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Identification of secreted proteins as novel antigenic vaccine candidates of *Haemophilus parasuis* serovar 5

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ABSTRACT

Haemophilus parasuis (*H. parasuis*) is a swine pathogen responsible for the Glässer's disease, which has received more attention in the past decade due to the increasing economic losses in the pig industry worldwide. As traditional inactive vaccine of *H. parasuis* has obvious disadvantage, to identify efficient immunoprotective antigens would undoubtedly contribute to the development of novel subunit vaccines. The putative secreted proteins of *H. parasuis* are potentially essential components of more potent vaccines. In the present study, six secreted proteins (PflA, Gcp, Ndk, HsdS, RnfC and HAPS_0017) were selected from the annotated *H. parasuis* serovar 5 genome as immunogenic protein with bioinformatic and experimental approaches. These proteins were successfully expressed in *Escherichia coli* and their immunogenicity was assessed in a mouse challenge model. The results showed that subcutaneous injection with the recombinant proteins resulted in the production of antibodies with high levels. Antigen-specific lymphoproliferative responses were detected in the splenocytes of the immunized animals. $CD4^+$ T-cell populations were higher in the vaccinated animals 3 weeks after the booster immunization than those of the control animals. A significant increase was observed in the cytokine levels of IL-2, IL-4 and IFN-γ in the culture supernatants of splenocytes. Furthermore, immunized mice conferred different levels of protection against challenge with a lethal dose of highly virulent serovar 5 strain (H46). Our results indicate that these six secreted proteins induced a good Th1 response and protection against *H. parasuis* infection, could be potential subunit vaccine candidates.

1. Introduction

Haemophilus parasuis (*H. parasuis*) is generally considered an important pathogen and the etiological agent of Glässer's disease, which is characterized by fibrinous polyserositis, polyarthritis, and meningitis [1]. To date, fifteen serovars of *H. parasuis* have been described, with apparent differences in virulence [2,3]. In China and North America, serotypes 4 and 5 are the most frequent isolate, and serotype 5 shows high virulence [4,5]. In recent years, with the changes in production methods, diseases caused by *H. parasuis* have become increasingly significant and result in huge financial loss worldwide.

Although *H. parasuis* is a serious disease of swine, there are no effective vaccines available with cross-protection against all pathogenic serovars. The currently commercially available vaccines for *H. parasuis* are mostly inactivated vaccines [6], however, traditional bacterium vaccine have the shortcomings that the presence of some complicated components probably induces side effects [7]. One promising strategy is based on subunit technology, which well-defined and highly purified recombinant antigens can induce a protective immune response against infection [8,9]. So the focus has shifted toward proteins as vaccine candidate. To develop a novel generation of subunit vaccine that can stimulate long-term immunity and provide cross-protection against infections, would be an effective strategy to control this disease [10,11].

Identification of conserved and broadly distributed proteins is crucial for developing subunit vaccines. Recent progresses in genomic and proteomic technologies have made it possible to

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perform global profiling of immunogenic proteins for bacterial pathogens. The studies have shown that outer membrane proteins (OMPs) and secretory proteins are usually good protective antigens [12,13]. So far, several OMPs of *H. parasuis* have been tested, including OmpP2, D15, PalA, HPS-06257, SmpA, YgiW, FOG, HPS-0675, GAPDH and OapA, which have shown partial protection against infection with the highly virulent *H. parasuis* [14–17]. However, little is known about the secreted proteins of *H. parasuis* as vaccine candidate protective antigens.

In this study, we predicted and identified immunogenic secretory proteins of *H. parasuis* with genome-wide strategy combined with bioinformatic and experimental approaches. Six potential candidate proteins (PflA, Gcp, Ndk, HsdS, RnfC and HAPS_0017) were successfully cloned, expressed and purified. The immunogenicity and protective efficacy of the recombinant proteins were evaluated in the mouse model. The results suggested that these proteins, especially Gcp, Ndk and RnfC have strong potential to become vaccine candidates.

2. Materials and methods

2.1. Bacterial strains, media and culture conditions

Escherichia coli (*E. coli*) DH5α was used as the host strain for cloning of plasmids. *E. coli* BL21 (DE3) was used as the host strain for protein expression. *E. coli* strains were cultured in Luria-Bertani (LB) medium supplemented with 100 μg/ml ampicillin. The *H. parasuis* H46 used in this study was a highly virulent strain of serovar 5 which isolated from separate pig farms located in Guangdong province. *H. parasuis* was cultured in tryptic soy agar (TSA) or tryptic soy broth (TSB) (Difco Laboratories, Detroit, MI, USA) supplemented with 10 mg/ml nicotinamide adenine dinucleotide (NAD) and 5% bovine serum at 37 °C.

2.2. Bioinformatic analysis of putative protective antigens

To identify the candidate secretory proteins of *H. parasuis* serovar 5, we used the strategy combining bioinformatic with experimental approaches. A coding sequence (CDS) set of the complete genome of *H. parasuis* serovar 5 strain SH0615 [18] was used to identify in silico possible secretory proteins. The CellPloc package (http://chou.med.harvard.edu/bioinf/Cell-PLoc/) was used to predict subcellular location of all bacteric proteins and extracellular proteins were selected. We focused our genome-wide screening strategy on the genes that encode secreted proteins and virulent-related factors simultaneously. Then, the gene sequences of selected antigens were collated from the genome sequence of *H. parasuis* strain SH0165. Signal peptide cleavage sites of open reading frames (ORFs) were predicted using the packages SignalP 4.0.

