Original Article

Identification and characterization of a novel protective antigen, SeseC_00396 of *Streptococcus equi* ssp. *Zooepidemicus*

Li Yi[1,2] Weiping Yang[1] Jinpeng Li[2,3] Shaohui Wang[4*] Yang Wang[2,3*]

Abstract

Streptococcus equi ssp. *zooepidemicus* (SEZ) is responsible for a wide variety of infections in many animal species. Studies aimed at developing safe and effective vaccines against SEZ infections are thus of great interest. SeseC_00396 was a novel protein identified in the previous study. In this study, recombinant SeseC_00396 of SEZ was expressed and showed a strong immunoreactivity with mini-pig convalescent sera. Studies in mice have revealed that the recombinant protein induced a marked antibody response which was associated with protection against SEZ infection. The hyperimmune sera against SeseC_00396 could efficiently kill the bacteria. In addition, it was also found that anti-SeseC_00396 antibodies can significantly inhibit the formation of SEZ biofilm. Data from this study suggests that SeseC_00396 identified in SEZ may represent immunogens of interest for vaccine development.

Keywords: *Streptococcus equi* ssp. *Zooepidemicus*, Immune protection, Immunogen, Vaccine

[1]*College of Life Science, Luoyang Normal University, Luoyang, China*
[2]*Key Laboratory of Molecular Pathogens and Immunology of Animals of Luoyang, Luoyang, China*
[3]*College of Animal Science and Technology, Henan University of Science and Technology, Luoyang, China*
[4]*Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Shanghai, China*
***Correspondence**: wangyocean@163.com, shwang0827@126.com* (Y. Wang, S. Wang)
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Introduction

The Gram-positive bacterium *Streptococcus equi* ssp. *zooepidemicus* (SEZ) is a member of the Lancefield group C streptococcus. It can infect many mammalian species, including humans, and causes respiratory tract infections, septicemia, meningitis, endocarditis and arthritis (Holden, Heather, Paillot, Steward, Webb, Ainslie, Jourdan, Bason, Holroyd, Mungall, Quail, Sanders, Simmonds, Willey, Brooks, Aanensen, Spratt, Jolley, Maiden, Kehoe, Chanter, Bentley, Robinson, Maskell, Parkhill and Waller 2009: e1000346, Yi, Wang, Ma, Lin, Xu, Grenier, Fan and Lu 2016a: 50). Mice can also be infected and show severe clinical signs such as a ruffled hair coat, a slow response to stimuli or depression before dying (Liang, Tang, Zhao, Deng, Yan, Zhai and Wei 2018: 788-93, Tang, Liang, Gao, Yan, Deng, Zhai, Yang and Wei 2019: 387-92). Higher titers of SEZ were also obtained from the blood, liver, lung and spleen tissue of the infected mice (Yi, Wang, Ma, Zhang, Li, Zheng, Yang, Lu and Fan 2013: 174-83). In China, SEZ is one of the most important pathogens in the swine industry (Feng and Hu 1977: 7-12). In 1975, more than 300,000 pigs died within two weeks of a SEZ pandemic that occurred in the Sichuan province (Feng and Hu 1977: 7-12, Mao, Fan and Lu 2008: 103-9). At present, the swine industry is still suffering from the streptococcal disease(B, H, X, L, M, P, H and Z 2019: 387-92).

Recently, drug resistance and drug residues are still a big problem in the animal product. To better control the SEZ infection, the development of an effective vaccine is required. Although some virulence factors produced by SEZ, including M-like protein (SzP) (Hong-Jie, Fu-yu, Ying and Cheng-ping 2009: 56-61) and fibronectin-binding protein (Fnz) (Yi, Wang, Ma, Zhang, Li, Zheng, Yang, Lu and Fan 2013: 174-83) have been proposed as subunit vaccines (Wei, Fu, Chen, Cong, Xiao, Mo, He and Liu 2012, Wei, Fu, Liu, Xiao, Lu and Chen 2012), it is of interest to identify novel protective antigens for the development of effective vaccines against SEZ.

