

Short Communication

Isolation and identification of *Clostridium septicum* from sheep-dung

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ABSTRACT

To study the existence of *Clostridium septicum* as probable agent associated with currently occurrence of black quarter in Urmia area, sheep-dung samples were collected. The bacteriological examinations on suspicious gram positive anaerobic spore-bearing bacilli which are in *Clostridia* genus include isolation and biochemical identification were performed. The results showed 28 different types of *Clostridia* contain of *Cl. Septicum* (6 isolates), *Cl. Perfringens* (5 isolates), *Cl. novyi* (4 isolates), *Cl. fallax* (4 isolates), *Cl. innocuum* (3 isolates), *Cl. subterminale* (2 isolates), *Cl. carnis* (2 isolates), *Cl. ramosum* (1 isolate), *Cl. bifforrentanse* (1 isolate). All six isolated of *Cl.septicum* were confirmed by indirect florescent antibody test while other species were negative. PCR was performed for confirmation of isolation by using designed primers with respect to a segment of hemolysin gene (alpha or lethal toxin). All six isolates showed 270-bp band on agarose gel electrophoresis, suggesting conserved sequence for lethal toxin in *Cl. septicum* isolates. In this work we have isolated different *Cl.septicum* from sheep-dung in Urmia and biochemical, indirect immunoflorescent antibody and polymerase chain reaction tests suggesting that PCR could be selected as specific and quick method for identification of this bacterium.

Keywords: *Clostridium septicum*, Isolation, PCR, Alpha toxin

INTRODUCTION*

Malignant edema or gas gangrene in all kind of animals and braxy in sheep are the acute fetal infectious cause by *Cl. septicum* commonly (Blood *et al* 2000, Assis *et al* 2002). Acute myonecrosis, meningitis, arthritis, bacteriemia, oosteomielitis, miocarditis and other infections with different kinds

of cancer can be caused by *Cl. septicum* in human (Larson *et al* 1995, Chu *et al* 1989, Pelletier *et al* 1999, Fernandez *et al* 1994). Molecular identification of *Cl.septicum* was performed based on alpha toxin gene (Takeuchi *et al* 1997, Ballard 1992, 1993, 1995, Imagawa *et al* 1994), flagellin gene (*fliC*) (Sasaki *et al* 2003), tetracycline-resistance gene (Sasaki *et al* 2001), 16S rRNA gene (Kuhnert *et al* 1996) and 16S-23S rDNA spacer region (Sasaki *et al* 2001). In Iran, *Cl. septicum* was

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detected (Ardehali *et al* 1969, 1984, 1988) and (Moosawi *et al* 1999) by isolation, biochemical and fluorescent antibody tests. In this study we compared different methods for identification of *Cl. septicum* and suggestion of relevant method for further study.

MATERIALS AND METHODS

Isolation of microorganism. Sheep-dung samples were collected from different area of Urmia using sterile normal saline. All the samples were treated at 80 °C for 10 min and then they were cultured on 10% fresh sheep blood agar at 37 °C for 72 hours in anaerobic condition. After gram staining, all expected bacterial colonies (gram positive bacilli with any growth on aerobic condition) were purified on another plate.

Biochemical tests. The isolated bacteria were characterized by biochemical tests according to Mac Faddin standards (Mac Faddin, 2000). The biochemical tests such as gelatinase, licitinase, lipase, nitrate reductase and amylase were performed. Also motility, milk digestion, indol, H₂S production, sugar fermentation for glucose, sucrose, lactose, maltose, salicin, mannitol and esculin were tested according to the controls.

Indirect fluorescent antibody test. The immunization of sheep was performed using 5ml formalin-hole culture of *Cl.septicum* strain, given by Anaerobic Department of Razi Vaccine and Serum Research Institute (cat. no: 915), by subcutaneous injection. First administration mixed with incomplete Frouds adjuvant and next two injections were done after 14 and 28 days without adjuvant. The animals were bleeding one week after the last injection and the serum was collected by centrifugation.

