwide. *H. parasuis* obtains transferrin-bound iron by expressing two surface receptors, transferrin-binding protein A and B (TbpA and B). The TbpA and B are capable of binding to transferrin, and an impairment of iron uptake mechanisms is likely to induce virulence. For this reason, these proteins can be useful as a candidate target for *H. parasuis* vaccination. Also, the live attenuated *Salmonella* Typhimurium expressing recombinant antigens from other pathogens are attractive vaccine vectors.

***Materials, Methods & Results*:** In this study, we constructed attenuated S. Typhimurium vaccine strain by porcine neurtophil passage method. By the passage, the ability of the neutrophil-adapted isolate to utilize d-xylose was lost, while the ability of the strain to ferment trehalose was delayed after 2 or more days of the culture. The *aspartate β-semialdehyde dehydrogenase* (*asd*) gene was eliminated from *S*. Typhimurium by one-step PCR. Deletion of *asd* region was confirmed by PCR using primers specific to the endpoints of the targeted region. TbpA fragment was amplified by PCR from genomic DNA of *H. parasuis* serotype 5. To construct TbpA expression plasmids, *tbpA* was subcloned downstream from the β-lactamase signal sequence in the multicopy asd^+ pYA3493 vector. This plasmid was subsequently electrotransformed into attenuated *S*. Typhimurium. The 636bp fragment of the tbpA gene of *H. parasuis* in attenuated *S*. Typhimurium was amplified by PCR and the in-frame fusion of the *tbpA* was confirmed by nucleotide sequencing. The used this strain with Asd^+ balanced-lethal plasmid pYA3493 vector to specify recombinant TbpA antigen, conserved immunogenic region of *H. parasuis*. Expression of the TbpA protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot. The size of TbpA protein was estimated at about 30kDa. Mice were administered orally in order to evaluate protective efficacy of this vaccine strain against *H. parasuis* serotype 5. For efficacy test, female ICR mice (5 weeks old) were orally injected, intraperitoneally challenged with a lethal dose ($4X10^5$ CFU/mouse) of *H. parasuis* serotype 5, and examined the survival rates compared with vaccination and non-vaccination group. The experiment was terminated two weeks post-challenge. The live attenuated *S*. Typhimurium conserved pYA3493-TbpA antigen vaccine protected 40% of immunized mice against challenge of *H. parasuis* serotype 5. This result suggested that the live attenuated *Salmonella* Typhimurium vaccine expressing TbpA might be protection for Glässer's disease outbreaks caused by *H. parasuis*.

***Discussion*:** This paper has shown protected mice that attenuated *S*. Typhimurium strain using pYA3493 expresses TbpA antigen against *H. parasuis*. Vaccination using bacterins is an efficient way to control outbreaks of Glässers disease, but significant variability has been reported. Therefore, the development of a new vaccine system to prevent Glässers disease using pYA3493-TbpA will avoid the disadvantages of the currently used vaccines. We need further works to enhance protection rate and to determine the potential of the vaccine in pigs.

Keywords: *Salmonella* Typhimurium, live vaccine, *Haemophilus parasuis*, transferrin-binding protein A (TbpA), Glässer's disease.

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INTRODUCTION

Haemophilus parasuis (*H. parsuis*), a respiratory disorder induced by bacterial pathogens, has been a serious problem in the last few years in modern swine husbandry and caused significant financial losses worldwide [5]. These outer membrane proteins (Omps) have been considered as vaccine immunogens for the development of attenuated live vaccines in relevant human or animal pathogens, such as *H. influenza* [12] or *A. pleuropneumoniae* [6] respectively. Both belong, as *H. parasuis*, to the *Pasteurellaceae* family because the impairment of iron uptake mechanisms is likely to reduce virulence. Transferrin-binding protein A (TbpA) has a serve as a channel for transport of iron across the outer membrane [2,18].