Many bacterial surface proteins are involved in host-pathogen interactions and are potential targets for effective vaccines aimed at preventing bacterial infections and diseases (Wei, Fu, Liu, Xiao, Lu and Chen 2012). SeseC_00396 (gi: 338846346, pyridine nucleotide-disulfide oxidoreductase) is a novel identified surface protein by immunoproteomic analysis of SEZ with pig convalescent sera (MAO, FAN Hong-jie, Yong-hua and Cheng-ping 2011: 1096-105, Yi, Wang, Ma, Lin, Xu, Grenier, Fan and Lu 2016a: 50) which may have the potential to be a vaccine candidate and needs further testing. In the present study, the protective efficacy of recombinant SeseC_00396 was systematically evaluated and proved to be a novel protective antigen in mice.

Materials and Methods

Animals: Three-week-old female ICR (Institute of Cancer Research) mice were purchased from the Experimental Animal Center of Zhengzhou University (Zhengzhou, China) and their health status was confirmed by this Research institute. Mice were housed in an appropriate environment at a temperature of 24–26 humidity of 60% and also free access to fresh water and food. The mice were acclimatized for one week after arrival before starting the experiment. Work with animals in this study was in agreement with the Ethical Principles for Animal Research established by Henan University of Science and Technology (Luoyang, China). All animal tests were approved by the Laboratory Animal Monitoring Committee of Henan University of Science and Technology (Luoyang, China).

Bacteria and culture conditions: *Streptococcus equi* ssp. *zooepidemicus* strain ATCC 35246, was initially isolated from a dead pig in Sichuan Province, China (Ma, Geng, Zhang, Yu, Yi, Lei, Lu, Fan and Hu 2011: 5583-4). Bacteria were cultured in Todd-Hewitt broth (THB, Oxoid) medium. *E. coli* strains DH5a and BL21 (Invitrogen, China) used for transformation and protein expression were cultured in Luria Bertani (LB) (Difco, USA) medium.

Expression and and western blotting assay of the recombinant protein: DNA fragments encoding SeseC_00396 were amplified by PCR from SEZ ATCC 35246 genomic DNA using the primer pairs of S（5′-CGCGAATTC ATGGAAGCATACGAGCTTAT-3′）and A（5′-CCCTCGAG TTAAAGGTTAAAGACATC AT-3′. The primers were designed to express the recombinant protein using Primer 5.0 software. Then, the amplified product was cloned in the pET28a （+） expression vector (Invitrogen, China) and transformed into *E. coli* BL21(Wu, Jiang, Tang, Lin, Lu and Yao 2012: 183-8). The His-tagged fusion proteins were purified using High-Affinity Ni-IDA resin column (GE Healthcare, USA) according to the manufacturer's instructions. The recombinant protein was induced with 1 mM isopropyl-d-1-thioglucopyranoside (IPTG) at 37 ℃ and then was purified using a Ni^{2+} affinity chromatography column. The purified recombinant protein was subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western Blot. Mini-pigs' convalescent sera prepared earlier (Yi, Wang, Ma, Lin, Xu, Grenier, Fan and Lu 2016a: 50) (1:1000 dilution) and horseradish peroxidase-labeled Staphylococcal protein A (1:5000) (Boster, China) were applied as the first and the second antibodies, respectively. Finally, the membrane was stained using 3,3′-diaminobenzidine (Tiangen, China) as a substrate.

Measurement of antibody titers: Forty 4-week-old ICR female mice were randomly divided into 4 groups; 10 in each group (10 mice per group). For the first group, the recombinant protein was emulsified with the ISA206 adjuvant (SEPPIC, France) and each mouse was immunized by an intramuscular injection of 200 μL of recombinant protein (100 μg/mL). For the second group, the mice were immunized with the inactivated SEZ ATCC 35246 ($(1 \times 10^9$ cfu/mL) emulsified with an equal volume of ISA206 adjuvant, then injected intramuscularly with 200 μL to each mouse. The third group served as a negative control group and mice received an intramuscular injection of PBS emulsified in the ISA206 adjuvant. The fourth Group serving as

blank controls were immunized with PBS alone. Second booster injections were given two weeks later. Blood was collected by tail vein bleeding on days 0, 7, 14, 21 and 28, and sera were harvested.

