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Evaluation of the protective efficacy of four novel identified membrane associated proteins of *Streptococcus suis* serotype 2

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ABSTRACT

Streptococcus suis serotype 2 (*S. suis* 2) is an important zoonotic pathogen that can also cause epidemics of life-threatening infections in humans. Surface proteins of pathogens play a critical role in the interaction with host system or environment, as they take part in processes like virulence, cytotoxicity, adhesion, signaling or transport, etc. Thus, surface proteins identified by the screening of immunoproteomic techniques are promising vaccine candidates or diagnostic markers. In this study, four membrane associated proteins (MAP) identified by immunoproteomic method were cloned and expressed as recombinant proteins with his-tag. Screening for vaccine candidates were firstly performed by protection assay in vivo and immunization with Sbp markedly protected mice against systemic *S. suis* 2 infection. The immune responses and protective of Sbp were further evaluated. The results showed that Sbp could elicit a strong humoral antibody response and protect mice from lethal challenge with *S. suis* 2. The antiserum against Sbp could efficiently impede survival of bacterial in whole blood killing assay and conferred significant protection against *S. suis* 2 infection in passive immunization assays. The findings indicate that Sbp may serve as an important factor in the pathogenesis of *S. suis* 2 and would be a promising subunit vaccine candidate.

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1. Introduction

Streptococcus suis is an important pathogen, associated with a variety of life-threatening infections that include meningitis, endocarditis, arthritis, septicemia and even sudden death in pigs [1]. The worldwide pig industry has been suffering great economic losses from it [2]. Among the 33 known serotypes, *Streptococcus suis* serotype 2 (*S. suis* 2) is considered the most prevalent capsular type as well as the most virulent isolated from diseased pigs. Although only sporadic cases of *S. suis* infection have been reported in humans, two outbreaks (14 deaths in Jiangsu in 1998 and 38 deaths in Sichuan in 2005) in China and other countries have raised considerable concerns among public health and food safety professionals in recent years [3]. However, the lack of effective vaccine and limited knowledge on the pathogenic mechanisms of *S. suis* impede the attempts to control this organism.

Surface proteins of pathogens have received increasing attention since these proteins have the highest chances to be recognized by the immune system, which may raise effective immune responses and become effective vaccine formulations against infection [4,5]. Previous studies have attempted to test several surface/extracellular proteins of *S. suis* as potential vaccine targets, such as extracellular protein factor, muramidase-released protein (MRP) [6] and suilysin [7]. However, clinical isolates from some geographical regions lacking these antigens may result in incomplete coverage when these proteins are served as a single ingredient [8–10]. Therefore, identification of new potential candidates is necessary to the development of monovalent or a multivalent subunit vaccine for *S. suis*.

For subunit vaccine development, the conventional approach depending on the process of evaluating potential candidates of pathogen one at a time. It has been revolutionized by proteome-scale, parallel strategies for discovering new candidates. For example, the proteomic platforms make the vaccine development

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of Group A *streptococcus* and *Streptococcus pneumoniae* more efficiently [11,12]. In *S. suis* 2, extracellular antigens were systematically identified by a combination of proteomic approach and Western-blot, using convalescent or hyperimmune sera [13–15]. More than twenty vaccine candidates have been discovered and the protective effect against the infection of *S. suis* 2 is worthy to be determined.

In this study, the protective effects of four membrane associated proteins, including L-lactate dehydrogenase (Ldh), Dihydrolipoamide dehydrogenase (Dldh), Pyruvate dehydrogenase E1 component, α subunit of (Pec) and amino acid ABC substrate binding protein (Sbp) identified by the immuno-proteomic approach were evaluated. Finally, Sbp and anti-Sbp serum in active and passive immunization assays were shown to protect mice against S. suis 2 infection and impede the survival of *S. suis* 2 in blood, indicating Sbp is a promising protective antigen against *S. suis* 2 infection.

2. Materials and methods

2.1. Bacterial strain and growth conditions

Strain SC19 (*S. suis* 2), which was isolated from the brain tissue of a diseased piglet in Sichuan Province in 2005, was used in the study. *S. suis* was cultured in tryptic soy broth or on tryptic soy agar (Difco, USA), in which, 5% newborn bovine sera was added (Sijiqing, China) at 37 °C in aerobic conditions. *E. coli* DH5a (TaKaRa, China) was used for cloning and *E. coli* BL21 (DE3) (Novagen, China) was used for expressing of the recombinant plasmid that embody the protein. Luria-bertani broth or agar (Oxoid, UK) added with kanamycin (50 μg/mL) was used to culture *E. coli* DH5a or *E. coli* BL21 strains at 37 °C in aerobic conditions.