In several reviews, they have identified attenuated *Salmonella* Typhimurium (*S*. Typhimurium) as the enteric pathogen that is most effective in acting as the oral delivery of heterologous antigens to the immune system, for its potential to induce immunity-mucosal, systemic, and humoral [1,4,9].

In this study, we developed an attenuated *S*. Typhimurium vaccine strain TbpA among the Omps as an oral live vaccine against *H. parasuis*. And then attenuated *S*. Typhimurium without asd gene vaccine strains would deliver to produce expressing TbpA antigen of *H. parasuis*, was cloned into a pYA3493 Asd^+ plasmid. Finally, in the present study we have protected properties of the vaccine that were evaluated in mice against *H. parasuis* serotype 5.

Here, this paper report the protective efficacy of a recombinant TbpA attenuated *S*. Typhimurium vaccine from *H. parasuis* serotype 5.

MATERIALS AND METHODS

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study were listed in previously published [16]. The pYA3493 vector has been described previously [7]. Isolation and culture of *H. parasuis* serotype 5 [14] and DNA preparation were carried out as described above [13]. In brief, *H. parasuis* serotype 5 isolate (APQA[1]) was inoculated in 5 mL of brain heart infusion broth[2] and NAD (50 μg/mL)[3] was added for their growth. Overnight culture was pelleted at 3,000 *g* for 15 min and chromosomal DNA was prepared on a QIAamp DNA Mini kit[4] observing the manufacturer's instruction.

Construction of an attenuated S. *Typhimurium vaccine strain with asd gene deletion (ST31Δasd)*

First we recently obtained an attenuated *S*. Typhimurium vaccine strain (ST31), capable of reducing pathogenic infection after oral inoculation of mice from Dr. Lee [10]. Second, the construction of the *asd* gene mutation was performed in an attenuated *S*. Typhimurium vaccine strain using the λ Red recombinase method. The basic strategy is to eliminate an asd gene that is generated by one-step polymerase chain reaction (PCR) by using specific primers from previous published articles [3,6]. Briefly, the kanamycin resistance gene *kan* flanked by two FRT sites was amplified by PCR using the template plasmid pKD4 and primers. For electroporation, the 1.4 kb PCR products were purified and electropoated into the *S*. Typhimurium ST31 strain in which the λ Red recombinase expression plasmid pKD46 was introduced. Mutants were directly selected as kanamycin resistant colonies after electroporation, they were grown 37°C loss of the helper plasmid pKD46. To eliminate the kan cassette, ST31*Δasd::kan* was transformed with the FLP recombinase expression plasmid pCP20 vector. The used *S*. Typhimurium vaccine strain, ST06-Chonnam31 (ST31), was constructed by deletion of the *asd* genes in wild-type *S*. Typhimurium, as described above.

Finally, the presence or absence of the asd deletion in attenuated ST31 (ST31*Δasd*) was confirmed by the inability of the strain to grow on a medium without DAP and by PCR using an asd primer set (5'- TTGCTTTCCAACTGCTGAGC-3' and 5'- TCCTATCTGCGTCGTC CTAC-3').

Construction, cloning, and expression of the tbpA gene in ST31Δasd

Extraction of bacterial genomic DNA of the *tbpA* gene from *H. parasuis* was the same as that described by del Rio *et al* [17]. Total genomic DNA of *H. parasuis* was isolated and DNA fragment encoding the open reading frames of the *tbpA* from *H. parasuis* was amplified by PCR using total genomic DNA as a template. In the experiment, the PCR was carried out with the specific primer which has sequence: TbpAF (5'-GAATTCTGGTGGCTTCTA TGGTCCAA-3') and tbpA33 (5'-AAGCTTGAAACTAAGGTACTCTAACAGCTG-3') from the previously designed primers [15]. Underlined nucleotide sequences represented the linker of EcoRI and SalI, respectively. The PCR conditions were as follows: after heating

