Rapid and Direct Detection of *Clostridium chauvoei* by PCR of the 16S-23S rDNA Spacer Region and Partial 23S rDNA Sequences

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ABSTRACT. *Clostridium chauvoei* causes blackleg, which is difficult to distinguish from the causative clostridia of malignant edema. Therefore, a single-step PCR system was developed for specific detection of *C. chauvoei* DNA using primers derived from the 16S-23S rDNA spacer region and partial 23S rDNA sequences. The specificity of the single-step PCR system was demonstrated by testing 37 strains of clostridia and 3 strains of other genera. A 509 bp PCR product, which is a *C. chauvoei*-specific PCR product, could be amplified from all of the *C. chauvoei* strains tested, but not from the other strains. Moreover, this single-step PCR system specifically detected *C. chauvoei* DNA in samples of muscle from mice 24 hr after inoculation with 100 spores of *C. chauvoei*, and in clinical materials from a cow affected with blackleg. These results suggest that our single-step PCR system may be useful for direct detection of *C. chauvoei* in culture and in clinical materials from animals affected with blackleg.

KEY WORDS: *Clostridium chauvoei*, PCR, 16S-23S rDNA spacer region.

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Clostridium chauvoei is an endospore-forming gram-positive bacterium, which causes blackleg, a fatal disease of cattle, sheep, and other ruminants associated with spore-contaminated soil. Blackleg needs to be distinguished from other severe diseases with similar symptoms, such as 'malignant edema' and anthrax caused by *Clostridium septicum*, *Clostridium novyi*, *Clostridium perfringens*, *Clostridium sor-dellii* or *Bacillus anthracis*. Typically, the diagnosis of blackleg depends on observation of clinical symptoms, followed by isolation and identification of the organism by immunological methods, such as immunofluorescence staining [10]. However, these diagnostic methods are time consuming and laborious.

Nucleic acid amplification of a specific target region of the bacterial genome by the polymerase chain reaction (PCR) is becoming widely used for detection and diagnostic purposes [18, 19]. The ability of PCR to amplify DNA specifically from low numbers of bacteria, as well as its simplicity, rapidity and reproducibility, offers advantages over conventional methods for identification. Kuhnert *et al.* [14] reported a PCR system for distinguishing *C. chauvoei* from *C. septicum* by using specific oligonucleotide primers for the 16S rRNA gene. This procedure is very effective in distinguishing *C. chauvoei* from *C. septicum*; however it requires restriction enzyme digestion of the PCR products for definitive identification of the organism, because the existence of only small differences in the 16S rRNA gene sequences, results in the primers amplifying the 16S rRNA genes of both bacteria. PCR amplification of the 16S-23S rDNA spacer region has been suggested as a target for a universal bacterial identification and typing system [3, 9, 12]. Recently, it has been reported that species-specific primers to *Actinobacillus seminis* were developed from the sequence of the 16S-23S rDNA

spacer region of *rrnB* for the identification and detection of *A. seminis* by PCR [1].

In the present study, we determined three distinct 16S-23S rDNA spacer regions and the partial 23S rDNA sequences of *C. chauvoei* and designed *C. chauvoei*-specific primers for the detection of *C. chauvoei*. To assess its applicability as a rapid and reliable diagnostic method for clinical blackleg in ruminants, we evaluated our single-step PCR system using specimens from mice experimentally infected with *C. chauvoei* and clinical materials from a cow affected with blackleg.

MATERIALS AND METHODS

Bacterial strains: Bacterial strains used are listed in Table 1.

DNA preparation: To isolate chromosomal DNA from bacteria, cells in 1 ml of a late logarithmic phase culture were pelleted by centrifugation and used as the source of chromosomal DNA. To isolate the DNA samples from mice and a cow, approximately 50 mg of each of the vital organs was homogenated and resuspended in 3 ml of phosphate buffered saline (PBS), then centrifuged at 800 × g for 10 min. The supernatant was centrifuged at 10,000 × g for 10 min. The pellets were washed once with in 1.5 ml of PBS, and resuspended in 150 µl of sucrose-TES (25% [w/v] sucrose, 10 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl) containing 4 mg/ml of lysozyme. The DNA extraction and purification was performed according to the methods of Takeuchi *et al.* [17].

