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A simple cost-effective method for purification of *Clostridium chauvoei* cell-surface proteins for detection of antibodies against blackleg disease vaccine

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Abstract

Cell-surface proteins of *Clostridium chauvoei* were purified using a simple method. Bacterial cultures were centrifuged and agitated vigorously in phosphate buffered saline with or without further glycine treatment and ammonium sulfate precipitation. Rabbits were immunized subcutaneously with a blackleg disease vaccine twice with a two-week interval. Immunized sera were collected one week after the second injection. Enzyme-linked immunosorbent assay (ELISA) was performed using the proteins purified by the second method as the coating antigen. Bradford assay results showed a higher protein concentration in the second than the first method. Sodium dodecyl-sulfate polyacrylamide gel electrophoresis analysis showed multiple bands for the cell-surface proteins of *C. chauvoei* in the first method and a sharp band equivalent to flagellin protein in the second method. The ELISA results indicated that the purified proteins were capable of detecting antibodies against Blackleg disease vaccine. The purified protein would be an alternative antigen for indirect ELISA in order to monitor the immune response in vaccinated farm animals.

Introduction

Clostridium chauvoei is rod-shaped gram-positive bacteria, motile by peritrichous flagella, arranged singularly or in short chains and responsible for blackleg disease especially in cattle.[1] Blackleg infection occurs through ingestion of bacterial spores by the animal. The bacterial spores spread to the skeletal muscle through the bloodstream. The spores germinate in the infected tissues in low oxygen conditions result in toxin production and muscle necrosis, and lead to death.[2] Dissimilar to most clostridial bacteria, the pathogenicity or antigenicity of *C. chauvoei* is not associated with only a single major toxin. *Clostridium chauvoei* toxin A (CctA) is the major virulence factor[3] which causes cell lysis by increase in permeability of the animal cell.[4] One of the other virulence factors associated with pathogenesis is flagella.

Flagella of *C. chauvoei* have drawn particular attentions towards characterization of this pathogen. Flagella are not directly involved in the pathogenesis of blackleg disease,[5] however, can contribute to the infection due to providing mobility to bacteria. Flagellar antigens (flagellin proteins) have been introduced as potential candidates for vaccines or adjuvants.[6,7] Flagellin has shown a protective as well as a diagnostic role for detection of *C. chauvoei* by enzyme-linked immunosorbent assay (ELISA).[8] Flagellin induces the immune response against other pathogenic bacteria such as *Salmonella enterica*.[9] Purification of flagellin from native bacteria has been limited by some disadvantages such as need for ultracentrifuge and multiple steps of purification.[10] Ultracentrifuges have been used in flagellin purification from *Clostridium tyrobutyricum*[11] and *C. chauvoei*.[12] The detection of *C. chauvoei*-specific antibodies by ELISA needs specific antigens such as recombinant proteins or sonicated whole proteins and it would be beneficial to run a simple method to obtain cell-surface proteins such as flagellin for use in detection and diagnosis. Different studies have used recombinant or partially purified flagellin proteins for antibody detection.[13,14] However,

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most of these methods were based on expensive technology or equipment.

Hence, the aim of the present study was to develop a cost-effective purification method for *C. chauvoei*-cell surface proteins and optimize an ELISA for detection of antibody titers against blackleg vaccine.