All serum antibody titers were determined by indirect ELISA (Imada, Mori, Daizoh, Kudoh and Sakano 2003: 5015-21). Briefly, the 96-well microplates were coated with recombinant protein（2 μg/mL） overnight at 4 °C. The protein solution was removed and PBS-BSA was added to each well, blocked at 37 ° C for 1 h, and washed with PBS containing 0.05% Tween 20 (PBST) three times. Then, the plates were incubated with serially diluted mice sera for 1 h at 37 °C. Finally, the plates were incubated with a secondary goat anti mouse IgG –HRP antibody (Beyotime, China) at a dilution of 1:3000 in blocking solution for 1 h. The plates were washed as before and were incubated with 3,3′-diaminobenzidine (Tiangen, China) for 15 mins. The absorbance at OD_{630nm} was measured using a microplate reader.

Determination of protection efficacy: Protection efficacy was performed as previously described (Yi, Wang, Ma, Lin, Xu, Grenier, Fan and Lu 2016a: 50). The experiment was performed 14 days after the booster immunization. Mice in group 1-3 were challenged with 2.5×10^5 CFU of SEZ. The control group mice were injected with sterile PBS. The clinical symptoms and mortality of mice were observed daily for up to seven days. After the test, the surviving mice were euthanized by cervical dislocation. The lung and liver tissues of mice were collected for histopathological analysis.

Histological assay: The lung and liver tissues of the tests were fixed with 4% paraformaldehyde. After paraffin embedding, samples were sliced serially into 4 mm sections and stained with hematoxylin and eosin. Histopathological observation was performed with a light microscope (Zeiss, Germany).

Bactericidal assay: An *in vitro* whole blood bactericidal test was performed according to a previous method with small modifications (Liu, Zhu, Zhang and Lei 2007: 3651-7, Shao, Pan, Li, Liu, Han, Wang, Wang, Zheng, Cao and Tang 2011: 174-82). The whole blood of healthy mice was collected prior to the test. Two weeks after the booster immunization, the blood of the mice was collected. SEZ ATCC 35246 was cultured to logarithmic growth phase, bacteria were harvested by centrifugation, washed three times in sterile PBS and then adjusted to a concentration of 10^4 CFU / mL. Healthy mouse blood (900 μL) was mixed with 100 μL of mouse anti- SeseC_00396 sera. The inactivated SEZ immune sera and pre-immune sera were regarded as positive and negative controls respectively. Then, 100 μL of strain suspension was added to the mixture and rotated at 37 °C for 90 mins. Finally, the bacteria were plated on THB plates. CFU were determined following a 24 h incubation at 37 °C. The results were expressed as survival rate. Each assay was performed in triplicate.

Biofilm formation assay: The biofilm formation assay was carried out as described before (Wang, Yi, Zhang, Qiu, Tan, Yu, Cheng and Ding 2017: 17-22, Wang, Zhang, Wu, Zhu and Lu 2011: 151-60). Overnight SEZ ATCC 35246 cultures were diluted in fresh THB liquid medium supplemented with 1% fibrinogen at a ratio of 1:100. The experiment included 4 groups. The first group contained 1% pooled mouse anti- SeseC_00396 sera; the second group contained 1% pooled non-immunized mouse sera. The whole bacteria and THB medium were applied as positive and negative controls respectively. After incubating at 37°C for 24 h, biofilm formation assays in 96-well plates were performed using the previously described method (Wang, Xia, Dai, Shi, Kou, Li, Bao and Lu 2011: 328-38, Wang, Zhang, Wu, Zhu and Lu 2011: 151-60). The biofilms were stained with 1% crystal violet for 30 minutes, washed four times with sterile PBS, air dried, dissolved in 95% ethanol solution, and quantified at 595 nm absorbance. All the assays were performed in triplicate.

Statistical analysis: Statistical analysis was performed using GraphPad Prism 5 software. Detection of specific antibody data was analyzed by Two-way ANOVA. The survival rate of the mice in the challenge protection test was evaluated by Kaplan-Meier. $P < 0.05$ was considered to be significant and $P < 0.01$ was considered to be extremely significant.

Results

Expression, purification and western blot analysis of the recombinant protein: The SeseC_00396 gene was successfully cloned and expressed in *E. coli*. Purified recombinant SeseC_00396 protein was recovered by Ni-NTA affinity chromatagraphy and the protein showed good immunoreactive to convalescent mini-pig sera against SEZ by Western blotting analysis (Figure. 1).

