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Short communication

Identification of *Streptococcus equi ssp. zooepidemicus* surface associated proteins by enzymatic shaving

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ABSTRACT

Streptococcus equi ssp. zooepidemicus (*Streptococcus zooepidemicus*, SEZ) is responsible for a wide variety of infections in many species. Attempts to control the infection caused by this agent are hampered by a lack of effective vaccines and useful diagnostic kits. Surface proteins of bacterial species are usually involved in interaction with host and hopefully act as biomarkers for serodiagnosis and subunit vaccine components. In this study, the surface proteins of SEZ C55138 strain were systematically identified by surface shaving with trypsin and a total of 20 surface associated proteins were found. Further analysis of five selected novel proteins (SzM, FBP, SAP, CSP and 5′-Nu) revealed that they all expressed *in vivo* and their recombinant derived proteins could be reactive with convalescent sera. These identified immunogenic surface proteins have potential as SEZ vaccine candidates and diagnostic markers.

1. Introduction

Streptococcus equi ssp. zooepidemicus (*Streptococcus zooepidemicus*, SEZ) is an important pathogen associated with a wide range of diseases in many mammalian species (Gaede et al., 2010; Priestnall and Erles, 2011). In addition, SEZ is a major cause of swine streptococcal diseases in China (Yan et al., 2009). The development of an effective vaccine is vital to circumvent the widespread economic losses in the event of a pandemic. Because of the side effects of the killed whole-cell vaccines and the live attenuated vaccines, subunit vaccine is preferred for vaccine development. However, attempts to control the SEZ infection are hindered by a lack of thorough knowledge of the virulence factors and protective antigens of the bacterium. Thus, it is necessary to identify other antigenic factors contributing to the development of a monovalent or a multivalent subunit vaccine to protect host against infection.

Bacterial surface proteins are involved in the interaction with host cells. Hence, surface proteins are potential targets of effective vaccines aimed at preventing bacterial infections and diseases (Rodriguez-Ortega et al., 2006). Previous studies showed that whole viable bacterial cell treatment with proteases shaving is the method of choice for identification of vaccine candidate in Gram-positive bacteria (Doro et al., 2009; Rodriguez-Ortega et al., 2008; Bohle et al., 2011).

In the present study, the surface associated proteins of SEZ were identified by enzymatic shaving and five novel vaccine candidate antigens have been discovered by Western blot method. The results could provide guidance in the development of novel effective passive and active immunotherapeutic approaches against SEZ.

2. Materials and methods

2.1. Bacterial strains and growth conditions

SEZ strain C55138 (China Institute of Veterinary Drug Control, Beijing, China) was originally recovered from diseased pig with septicemia in Sichuan, China. It was grown on tryptone soya broth (TSB) (Oxoid, Wesel,

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Germany) or tryptone soya agar (TSA) (Difco Laboratories, Detroit) plus 5% Newborn Calf Serum at 37 °C under aerobic conditions. The capsule deficient mutant ΔhasB was constructed in our laboratory by disruption of capsule synthesis *hasB* gene (data not shown).

Laboratory *E. coli* strains DH5α and BL21 were used as non-adherent and non-invasive recipients of recombinant protein-containing plasmids pET-32a (+).

2.2. Surface digestion of live cells

100 ml of bacteria from mid-exponential growth phase were harvested by centrifugation (at 8000 × *g* for wild-type strain and at 3500 × *g* for ΔhasB strain) for 10 min at 4 °C, and washed three times with PBS (pH 7.2). Cells were resuspended in 1 ml of incubation buffer consisting of PBS/30% sucrose (pH 7.2). Proteolytic reactions at 37 °C were carried out with trypsin (Promega, USA) at 10 μg/ml for 15 min. Controls were carried out without adding any enzyme. The number of surviving bacteria was calculated by plating serial dilutions from 100 μl mixture. The residual digestion mixtures were centrifuged at 8000 × *g* for 10 min at 4 °C, and the supernatants were filtered using 0.22-μm pore-size filters (Millipore, USA). Then the digestion reaction was re-digested with same enzyme and concentration for 20 min. Protease reactions were stopped with formic acid at 0.1% final concentration. Peptide fractions were concentrated with a Speed-vac centrifuge (Savant), and kept at −20 °C until further analysis.