Materials and Methods

Bacterial cultures and protein purification. *Clostridium chauvoei* and *C. septicum* vaccinal strains were cultured in liver infusion nutrient broth (Merck, Rahway, USA) under anaerobic condition at 37.00 ˚C overnight in 500-mL containers. Purification of cell-surface proteins was performed in two different methods. Amounts of 100 mL culture was centrifuged at 4,000 rpm for 5 min and the pellets were washed by adding phosphate buffered saline (PBS) and centrifuged. In the first method, PBS was added to the pellets and agitated vigorously for 10 min. Then, it was centrifuged for 20 min at 15,000 rpm. The supernatant was preserved at – 70.00 ˚C until use. In the second method, 0.20 M glycine (Merck)[15] pH: 2.20 was added to the pellets and agitated vigorously for 15 min. After a second 15 min incubation at room temperature, the mixtures were centrifuged at 15,000 rpm for 10 min. Then, the supernatants were collected and ammonium sulfate, (Merck, Darmstadt, Germany) powder was added to make a 70.00% saturated solution at room temperature. After centrifugation at 15,000 rpm, 4.00 ˚C for 10 min, the supernatants were discarded and the pellets were dried at room temperature. The PBS was added to the pellets and the proteins were kept at – 70.00 ˚C until use.

Protein analysis. Purified samples were analyzed on 12.00% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the protein bands were visualized. Concentration of protein samples was calculated by Bradford assay using bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, USA) as standard.

Production of polyclonal antibody. A potent formalin-inactivated whole-culture blackleg disease vaccine (Razi Vaccine and Research Institute, Karaj, Iran) was used for production of polyclonal antibody against *C. chauvoei.* New Zealand White rabbits weighing 2.00 - 3.00 kg were injected subcutaneously with 2.00 mL vaccine and boostered 2 weeks later according to the vaccine instructions. Serum samples were collected on day seven after the last injection.[16]

Indirect ELISA. For detection of antibodies against the *C. chauvoei* cell-surface proteins of blackleg vaccine, an indirect ELISA was implemented. The amount of coating antigen is variable (1.00 to 15.00 µg mL^{-1}) in similar studies.[13,14] Hence, in order to optimize the ELISA condition, checkerboard titration was performed using serial dilutions of antigens and sera. Plates (JetBiofil, Guangzhou, China) were coated with 200 µL serial dilutions (0.50, 1.00, 2.00, 4.00, 8.00 µg mL^{-1}) of *C. chauvoei* protein purified by the second method in carbonate buffer, pH 9.60, and incubated for 16 hr at 4.00 ˚C. Plates were washed with PBS containing 0.05% Tween 20 (PBST), Fluka, Seelze, Germany) for five times. Then, unbound sites were blocked using PBST with 2.00% BSA and 4.00% sucrose (Merck), for 1 hr at room temperature. Serum samples were diluted two folds at 1:100 to 1:3,200 in PBST containing 1.00% BSA, added to wells and incubated for 30 min at room temperature. Pre-injection sera were used as negative control. After five washes, 100 µL horseradish peroxidase-conjugated goat anti rabbit antibody (Sigma-Aldrich) was added to the wells in the same dilution buffer at appropriate working concentration (1:15,000) and incubated at 37.00 ˚C for 30 min. After five washes 3, 30, 5, 50-tetramethyl-benzidine/H_2O_2 (Pishtaz Teb Zaman, Karaj, Iran) was added to wells and incubated in the dark. After 15 min, the reaction was stopped by the addition of 2.00 M H_2SO_4 (Merck).

The optical density (OD) of each well was read at 450 nm using ELISA microplate reader (BioTek, Winooski, USA). The lowest concentrations of antigen and serum giving a good OD result (OD >1.00) were considered as optimal. All experiments involving animals were performed in accordance with animal ethics guidelines and protocols and approved by Research Ethics Committees of Razi Vaccine and Serum Research Institute, Iran (Approval ID: IR.RVSRI.REC.1400.006).

Results

The SDS-PAGE analysis indicated that both methods were mostly successful to purify cell-surface proteins from *C. chauvoei* and *C. septicum* strains as observed in Figure 1. Flagellin as one of the most important and immunogenic proteins of *C. chauvoei* has 413 aminoacids and the flagellin protein of *C. septicum* has 388 aminoacids. The expected bands can be observed around 45.00 kDa in SDS-PAGE (Fig. 1). For purification of cell-surface proteins, it seems that the first method was not successful for *C. septicum*. Both methods were appropriate for purification of cell-surface proteins from *C. chauvoei.* However, a stronger band in accordance with flagellin protein size was observed in the second method. In the first method the protein concentration calculated by Bradford assay was about 40.00 µg mL^{-1}, however, it was about 500 µg mL^{-1} in the second method.