2.2. Cloning, expressing and purification of the recombinant proteins

Genomic DNA was isolated from strain SC19. The DNA region encoding the memberane associated proteins without the putative secreted signal or transmembrane domain were amplified using the primers as listed in Table 1, with PrimeSTAR HS DNA polymerase (TaKaRa). The PCR products, digested with *Eco*RI/*Sal*I (TaKaRa), were cloned into pET30a (Novagen) and transformed into *E. coli* BL21 strain for expression. The recombinant proteins were induced at 37 °C in LB grown to log phase by the addition of 0.1 mM isopropyl-beta-D-thiogalactopyranoside (Sigma, USA) and incubation for 3 h. According to the QIAexpress manual (Qiagen), the whole lysates were centrifuged after sonication on ice and the recombinant protein from the supernatants was purified by Ni-NTA affinity chromatography. Endotoxin was removed by ToxinEraser™ Endotoxin Removal Kit (Genscript, USA). The quantity and quality of the recombinant proteins were determined by the Bradford method and SDS-PAGE analysis, respectively. Finally, the purified proteins were concentrated by membrane ultrafiltration (Millipore) and stored in PBS at –80 °C.

2.3. SDS-PAGE and Western blotting

In SDS-PAGE analysis, polyacrylamide vertical slab gel (12%) with stacking gel (5%) was used to separate recombinant proteins. For Western-blot, the gel was electrotransfered to a PVDF membrane (Invitrogen). The PVDF membrane was blocked at 4 °C with 0.5% skimmed milk in TBST (20 mM Tris–HCl, 150 mM NaCl and 0.05% Tween-20) overnight. Convalescent sera against *S. suis* 2 and goat anti-IgG (H + L)-HRP (1:5000) (Southern Biotech, USA) were used as the first and the second antibody to determine the specific band. After washing, the PVDF membrane was developed using substrate solution 3, 3′-diaminobenzidine (Sigma).

2.4. Mice immunization and challenge

All the animal experimental protocols performed in the study were approved by the Laboratory Animal Monitoring Committee of Huazhong Agricultural University and performed accordingly. The animals were euthanized when moribund during the experiment or at the end of the experiments.

4 week old Female BALB/c mice were used in this study as described previously [16]. For immunization assay, 50 μg purified recombinant protein in 100 μL PBS emulsified with 150 μL aluminum hydroxide $Al(OH)_3$ adjuvant was used to immune mice by intraperitoneal injection. Mice immunized with PBS emulsified in $Al(OH)_3$ were considered as negative control and mice injected with PBS were considered as blank control. The primary immune and subquent booster immune have a 14 days interval. 10 days after the booster immune, blood samples were collected from tail vain and then all the mice in each group were intraperitoneally infected with 5×10^9 CFU (2×10^9 CFU for primary screening) *S. suis* SC19 strain in 200 μL PBS. All the mice were monitored for two weeks after infection. Meanwhile morbidity and mortality were recorded.

Passive immune assays were carried out as described with some modifications [17]. Groups of ten female 6 week old BABL/c mice were immunized with 100 μL of hyperimmune serum specific for amino acid ABC substrate binding protein (Sbp) by intraperitoneal injection, while the serum from PBS (PBS emulsified in $Al(OH)_3$) immune mice served as negative control and PBS serve as blank control. At 24 h after immunization, all the mice were infected with 5×10^9 CFU of *S. suis* SC19 strain by intraperitoneal injection.

2.5. Antibody determination and FACS analysis

The IgG titers of serum from immunized mice were determined by enzyme-linked immunosorbent assay (ELISA) as described previously [17]. IgG isotype (IgG1 and IgG2a) was also determined by ELISA method. Briefly, 96 well microtiter plates were coated with purified proteins (250 ng/100 μL) diluted in sodium carbonate buffer (PH 9.6) at 4 °C overnight. The plates were incubated with 0.5% skim milk at 37 °C for 1 h and washed with PBST (PBS containing 0.05% Tween-20). Serially diluted serum were added and incubated for 30 min at 37 °C. After washing three times with PBST, goat anti-mouse IgG-HRP (Southern biotech, USA), rabbit anti

Table 1
Amplification primers of gene sequence.

Gene	Primer sequence used for clone (5′ to 3′)
L-lactate dehydrogenase (Ldh)	Forward: CGCCGAATTCATGACTGCAACTAAACAACACAAA Reverse: CCCCGTCGACTTAGTTTTTTACACCAGCTGCAA
Dihydrolipoamide dehydrogenase (Dldh)	Forward: CGCCGAATTCATGGCAATTGAAATTATTATGCC Reverse: CCCCGTCGACTTATTTGCGTTTTGGTGGGT
Pyruvate dehydrogenase E1 component, α subunit of (Pec)	Forward: CGCCGAATTCATGGTATCTATCACAAAAGAACAACA Reverse: CCCCGTCGACCTAGTCTACAAACACATCCTCATAAGC
Amino acid ABC substrate binding protein (Sbp)	Forward: CGCCGAATTCGGTACATCGAATAGTACAGACCAAA Reverse: CCCCGTCGACTTACTTAGCTTTTGATACGTCTTCAC

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