2.3. Expression and purification of immunogenic proteins

Primers used for amplifying the candidate genes were designed by referring to the sequence of *H. parasuis* SH0165 listed in Table 1. The DNA from H46 strain was extracted according to the instructions from Takara MiniBEST Bacterial Genomic DNA Extraction kit (Takara, China). The PCR products were cloned into pET-30a(+) vector and transformed into *E. coli* DH5α. Then, the positive recombinant plasmids were transformed into laboratory *E. coli* strain BL21 (DE3) for expression. Overnight cultures were reinoculated into 500 ml of LB medium containing 100 μg/ml of ampicillin. When the concentration of cell suspension reached an OD_{600} 0.5–0.6, 0.5 mM IPTG was added to induce protein expression at 37 °C for 4 h. The bacterial cells were harvested by centrifugation at 9000 × *g* and the pellets were resuspended in lysis buffer (50 mM Tris–HCl, 100 mM NaCl, pH 8.0). Then the cells were disrupted by sonication on ice. The lysate was centrifuged at 10,000 × *g* for 30 min and the suspension was saved for purifying target protein over a His-Ni-resin column (QiAGEN, Valencia, CA). The protein samples were stored at −20 °C.

Table 1
Primer sequences and cloning sites used for gene expression.

Gene name	Primer sequence
Gcp	5′-GCC*GGATCC*AAAATTCTCGGTATTGAAAC-3′
	5′-GG*CTCGAGC*TATACATCGATGGGACAAA-3′
Ndk	5′-GCC*GGATCC*ATCCAACAAACTCTCGCCAT-3′
	5′-GG*CTCGAG*TTACACAATCTCACTCGGCG-3′
HsdS	5′-GCC*GGATCC*ACTAATCTCAATAGAAATGG-3′
	5′-GG*CTCGAGC*TATATAAAAACTCTCTGCA-3′
RnfC	5′-GCC*GGATCC*CAAGCTGATGTATTAAGCCG-3′
	5′-GG*CTCGAGC*TATTTCTGAGCTTCTTTTTTCGCC-3′
PflA	5′ GCC*GGATCC*CTGACGTTTACTACGCCAGC-3′
	5′GG*CTCGAG*TTACTTAAATTGATGAATAG-3′
HAPS_0017	5′-GCC*GATATC*GAAGACGCAGAAGAGAAATT-3′
	5′-GG*CTCGAG*TTATTTCCCCTCATTTAATG-3′

2.4. SDS–PAGE and Western blot analysis

The purified proteins were separated on a 12% (v/v) polyacrylamide vertical slab gel with a 4% (v/v) stacking gel. Then the gel was transferred onto a PVDF membrane. The membrane was blocked with 0.5% skimmed milk in PBST (PBS containing 0.05% Tween-20) overnight at 4 °C. Convalescent swine sera (1:500) against *H. parasuis* and Horseradish peroxidase (HRP)-conjugated goat anti-porcine IgG (1:5000) (Sigma, USA) were used as the first and the second antibody, respectively. After washing with PBST three times, the membrane was developed with ECL liquid A and B following the manufacturer's instructions.

2.5. Mice immunization and challenge

All animal experiments were carried out in strict accordance with the recommendations in the China Regulations for the Administration of Affairs Concerning Experimental Animals 1988. The protocol was approved by China Guangdong Province Science and Technology Department (permit number: SYXK(Yue) 2011–0116). Seven-week-old female BALB/c mice (purchased from Guangdong Medical Laboratory Animal Center) were randomly divided into seven groups (with fifteen mice in each group). One group was injected subcutaneously recombinant protein 50 μg emulsified in 200 μl of complete Freund's adjuvant. At three weeks following the primary immunization, the mice were boosted with the same dose of antigens and incomplete Freund's adjuvant. The mice inoculated with equal PBS emulsified in the same adjuvant were served as negative control. Three weeks after the second immunization, ten mice from each group were challenged intraperitoneally with a lethal dose of 2.0×10^9 CFU ($2 \times LD_{50}$) of log-phase *H. parasuis* strain H46.

2.6. Antibody detection by enzyme-linked immunosorbent assay

Serum from immunized mouse was examined by the indirect enzyme-linked immunosorbent assay (ELISA) for IgG levels. In brief, 96-well plates were coated with 200 ng/100 μl of purified recombinant protein diluted in 0.02 M carbonate–bicarbonate buffer (pH 9.6) overnight at 4 °C. Wells were washed thrice with PBST, then blocked with 1% (w/v) bovine serum albumin (BSA) in PBST for 2 h at 37 °C. The plates were washed three times and incubated with 100 μl sera diluted in 1:100 for 1 h at 37 °C. After three rinses, goat anti-mouse HRP–IgG (Sigma, USA), diluted 1:5000 in PBS, was used as the secondary antibody and incubated for 1 h at 37 °C. Plates were washed three times with PBST and added TMB for 10 min in the dark

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