at 94°C for 5 min, amplification was performed at 94°C for 1 min, 50°C for 1 min, and 72°C for 3 min. This cycle was then carried out for 30 cycles. The final extension step, furthermore, was continued at 72°C for 10 min. The PCR product was gel purified, digested with *EcoR*I and *Sal*I and cloned into the Asd+ vector pYA3493 that was digested with the same restriction enzymes. The ligation reaction was done overnight at 16°C in the presence of T4 DNA ligase5. The 0.7-kb size of the ligated product was checked by electrophoresis in a 1.0% agarose gel. The identity of the recombinant plasmid was confirmed by restriction digestion analysis with *EcoR*I and *Sal*I. The recombinant plasmid was then transformed into the ST31*Δasd* by electroporation. Initial selection of the recombinant clones was on LB agar plates without DAP since only clones harboring the recombinant plasmid would grow.

Furthermore, the presence of the tbpA gene in ST31*Δasd* was confirmed by PCR using a two primer which was the selected fragment from 303 to 903 bp of *H. parausis tbpA* gene (F: 5'- GGCTTGGCATTG-GATGGGTTG-3' and R: 5'- AACCAACCACGAAT CAGATTT-3').

Also, construction and purification of recombinant TbpA protein were performed [8]. Briefly, the mature form of TbpA protein was subcloned into pET15b plasmid[6] at *EcoR*I and *Sal*I sites (pET15b-TbpA) in which target protein is expressed as a C-terminally 6-histidine (6His) fusion protein. The plasmid was transformed into BL21(DE3) *E. coli* bacteria and grown at 37°C in 1 L of Luria-Bertani (LB) medium containing 100 μg/mL ampicillin to an OD_{600} of 0.7. The cells were then induced to overexpress the target by the addition of 1 mM IPTG at 37°C for an additional 4 to 5 h. Cell pellets were dissolved in 10 mL of phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) and treated with lysozyme (0.5 mg/mL) at 4°C, with stirring for 30 to 60 min, followed by ultrasonication. The extracts were loaded onto Ni-affinity column[7], which was pre-equilibrated with the same buffer. The column was washed with buffer (20 mM Tris-HCl, 300 mM NaCl, 20 mM imidazole, 1 mM β-ME, pH 8.0) and rTbpA was eluted by a 0.02-0.5 M linear gradient of imidazole and dialyzed in the buffer containing 20 mM Tris-HCl, pH 8.0.

Production of polyclonal antibodies against H. parasuis *serotype 5*

The inactivated *H. parasuis* serotype 5 (HPS 5) by formaldehyde was used as an antigen for mice immunization. Individual 5-week-old ICR female mice were injected i.v. via the tail vein with 0.3 mL of purified 6His-tagged TbpA. The immunization was repeated 2, 3 and 4 weeks after the first injection. The animals were bled 2 weeks after the third injection, and serum was collected by centrifugation and stored at -80°C. To test for production of anti-HPS 5 polyclonal antibodies in mice, we used the agglutination method. The mice's serum is mixed with the particulate antigen and a positive test is indicated by the agglutination of particulate antigen.

SDS-PAGE and Western blot

The expression of TbpA protein was analyzed by sodium dodecyl sulfate polyacrylamide gel elextrophoresis (SDS-PAGE) and Western blot with the anti-His and HPS antibodies. The proteins separated by 12% SDS-PAGE were transferred on to nitrocellulose (NC) membrane by iBlot 7-Minute Blotting system[8] adhering to manufacturer's protocol. After transfer the membrane was blocked with 2% BSA in TBST (Tris-buffered saline containing 0.1% Tween-20) at RT for 1 h. The TbpA was detected using anti-HPS serum as primary antibody at a dilution of 1:500 in TBST buffer for 1hrs at RT. And then secondary antibodies, anti-mouse IgG HRP conjugate were bound at a dilution of 1:1,000 along with chemiluminescent detection using the ECL system[6].

