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Research paper

Immunogenicity of the recombinant HxuCBA proteins encoded by *hxuCBA* gene cluster of *Haemophilus parasuis* in mice

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ABSTRACT

Haemophilus parasuis causes serious economic losses in pigs, which is the etiology of *Glässer's* disease. In this study we studied the immunogenicity of proteins encoded by the *hxuCBA* gene cluster in *H. parasuis*. Through bioinformatics analysis, HxuC, HxuB, and HxuA proteins were found that they might have strong antigenicity, with 31 putative cytotoxic T-lymphocyte (CTL) epitopes and multiple B-cell antigenic determinants. Western blotting assay indicated that all these antigens are highly immunogenic. The antibody levels and the levels of IL-2, IL-4, IFN-γ in the groups of HxuA, HxuB, HxuC, HxuCBA (include HxuC, HxuB and HxuA proteins), and M-3 were observed to significantly increase with time post vaccination. HxuC, HxuB, HxuCBA and *H. parasuis* M-3 vaccinated groups showed a strong immune response and protection against challenge with 6.5×10^9 cfu ($5 \times LD_{50}$) of *H. parasuis* M-3 strain in a mouse model, but HxuA group showed only a low level protection. Additionally, the immune response induced by all of the proteins reduced histopathological lesions and their antisera could inhibit the growth of *H. parasuis*. We concluded that HxuC, HxuB and HxuCBA may have potential for use as a recombinant subunit vaccine against *H. parasuis* challenge.

1. Introduction

Haemophilus parasuis is a polymorphous, NAD-dependent, Gram-negative bacterium of *Pasteurellaceae* family that causes *Glässer's* disease. This disease is characterized by fibrinous polyserositis, polyarthritis, meningitis, acute pneumonia without polyserositis and acute septicemia in swine (Nedbalcova et al., 2005; Brockmeier et al., 2013; Frandoloso et al., 2012). *H. parasuis* has 15 serotypes with apparent differences in virulence, and many nontypeable strains exhibit high heterogeneity at the molecular level (Kielstein and Rappgabrielson, 1992).

Due to many serotypes plus nontypeable strains, this has hindered the development of a cross protective vaccine against *H. parasuis* infection. The search of the outer membrane proteins (OMPs) to be used as vaccine candidates has become popularly. The antisera against the outer membrane proteins PalA, Wza, Omp2, D15 and HPS_06257 could inhibit the growth of *H. parasuis in vitro*, while the PalA, Omp2, D15 and HPS_06257 subunit vaccine could induce protection against *H. parasuis* challenge in a mouse model (Zhou et al., 2009). It has been shown that three putative OMPs (SmpA, YgiW and FOG) of *H. parasuis* SH0165 improved the survival rate of mice and reduced the tissue damages in mice against the challenge with serovar 5 strain (SH0165) or serovar 4 strain (MD0322) (Yuan et al., 2012). The OMPs of *H. parasuis* have been shown greater potential as protective antigen candidates than other components of this bacterium (Miniats et al., 1991; Ruiz et al., 2001).

Heme is an important iron source for microorganisms. Gram negative bacteria can acquire heme from the host by using many routes, such as *hxu* system (Cope et al., 1995), *has* system (Wandersman and Delepelaire, 2004), and others. The *hxu* system consists of *hxuC*, *hxuB*, and *hxuA* genes which encode HxuC, HxuB, and HxuA proteins, respectively. This system has been widely studied in *Haemophilus influenzae*, which was first identified in *H. influenzae* type b (Hanson et al., 1992). This system acquires heme from the heme-hemopexin complex by protein–protein interaction. Briefly, heme is released into the medium and binds with the outer membrane receptor HxuC by the interaction among HxuB, HxuA and heme-hemopexin complex. HxuB protein is likely related with the release of soluble HxuA (Cope et al., 1995). In addition, HxuC protein can also acquire low concentrations of free heme, heme-albumin complex and hemoglobin (Morton et al., 2007). Hemopexin is the major heme transportation vehicle present in the

Abbreviations: NAD, nicotinamide-adenine dinucleotide; OMP, outer membrane proteins; BSA, Bull Serum Albumin; SDS, sodium dodecyl sulfate; PAGE, PA-gel electrophoresis; PBS, Phosphate Buffer Saline; ELISA, enzyme-linked immuno sorbent assay; CTL, cytotoxic T cell; kDa, kilodalton(s).