DNA amplification and gel electrophoresis: PCR was performed in a reaction volume of 100 µl containing 1 × PCR buffer (Mg²⁺ free), 2.5 mM MgCl₂, 0.2 mM dNTP mixture, 2.5 units of LA Taq DNA polymerase (TaKaRa LA Taq kit; TaKaRa Shuzo Co., Ltd., Kyoto, Japan), 10 pmol of each

Table 1 The list of bacterial strains and results of PCR

Bacterial strains	Source	PCR results ^{d)}
<i>C. chauvoei</i>		
ATCC10092 ^{a)}	Type strain	+ (1)
ATCC11956		+ (2)
ATCC11958		+ (3)
ATCC11957		+ (4)
ATCC19399		+ (5)
Okinawa	Japanese vaccine strain	+ (6)
Chousen	Clinical isolated	+
Souya	Clinical isolated	+
Tochigi	Clinical isolated	+
Iwate	Clinical isolated	+
Ouita	Clinical isolated	+
Yamanashi	Clinical isolated	+
S4269A	Clinical isolated	+
C6H	Clinical isolated	+
<i>C. septicum</i>		
JCM8144 ^{b)}	Type strain	– (7)
NCTC551 ^{c)}		– (8)
NCTC286		– (9)
NCTC282		– (10)
NCTC504		–
44	Japanese vaccine strain	–
<i>C. novyi</i>		
JCM1406	Type strain	– (11)
ATCC25758	Type B	–
CN1025	Japanese vaccine strain	–
ATCC19402	Type A	–
<i>C. perfringens</i>		
JCM1290	Type strain	– (12)
PB6K	Type A	–
<i>C. sordelli</i>		
JCM3814	Type strain	– (13)
CL-2	Clinical isolated	–
<i>C. haemolyticum</i>		
ATCC9650	Type strain	–
ATCC9652		–
<i>C. carnis</i>		
JCM1393	Type strain	– (15)
<i>C. difficile</i>		
JCM1296	Type strain	–
<i>C. tetani</i>		
T3	Clinical isolate	–
harvard	Clinical isolate	–
<i>C. sporogenes</i>		
80011	Clinical isolated	–
<i>C. acetobutylicum</i>		
JCM1419	Type strain	–
<i>C. butylicum</i>		
Miyairii		–
<i>B. anthracis</i>		
17JB	Japanese challenge strain	– (14)
34F ₂	Japanese vaccine strain	–
<i>B. subtilis</i>		
ATCC6633		–

a) ATCC, American Type Culture Collection, USA.

b) JCM, Japan Collection of Microorganisms, Japan.

c) NCTC, National Collection of Type Culture, UK.

d) (), Lane no. in Fig. 2.

primer, and 1 μ l of each template DNA solution. The primers are shown in Table 2 and Fig. 1C. Cycle conditions were

an initial 5 min denaturation at 94°C, followed by 30 cycles, each consisting of 1 min denaturation at 94°C, 1 min annealing at 54°C, and 1.5 min extension at 72°C. These 30 cycles were followed by 7 min extension at 72°C. The amplified products were separated by electrophoresis in 2.0% (w/v) agarose gels and stained with ethidium bromide. The identity of the PCR products was confirmed by cleavage with *Hind* III (TaKaRa Shuzo Co., Ltd., Kyoto, Japan).

Sequencing of the PCR-amplified 16S-23S rDNA spacer region genes and partial 23S rRNA gene: The amplified products of *C. chauvoei* type strain ATCC10092^T (T = Type strain) were recovered from an agarose gel and were cloned into the pCR II plasmid vector using the TA cloning system version 2.2 (Invitrogen Corp., San Diego, Calif.) according to the manufacture's instructions. Automated DNA sequencing of samples of purified plasmid DNA were performed on a DNA sequencer (model 373A, PE Biosystems, Co., Ltd., U.S.A.). The *rrnA* sequences of the other strains and partial 23S rDNA gene sequence of the type strain were determined by direct sequencing. All the sequences were confirmed by sequencing both strands. The sequences have been registered in the DDBJ database under the accession numbers: AB040539 (23S rRNA gene), AB040540 (*rrnA*), AB040541 (*rrnB*), and AB040542 (*rrnC*).