Oral immunization and challenge of mice

The recombinant attenuated *S*. Typhimurium vaccine expressing TbpA of *H. parasuis* serotype 5 (pYA3493-TbpA) grown in 5 mL of BHI broth and NAD (10 mg/L)[3] was orally administered in 5-week-old female ICR mice. To determine the efficacy of pYS3493-TbpA as a live vaccine against *H. parasuis*, ICR mice were orally immunized. Control groups were immunized with plasmid pYS3493 (TbpA no-expression) or with PBS. Mice received a booster immunization after 21 days. Twenty-one days after the last immunization, the mice were challenged with a lethal dose of *H. parasuis* 5, (4X105 CFU per mouse), by intraperitoneal (i.p.) administration.

RESULTS

Deletion of asd gene for attenuated S. *Typhimurium mutants (ST31Δasd)*

To deletion of *asd* gene in attenuated strain of ST31, here we used the systematic construction of well-defined [3], in-frame, single-gene deletions in *E. coli* K-12. Deletion of *asd* gene was performed in *S*. Typhimurium ST31 vaccine strain by use of the one-step chromosomal gene inactivation technique. After culture at 37°C, the resulting mutants, ST31Δasd were confirmed by PCR using primers specific to the endpoints of the targeted region (Figure 2).

Expression and purification of rTbpA plasmid

The pYA3493-TbpA recombinant plasmid was transformed into ST31*Δasd* by electroporation (Figure 1).

To obtain the TbpA-expressing proteins, we first constructed a bacterial overexpression system of rTbpA in pET15b vector, which produces the target protein as a C-terminally 6His-tag fusion protein. The recombinant pET15b-TbpA plasmid was used as positive control for confirmation of TbpA molecular size (Figure 3).

The protein expression was induced by addition of 1 mM IPTG at 37°C for 4-5 hrs. Dissolved extracts were loaded on a Ni-affinity column at room temperature and washed with the washing buffer. The protein was eluted afterwards. After purification, elution fractions were analyzed for TbpA production by SDS-PAGE and there was clear evidence of production of a 30 kDa protein on the western blot assay using anti-His antibody (Figure 3). This result is comparable with the attenuated *S*. Typhimurium vaccine expressing TbpA recombinant protein (rTbpA) whether it is located in the same position (about 30 kDa). Next, the pYA3493-TbpA recombinant protein was found predominantly in the soluble fraction. The bacteria were harvested by centrifugation. The supernatants from overnight cultures were filtered to remove bacterial cells and centrifuged. Proteins in the supernatants were obtained by trichloroacetic acid (TCA) precipitation. And then, the precipitated proteins were resuspended in Tris-HCl buffer. These proteins were separated by SDS-PAGE gel and analyzed by Western blot assay with a polyclonal anti-HPS antiserum. The result showed bands with expected sizes, 30 kDa for TbpA (Figure 3).

Evaluation of protective immunity

To examine the ability of attenuated *S*. Typhimurium expressing TbpA of H. parasuis vaccines to protect against *H. parausis* serotype 5 infection, ICR mice were immunized with attenuated *S*. Typhimurium (ST31) expressing TbpA (pYA3493-TbpA) vaccine (does of 1.0 X 10^9 CFU/mL). At 3 weeks after the initial immunization, a second dose of 10^9 CFU/ml of each vaccine was administered orally. We did not detect weakness or disease signs in vaccinated mice during the immunization periods. At 3 weeks after the last immunization, mice were challenged intraperitoneally with 4.0 X 10^5 CFU/mouse of *H. parasuis* serotype 5. This challenge dose killed 100% of unimmunized. Seven days after the *H. parasuis* challenge, 40% of the mice vaccinated with pYS3493-TbpA survived. In contrast, 0% of the mice injected with PBS or pYS3493 survived the lethal challenge (Table 1).