Indirect fluorescent antibody test was done after fixating the antigen (bacterial samples) on slide by mixture of methanol-acetone (75:25) for 20 min. Then the collected serum (antibody) was added to the fixed antigen and incubated at 37 °C for 30 min in wet chamber. The slide with antigen-antibody

complex was washed with PBS and then anti-sheep conjugated antibody (VMRD, Inc) was added and incubated at 37 °C for 30 min in wet chamber. After proper washing the slide and drying in room temperature, the results were visualized by fluorescence microscopy.

DNA extraction. The bacteria were grown in 5 ml LB for 20 h under anaerobic condition and then it was centrifuged at 5000 rpm for 5 min. The cell pellet was suspended by 300 µl of TE buffer (Tris 10mM, EDTA 1mM pH 8.0). The suspension was treated with 100 µg/ml lysozyme (Roche) (final concentration) for 10 min at 37 °C. SDS was added to 1% for final concentration along with 100 µg/ml proteinase K (Fermentas). After 20-30 min incubation at 37 °C, phenol-chloroform extraction of DNA was performed as Sambrook (1989, 2002) and then extracted DNA dissolved in appropriate amount of distilled water. Quality and quantity of DNA was determined by 1% gel agarose electrophoresis and absorption at 260/280 nm.

PCR procedure. The primer set (prepared by Fermentas) was used to amplify a 270 bp DNA fragment according to Takeuchi *et al.* (1997). The forward primer was 5'-AAT TCA GTG TGC GGC AGT AG-3' (which has position on hemolysin gene from pb 611-631) and the reverse primer was, 5'-CCT GCC CCA ACT TCT CTT TT-3' complimentary to 861-880 of hemolysin gene. T_m of each primer was calculated according to the following formula $T_m = 4(G+C) + 2(A+T)$.

The reaction mixture for PCR was 100 µl, contained 2 µg of DNA sample, 20 pmol of each primer, 200 µM of mixed dNTPs (Fermentas), 5U of Taq DNA Polymerase (Recombinant-CinnaGen), 10 µl of PCR buffer 10X, 3 mM Mg²⁺ and the rest of the volume was filled with water. The reactions were performed by thermal cycler (TECHNE, Techne Ltd, USA) using 30 cycles and initiated with 94 °C for 4 min as first denaturation. Each cycle was started with denaturation at 94 °C for one min, annealing at 55 °C for one min and extension at 72

°C for one min. Final extension was followed by an extension at 72 °C for five min. *Cl. perfringens* (vaccine strains) was used as negative control and the positive control was vaccine strain of *Cl. septicum*. The PCR products were analyzed by loading 5 µl of each PCR reaction sample on 1% agarose gel follow by electrophoresis started with 60V. Molecular Marker with 100-bp DNA ladder was used and after electrophoresis the gel was stained with ethidium bromide (0.5 µg/ml) and visualized by UV laminator (Transilluminator, UVP, USA).

RESULTS AND DISCUSSION

Out of one hundred sheep-dung samples 28 suspected gram positive spore-bearing anaerobic bacilli were isolated which characterized as *Clostridium*. The biochemical tests data showed six strains of *Cl. septicum*, plus five strains of *Cl. perfringens*, four strains of *Cl. novyi*, four of *Cl. fallax*, three of *Cl. innocuum*, two of *Cl. carnis*, two of *Cl. subterminale*, one of *Cl. bifementance* and one of *Cl. Ramusum*.