Antibody response to recombinant proteins following vaccination in mice: The levels of antibody titers against Sec_00396 were significantly higher ($P < 0.01$) than in the PBS control group. There were no significant differences between the fusion protein-vaccine and the inactivated SEZ vaccine ($P > 0.05$) (Figure. 2).

Immunoprotection provided by recombinant protein following vaccination in mice: All mice in the PBS control died successively within 2 days. Comparatively, two out of ten mice in the inactivated SEZ vaccine group died on day 3. Meanwhile, only one out of ten mice died in the group immunized with SeseC_00396 (Figure. 3). Pathological anatomy showed that the lung and liver tissues appeared to be hemorrhagic and congestive in the PBS control group challenged with SEZ. Histopathological changes were also found in the lung and liver tissues, such as hemorrhage, congestion, and inflammatory cell infiltration. The SeseC_00396 protein and inactivated vaccine group showed no such pathological damage following SEZ challenge (Figure 4).

Survival of SEZ in whole blood: The results showed that the bactericidal activity of the SeseC_00396 group

and the positive control group was significantly higher than that of the PBS control group ($P < 0.01$). Only 35.30% ±6.08% of bacteria survived with hyperimmune sera against SEZ inactive vaccine while 90.20% ± 5.78% of bacteria survived in the absence of specific humoral response. However, there was no significant difference between the SeseC_00396 protein group (43.70% ± 5.25%) and inactivated SEZ group ($P > 0.05$) (Figure. 5).

Biofilm inhibition by the anti- SeseC_00396 antibody in vitro: Biofilm formation by SEZ in the presence of antisera was reported. It was found that the anti-SeseC_00396 sera reduced the ability of SEZ to form biofilm. As shown in Figure.6, Biofilm formation by SEZ cultured in THB medium contained anti-SeseC_00396 sera (0.32 ± 0.05) was significantly lower ($P < 0.01$) than that of SEZ cultured in THB medium alone (1.13 ± 0.05) or in THB medium contained non-immunized mouse sera (0.94 ± 0.11).