Detection of *C. chauvoei* DNA in mice experimentally infected with *C. chauvoei* and in a cow affected with blackleg: Eight-week-old female ddY mice were supplied by SLC, Co., Ltd. (Shizuoka, Japan). Three mice were inoculated in the middle of the right thigh with 100 spores of ATCC10092^T in 0.25 ml of 6% (w/v) calcium chloride solution. Mice were sacrificed to obtain samples at 24 hr after inoculation. Clinical materials (muscle, blood and liver) were collected from a cow that died in an outbreak of blackleg in Tochigi Prefecture, Japan. The cow was diagnosed as having blackleg by clinical and pathological findings and *C. chauvoei* were detected in the clinical materials by the immunofluorescence technique. Samples from the cow affected with blackleg were cultured on CLA agar [2] supplemented with 5% (v/v) defibrinated sheep blood (CLA medium), and in CLB broth [2] under an anaerobic condition. Colonies forming a hemolytic zone were picked, and then cultured three times in a new CLA medium. The isolated cells were used as the source of chromosomal DNA. Muscle samples from a normal mouse and a normal cow were used as negative controls. DNA isolation and PCR were performed as described above.

RESULTS

16S-23S rDNA spacer regions of *C. chauvoei*: The PCR was performed with total DNA from 3 strains of *C. chauvoei* including type strain ATCC10092^T using the primers 16-23S and 16-23AS. As shown in Fig. 1A, all the *C. chauvoei* strains had at least three sizes of PCR products. The bands of 286 bp, 363 bp and 441-bp were designated as *rrnA*, *rrnB* and *rrnC*, respectively. The PCR products of type strain ATCC10092^T were sequenced (Fig. 1B). The three PCR products share common sequences, however DNA

Fig. 1. (A) 16S-23S rDNA spacer region patterns of *C. chauvoei* strains. PCR was done using the primers 16-23S and 16-23AS. Lane M: molecular size markers (100 bp DNA ladder), lane 1: ATCC10092^T, lane 2: ATCC11958, lane 3: ATCC11956. (B) Sequences of three spacer regions. 1–30, 16S rDNA partial sequence; 131–206, tRNA^{Ala} sequence; 209–285, tRNA^{Ile} sequence; 413–441, 23S rDNA partial sequence. (C) Structure of the 16S–23S rDNA spacer regions of *C. chauvoei*., Deleted region. ■, 16S-23S rDNA spacer region.

Table 2. Primers used for amplification and/or sequencing

Primer	Sequence	Region	Position
16-23S	5'GAGAACCTGCGGCTGGATC 3'	16S	1381-1499 ^{a)}
16-23AS	5'TTCACCATGCGCCCTTTGTAG 3'	23S	28-8 ^{b)}
LR488	5'CCTTTCCTCACGGTACTG 3'	23S	520-502 ^{c)}
IGSC4	5'GAATTAACAACACTTTATTAACAAATG 3'	16S-23S rDNA spacer region	308-334 ^{d)}
23UPCH	5'GGATCAGAACTCTAAACCTTTCT 3'	23S	403-381 ^{e)}

Positions according to: a) the 16S rDNA sequence from *C. chauvoei* (Genbank accession no. U51843), b) the 23S rDNA sequence from *C. perfringens* (Genbank accession no. M69264), c) the primer was reported by Gürtler [7], d) the aligned spacer sequence for *C. chauvoei* (Fig. 1B), e) the 23S rDNA sequence from *C. chauvoei* (AB040539)