2.3. Protein identification by nano-LC/MS/MS

The procedure was carried out according to Rodriguez-Ortega et al. (2008) with some modification. All analyses were performed with a Finnigan Surveyor HPLC System in tandem with a Finnigan LTQ mass spectrometer (Thermo Electron, San Jose, CA). Briefly, the peptide fractions were desalted by Zip Tip C18 (Millipore, USA) according to the manufacturer's instructions and then were separated on a reversed phase C18, 3 μm-resin, column (0.10 mm × 100 mm, Column Technology). The mobile phase consisted of acetonitrile and MS grade water, both containing 0.1% formic acid. Chromatographic separation was achieved using a line gradient of 5–35% acetonitrile in 120 min. The flow rate of 300 nl/min was provided by the nanoflow pump. LC–MS/MS measurements were made with a linear trap quadrupole (LTQ) mass spectrometer equipped with a microspray source. MS data (Full Scan) were acquired in the positive ion mode over the 400–2000 *m*/*z* range. MS/MS data were acquired in dependent scan mode, selecting automatically the five most intense ions for fragmentation, with dynamic exclusion set to on. In all cases, a spray voltage of 1.85 kV was used.

Search and identification of peptides were performed in batch mode with MASCOT. The following search parameters were applied; species, S. zooepidemicus sequences; allowed number of missed cleavages, 2; Fixed modifications, Carbamidomethyl (C); dynamic modifications, N-term acetyl, oxidation (M); peptide mass tolerance, 30 p.p.m.; fragment mass tolerance, 0.6 Da; Mass values, Monoisotopic; Significance threshold, 0.05.

2.4. Bioinformatic analysis of the identified proteins

Computational predictions of subcellular localization were carried out using the web-based algorithm PSORTb v 2.0 http://www.psort.org/psortb (Gardy et al., 2005). When it returned an "unknown" output, other algorithms were used: TMHMM 2.0 http://www.cbs.dtu.dk/services/TMHMM-2.0 (Krogh et al., 2001) for searching transmembrane helices; SignalP 3.0 http://www.cbs.dtu.dk/services/SignalP (Bendtsen et al., 2004) for type-I signal peptides, those proteins containing only a cleavable type-I signal peptide as featured sequence were classed as secreted; LipoP http://www.cbs.dtu.dk/services/LipoP (Juncker et al., 2003) for identifying type-II signal peptides, which are characteristic of lipoproteins.

2.5. Cloning, expression, purification and immunoblot analysis of surface antigens

Cloning, expression, and purification of recombinant proteins were performed as described (Zhou et al., 2009). The corresponding genes of the selected proteins were amplified by PCR from SEZ C55138 genomic DNA using the primers listed in Table 1. The primers were designed based on the DNA sequence data of the corresponding protein ID in NCBI website. PCR was performed in a thermal cycler using SEZ genomic DNA of C55138 as a template with following conditions: denaturing at 95 °C for 30 s, annealing at 56 °C for 30 s and extension for 1.5 min at 72 °C for a total of 30 cycles. The PCR products were digested with appropriate restriction enzymes and cloned into pET-32a (+). Transformed *E. coli* containing the selected construct was incubated at 37 °C until exponential phase, and the recombinant proteins were induced by adding 1 mM IPTG for 3 h at 37 °C. Cells were harvested and the interest protein from the supernatant was purified by Ni-NTA chromatography as described in the QIAexpress manual. Purified recombinant proteins (4 μg) were resolved by SDS-PAGE and analyzed by Western Blot using the convalescent porcine sera against SEZ infection.

Eight-week-old piglets with an average weight of 13 kg from a herd that was free of SEZ were randomly assigned to two groups. One group with twenty pigs inoculated intravenously with SEZ strain C55138 at a dose of 1×10^8 CFU/pig were used to generate convalescent sera, of which fifteen survived and their sera were collected a month after the inoculation. Ten pigs in the other group were mock-inoculated with sterile PBS as control and their sera were collected to generate negative sera.

2.6. In vivo characterization of identified immunogenic antigens

The expression of the immunogenic antigens *in vivo* was measured using ELISA according to Zhang et al. (2008). Microtitre plates (Nunc, USA) were coated overnight at 4 °C with 100 ng/100 μL of purified recombinant protein respectively diluted in sodium carbonate buffer (pH 9.6), and washed three times with PBS containing 0.05% Tween-20. After being blocked with 1% BSA, 100 μL swine sera (15 convalescent sera against SEZ infection and 10 negative