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Identification of *Clostridium septicum* in Clinical Cases Suspected to Braxy and Malignant Edema by Traditional and Molecular Method and Evaluation of Toxigenic Isolates for Vaccine Production

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Authors' contributions

This work was carried out in collaboration between both authors. Author LAK designed the study, wrote the protocol and wrote the first draft of the manuscript. Author AP managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Clostridium septicum is the causative agent of the acute fetal disease braxy and gas gangrene with major economic losses in the dairy industry. Accurate and rapid detection is great importance in this regard. Vaccination is an effective process for protection against *C. septicum* infection. It is necessary to identify and evaluate toxigenic Iranian isolates to produce a vaccine. This study aimed to detect Iranian isolates and evaluate toxigenic isolates as candidates for vaccine production. To this end, a total of 17 samples of animals, clinically suspected to braxy and malignant edema, were obtained. All samples were then cultured on media and microbiological and biochemical tests were performed on the colonies. The test results were confirmed by PCR amplification of the alpha-toxin gene. The toxigenic isolates were then evaluated using MLD. The experimental vaccine was produced and evaluated according to the British Pharmacopoeia Standard. According to the results, out of 17 samples, 15 samples were considered *C. septicum*. All samples were confirmed by PCR

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amplification. The most toxigenic isolate was used for experimental vaccine production. The result was successful. The Iranian isolate could be, therefore, used for vaccine production although further studies should be conducted in this regard.

Keywords: Clostridium septicum; pcr; MLD; vaccine; pathogenicity test.

1. INTRODUCTION

Clostridium septicum (*C. septicum*) is a gram-positive, rod, anaerobic, and spore-forming bacterium that produces alpha-toxin. Alpha-toxin gene (*csa*) is located on a chromosome which is the most virulence factor [1]. Alpha-toxin is secreted as a protoxin containing 443 aa (with an approximate molecular weight of 47 kDa) and activated by proteases as a 45 amino acid [2]. Alpha-toxin is lethal, haemolytic, and necrotizing [3]. Alpha-toxin binds to glycosyl phosphatidylinositol membrane proteins [4] and induces the oligomerization and formation of channels [5]. *C. septicum* causes myonecrosis, braxy, malignant edema (gas gangrene) and clostridial dermatitis [6]. Malignant edema (gas gangrene) and braxy are acute fetal disease leading to damage to the abomasum caused by the ingestion of feedstuffs including frozen grass. The characterization of the disease can be mentioned as sudden death and presence of inflammation and necrosis in the mucous membrane intestinal tract [7]. Vaccination is an effective process for protection against *C. septicum* infection. It is essential to identify and evaluate toxigenic Iranian isolates for vaccine production. However, there are a few references in this regard. Several methods are used for identification of infection cases including microbiological and biochemical tests, Immunological methods, DNA-based PCR and Real-time PCR assay. Molecular detection was carried out based on alpha-toxin and other virulence factor gene [8]. In Iran, *C. septicum* isolates have been isolated from braxy and malignant oedema from animals [7,9,10]. This study aims to identify Iranian *C. septicum* isolates using traditional and molecular methods and evaluate toxigenic isolates as candidates for vaccine production.

2. MATERIALS AND METHODS

2.1 Identification

A total of 17 samples of animals, clinically suspected to braxy and malignant edema, were obtained and lyophilized in Razi Institute Anaerobic Research and Production Department. All samples were inoculated on the liver under anaerobic condition using anoxomat (Mart® microbiology, Netherlands) at 37 °C overnight. The samples were then sub-cultured on the liver extract under anaerobic condition overnight. After growth, 1 mL of the culture suspension plus 2.5% calcium dichloride were injected intramuscularly into the legs of two guinea pigs. After 24 h, both the inoculated guinea pigs were killed. The animals were necropsied and the bacterium was isolated from the heart (H), muscle (M) and liver (L) as follows. The heart, muscle and liver lesions were inoculated onto the fresh blood agar plates with 5% defibrinated sheep blood and incubated under anaerobic condition at 37 °C for 48 h. Any cases resembling *C. septicum* based on the shape, color, and type of hemolysis, were examined for gram-staining under a light microscope in ×100 objective magnification. The colony was also examined by reactions to 3% wt/vol hydrogen peroxide and biochemical tests including the fermentation test, lecithinase, lipase, gelatinase, indole, motility and skim milk coagulation (stormy reaction).