Colonies of *Cl. septicum* on blood agar after two days of incubation shows 2-3 mm in diameter, slightly raised, semitranslucent, grayish, with markedly irregular to rhizoid margins. The characterized *Cl. septicum* shows long cylindrical bacilli in 0.8×3-5 µm or longer. The colonies shown zone of clearance on blood agar plate indicating β-hemolysis activity. Each colony could also be surrounded by a zone of swarming organisms (Figure 1). All strains of *Cl. septicum* liquefied gelatin and changed milk to clot and produced acid but did not produce hydrogen sulfide and indol. Urease was negative, lecithinase and lipase did not form on egg yolk agar but they produced nitrate reductase in nitrate broth. All six strains also fermented salicin, maltose and glucose but not esculin, sucrose, lactose and mannitol.

IFA results confirmed that all six isolated bacteria

are *Cl. septicum* when compared with the vaccine strain as positive control. Other isolated clostridia were shown negative against *Cl. septicum* specific serum (table 1).

Table 1. Results of indirect fluorescent antibody tests of the Clostridia isolated from Urmia sheep dung samples (Antibody prepared against *Cl. septicum*)

No	Species	Number of isolates	Results
1	<i>Cl. septicum</i>	6	+
2	<i>Cl. perfringens</i>	5	-
3	<i>Cl. novyi</i>	4	-
4	<i>Cl. fallax</i>	4	-
5	<i>Cl. innocuum</i>	3	-
6	<i>Cl. subterminale</i>	2	-
7	<i>Cl. carnis</i>	2	-
8	<i>Cl. ramosum</i>	1	-
9	<i>Cl. bifformentanse</i>	1	-
10	<i>Cl. septicum</i> (vaccine)	1	+

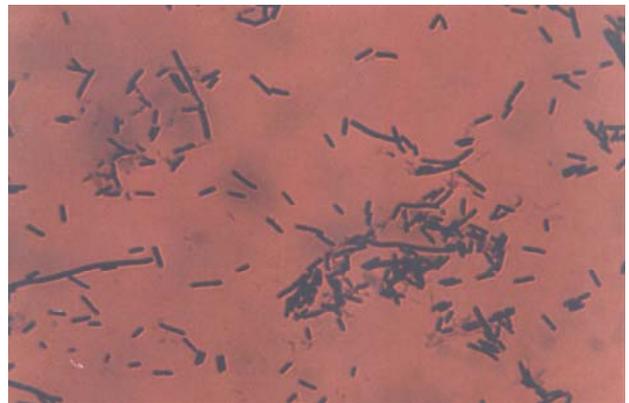


Figure 1. Long Gram positive cylindrical bacilli of *Cl. septicum* after 12 hours on blood agar plate. Using Gram staining (X1000).

With respect to the designed primer, all positive strain of *Cl. septicum* in the least experiment (IFA test) were shown the same size of DNA fragments (270 bp) on agarose gel. Comparison of different techniques used in this work suggested that PCR technique is faster and more specific than the other one in characterization of *Cl. septicum* with respect

to hemolysin gene of this bacterium.

Amplification of the expected size from all *Cl. septicum* indicates that the selected sequence is conserved in all *Cl. septicum* strains. The specificity of the PCR procedure to amplify the 270-bp fragment was examined by using DNA templates isolated from *Cl. perfringens*, *Cl. novyi*, *Cl. fallax*, *Cl. innocum*, *Cl. carnis*, *Cl. subterminale*, *Cl. bifermentans* and *Cl. ramusum* as shown in figure. The expected fragment in *Cl. septicum* for hemolysin was not observed for the control samples (Figure 2). *Cl. septicum* is a causative agent of malignant edema in animals and gas gangrene in human. The organism is closely related to *Cl. chauvei* which causes blackleg in cattle and sheep (Hatheway & Johnson 2000, Kelser & Schoening 1975). These species produce four major toxins (alpha, beta, gamma and delta toxins).

