Haemophilus parasuis is a Gram-negative bacterium belonging to the family Pasteurellaceae and a commensal organism of the upper respiratory tract of healthy pigs [3, 8]. Under appropriate conditions, some strains can be invasive and cause severe systemic disease characterized by fibrinous polyserositis, arthritis and meningitis [8]. Recently, large numbers of virulence genes have been screened. However, most of them are yet to be understood [12, 20].

The autotransporter (AT) protein family is the largest family of Gram-negative bacterial extracellular proteins [13]. In this family, there are more than 700 members [7]. These proteins are synthesized as precursor proteins with three common functional domains, an N-terminal signal peptide, a passenger domain and a C-terminal translocator domain, which is embedded in the outer membrane as the pore-forming structure to facilitate delivery of the internal passenger domain to the bacterial surface [3, 19]. The trimeric autotransporters, a subfamily of the autotransporter protein family named VtaAs (virulence-associated trimeric autotransporters) of H. parasuis, have been reported by Pina et al. [14], while the existence of many other autotransporter protein family members has been suggested.

In this study, we selected a putative extracellular serine protease (ESP) gene, espP2, annotated in a published genome sequence of H. parasuis [17]. This putative AT protein of H. parasuis was cloned from a serotype 5 field strain, expressed and then characterized immunologically. The recombinant ESP-like protein-based vaccine conferred partial protection when compared with a formalin-inactivated bacterin.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions:** The reference strain of H. parasuis serovar 5 (strain Nagasaki) was kindly presented by Dr. Pat Blackall, QLD DPI, Animal Research Institute, Australia. H. parasuis strain HPS0819 originated from a diseased pig on a farm in Northwest China and was serotyped as serovar 5 by the gel diffusion (GD) and indirect hemagglutination (IHA) tests as described by Cai et al. [5]. Tryptone soya agar (TSA) and tryptone soya broth (TSB) medium were used with the addition of a final concentration of 10% horse serum, 5% yeast extract (Becton, Dickinson and Co., San Jose, CA, U.S.A.) and 0.05% NAD (Roche, Shanghai, China) to culture H. parasuis at 37°C in 5% CO2. When the OD600 reached 0.6–1.0, the bacteria were centrifuged and were suspended in sterile PBS. E. coli strains were cultured in Luria-Bertani medium (LB) at 37°C. When required, the LB was supplemented with kanamycin at a final concentration of 50 μg/ml.

**Sera preparation from pigs experimentally infected with H. parasuis:** Sera were obtained from three eight-week-old pigs, which were rearmed in accordance with Blanco et al. [4], and were experimentally infected with H. parasuis. Each
animal was intratracheally inoculated with 4-ml inoculums containing 10^6 CFU (subclinical dose) of H. parasuis strain HPS0819. Sera were collected at 7 days pre-infection and 7, 14, 22 and 42 days post infection. When the OD450 value of collected sera was above 0.40 as measured by ELISA as described by Martin et al. [9], the sera were used to analyze the immunogenicity of ESP-like protein by western blotting in this study.

Cloning of the putative espP2 gene: Extraction of bacterial genomic DNA of HPS0819 was carried out as reported previously [6]. Forward primer espF (5'-GGCCGAATTC-GAAACCTTATCTTTCGAA-3', restriction sites are shown in italics) and reverse primer espR (5'-CCCTCGAGTAGAACGAGATCTTTAC-3'), designed in this study based on the nucleotide sequence from the H. parasuis SH0165 strain (locus_tag in SH0165 genome: HAPS_1381), were used for PCR amplification of the putative espP2 gene. The primers were synthesized by Takara, Dalian, China. PCR reactions were carried out with Premix Taq version 2.0 (Takara) according to the manufacturer’s instructions, and the reaction conditions were 5 min at 94°C; 35 cycles of 60 sec at 94°C, 50 sec at 48°C and 2 min 50 sec at 72°C; and a final extension at 72°C for 10 min. The PCR product was then cloned into pMD19-T vector (Takara). The recombinant plasmid, named pMD-ESP, was transformed into E. coli strain JM109 (Invitrogen, Carlsbad, CA, U.S.A.) for propagation.