Fermentation media used in this study contained glucose, maltose, lactose, sucrose, mannitol and salicin were used in this study. The vaccine strain (CN 913) was used as a positive control for confirmation. The final confirmation was performed by PCR amplification of alpha-toxin gene as previously described.

2.2 Isolation of Genomic DNA

Briefly, the bacterial cells were centrifuged and diluted in TE containing 1 mg/mL lysozyme before adding 10% SDS and then incubated for at least 30 min at 37 °C. Proteinase K (50 mg/mL) was then added and incubated at 56 °C for 1 h. Extraction was performed by the equivalent phenol and chloroform solutions. Sodium acetate (1:10 v/v) and isopropanol (1 v/v) were then added to the mixture and incubated at -20 °C overnight. The DNA was then centrifuged for 10 min at 12500 rpm at 4 °C. The sediment was washed by ethanol 70%, dried, and

dissolved in TE buffer. The quality and quantity of the DNA were measured using a NanoDrop (Nano-Drop Technologies, Wilmington, DE, USA).

2.3 PCR Amplification

The volume of the final reaction mixture was 30 µL containing 1.8 µL of MgCl2 25 mM, 3 µL PCR buffer 10X, 0.6 µL of 10 mmol dNTPs, 0.5 µL *Taq* DNA polymerase (5 units / mL) (fermentase, Germany), 2.5 µL DNA template (100 ng/µL), 1 µL of each primer (10 pmol/mL) and 20 µL pure water. Pure water and type D *C. perfringens* were used as a negative control. The *C. septicum* (CN 913) vaccine strain was used as a positive control. The sequences of forward and reverse primers were 5-ATCGGAAACATGAGTGCTGC-3 and 5´-AGTCTTTATGCTTCCGCTAG-3´, respectively. The PCR program includes initial denaturation at 94 °C for 3 min and 30 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min followed by a final extension at 72 °C for 5 min. The PCR product was then mixed with 2 µL ×6 gel loading dye, electrophoresed, stained with ethidium bromide (0.5 µg/mL), and visualized by the UV-light transilluminator (GelDoc, BioRad, USA).

2.4 Increase of Pathogenicity

The reference strain of *C. septicum* (CN 913) was used to increase pathogenicity. The *C. septicum* strains were cultured on the liver extract medium under anaerobic conditions overnight. The culture suspension plus 2.5% calcium dichloride of 1:1 ratio were injected deep intramuscularly into two guinea pigs. Both the inoculated guinea pigs were killed the next day. The animals were necropsied and the bacteria were taken from the heart, muscle, and liver. The heart and liver lesions were inoculated onto the fresh liver extract broth medium under the previous conditions. The samples were serially passaged (seven times) through guinea pigs.

2.5 Evaluation and Selection of Culture Media

The vaccine strains of *C. septicum* grown on three media of brain-heart infusion (BHI), BHI plus casein hydrolysate, and BHI plus multivitamins & trace elements were cultured, and MLD was performed after their growth.

2.6 Minimum Lethal Dose

The minimum lethal dose (MLD) was determined for the alpha-toxin. Briefly, the bacterial cells were centrifuged for 15 min at 4500 rpm and diluted with physiological saline (1/10 through 1/70 with 10 intervals). Two *NMRI* mice (17–22 g body weight) were immediately injected intravenously by 0.5 mL of each dilution. The inoculated animals were observed for 72 h and the toxin level was calculated.

2.7 Pathogenicity Test

The samples were diluted (1/10 through 1/1000000 with 10X intervals) (Fig. 1). The diluted suspension plus calcium_dichloride were injected intravenously by 0.5 mL of each dilution. The inoculated animals were observed for 72 h.

