Detection of *Clostridium septicum* Hemolysin Gene by Polymerase Chain Reaction

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ABSTRACT. A polymerase chain reaction (PCR) was developed for the detection of the hemolysin (alpha toxin) gene of *Clostridium septicum*. The PCR primers were designed from the sequence of the hemolysin gene and synthesized. A DNA fragment of 270 bp was amplified from 10 strains of *C. septicum*, but was not from strains of *C. chauvoei*, *C. perfringens*, *C. novyi*, or *C. haemolyticum*. When the PCR product was digested with *Sau3A*I, two DNA fragments of the expected 148 bp and 122 bp were recognized. The lowest detectable threshold of PCR for the hemolysin gene was 3.8×10^3 cells/ml. The PCR technique may be useful for rapid detection or identification of *C. septicum* associated with malignant edema. — **KEY WORDS:** alpha toxin, *Clostridium septicum*, PCR.

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Clostridium septicum is a causative agent of malignant edema in animals and gas gangrene in human. The organism is closely related to *Clostridium chauvoei* which causes blackleg in cattle and sheep [17, 21]. These species produce four major toxins (alpha, beta, gamma and delta toxins) that correspond to each other in biological activity. Moreover, they possess common antigens, which are detected by a complement fixation test, immunofluorescence and enzyme-linked immunosorbent assay [9, 11, 15]. At present, the diagnosis of the diseases by these organisms is confirmed by clinical symptoms, detection of toxins, and isolation and identification of the organisms. However, these procedures are complexity and take several days to complete.

The polymerase chain reaction (PCR) is a molecular genetic technique for *in vitro* amplification of specific nucleic acid sequences. By this technique, organisms can be detected and identified directly from clinical specimens or environmental samples. Recently, PCR has been used for the detection of toxin genes, including neurotoxins of *C. botulinum* type A to F [4–7, 18–20], major toxins and enterotoxin of *C. perfringens* [3, 10, 16], and toxin A of *C. difficile* [8, 13–14].

In the present study, we report the development of a PCR technique which will rapidly and specifically detect the gene for hemolysin (alpha toxin) of *C. septicum*. The PCR technique may be useful for rapid detection or identification of *C. septicum* associated with malignant edema in cattle.

The bacteria used in this study are listed in Table 1, including 10 strains of *C. septicum*, 6 strains of *C. perfringens*, 4 strains of *C. chauvoei*, 2 strains of *C. novyi*, and 1 strain of *C. haemolyticum*. Clinical isolates of *Staphylococcus aureus* and *Actinomyces pyogenes* were also used. All organisms, except *S. aureus*, were cultivated anaerobically at 37°C for 18 hr by the CO₂ gas jet method, modified by Azuma and Suto [1] from the original description of Hungate.

After centrifugation, the bacterial cells were resuspended in 1.5 ml of TES buffer (10 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl) containing 25% (W/V) sucrose and 2 mg/ml lysozyme and incubated at 37°C for 1 hr. Then, 150 µl of 10% SDS solution was added to the suspension and it was incubated at 37°C for 10 min. The lysed cell suspension

was diluted with 3 ml of TES buffer. Nucleic acids in the diluted cell suspension were extracted twice with phenol and once with phenol/chloroform/isoamyl-alcohol (50/48/2) and then precipitated with ethanol. After drying, the precipitates were dissolved in 100 µl of TE buffer and used for the template DNA.

From the sequence of *C. septicum* hemolysin gene reported by Imagawa *et al.* [12], two oligonucleotides were synthesized as primers to amplify a DNA fragment of 270 bp. The sequences of the synthesized primers were 5'-AATTCAGTGTGCGGCAGTAG-3' (nucleotides 611–630) and 5'-CCTGCCCAACTTCTCTTTT-3' (complementary to nucleotides 880–861).

PCR amplification was performed in a total of 100 µl containing 10 µg of the template DNA, 20 pmol of each primer, 2.5 mM of the four deoxynucleotides, and 2.5 units of Taq polymerase (Takara Shuzo Co., Ltd.). The reaction mixtures were overlaid with mineral oil and subjected to 35 cycles of amplification in a PCR thermal cycler (Takara Shuzo Co., Ltd.). Each amplification cycle performed was at 94°C for 1 min for denaturing, at 55°C for 1 min for annealing, and at 72°C for 1 min for extension. After amplification, the PCR products were analyzed by agarose gel electrophoresis.

Figure 1A shows the electrophoretic pattern of the PCR products obtained from 10 strains of *C. septicum*. The 270-bp band of the hemolysin gene was obtained in reference strains (lanes 1 to 7) and clinical isolates (lanes 8 to 10). These PCR products were digested with restriction enzyme *Sau3A*I and electrophoresed. As shown in Fig. 1B, two bands of the expected lengths (148 and 122 bp) were recognized. This result indicates that the PCR products did indeed arise from the sequence of hemolysin gene of *C. septicum*.