Sequence analysis: The obtained sequence was analyzed by the BLAST program (http://blast.ncbi.nlm.nih.gov/) [18] and MEME program (http://meme.sdsc.edu/meme4_6_1/cgi-bin/meme.cgi) [2].

Plasmid construction and expression of the putative espP2 gene: The recombinant plasmid pMD-ESP was purified and digested by Eco RI and Xho I (Takara). Then, the putative espP2 gene was cloned into the expression vector pET-30a (Invitrogen) to yield the recombinant plasmid pET-ESP. The plasmid pET-ESP was transformed into E. coli strain BL21 (DE3) (Invitrogen) for expression of the target protein. BL21 (DE3)/pET-ESP was cultured at 37°C in LB supplemented with kanamycin until the OD600 reached 0.6–1.0. The optimized conditions for expression were at a final concentration of 0.8 mM IPTG (Takara) and shaking for 8 hr at 32°C. Whole cell proteins from E. coli BL21 (DE3)/pET-ESP were separated by SDS-PAGE and stained with Cooamassie brilliant blue.

Purification and immunoblotting analysis of the ESP-like protein: The recombinant protein was purified using Ni-NTA His Bind resin (Invitrogen) according to the manufacturer’s instructions. Purified protein, the total protein of E.coli BL21(DE3)/pET30a and BL21 (DE3)/pET-ESP were separated on SDS-PAGE gel and electrotransferred onto nitrocellulose (NC) membranes (Pall, Pensacola, FL, U.S.A.), respectively. Nonspecific binding sites of the NC membranes were blocked in TBS (150 mM NaCl, 10 mM Tris-HCl) containing 5% bovine serum albumin (BSA) for 1 hr at room temperature (RT). The NC membranes were then incubated with the sera obtained from the pigs experimentally infected with H. parasuis (dilution of 1:2,000) for 1 hr at RT. After being washed 3 times with TBST (0.5% Tween 20 in TBS), the membranes were incubated with goat anti-pig IgG-alkaline phosphatase antibody (Sigma, St. Louis, MO, U.S.A.) for 1 hr at RT. After washing off unbound second antibody, the specific antigen-bound antibody was visualized using a BCIP/NBT kit (Invitrogen).

Vaccine formulations and experimental challenge: Two vaccine formulations were compared. Formulation I consisted of 100 ng/ml of recombinant ESP-like protein from the HPS0819 strain. The concentration of the antigen was referenced from the TbpB-vaccine reported by Martin et al. [9]. Formulation II contained the inactivated HPS0819 strain, which was manufactured as described by Olvera et al. [12]. Both formulations were adjuvanted using ISA206 emulsion (SEPPIC, Paris, France).

Thirty specific pathogen free (SPF) guinea pigs, obtained from the animal farm of Lanzhou Veterinary Research Institute [ Licence number: SYXX (Gan) 2004–2005], were randomly allotted into three experimental groups. The animals in group I (n=10) were immunized through dorsal subcutaneous injection with 1.0 ml of formulation I, and the group II animals (n=10) were immunized with formulation II. A booster immunization was administered 7 days later in both groups and every 7 days thereafter for a total of 4 times. The group III animals (n=10) were inoculated with an identical volume of saline as a control. Seven days after the last immunization, all groups were challenged intraperitoneally with 5 × 10^6 CFU of the HPS0819 strain. Clinical symptoms were observed for 48 hr. Animals that survived for 48 hr were euthanized.

Serum collection and detection: Blood samples were collected by cardiocentesis from two randomly selected guinea pigs in each group at day 0 (before the first immunization) and day 35 (before the challenge). Sera were obtained after centrifugation and stored at −20°C until use. Specific antibodies to ESP-like protein were detected in sera from immunized guinea pigs by western blotting, which was performed using anti-guinea pig IgG-alkaline phosphatase antibody (Sigma) as the second antibody, and ELISA [9].

