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Characterization of SeseC_01411 as a surface protective antigen of *Streptococcus equi* ssp. *zooepidemicus*

Honglin Xie[a], Zigong Wei[b], Chunquan Ma[a], Shun Li[a], Xiaohong Liu[b], Qiang Fu[a,b,*]

[a] School of Life Science, Foshan University, Guangdong 528225, PR China
[b] State Key Laboratory of Biocontrol, School of Life Sciences, Sun Yat-sen University, Guangzhou 510006, PR China

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ABSTRACT

Streptococcus equi ssp. *zooepidemicus* (*Streptococcus zooepidemicus*, SEZ) is a commensal bacterium related to opportunistic infections of many species, including humans, dogs, cats, and pigs. SeseC_01411 has been proven to be immunogenic. However, its protective efficacy remained to be evaluated. In the present study, the purified recombinant SeseC_01411 could elicit a strong humoral antibody response and protect against lethal challenge with virulent SEZ in mice. Our finding confirmed that SeseC_01411 distributes on the surface of SEZ. In addition, the hyperimmune sera against SeseC_01411 could efficiently kill the bacteria in the phagocytosis test. The present study identified the immunogenic protein, SeseC_01411, as a novel surface protective antigen of SEZ.

1. Introduction

Streptococcus equi ssp. *zooepidemicus* (SEZ), a member of Lancefield group C streptococcus, is a frequently isolated opportunist pathogen from a variety of animal hosts, including cats, mare, cows, sheep, horses, and pigs (Lyle, 2014; Polak et al., 2014; Priestnall et al., 2014). It can also infect humans *via* zoonotic transmission from the infected animals and cause invasive infections such as septicemia and meningitis (Gruszynski et al., 2015; Madzar et al., 2015; Watson et al., 2015).

Undoubtedly, development of an effective vaccine was a predominant strategy to control SEZ diseases. Although some virulence associated factors have used for subunit vaccines to protect against SEZ infection (Timoney et al., 1995, Fu et al., 2013a, 2013b, Velineni and Timoney, 2013), identifying more novel antigenic factors is necessary to develop a monovalent or a multivalent subunit vaccine in protecting against SEZ infection.

SeseC_01411 (GI: 338846021) is a novel clear immunogenic protein, which was similar to LPXTG-like motifs (Wei et al., 2012). The protein BLAST search revealed that SeseC_01411 of SEZ ATCC 35246 shared 53% amino acid identity with LrrG of S.agalactiae. Recombinant LrrG was shown to adhere to epithelial cells, indicating that it might play a potential role in pathogenicity (Seepersaud et al., 2005). Immunization with Recombinant LrrG protected against lethal challenge with Group B streptococci. These findings revealed that SeseC_01411 can be considered as a candidate vaccine prevent SEZ. In this research, we evaluated the protective quality of SeseC_01411 systematically, and demonstrated it was a new protective antigen.

2. Materials and methods

2.1. Strains growth and culture

We cultured SEZ strain C55138 with tryptone soya broth (TSB) or tryptone soya agar (TSA) add 5% Newborn Calf Serum at 37 °C (Oxoid, Wesel, Germany). The strain from China Institute of Veterinary Drug Control (Beijing, China). *E. coli* strains DH5α and BL21 were used as non-adherent and non-invasive recipients of recombinant protein-containing plasmids pET-28a (+).

2.2. Mouse immunization and challenge

The study was performed accordance with the Guide for the Animal Care and Use Committee of Guangdong Province and performed accordingly. The approval ID or permit numbers were *SCXK* (Guangdong) 2011–0029 and *SYXK* (Guangdong) 2011–0112. We allocated Forty BALB/c mice (4-week-old female) into 4 groups randomly. Then, 50 μg purified recombinant SeseC_01411 emulsified in a 200 μL volume of Marcol 52 (ESSO, USA)-based adjuvant was applied to immunizing mice in group 1. Group 2 mice contain SzP were regarded as the positive control. Group 3 mice inoculated with phosphate-buffered saline (PBS) emulsified in Marcol 52 adjuvant were negative control. Blank control was mice in group 4 immunized with PBS alone.

All of the mice were immunized through intraperitoneally (i.p.) injection. Then booster immunization after ten days, blood samples were drawn by tail vein bleeding. Then, all mice in each group were

* Corresponding author at: No. 33 Guangyun Road, School of Life Science, Foshan University, Guangdong 528225, PR China.
E-mail address: fuqiang@fosu.edu.cn (Q. Fu).

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challenged by i.p. injection with 2×10^5 CFU of SEZ, and recorded 14 days to calculate the morbidity and mortality. Mice exhibiting extreme lethargy were considered to be moribund and were euthanized using carbon dioxide for ethical reasons.

2.3. Antibody titers determination

Specific antibody titers in sera were examined by ELISA using Microtitre plates (Nunc, Roskilde, Denmark) coated with purified recombinant SeseC_01411 (200 ng/100 μL) as previously described (Fu et al., 2013a, 2013b). After saturation of the plates with 0.5% BSA for 2 h at 37 °C, serially diluted mouse sera were added and cultured for 30 min at 37 °C. Bound antibodies against immunoglobulin subtypes were confirm by rabbit anti mouse IgG-HRP, IgG1-HRP or IgG2a-HRP (Southern Biotech, Birmingham, AL). The plates were read with a micro ELISA reader at an optical density (OD) of 630 nm. End-point titers were calculated as the reciprocal of the last serum dilution yielding 50% of the maximum OD value above the background.