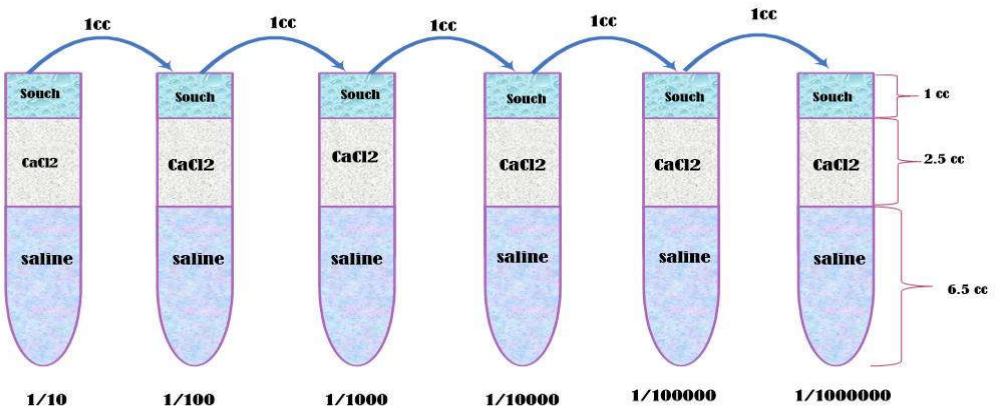

Fig. 1. Preparation of serial dilution for pathogenicity test

2.8 Sequencing

Nucleotide sequencing was carried out for the most toxigenic isolate by SEQLAB (Sequence Laboratories Goettingen GmbH).

After confirmation, experimental anaculture vaccines were prepared from the toxigenic Iranian isolate and vaccine strain.

2.9 Vaccine Production

The vaccine was produced by culturing of *C. septicum* strains on the CAM media in a fermenter using the complex media containing meat peptones, meat extract, tryptone, yeast extract, casein hydrolysate, glucose, trace elements and vitamins, L-cysteine hydrochloride, Na_2HPO4, and NaOH at 37 °C (pH 7.2-7.4) until growth completion and inactivation by formaldehyde (1.5 mg/mL) [11]. The experimental vaccines were incubated for 60 days in a refrigerator. The sterility, residual toxicity, safety, and pathogenicity test were performed after adjusting formaldehyde (%0.6) and pH.

2.10 Sterility

The vaccines were inoculated onto the fresh blood agar plates, thioglycolate broth, and trypticase soy broth (TSB) and then incubated for 14 days [12].

2.11 Residual Toxicity

Five mice were injected subcutaneously with 0.5 mL of the experimental vaccines and observed for 7 days [12].

2.12 Safety

Three healthy sheep were injected subcutaneously with 3 mL of the vaccine and observed for 14 days [12].

2.13 Challenge

Twelve guinea pigs, weighing 300 and 350 g, were injected subcutaneously with 2 mL of the experimental vaccine. The second injection was carried out after four weeks as a booster in the same manner. After 14 days, the vaccinated guinea pigs were challenged intramuscularly with a virulent culture of C. *septicum* As follows: 1 mL of the culture suspension plus calcium dichloride was injected intramuscularly into the legs of twelve guinea pigs by using a needle. Three and two guinea pigs were injected with 1 mL virulent strain and PBS as respectively as positive and negative controls. All animals were observed for five days.

3. RESULTS

Out of 17 samples of animals, clinically suspected to braxy and malignant edema, 16 samples were identified as typical colonies with β-hemolysis on media (Fig. 2), gram-positive bacilli on staining, and reactions to hydrogen peroxide (negative), which were considered *Clostridium*. The microbiological and biochemical examinations also showed 15 isolates as *C. septicum*. All samples liquefied gelatin and modified milk to clot. The catalase activity was positive but indole, lecithinase, and lipase were negative. All samples fermented salicin, lactose, maltose, and glucose but others were negative (Table 1).

The samples were also confirmed by PCR amplification of alpha-toxin (Fig. 3).