Moreover, they possess common antigens which are detected by a complement fixation test, immunofluorescent and enzyme linked immunosorbant assays (Takeuchi et al 1997). Nowadays the diagnosis of the disease by these organisms is confirmed by clinical symptoms, detection of toxins, isolation and identification of the germs. These procedures are complexity and take several days to complete. In present study, we use a PCR technique which will rapidly and specifically detect the gene of alpha toxin of *Cl. septicum*. The PCR can be useful for rapid detection or identification of *Cl. septicum* in clinical or from environmental samples. The DNA fragment of 270-bp was not amplified for *Cl. perfringens*, *Cl. novyi*, *Cl. fallax*, *Cl. innocum*, *Cl. carnis*, *Cl. subterminale*, *Cl. bifermentans* and *Cl. ramusum*, confirmed specificity of this gene. Also in a DNA sequence homology, significant sequence homology could not be found between the primers and any other hemolysin genes in other bacteria. The same results were reported by Takeuchi who design the primers. Molecular detection of *Cl. septicum* was performed also by other researchers based on alpha

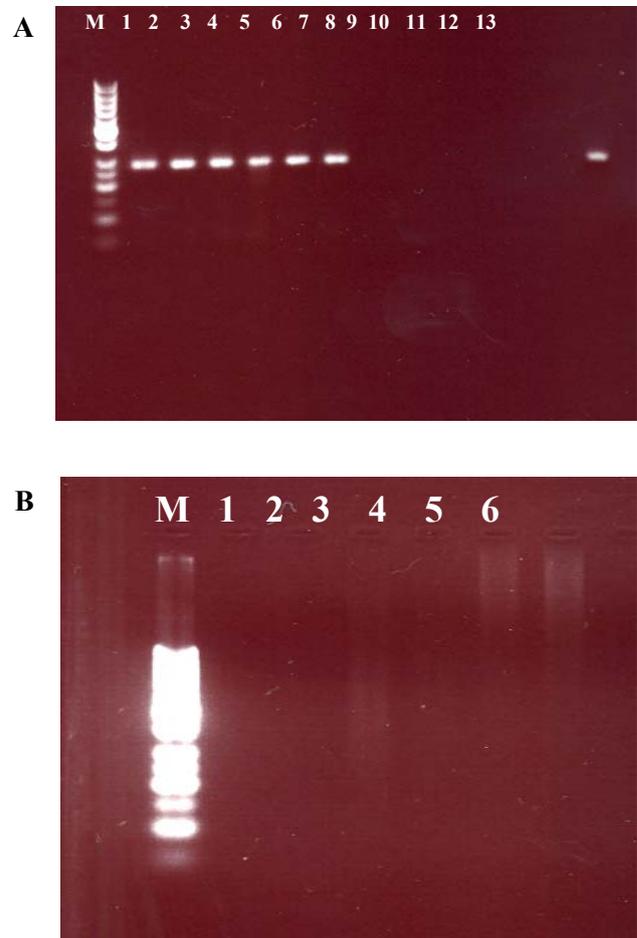


Figure 2. Electrophoresis analysis of PCR product using 1% (w/v) agarose gel. DNA molecules were visualized by ethidium bromide staining as mentioned in methods and materials. Molecular marker (M) was used with 100-bp. **A):** Lanes 1-6: *Cl. septicum* strains, Lanes 7-9: *Cl. perfringens* types B, C, D (vaccine strains) accordingly, Lane 10: *Cl. novyi* type B, Lane 11: *Cl. fallax*, Lane 12: *Cl. bifermentans*, Lane 13: *Cl. septicum* (vaccine strains). **B):** Lane 1: *Cl. innocum*, lane 2: *Cl. ramusum*, Lane 3: *Cl. carnis*, Lanes 4-5: *Cl. perfringens* strains. For further detail refer to the text.

toxin gene (Ballard et al 1992, 1993, 1995, Imagawa et al 1994), flagellin gene {fliC} (Sasaki et al 2003), tetracycline-resistance gene (Sasaki et al 2001), 16S rRNA gene (Kuhnert et al 1996) and 16S-23S rDNA spacer region (Sasaki et al 2001). According to our best knowledge this work is the first report of molecular detection of *Cl. septicum* in Iran.

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