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plasma, which can prevent heme-mediated oxidative stress and heme-bound iron loss (Tolosano and Altruda, 2002). The gene cluster (*hxuCBA*) is a virulence factor of *H. influenzae*. It has been shown that *hxuCBA* mutant of *H. influenzae* was less pathogenic than that of a wild-type strain in a 5-day-old rat infection model (Morton et al., 2007). However, there is no report about *hxu* system in *H. parasuis*.

By using the bioinformatics analysis, we have identified a homolog of HxuC, HxuB, HxuA from *H. parasuis* genome. In this study, we have cloned and expressed these three proteins in *Escherichia coli*. The immunogenicity of these recombinant proteins was studied in a mouse model.

2. Materials andmethods

2.1. Bacterial strains and culture conditions

The *H. parasuis* M-3 strain, a highly virulent strain of serovar 5, was isolated from the joint fluid of an infected commercial piglet. Tryptic soy agar (TSA, Difco, Detroit, USA) and tryptic soy broth (TSB, Difco, Detroit, USA) media, supplemented with 10 μg/mL nicotinamide adenine dinucleotide (NAD, Sigma Aldrich, Missouri, USA) and 5% calf serum (Gibco, USA) were used to culture the *H. parasuis* strain at 37 °C. The *E. coli* DH5α and BL21 strains were cultured by Luria–Bertani (LB) and LB containing 1.5% (wt/vol) agar. Rabbit antiserum against *H. parasuis* M-3 was prepared in the early research. The five to six weeks old pathogen-free KM mice were obtained from Chengdu Dossy Experimental Animals Company, Ltd.

2.2. Bioinformatics analysis of HxuC, HxuB, HxuA

The secondary structure, T cell epitopes, B cell epitopes, and subcellular location of HxuC (GenBank: ACL33614.1), HxuB (GenBank: ACL33615.1), and HxuA (GenBank: ACL33616.1) of *H. parasuis* SH0165 were analyzed by SOPMA (Geourjon and Deléage, 1995; Combet et al., 2000), CTL epitope-Finder ver. 1.1 (Bhasin et al., 2003; Bhasin and Raghava, 2004), BepiPred 1.0 Server (Larsen et al., 2006), and PSORTb (Yu et al., 2010) bioinformatics tools, respectively.

2.3. Cloning, expression, and purification of recombinant proteins

Based on *HAPS_RS10585* (*hxuC*, Gene ID: 7277722, Genbank), *HAPS_RS10590* (*hxuB*, Gene ID: 7277723, Genbank), and *HAPS_RS10595* (*hxuA*, Gene ID: 7277724, Genbank) gene sequences of *H. parasuis* SH0165 strain, three pairs of primers were designed and ordered from Invitrogen, USA (Table.1). These three genes were amplified from the genome of *H. parasuis* M-3 strain by polymerase chain reaction (PCR). The PCR products were cloned into pMD19-T cloning vector (TaKaRa, Japan). The recombinant plasmids were identified by *NcoI-XhoI* restriction enzyme cutting (TaKaRa, Japan) and sequenced. The sequences of these three genes in *H. parasuis* M-3 were aligned with those of *H. parasuis* SH0165 strain by DNAMAN software. The recombinant proteins were expressed and purified as previously described with slight modification (Fu et al., 2011). The recombinant plasmids were digested by *NcoI-XhoI*, which were ligated with pET-39b expression vector. The positive bacteria pellets which were induced by IPTG were suspended in Tris-HCl buffer (50 mM, pH 8.0) to be disrupted by sonication on ice. The lysate was centrifuged to collect the precipitate for protein purification using Ni–NTA affinity chromatography. The purified protein was dialyzed by using 50 mM Tris-HCl buffer to eliminate any residual imidazole and urea. The proteins were quantified by different concentrations of BSA solutions in which were 0, 0.5, 1.0, 2.0, 4.0, 8.0 mg/mL. with SDS-PAGE. The last calculated were using gel imaging system.

