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Cloning, expression and characterization of a cell wall surface protein, 6-phosphogluconate dehydrogenase, of *Haemophilus parasuis*

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ABSTRACT

Haemophilus parasuis (*H. parasuis*) is a swine pathogen responsible for the Glässer's disease. In order to understand the pathogenesis of the *H. parasuis* infection, the *gnd* gene encoding a cell surface protein, 6-phosphogluconate-dehydrogenase (6PGD) of *H. parasuis* was inducibly expressed in *Escherichia coli* BL21 with a hexahistidyl N-terminus to permit its purification. Western blotting using the r6PGD-specific antiserum showed that the 6PGD protein is on the cell surface of *H. parasuis*. The characterization of 6PGD in *H. parasuis* pathogenesis involved as an adhesion and its immunogenicity in mice was further investigated. The adherence assay with *H. parasuis* and swine alveolar epithelial cells (SJPLC) pre-incubated with $(His)_6$6PGD and non-incubated SJPLC showed a noticeable reduction in the adhesion of *H. parasuis* in the $(His)_6$6PGD pre-incubated SJPLC compared to the non-incubated SJPLC. Further, the r6PGD protein induces the production of IL-8 and IL-6 by SJPLC. Furthermore, immunization with the r6PGD protein can provide the protective efficacy by 75% following intraperitoneal administration of a $5 \times LD_{50}$ dose of *H. parasuis* SH0165, and elicited a good protective immune response, which demonstrated the importance of 6PGD to bacterial pathogenesis. Identification and characterization of the role of *H. parasuis* 6PGD in adhesion and immunogenicity will allow us to use this protein to develop new antimicrobial therapies and/or vaccines.

1. Introduction

Haemophilus parasuis is a commensal organism of the upper respiratory tract of swine. And at the same time it is the causative agent of Glässer's disease, which is characterized by fibrinous polyserositis, polyarthritis, meningitis as well as other important conditions (MacInnes and Desrosiers, 1999; Oliveira and Pijoan, 2004; Bouchet et al., 2008). To date, *H. parasuis* 15 serovars have been identified (Kielstein and Rapp-Gabrielson, 1992) and serovar 4 and serovar 5 are the most prevalent serovar in China and minimal cross-protection is observed between strains (Cai et al., 2005). It is so common for observation of a single herd colonizing with several different strains and isolates recovered from systemic sites tend to be distinct from those recovered from the lung or nasal cavity (Rapp-Gabrielson and Gabrielson, 1992; Olvera et al., 2007). The organism is early colonizer, so it is difficult to control by management procedures such as segregated early weaning. Vaccination, serodiagnostic testing, and even serotyping are complicated by the presence of multiple serotypes, cross-reactive antigens, and the absence of clear markers for virulence (MacInnes and Desrosiers, 1999).

Although some possible virulence related factors involved in *H. parasuis* pathogenicity process have been identified, so far little is known about the pathogenesis of *H. parasuis* infection. The lipooligosaccharide (LOS) of *H. parasuis* is partially involved in adhesion of *H. parasuis* porcine brain microvascular endothelial cells PBMEC and induces PBMEC apoptosis (Bouchet et al., 2008). Some research results showed that certain outer membrane protein may be associated with virulence (Ruiz et al., 2001). Neuraminidase may functions in *H. parasuis* colonization or disease and interfering with innate and immune defense systems of the host (Crocker and Varki, 2001; Lichtensteiger and Vimr, 2003).

Recent studies have demonstrated that bacterial adherence to host cells is important for pathogenicity. Some virulence factors associated with bacterial adherence may play a role in the early stages of infection. *H. parasuis* has the ability to adhere to many different epithelial and endothelial cell lines. Therefore, searching for new potential virulence factors and protective antigens involved in

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H. parasuis interaction with host cells and the immune system is necessary for understanding pathogenic and immunity mechanisms. Our efforts have focused on understanding the pathogenic mechanism of *H. parasuis* and searching for a protein that will be useful in the development of a vaccine and and/or antimicrobial therapies.

6-Phosphogluconate dehydrogenase is the rate-limiting enzyme in the pentose phosphate pathway and catalyzes the first reaction in the pathway which by converting glucose-6-phosphate to 6-phosphogluconolactone (Glock and McLean, 1955; Tepperman and Tepperman, 1964; Rognstad, 1979) and 6-phosphogluconate dehydrogenase maintains cellular NADPH levels, which are essential for cellular functions, such as vascular endothelial growth factor (VEGF)-induced angiogenesis (Pan et al., 2009). Some studies revealed that *gnd* gene was present in prokaryotic and eukaryotic organisms (Tsui et al., 1996). Generally they are very conserved in numerous species and have important function in tricarboxylic acid cycle (TCA). And the researcher George by analyzing amino acid variability of 37 6PGD sequences revealed that all regions important for the catalytic activity (Goulielmos et al., 2004).