2.4. Immunofluorescence microscopy analysis

The immunofluorescence microscopy analysis of SeseC_01411expression adopted as previously report with some modification (Becherelli et al., 2012). SEZ was applied in duplicate to multiwell slides. The slides were air dried and then fixed in 100% methanol for 10 min at −20 °C. The slides were cultureed with the mouse sera against SeseC_01411 (1:20), and negative control was pre-immune mouse sera. After washing, the slides were incubated with FITC-labeled affinity purified antibody to mouse IgG (H + L) (SANTA CRUZ, USA). The slides were examined with a fluorescence microscope (Zeiss, Germany) after final washing.

2.5. Flow cytometry analysis

The surface expression of SeseC_01411 by SEZ was determined as previously described with some modification (Rubinsztein-Dunlop et al., 2005). About 5×10^6 bacteria were cultured with the mouse sera against SeseC_01411 or preimmune mouse sera to detect membrane expression of the protein. Sera was diluted 10-fold in PBS-BSA and incubated at room temperature with strain in a total of 500 μL for 45 min. The strain were harvested by centrifugation at 6000 ×*g* (5 min) and washed with PBS-BSA. Goat anti-mouse IgG-FITC (10 μg) (SANTA CRUZ, USA) was added, then the strain were cultured for 45 min at room temperature, washed and analyzed by FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA). The mean fluorescence intensity was calculated for each sample.

2.6. Quantitative real-time PCR measure expression

Bacteria recovered from three SEZ-infected mice, the total RNA from *in vitro* and *in vivo* harvested strain were prepared as previously described (Ogunniyi et al., 2002). We used Reverse Transcription System (Promega, USA) to synthesize cDNAs. Each cDNA sample (template) performed a real-time PCR in an amplification mixture containing SYBR Green (TaKaRa, Dalian, China). All samples were performed on the LightCycler 480 with three replications (Roche, Indianapolis, IN). For each reaction, the *Ct* value of the endogenous control *16S rRNA* gene was subtracted from the *Ct* value of each gene ($\Delta Ct = Ct\ SeseC\_01411 - Ct\ 16S\ rRNA$) to normalize the amount of sample cDNA added to each reaction. For a comparison of the expression of each gene *in vitro* and *in vivo*, the ΔCt value of the gene *in vitro* was subtracted from the ΔCt value of the gene *in vivo* ($\Delta\Delta Ct = \Delta Ct\ in\ vivo - \Delta Ct\ in\ vitro$). The fold changes were calculated using the formula of $2^{-\Delta\Delta Ct}$ described by Livak and Schmittgen (2001). Data are means ± standard definition (SD) of triplicate reactions for each gene transcript.

2.7. Passive protection assays

Passive immunization was referenced as previously report with some modification (Li et al., 2011). There were ten 6-week-old female BALB/c mice were immunized with hyperimmune sera (100 μL) raised against SeseC_01411, and then carry out by i.p. injection with 2×10^5 CFU of SEZ. Mice passively immunized with hyperimmune sera against SEZ inactive vaccine or normal sera were regard as positive and negative control respectively. The mice were performed with 2×10^5 CFU of SEZ twenty four hours after the immunization.

2.8. Whole blood killing assay

The whole blood killing assay was performed as described previously (Voyich et al., 2009). Three BALB/c mice (6-week-old female) were used to extracted healthy mouse whole blood. SEZ was adjusted to a concentration of 10^4 CFU/mL. Healthy mouse blood (900 μL) was mixed with 100 μL of mouse anti-SeseC_01411 sera. SEZ inactive vaccine immune sera and pre-immune sera were regard as positive and negative control respectively. Then, 100 μL of strain suspension was added to the mixture and rotated at 37 °C for 90 min. Each blood sample was spread onto TSA to enumerate surviving strain at the start and end of the culture. Survival rate was calculated as a percentage of remaining strain relative to the starting inoculums.

2.9. Statistical analysis

All data were shown as means ± SD and were analyzed by Student's *t*-test. Survival curves were compared using the log-rank test. Statistical significance was defined at $P < 0.05$.

3. Results

3.1. Immune response

Antibodies against SeseC_01411 were monitored in sera obtained from mice on day 10 after booster immunization. Compared to negative control group, specific IgG titers against SeseC_01411 were much higher in the immunized group ($P < 0.001$) (Fig. 1A). The subclasses IgG1 and IgG2a were further tested to be used as a surrogate marker to indicate T helper 2 (Th2) and T helper 1 (Th1) immune responses in order to prove the type of immune response. The result did indicated that SeseC_01411 could induce significant Th1-/Th2-immune responses and that IgG1 response predominated over IgG2 responses in mice (Fig. 1B), although our experiments did not allow an accurate quantification of different immunoglobulin subclasses.

3.2. Protection of mice against lethal challenge by immunization with SeseC_01411

Three mice in the blank control group died on day 3 post-challenge. All of the remaining mice showed significant clinical signs, including ruffled hair coat, a slow response to stimuli, or depression, and four died off within 7 days. Negative control group mice were found obvious clinical signs. There were half of the mice died off within 8 days, and the remain mice were gradually recover. Meanwhile, no mice died (9 day) to the end of this research. Mice immunized with SeseC_01411 or SzP showed minor clinical signs than mice in the blank and negative control, while only one mouse died on day 4 post-challenge in SeseC_01411 group, and no mice died from day 5 to the end of study (Fig. 2A). Our result proved that SeseC_01411 could protect mice against SEZ infection.

3.3. Surface expression of SeseC_01411 by SEZ

Immunofluorescence assay was carried out to confirm that