Table 1
Primer sequences of *hxuC*, *hxuB*, *hxuA*.

Gene	Primer sequences
hxuC-F	5′-CATGCCATGGATAGCAATGTTTCAGCTG-3′
hxuC-R	5′-CCGCTCGAGGAACTGATAAGACGCGTT-3′
hxuB-F	5′-CATGCCATGGATCCAATACCAGATTCAGG-3′
hxuB-R	5′-CCGCTCGAGAAATGTTTTAATTGCAGATAAC-3′
hxuA-F	5′-CATGCCATGGCTGATTTACCTCAAAACCAT-3′
hxuA-R	5′-CCGCTCGAGACGAATTTCACCTACCG-3′

Note: The underline portions of the primer sequences was the restriction enzyme cutting site (F (forward): *Nco*I; R (reverse): *Xho*I).

2.4. Immunoblotting analysis of recombinant proteins

The preparation method of rabbit anti-M-3 serum was as follows. The immunization of the rabbit was 2 mL bacteria liquid (1.0×10^{10} cfu/mL) inactivated by 0.2% formalin mixed with 2 mL complete Freund's adjuvant (Sigma, USA) on the first day and incomplete Freund's adjuvant (Sigma, USA) on the fourteenth day by subcutaneous injection. Then the booster immunization was 2 mL activated bacteria liquid (5×10^9 cfu/mL) on the twenty-first day and twenty-seventh day by subcutaneous injection. Seven days after the forth immunization, the blood sample was collected from the heart. The serum was separated by centrifugation. The immunoblotting analysis of recombinant proteins was carried out according to Zhang et al. (Zhang et al., 2012). The purified proteins were concentrated and separated by 5% stacking gel and 12% separation gel. Then the proteins were blotted onto PVDF membranes (BioRad, USA) using a semi dry electrophoretic transfer apparatus. After transfer, the membranes were blocked at room temperature for 30 min with 5% skim milk. The rabbit anti-M-3 serum (1:500) and goat anti-rabbit IgG/HRP (1:10,000, Bioss, China) antibodies were applied for 1 h and 30 min at room temperature, respectively. Enhanced chemiluminescence (ECL, BioRad, USA) reagents were used to visualize the blot by exposing the blot to autoradiographic film.

2.5. Immunization and challenge of mice

The immunization and challenge study was carried out as previously described with slight modification (Tadjine et al., 2004). The mice were assigned to groups of HxuC, HxuB, HxuA, HxuCBA, M-3, adjuvant, and PBS randomly. There were 8 mice in each group. The immunization of each mouse was 50 μg recombinant protein mixed with 100 μL complete Freund's adjuvant (Sigma, USA) on the first day or incomplete Freund's adjuvant (Sigma, USA) on the fourteenth day by subcutaneous injection. The HxuCBA vaccine was prepared with adjuvant and 50 μg of each protein. In M-3 group, the immunizing doses of inactivated *H. parasuis* M-3 was 1×10^9 cfu per mouse. Blood samples from vaccinated groups were collected on day 0, 10, and 24 by tail vein bleeding. The LD_{50} of *H. parasuis* M-3 in mice was measured by the laboratory. Ten days after second immunization, the mice of each group were challenged with 6.5×10^9 cfu ($5 \times LD_{50}$) of *H. parasuis* M-3 by intraperitoneal injection, Then the mice were monitored daily after challenge. Body weights were measured at the same time each day for seven days. Each mouse was euthanized by intraperitoneal injection of overdose (100 mg/kg) of pentobarbital, when they were moribund. Spleen, lungs and brain tissue samples were histologically examined after euthanasia. The mortality of mice was observed and recorded over a post-challenge period of seven days. The animals had *ad libitum* access to a commercial pelleted ration and drinking water. Experiments were conducted according to the protocol approved by IACUC (Institutional Animal Care and Use Committee) at Sichuan Agricultural University.

2.6. Detection of antibody and cytokine levels against the recombinant proteins

The antibody levels were detected by indirect enzyme-linked immunosorbent assay (ELISA) as previously described with slight modification (Zhang et al., 2008). Briefly, the purified proteins (500 ng/well) and *H. parasuis* M-3 bacterial cells (1.667×10^7 cfu/well) were used to