Imagawa *et al.* [12] mentioned that the nucleotide sequence of the *C. septicum* hemolysin was unique, because no significant sequence homology has been found between this gene and the GenBank and EMBL nucleotide sequence data base, including the toxins of *C. perfringens* or *Streptococcus pyogenes*. On the other hand, Bourgeau *et al.* [2] cloned and sequenced the gene for alpha toxin of *C. septicum* BX96. In addition, they found that the DNA-

Fig. 1. Agarose gel electrophoresis. (A) The PCR products from *C. septicum*. (B) The PCR products restricted with *Sau3AI*. Lanes 1–7: reference strains (NCTC 281, NCTC 282, NCTC 286, NCTC 501, NCTC 504, NCTC 551, NCTC 12464), lanes 8–10: clinical isolates (44, Chiba, Miyazaki), M: molecular marker.

derived primary sequence of alpha toxin exhibited 27% identity with aerolysin of *Aeromonas hydrophila* over a contiguous region of 387 amino acids.

In the present study, the DNA fragment of 270 bp was not amplified from *C. perfringens*, *C. chauvoei*, *C. haemolyticum*, *C. novyi*, *A. pyogenes* or *S. aureus*, which produce various hemolytic toxins (Table 1). Also in a DNA sequence homology research, significant sequence homology could not be found between our primers and *A. hydrophila* aerolysin (M29818), *A. hydrophila* alpha hemolysin (L36462), *Aeromonas salmonicida* beta hemolysin (X65049), *Bacillus cereus* hemolysin (U94743), *C. perfringens* perfringolysin O (M36704), *Listeria monocytogenes* listeriolysin (U25443), *Paenibacillus alvei* alveolysin (M62709), *Pseudomonas paucimobilis* beta hemolysin (L01270), *S. aureus* alpha hemolysin (M90536), *S. aureus* beta hemolysin (X13404), *Streptococcus canis* streptolysin O (D16825), *Streptococcus equisimilis* streptolysin O (D16824), or *Streptococcus pneumoniae* pneumolysin (M17717). From the results, it seems that the PCR technique is specific for the hemolysin gene of *C. septicum*.

The lowest detectable threshold of the PCR for the hemolysin gene was examined as follows. After overnight cultivation, bacterial cell numbers were determined by direct microscopic count with a hemocytometer (Thoma type). After that, the culture fluid was centrifuged and resulting cells were resuspended in TES buffer. The cell suspension was diluted serially 10-fold with TES buffer and heated at 95°C for 10 min. After centrifugation, the supernatant was used as a template. As a result, a positive DNA band of hemolysin gene was observed in PCR products ranging from 3.8×10^1 to 3.8×10^5 cells, but not from 3.8×10^0 cells per $10 \mu\text{l}$ sample (data not shown). The result indicates that the lowest detectable threshold is 3.8×10^1 cells per $10 \mu\text{l}$ (3.8×10^3 cells/ml).

For the detection of *C. septicum* in organs, the culture fluid of *C. septicum* was inoculated intraperitoneally into a

Table 1. List of bacterial strains, source and the results of PCR

Bacterial strains	Source	PCR results
<i>Clostridium septicum</i>	NCTC 281	+
<i>Clostridium septicum</i>	NCTC 282	+
<i>Clostridium septicum</i>	NCTC 286	+
<i>Clostridium septicum</i>	NCTC 501	+
<i>Clostridium septicum</i>	NCTC 504	+
<i>Clostridium septicum</i>	NCTC 551	+
<i>Clostridium septicum</i>	NCTC 12464	+
<i>Clostridium septicum</i>	isolate (44)	+
<i>Clostridium septicum</i>	isolate (Chiba)	+
<i>Clostridium septicum</i>	isolate (Miyazaki)	+
<i>Clostridium chauvoei</i>	ATCC 10092	–
<i>Clostridium chauvoei</i>	ATCC 11957	–
<i>Clostridium chauvoei</i>	isolate (C6H)	–
<i>Clostridium chauvoei</i>	isolate (Okinawa)	–
<i>Clostridium novyi</i>	ATCC 19402 (type A)	–
<i>Clostridium novyi</i>	ATCC 25758 (type B)	–
<i>Clostridium haemolyticum</i>	ATCC 9650	–
<i>Clostridium perfringens</i>	isolate (C-1)	–
<i>Clostridium perfringens</i>	isolate (C-2)	–
<i>Clostridium perfringens</i>	isolate (B-1)	–
<i>Clostridium perfringens</i>	isolate (B-2)	–
<i>Clostridium perfringens</i>	isolate (E-1)	–
<i>Clostridium perfringens</i>	isolate (E-2)	–
<i>Actinomyces pyogenes</i>	isolate (42)	–
<i>Staphylococcus aureus</i>	isolate (CH-91)	–

mouse. After 24 hr inoculation, the mouse was sacrificed and its heart, lung, liver, kidney and spleen was harvested. These organs were triturated in 1 ml of TE buffer in a glass homogenizer and then heated at 95°C for 10 min. After centrifugation, the resulting supernatants were used in the PCR amplification as a template. However, the 270 bp-band of hemolysin gene was not detected in these organs. Moreover, the inoculated organism was not recognized in the homogenized organs by Giemsa's staining. Therefore, *C. septicum* was added in the homogenized organs of other mouse to a final concentration of 5×10^3 cells/ml and then

heated at 95°C for 10 min. After centrifugation, the resulting supernatants were used in the PCR amplification. As a result, the 270-bp band of hemolysin gene was observed in heart and lung, but was not in liver, spleen and kidney (data not shown). From the result, it is conjectured that inhibitors in enzymatic reaction of PCR amplification exist in the liver, spleen and kidney of mice. Therefore, further study is necessary to remove the inhibitors for the direct detection of *C. septicum* in organs by the PCR technique.

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