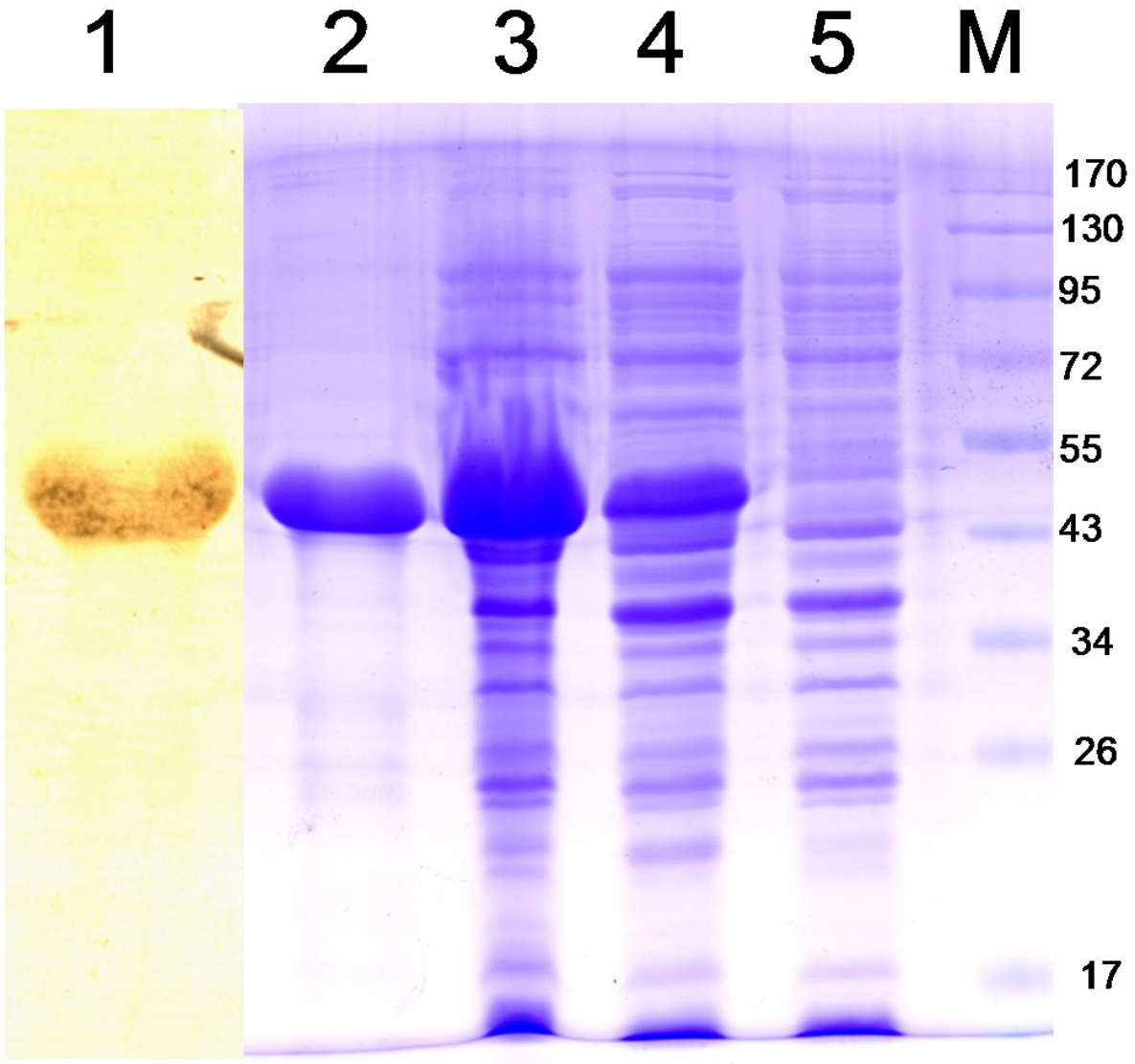

Figure 1 SDS-PAGE and Western blot analysis of fusion protein SeseC_00396 expressed in *E. coli* BL21.
Lane M, protein molecular mass marker; lane 1, Western blot analysis of purified SeseC_00396 protein using mini-pig convalescent sera; lane 2, purified SeseC_00396 ; lane 3, SeseC_00396 protein in *E. coli* BL21induced with IPTG ; lane 4, SeseC_00396 protein without IPTG ; lane 5, empty expression vector pET28a (+).

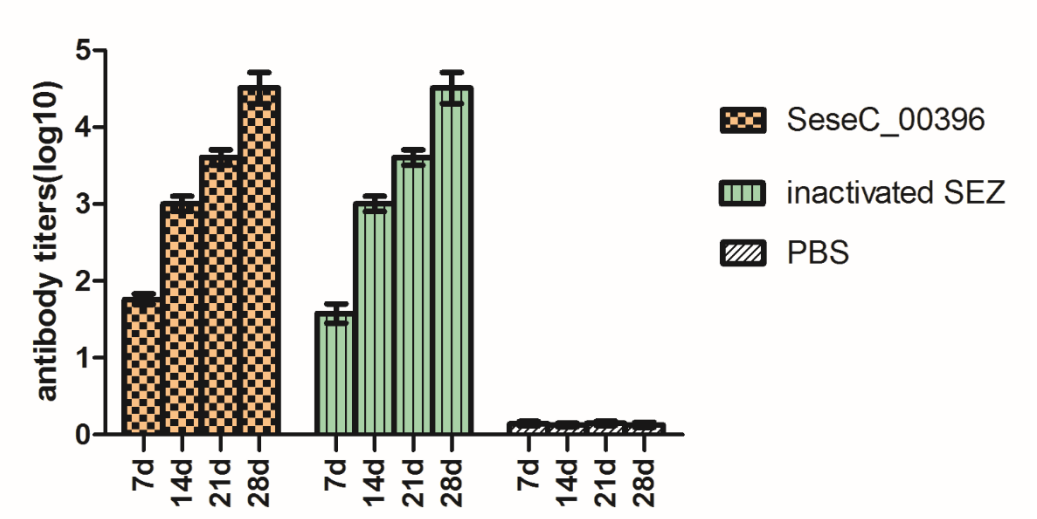

Figure 2 Antibody response following vaccination in mice. The antibody titers of the SeseC_00396 protein-vaccinated group were significantly higher ($P < 0.01$) than those of the PBS control group. No significant differences were found between the SeseC_00396 group and the inactivated SEZ group ($P > 0.05$).