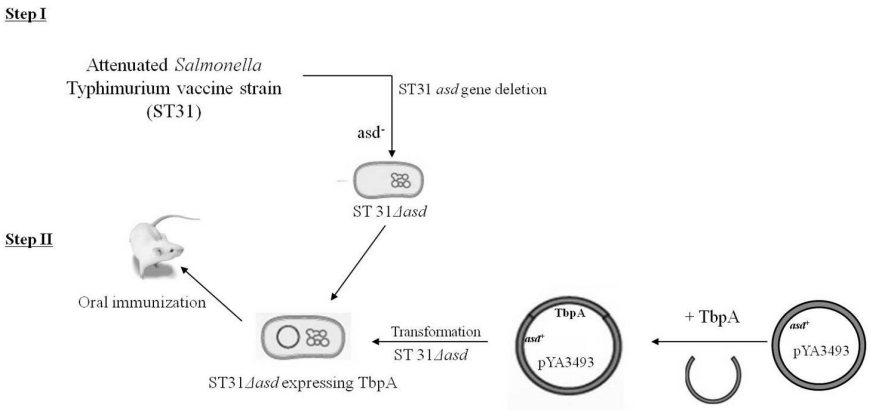

Figure 1. Schematic diagram of the experimental approach for attenuated *S*. Typhimurium expressing TbpA of *H. parasuis*. To construct an attenuated *S*. Typhimurium vaccine strain (ST31) expressing TbpA of *H. parasuis*, ST31 was attenuated by several passages of porcin neutrophil and then the attenuated ST31 was eliminated *asd* gene (λ Red recombinase method) (StepI). The attenuated ST31 were transformed expressing TbpA of *H. parasuis* serotype 5 in multicopy Asd$^+$ plasmid pYA3493 vector (StepII) and analyzed to determine the sequence corresponding to the TbpA as described in Materials and Methods.

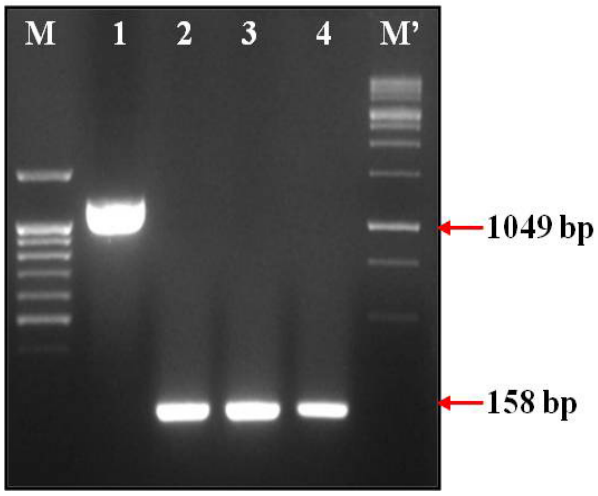

Figure 2. Genetic confirmation of *asd* deletion. DNA fragments were amplified using PCR from the wild-type attenuated *S*. Typhimurium strain and three randomly picked *asd* deletion. Before (lane 1) and after (lanes 2 to 4) DNA elimination by one-step PCR, DNA fragments were separated on a 1.0% agarose gel. M: 100 bp size marker; M': 1 kb size marker; Lane 1: attenuated *S*. Typhimurium strain with *asd*; Lanes 2 to 4: attenuated *S*. Typhimurium strain without asd.