Checkerboard titration data are presented in Figure 2. Results showed that the purified cell surface proteins have the ability to detect antibodies against *C. chauvoei*. The optimal condition for detection of antibodies was 1.00 µg mL^{-1} purified proteins and 1:100 dilution of vaccinated sera.

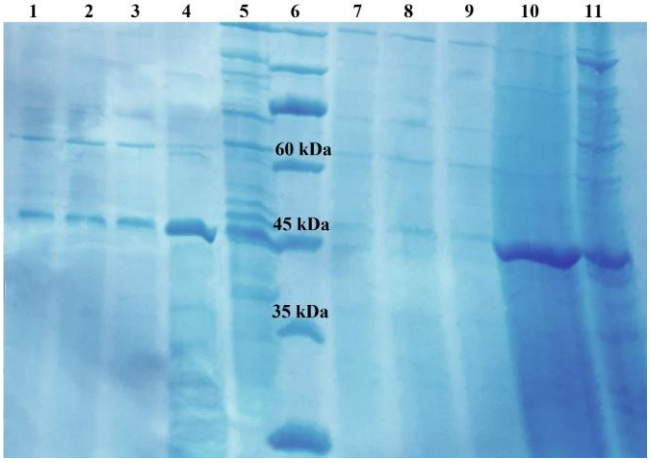

Fig. 1. Sodium dodecyl-sulfate polyacrylamide gel electrophoresis analysis of the purification of *Clostridium chauvoei* and *C. septicum* cell-surface proteins. Lanes 1-3: Proteins purified by the first method from *C. chauvoei.* Lane 4: Proteins purified by the second method from *C. chauvoei*. Lane 5: *C. chauvoei* cell lysate, Lane 6: 10.00 - 180 kDa protein ladder, Lanes 7-9: Proteins purified by the first method from *C. septicum,* Lane 10: Proteins purified by the second method from *C. septicum,* Lane 11: *C. septicum* cell lysate.

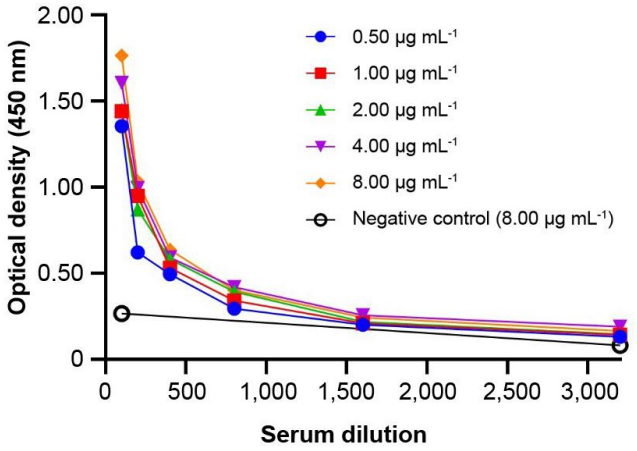

Fig. 2. Reactivity of sera at different dilutions with different concentrations of antigen (*Clostridium chauvoei* cell-surface proteins) in indirect enzyme-linked immunosorbent assay.