Clinical and pathological examinations: Clinical signs were monitored for 48 hr after challenge. All the animals were necropsied, and the macroscopic lesions were recorded. Spleen specimens from all dead and surviving guinea pigs were cultured on TSA plates to isolate H. parasuis. The cultured colonies were then identified by the PCR method as described by Angen et al. [1].

RESULTS

Cloning of the putative espP2 gene and sequence analysis: A fragment of putative serine protease gene was amplified by PCR. The agarose gel electrophoresis analysis showed that the size of the PCR product was approximately 2,300 bp, which coincided with the expected length of the target gene (Fig. 1). The nucleotide sequence analysis showed that the cloned gene fragment codes for a polypeptide of 780 amino acids containing a complete open reading frame and having 100% identity with the putative espP2 gene sequence of H.
parasuis SH0165 (GenBank No. ACL32961). The amino acid sequence of the predicted ESP-like protein was shown to have homology with *E. coli* IgA protease (Protein Data Bank accession: 2QOM_A) at the C-terminal putative translocation domain (ESP-like protein residues 507–780 and IgA protease residues 1–277) with 44% similarity. MEME analysis results demonstrated a motif sequence, EMNN-LNKRMGELRG, at ESP-like protein residues 504–518, which is similar to the sequence, EVNNLNKRMGDLRD, conserved among members of the serine protease family. The ESP-like protein was shown to have no sequence similarity with the known ATs of *H. parasuis* (VtaAs).

Immunological analysis of the ESP-like protein: SDS-PAGE of the induced BL21(DE3)/pET-ESP showed that the molecular weight of the recombinant ESP-like protein was approximately 85 kilodaltons (kDa) (Fig. 2), which coincided with the theoretical value. SDS-PAGE of the purified ESP-like protein showed the abundant target protein appeared in elution buffer B (pH 6.0).

In Western blot analysis, ESP-like protein reacted with infection in selected serum from pigs with experimental *H. parasuis* infection (Fig. 3), whereas no band at the predicted size was detected in the *E. coli* without expression of ESP-like protein. Meanwhile, one positive band at the position of approximately 85 kDa from the whole cell proteins of *H. parasuis* was recognized with the serum from guinea pigs immunized with the purified recombinant ESP-like protein (Fig. 3).

Clinical and pathological signs: Eight non-immunized guinea pigs (group III) died within 12 hr after challenge. Two guinea pigs in group II and four from group I also died in 24 hr post infection. At 48 hr, the end of the experiment, one guinea pig in group II and two in group III died. All the dead guinea pigs had shown trembling, prostration, rough hair, anorexia, dyspnea and depression. The pathological analysis showed the typical pericarditis and peritonitis. None of the survivors showed any remarkable clinical signs or lesions.

Bacteriological examination and antibody detection results: *H. parasuis* was obligate to the dead animals (Table 1). The two non-immunized control guinea pigs (group III) remained seronegative until day 35. The antibody response in the recombinant protein vaccinated group (group I) at day 35 increased when compared with that at day 0 (mean OD of 0.29 versus 0.09). The mean antibody level also rose from 0.12 (day 0) to 0.41 (day 35) in group II (Table 1).

DISCUSSION

*H. parasuis* produces a group of virulence-associated AT proteins known as VtaAs [12, 14]. This pathogen has been shown to possess several genes encoding other putative
Table 1. Results of challenge and antibody detection in guinea pigs from groups immunized with different vaccine formulations

<table>
<thead>
<tr>
<th>Group (n=10)</th>
<th>Numbers of guinea pigs</th>
<th>Death</th>
<th>H. parasuis re-isolation</th>
<th>OD450 of antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>4</td>
<td>4</td>
<td>0.09 ± 0.04</td>
<td>0.29 ± 0.01</td>
</tr>
<tr>
<td>Group II</td>
<td>3</td>
<td>3</td>
<td>0.12 ± 0.06</td>
<td>0.41 ± 0.02</td>
</tr>
<tr>
<td>Group III</td>
<td>10</td>
<td>10</td>
<td>0.11 ± 0.03</td>
<td>0.08 ± 0.04</td>
</tr>
</tbody>
</table>

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REFERENCES