The result of bacterial isolation from several organs of infected guinea pigs including the heart, liver, and muscle, which serially passages through guinea pigs, were satisfactory. BHI plus multivitamins & trace elements was the best culture medium. The toxin was produced by strains No. 901, 905, 907, 910, and 912. The maximum toxin level was produced by strain No. 907 (1/60 M.L.D. per mL) more than vaccine strain as shown in Table 2. According to the results of the pathogenicity test, strains No. 907 and 912 were at their desired level.

The experimental vaccine was produced on CAM media successfully. The results of the sterility tests showed that experimental vaccines were pure without any contamination (without turbidity). None of injected mice and sheep showed any systemic or local reactions death including fever, swelling, Inflammation, and necrosis. Most vaccinated guinea pigs resisted the challenge with the virulent strain like the vaccine strain, while the positive control guinea pigs died (Table 3).

Table 1. The result of microbiological and biochemical tests for *C. septicum* isolates and vaccine strain

Isolates & vaccine strain (positive samples)	Characterization
Gram-staning	rod-shaped bacterium
Morphology on B.A	Convex with β-hemolysis
Motility on SIM	Positive
Catalase	Negative
indole	Negative
lecithinase	Negative
lipase	Negative
salicin	Positive
lactose	Positive
maltose	Positive
glucose	Positive
Sucrose	Negative
Manitol	Negative
Milk digestion	Clot produced
Gelatinase	Positive

Fig. 2. Beta hemolytic colonies of *C. septicum* onto the fresh blood agar plates with 5% defibrinated sheep blood

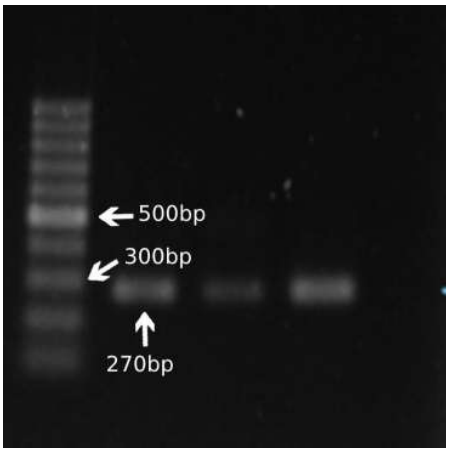

Fig. 3. PCR amplification of alpha-toxin (haemolysin) gene using the specific primers showing an amplicon size of 270 bp

Lane 1, 100bp plus DNA size marker, lane 2-3, PCR product of Iranian isolates, lane 4, PCR product alpha-toxin C. septicum (CN 913) (positive control), lane 5, Pure water (Negative control)

Table 2. The result of minimum lethal dose on *NMRI* mice among Iranian isolates

$\frac{1}{70}$	$\frac{1}{60}$	$\frac{1}{50}$	$\frac{1}{40}$	$\frac{1}{30}$	$\frac{1}{20}$	$\frac{1}{10}$	Dilution isolate
AA	AA	AA	DD	DD	DD	DD	vaccine strain
AA	AA	AA	AA	AA	DA	DD	901
AA	AA	AA	AA	AA	DA	DD	905
AA	DD	DD	DD	DD	DD	DD	907
AA	AA	AA	AA	AA	AA	DA	910
AA	AA	AA	AA	AA	DD	DD	912
AA	AA	AA	AA	AA	AA	AA	904
AA	AA	AA	AA	AA	AA	AA	910
AA	AA	AA	AA	AA	AA	AA	915
AA	AA	AA	AA	AA	AA	AA	728
AA	AA	AA	AA	AA	AA	AA	909
AA	AA	AA	AA	AA	AA	AA	913
AA	AA	AA	AA	AA	AA	AA	922
AA	AA	AA	AA	AA	AA	AA	721
AA	AA	AA	AA	AA	AA	AA	924
AA	AA	AA	AA	AA	AA	AA	923

D=Dead, A=Alive

Table 3. The result of challenge test among vaccine stain and Iranian isolates (No. 907)

Strain						Positive control	Negative control
Vaccine strain	DD	DD	AA	AA	AA	DDD	AA
907	DD	DD	AA	AA	AA	DDD	AA