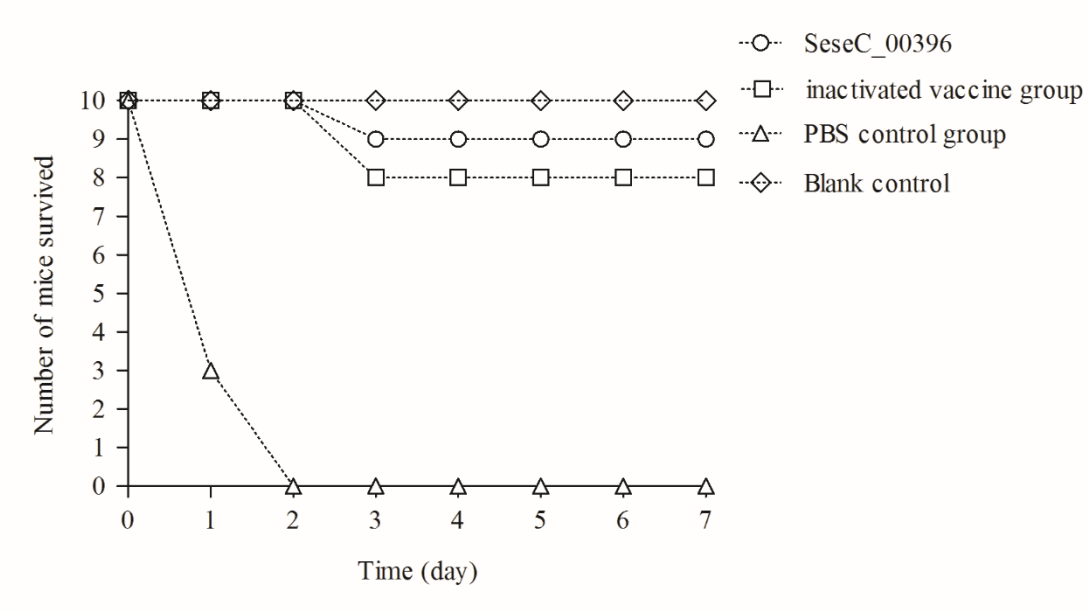

Figure 3 Survival of mice immunized with SeseC_00396, inactivated vaccine (positive control) and PBS control (negative control) following challenge with SEZ ATCC35246.

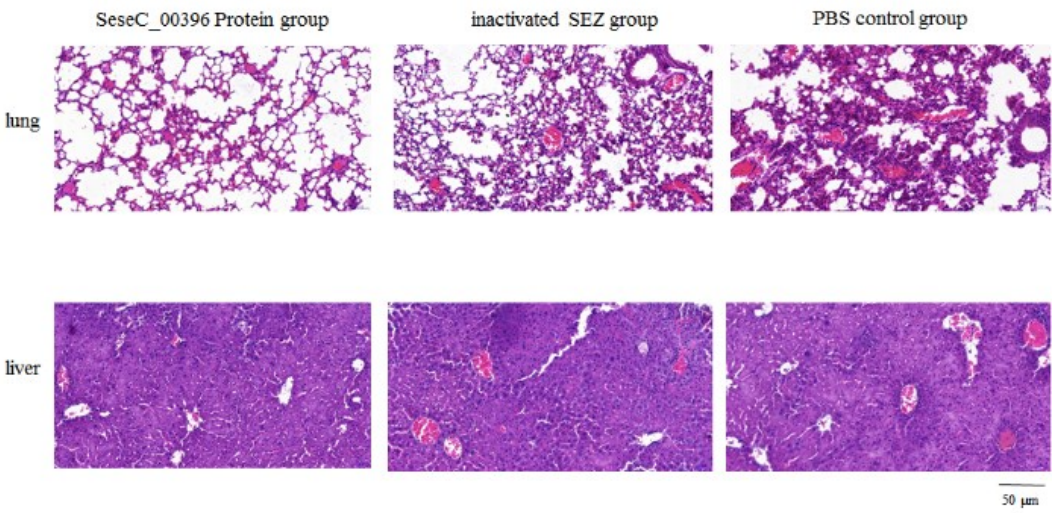

Figure 4 Pathological changes observed in the lung and liver tissues of mice immunized with SeseC_00396, inactivated vaccine and PBS control following challenge with SEZ. Scale, 50 μm.