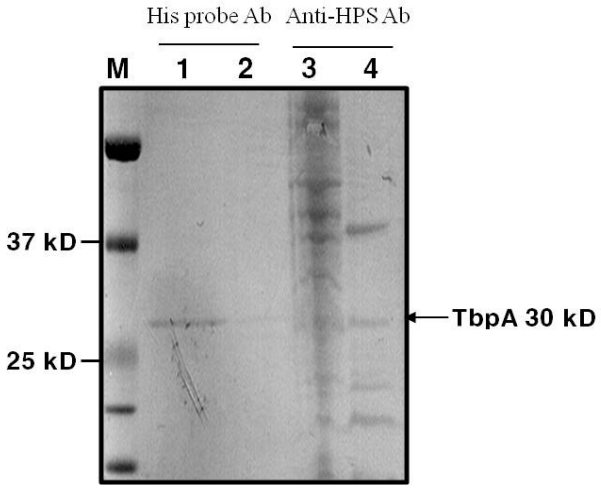

Figure 3. Expression of rTbpA protein in the attenuated *S*. Typhimurium. Protein samples were prepared from cells grown in LB at 37°C by the procedures described in Materials and Methods. Fractions equivalent to 25 mL volume of 0.7 OD_{600} culture were analyzed by SDS-PAGE and the rTbpA was detected by immunoblot with HPS specific monoclonal antibody and His probe polyclonal antibody. Lane 1: rTbpA eluted from nickel affinity columns. *E. coli* BL21(DE3) cells transformed with rTbpA (pET15b-TbpA-His_6); Lanes 2 and 3: *H. parasuis* produced by reference strain of serotype 5 (Positive control); Lane 4: *S*. Typhimurium transformed with pYA3493-TbpA.

Table 1. Oral immunization with TbpA-expressing *S*. Typhimurium (pYA3493-TbpA) vaccine protects ICR/c mice against challenge with *H. parasuis* serotype 5.

Vaccine[a]	TbpA expression[b]	Protection[c] (% alive)	Days to death
Attenuated *S*. Typimurium (pYA3493-TbpA)	+	40	>14, >14, >14
Attenuated *S*. Typimurium (pYA3493)	-	0	1
None (unimmunized)	NA	0	1

[a]Mice were orally immunized a total of two times at 3-week intervals with ~10^9 CFU of indicated vaccine strain per dose. [b]+:TbpA expreesed; [b]-: TbA not expressed; [b]NA: not applicable. [c]Three weeks after the second immunization, mice (n=30) were challenged intraperitoneally with approximately 4.0 X 10^5 CFU/mouse of *H. parasuis* serotype 5. The LD_{50} of *H. parasuis* serotype 5 by intraperitoneal infection in unimmunized ICR/c mice was <10^4 (data not shown). Mortality was monitored for two weeks after *H. parasuis* challenge.

DISCUSSION

The aim of this study was to assess the protective efficacy of expressing TbpA against an *H. parasuis* infection, using attenuated *S*. Typhimurium strains as carrier.

Recent interest has been focused on Tbps because they have long been known to be important for bacterial virulence [20]. Among these Tbps, TbpA is a member of the family of TonB-dependent outer membrane receptors [11], involved in transferrin iron uptake and composed of *tonB*, *exbB*, *exbD*, *tbpB* and *tbpA* genes. That pathway is still not fully understood, but how the expression of TbpA is known to affect on pathogenicity of *H. parasuis* has previously been described [15].

In this study, the attenuated *S*. Typhimurium vaccine strain ST31 was transformed with plasmid pYA3493-TbpA, characterized for expression of TbpA, and examined for efficacy as an oral vaccine in mice. The studies have demonstrated that oral immunization with a pYA3493-TbpA can be used to elicit protection of about *H. parasuis* serotype 5. Vaccination of

attenuated *Salmonella* vaccine expressing TbpA was induced in a similar or poor protective efficacy against *H. parasuis* serotype 5 than previously resulted about live vaccine [1,4,13]. The lack of protection may be due to low expression of TbpA protein as a vaccine candidate, although this has not been studied. Also, the bacteria are not perfectly protected by single TbpA protein, so it may not be enough to protect it against *H. parasuis* challenge.

CONCLUSIONS

In summary, the results of this study demonstrate that a recombinant attenuated ST31 vaccine expressing rTbpA antigen protected mice against *H. parasuis* serotype 5 challenges. Recombinant attenuated ST31-rTbpA vaccines may have great potential for use to induce effective protection against Glässer's disease. However, there are some experiments that need to be evaluated in future studies. Further work is needed to determine the potential of the vaccine in pigs before comprehensive evaluation and practical application are started. In addition, it would be interesting to compare the protective efficacy of vaccination with expressing TbpA to that of several expressing proteins.

Our observation might provide an opportunity to construct novel potent live vaccines with an increased polyvalency.

MANUFACTURERS

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