D=Dead, A=Alive

4. DISCUSSION

Microbiological and biochemical identification is time-consuming process. moreover, it is difficult to differentiate Clostridia especially *C. chauvoei* and *C. septicum* because of their similarities [13]. The fluorescence antibody test (FAT) was used for identifying and differentiating *C. chauvoei* and *C. septicum* [14]. However, FAT suffers from several disadvantages including the need for a fluorescent microscope and labelled antibodies, and short-term maintenance of sections [15]. The immunohistochemistry (IHC) technique, however, does not suffer from disadvantages, and thus was used for the identification of Clostridia spp. [16]. The streptavidin-biotin peroxidase technique (SBPT) is highly sensitive for detecting *C. septicum* in animal samples [17]. The Immunological methods can also be used for the identification despite disadvantages such as cross-reactions between the two species [18] and the need for laboratory animals for antiserum production. Other methods can be used for identification including complement fixation test [8], the MALDI-TOF MS technology [19], and single PCR, [20] and real-time PCR [21]. In this study, all positive strains of *C. septicum* in the biochemical and microbiologic test showed the PCR product size (270 bp) observed in the molecular examination confirming the presence of the bacterium in the samples. *C. septicum* alpha-toxin is similar to *C. chauvoei* alpha-toxin and can only be differentiated by the PCR assay not by immunological methods. The sequence of the hemolysin gene has not observed in other clostridia specious and has not homology with other toxins [22]. This sequence seems specific and suitable for identifying *C. septicum*. in comparison with other methods such as the complement fixation test and immunofluorescent and enzyme-linked immunosorbent assays, [8] PCR is faster and its results are consistent with those reported in previous studies [8]. On the other hand, a small concentration of genomic DNA can detect *C. septicum* in samples. Moreover, molecular detection is more specific and sensitive to other methods. Other sequences have been used in the literature for the design of

primers such as the flagellin gene, which can be used for rapid detection of *C. chauvoei* and *C. septicum* alpha-toxin gene [22] or other sequences [20]. It seems that the PCR assay should be confirmed for rapid detection of *C. septicum* cases. The reference strain of *C. septicum* was cultured and serially passages through guinea pigs to increase pathogenicity. According to the results, the changes reached a maximum severity by seven passages in the heart and liver lesions of guinea pigs. Formulations of three media were compared in this study in terms of toxin production. The multivitamins & trace elements solution increased α-toxin secretion which was confirmed by the MLD test. The result of another study also showed an increase in α-toxin secretion by magnesium, multivitamins & trace elements solution in the culture medium [23]. Alpha-toxin secretion was determined in this study using the minimal lethal dose (MLD) on *NMRI* mice. Other methods can be used such as hemolysin test [24]. However, lethality is depends on a variety of virulence factor including alpha-toxin secretion and other factors. According to Ballard, alpha-toxin was estimated to contribute to lethality by 70%. Therefore, LD50 or MLD tests are not sufficient for evaluating alpha-toxin secretion. The results showed that the maximum toxin level was produced by strain No. 907 (1/65 M.L.D. per mL) more than the vaccine strain. The pathogenicity test showed the same result. The experimental vaccine was prepared and inactivated by formalin as previously discussed by Hang's study [25]. The results also showed that the experimental vaccine passed the sterility, residual toxicity, and safety tests according to the British Pharmacopoeia Standard. According to the results of the challenge test, most vaccinated guinea pigs resisted the challenge with the virulent strain like vaccine strain.

5. CONCLUSION

The results of this study suggest that this Iranian isolate (No. 907) could be used for vaccine production. However, further studies should be performed in the future to determine the amount of antibody produced after injection in the target animal and the amount of antibodies to achieve immunity according to the international unit.

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ETHICAL APPROVAL

Animal Ethic committee approval has been taken to carry out this study. Experimental procedures were approved by Razi Vaccine and Serum Research Institute (Documentary Proof of Ethical Clearance no: RVSRI–2-18-18-86024).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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