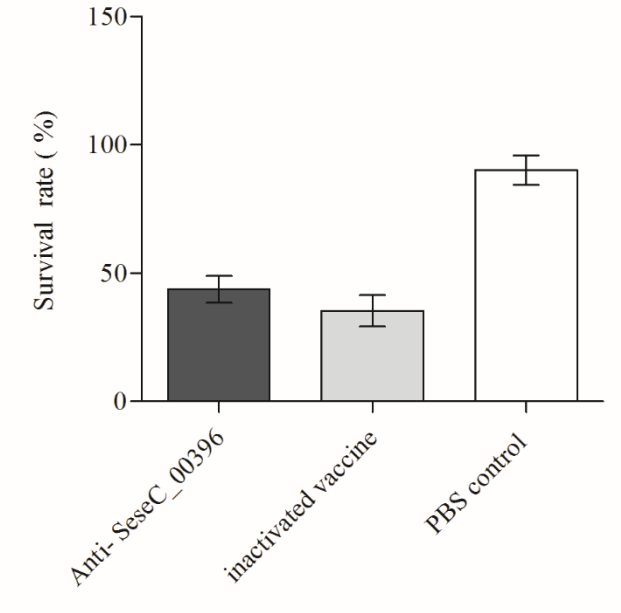

Figure 5 Whole blood bactericidal activity of specific antibody elicited in mice by recombinant SeseC_00396 protein.

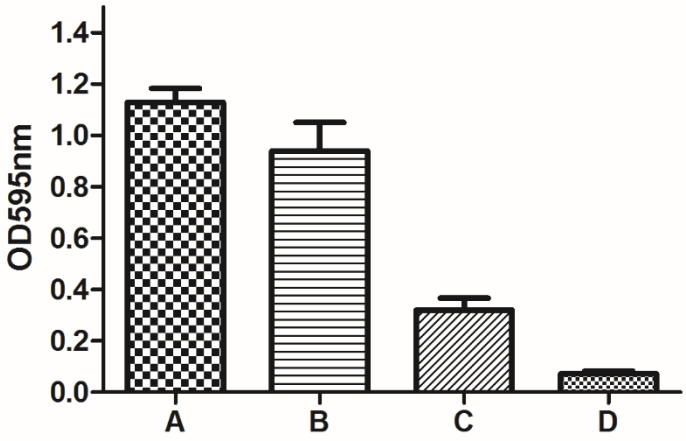

Figure 6 Quantitative microtiter plate assay for biofilm production by SEZ. (A) Biofilm formation in THB medium (control); (B) Biofilm formation in THB medium supplemented with normal mouse sera; (C) Biofilm formation in THB medium supplemented with mouse SeseC_00396-antisera; (D) THB medium.

Discussion

SEZ is associated with a wide variety of infections in many species and is also known as a zoonotic agent. The lack of efficient vaccines and rapid diagnostic methods is still the bottleneck to controlling this infection. Recognition of more protective antigens is necessary to develop a novel generation of vaccine for SEZ infection. Many bacteria surface proteins are considered as potential virulence factors and are promising vaccine candidate surface-associated proteins. SeseC_00396 (Pyridine nucleotide disulfide oxidoreductase) belongs to sulfur metabolism protein (Harnvoravongchai, Kobori, Orita, Nakamura, Imanaka and Fukui 2014: 603-16). Pyridine nucleotide-disulfide oxidoreductase, part of the heterodisulfide complex operon (HDR), could work in reverse and further oxidize the disulfide intermediates from sulfur oxidation to sulfite, delivering the collected electrons to the membrane quinine pool (Elagib, Tengner, Levi, Jonsson, Thompson, Natvig and Wahren-Herlenius 1999: 2471-81). SeseC_00396 was the surface protein of SEZ previously identified in an immunoproteomic analysis (MAO, FAN Hong-jie, Yong-hua and Cheng-ping 2011: 1096-105). In this study, we further investigated SeseC_00396 for its potential as a vaccine candidate against SEZ infection.

In the present study, recombinant SeseC_00396 could induce high titer of antibodies, indicating the good immunogenic properties of the protein. In addition, animal challenge experiments with the SEZ ATCC35246 strain showed that 90% of the immunized mice were protected. The protection rate was higher than that of the SEZ inactive vaccine. Physiological, pathological changes were not found after inoculation of SEZ in the target organ. It is suggested that the SeseC_00396 protein has potential as a vaccine candidate molecule.