The aim of this study was to understand the pathogenesis of *H. parasuis* induced by 6PGD. In this study, a new cell wall surface protein, 6-phosphogluconate dehydrogenase (6PGD), of a virulent strain *H. parasuis* SH0165 strain was identified and evaluated adhesion characteristics to SJPLC, immunogenicity in mice and capability of inducing pro-inflammatory cytokine production for SJPLC. The results showed that 6-phosphogluconate dehydrogenase was involved in bacterial adhesion to host cells as an adhesion and a protective antigen in *H. parasuis* SH0165 infection process. Therefore, all these results showed that 6PGD may function as a new adhesion and protective antigen in *H. parasuis* infection and immunity. This study has contributed to the understanding of the molecular interactions of *H. parasuis* with host cells, and forms the basis of potential development of new antimicrobial therapies and vaccine based on r6PGD.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The *H. parasuis* SH0165 used in this study which is a highly virulent strain of serovar 5 as the source of chromosomal DNA for polymerase chain reaction (PCR) was isolated from the lung of a commercial pig with hemorrhagic pneumonia, fibrinous polyserositis, arthritis and meningitis. *H. parasuis* was cultured on tryptic soy agar (TSA) or in tryptic soy broth (TSB, Difco) supplemented with 10 μg/mL nicotinamide adenine dinucleotide (NAD) and 5% fetal calf serum (Gibco) at 37 °C. The *Escherichia coli* strains which were used for plasmids construction and expression in *E. coli* BL21 of *6pgd* were grown in Luria–Bertani medium (LB) and plated on LB containing 1.5% (wt/vol) agar. For the analysis of the clones, the *E. coli* strains were cultured in Luria–Bertani medium (LB) supplemented with 50 μg/mL of ampicillin and with 50 μg/mL of kanamycin to select plasmids.

2.2. Isolation of chromosomal DNA

The *H. parasuis* SH0165 DNA was isolated as described by del Rio et al. (2005) with some modifications. Briefly, the strains were grown as described above and harvested with tryptic soy broth. The cells were centrifuged at 10,000 *g* for 10 min at 4 °C, washed twice with TE buffer (10 mM Tris–HCl [pH 8.0], 1 mM EDTA), and finally lysed in buffer (0.5 M EDTA [pH 8.0], 20% SDS, 50 mg/mL of proteinase K [Sigma]) for 1 h at 55 °C. For RNA removal, DNAse free RNAse (100 μg/mL, Sigma) was added and incubated for 30 min at 37 °C. Proteins were removed by adding 0.25% phenol equilibrated in TE buffer (pH 7.8). The DNA was then purified by repeated chloroform–isopropanol (24:1) extraction, precipitated by adding 0.1 vol of 3 M sodium acetate (pH 5.2), and 1 vol of isopropanol. The DNA was washed twice in 70% ethanol, allowed to dry and resuspended in double-distilled water.

2.3. Cloning of the gnd gene and sequencing the DNA

The *gnd* gene was amplified from chromosomal *H. parasuis* SH0165 DNA by PCR primed with the following primer pair: forward: 5′-GCG GGA TCC ATG TCA GTA AAA GGC GAT-3′; reverse: 5′-CCG GTC GAC TTA TAC ATC ATA AGT CGT-3′. The primers were designed by using the whole genome sequence of *H. parasuis* SH0165 (CP001321). The forward and reverse primers contained *Bam*HI and *Sal*I recognition sequences, respectively. The conditions for the PCR of 30 cycles were 10 min at 94 °C for the initial denaturation, 1 min at 94 °C for the denaturation, 45 s at 57 °C for the annealing, 1 min 30 s for the extension and 10 min for the final extension at 72 °C. The 1450 bp PCR product was inserted into the TA cloning vector (TakaRa), according to the instructions of the manufacturer. Plasmid DNA containing *gnd* of *H. parasuis* was purified with the TIANprep Mini Plasmid Kit (TIANGEN, Ltd.). The ligation product was used to transform into *E. coli* DH5a. Positive clones containing the *gnd* gene of *H. parasuis* SH0165 were sequenced.

2.4. Expression and purification of His-tag recombinant 6PGD (r6PGD)

For expression the protein of r6PGD, the *Bam*HI–*Sal*I digested TA vector contained *gnd* and the plasmid pET28a (Novagen). Then the ligation of the insert *gnd* and the prepared vector pET28a was carried out using T4 DNA ligase (TakaRa) under standard conditions. Then, it was transformed into *E. coli* BL21(DE3) (Novagen) cells and after induction with 0.8 mM isopropyl-beta-D-thiogalactopyranoside (IPTG, Sigma), the strain BL21(DE3) [pET28a–*gnd*] overproduced 6PGD as N-terminal hexahistidyl derivate [$(His)_6$6PGD]. The protein of r6PGD was purified as described by Brassard et al. (2004) with some modifications. Briefly, *E. coli* BL21(DE3) [pET28a–*gnd*] was inoculated in 100 mL of LB broth with kanamycin (50 μg/mL) and incubated at 37 °C until an optical density of 0.6–1.0 at 600 nm (OD600) was reached. The bacterial suspension was supplied with 0.8 mM IPTG and continued to be incubated at 37 °C for 3–5 h. The cells were centrifuged and the pellet was resuspended in 10 mL 50 mM sodium phosphate (pH 8.0) containing 300 mM NaCl, 10 mM imidazole and 5 mg lysozyme and incubated on ice for 1 h. The bacterial cells were disrupted by sonication on ice. The lysate was centrifuged at 11,400*g* for 45 min and the supernatant was saved for the purification of the $(His)_6$6PGD by Ni–NTA affinity chromatography as described in the QIAexpress manual. The fractions of $(His)_6$6PGD were pooled, and stored at −20 °C.

2.5. Production of rabbit antiserum against recombinant 6PGD (r6PGD)

An 7-week-old Japan white rabbit was immunized subcutaneously with 0.8 mg of r6PGD and 1 mL of complete Freund's adjuvant (Sigma, St. Louis, MO) on day 0 (primary immunization). At days 14, 21, 28, and 35, the rabbit was immunized with 0.8 mg of r6PGD and 1 mL of incomplete Freund's adjuvant (subcutaneous immunization), respectively. Ten days after the last subcutaneous immunization, blood samples were collected and the sera were pooled and stored at −80 °C for biological activity assays.

2.6. Extraction and isolation of H. parasuis cell wall proteins

The cell wall proteins were isolated as described by Siegel et al. (1981) with some modifications. Briefly, the bacterial cells were