Discussion

Cellular antigens are immunogenic components that are critical in the protection against *C. chauvoei* infection. That is why most of blackleg disease vaccines contain bacterins or whole formalized cultures as polyvalent formulations.[17] Protective immunity against *C. chauvoei* is mainly considered due to the cellular and flagellar antigens.[18]

Flagellin has shown protective immunity in mice by opsonic activity, through inducing the polymorphonuclear leukocytes to clear *C. chauvoei*.[19] Flagellin as an immuno-dominant surface protein of the *C. chauvoei* is proposed to have a protective role against blackleg disease infection and also a diagnostic potential.[8]

Khani and Bagheri have developed a cost-effective method for purification of bacterial flagellin from some gram negative bacteria.[20] However, most of the gram positive bacteria such as *C. difficile and C. chauvoei* have been under ultracentrifugation for removal of flagellin.[11,12] Cell-surface associated proteins of *C. chauvoei* have been isolated by glycine and lysozyme methods.[15] In the lysozyme method, mostly low molecular weight cell-surface proteins are isolated with a high quantity, however, in the glycine method, both low and high molecular weight cell-surface proteins are purified. Glycine treatment is milder than lysozyme treatment and predominantly releases proteins with signal peptides or those exported from the cell, rather than proteins derived from the cytoplasm, which are extracted through digestion of the peptidoglycan and partial cell lysis in the lysozyme method.[21]

One of the significant proteins identified through glycine method was flagellin with a molecular mass of 43.00 kD.[15] The glycine method that we used is different in equipment and materials, and can be performed in common centrifuges in a faster time with a good yield. A sharp band of about 45.00 kD was observed in our second (glycine) method.

Clostridium chauvoei and C. septicum were studies in this research for cell-surface protein purification in order to make a comparison. According to the expected band size of flagellin, both of the used methods yielded a sharp band in *C. chauvoei,* although the second (glycine) method extracted a band in accordance with flagellin with higher concentration and purity. However, only the second method worked for purification of the similar band from *C. septicum.* Results showed that *C. septicum* flagellin needed a harsher mechanical method with more chemical steps to be removed in comparison with *C. chauvoei*.

Due to the function of flagellin in inducing innate immunity, it has been suggested for use as a mucosal or systemic adjuvant to enhance the immunity in different immunization regimes such as anti-influenza mucosal vaccination[22] or mucosal vaccination strategy against *C. difficile* infections.[23] Cell-surface proteins extracted from *C. chauvoei* ATCC 10092 strain using glycine method has been analyzed by mass spectrometry and a sharp band of about 43.00 kDa was confirmed as the flagellin protein.[23] In our experiment, the second method of cell-surface protein extraction for *C. chauvoei* seemed to be successful in the purification of protein possibly related to flagellin band size with high purity (Fig. 1). Native flagellin purified from *C. chauvoei* using a simple and non-expensive method might have the potential to be used as an adjuvant for clostridial toxoid vaccines.

Different studies have shown the detection of *C. chauvoei* antibacterial antibody using ELISA method by application of *C. chauvoei* whole antigen[24] or flagellin[14] as the coating antigen. In this research, the efficiency of cell-

surface proteins of *C. chauvoei* was tested by implementing an indirect ELISA test on the rabbit sera immunized with Blackleg vaccine. Recognition of antibodies against *C. chauvoei* by the cell surface antigens extracted by a highly acidic solution, suggested that the linear epitopes of the proteins are critical in detection of the immune response. Since there is no major toxin for *C. chauvoei,* evaluation of vaccine efficacy in farm animals and development, persistence and duration of the antibody response would be difficult and along with challenges. It has been shown that recombinant flagellin can be used in ELISA for detection of *C. chauvoei* bacteria[8] or evaluation of vaccine immunogenicity.[14] Also, recombinant CctA may have the potential to be used as antigen for ELISA detection of antibodies induced by vaccination. However, ELISA was not able to specifically detect antibodies against CctA in cattle sera due to strong cross-reactivity with antibodies against other proteins in pre-vaccination sera.[4] On the other hand, recombinant technology is laborious, time consuming and expensive. Finding a simple and cost-effective way to produce cell-surface proteins including native flagellin from *C. chauvoei* for application as an alternative antigen in indirect ELISA would be useful in detection of blackleg disease vaccine immunogenicity in animal models or even farm animals.

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Conflict of interest

The authors declare no competing interest.

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