We then conducted a whole blood killing assay to further evaluate the contribution of antibodies to the bacterial killing and results showed that the relative survival rate of SEZ in whole blood containing SeseC_00396 antisera and whole bacterial antisera decreased by 35.3% and 43.7%, respectively, indicating that the antisera to SeseC_00396 can significantly promote whole blood bactericidal activity. In this

whole blood killing assay, an antigen-antibody complex formed by bacteria and immune serum, produces a bacteriostatic action by activating complement in the blood, and the bactericidal action of the blood is caused by a non-specific reaction of macrophages and neutrophils in the blood. This suggests that the antibodies induced by SeseC_00396 were effective at mediating SEZ opsonophagocytosis. The result of vaccination with SeseC_00396 was consistent with those of the bactericidal assays, in that protection against SEZ challenge was correlated with bactericidal activity of specific antibodies.

SEZ can form biofilms and may play a key role in causing chronic infections (Yi, Wang, Ma, Zhang, Li, Zheng, Yang, Fan and Lu 2014: 227-33). SeseC_00396 of SEZ appeared to be involved in biofilm formation (Yi, Wang, Ma, Lin, Xu, Grenier, Fan and Lu 2016b: 50). Zarankiewicz *et al*., found that Hsp60 antibodies inhibited the formation of *Histophilus somni* biofilm (Zarankiewicz, Madej, Galli, Bajzert and Stefaniak 2012: 373-8). It was also found that anti-SeseC_00396 antibodies can significantly inhibit the formation of SEZ biofilm. Multiple factors contribute to biofilm formation, including extracellular polysaccharides, eDNA and proteins(Nobbs, Lamont and Jenkinson 2009: 407-50, Table of Contents). Since disulfide bonds are important for the folding and stability of extracytoplasmic proteins, inactivation of a disulfide catalyst can affect multiple proteins on the cell surface (Davey, Halperin and Lee 2016: e0166656). This has the potential to alter surface adhesion and biofilm initiation, in addition to creating a stress response. For example, inactivation of the disulfide bond forming enzymes eliminates biofilm formation in pathogenic *E. coli* and *Pseudomonas aeruginosa* (Arts, Ball, Leverrier, Garvis, Nicolaes, Vertommen, Ize, Tamu Dufe, Messens, Voulhoux and Collet 2013: e00912-13, Lee, Kim, Yeom, Kim, Park, Jeon and Park 2008: 213-22). The primary functions of antibodies are neutralization and opsonization (Shahrooei, Hira, Khodaparast, Khodaparast, Stijlemans, Kucharikova, Burghout, Hermans and Van Eldere 2012: 3660-8). The effect of SeseC-00396 antibody on SEZ biofilms *in vitro*, in the absence of immune system components suggested the neutralizing effect of SeseC-00396 antibody or the blocking of the function of SeseC protein. The SeseC_00396 may thus be a promising target for the prevention/treatment of SEZ biofilm-related infections.

This study revealed strong evidence that the SeseC_00396 is a promising candidate for the development of an effective and novel vaccine against SEZ infections.

In conclusions, in this study, we investigated SeseC_00396 for its potential as a vaccine candidate against SEZ infection. Western blotting analysis revealed good immunoreactivity to convalescent mini-pig sera against SEZ. Bactericidal assays show that the results of vaccination with SeseC_00396 were consistent. Furthermore, anti-SeseC_00396 antibodies can significantly inhibit the formation of SEZ biofilm.

Ethics approval and consent to participate: All animal experiments were based on animal welfare consideration. This study is in agreement with the Ethical Principles for Animal Research established by Henan University of Science and Technology (Luoyang, China). All animal tests were approved by the Laboratory Animal Monitoring Committee of Henan University of Science and Technology (Luoyang, China). Blood sampling of mice to obtain serum was performed following good veterinary practices.

Declaration of competing interest: The authors declare that they have no competing interests.

Authors' contributions: Conceiving and designing the experiments: YW and SHW. Performing the experiments: LY, WPY and JPL. Analyzing the data: LY, WPY, JPL and SHW. Contributing reagents/materials/analysis tools: LY, WPY and JPL. Writing the paper: LY, SHW and YW. All authors read and approved the manuscript.

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