sequences with 77 and 155 nucleotides were inserted between the common sequences of *rrnB* and *rrnC*, respectively (Fig. 1C). The *rrnC* contained nucleotide sequences of alanine tRNA (tRNA^{Ala}) and isoleucine tRNA (tRNA^{Ile}) between nucleotide positions 131 and 206, and between nucleotide positions 209 and 285. The *rrnB* had a tRNA^{Ile} gene between nucleotide positions 209 and 285, respectively. The tRNA^{Ala} and tRNA^{Ile} sequences show 79% and 95% identity with the those of the related gram-positive bacteria *Staphylococcus aureus* (Genbank accession no. U11781), respectively. The *rrnA* had no tRNA genes. In short, *rrnA* is a fundamental 16S-23S rDNA spacer region in *C. chauvoei*, and *rrnB* or *rrnC* was inserted with one or two tRNA genes into this fundamental region, i.e. the *rrnA* (Fig. 1C). In order to confirm that the *rrnA* sequence is conserved in *C. chauvoei* strains, the *rrnA* sequences of five other strains (Okinawa, ATCC11958, ATCC11956, Souya and Chousen) were also sequenced, and they were completely identical with the *rrnA* sequence of ATCC10092^T (data not shown). This result revealed that *C. chauvoei*-specific primers could be designed from the *rrnA* sequence.

Design of *C. chauvoei*-specific primers: The sequences of the 16S-23S rDNA spacer regions of *C. chauvoei* were found partially conserved in many bacteria, including clostridia, according to the database (data not shown). The similarity was confined mainly to the 16S and 5' end of 23S rRNA genes and the tRNA gene. *C. chauvoei*-specific sequences of the 16S-23S rDNA spacer region genes were too narrow as a target for a single-step PCR system. Therefore, in order to establish *C. chauvoei*-specific primers for a single-step PCR system, the partial 23S rDNA sequence was determined with total DNA from ATCC10092^T using the primers 16-23S and LR488. Two sequences, IGSC4 and 23UPCH, were chosen as specific primers (Table 2). The IGSC4 was designed from the sequences of the 16S-23S rDNA spacer regions, and 23UPCH was designed from the partial 23S rDNA sequence (Fig. 1). The size of the PCR product predicted with these primers was 509 bp. The PCR product from total DNA of ATCC10092^T was sequenced and was identified as the target sequence (data not shown). To assess its specificity, our single-step PCR system was performed with total DNA from 37 strains of clostridia and 3 strains of other genera. All of the 14 *C. chauvoei* strains had a 509 bp PCR product, which was

not amplified from the other bacteria tested (Fig. 2A and Table 1). In order to confirm that the PCR products were the target sequences, these PCR products were digested with the restriction enzyme *Hind* III. As shown in Fig. 2B, two bands of the expected lengths (330 bp and 179 bp) were recognized.

Detection of *C. chauvoei* DNA in mice experimentally infected with *C. chauvoei* and in a cow affected with blackleg: To confirm the applicability of the single-step PCR system using primers IGSC4 and 23UPCH, for routine diagnosis of blackleg disease, we examined samples from mice infected with *C. chauvoei* (Fig. 3A). Mice were sacrificed at 24 hr after inoculation with 100 spores of ATCC10092^T. Samples of affected muscle were collected from three mice infected with *C. chauvoei*. All of the samples from the infected mice yielded the 509 bp PCR product, however a sample from a negative control mouse did not (Fig. 3A). The single-step PCR system described above was also applied to samples from a cow affected with blackleg (Fig. 3B). Although a sample from the liver had a faint band of 509 bp PCR product, samples from the muscle and blood had strong bands of 509 bp PCR product. The isolate from the liver named Tochigi also had a band of 509 bp PCR product. A sample from a negative control cow did not have the PCR product.

DISCUSSION

For detection of human and animal pathogens by PCR, most studies have targeted either species-specific genes, such as the toxin genes of *C. perfringens* [4, 11, 21], the alpha-toxin gene of *C. septicum* [17] and the urease subunit gene of *Helicobacter pylori* [5]. However there are no reports of the gene sequences of *C. chauvoei*, except for the 16S rRNA gene sequence. In general, it is difficult to identify species-specific genes. On the other hand, the rRNA genes are essential for the survival of all organisms and are highly conserved in the bacterial and other kingdoms. It is easy to determine the 16S-23S rDNA spacer region sequences using common primers designed for the highly conserved regions of the 16S rRNA gene and the 23S rRNA gene sequences. Therefore, we determined the 16S-23S rDNA spacer region sequences of *C. chauvoei*. We clarified that there are at least three rRNA operons in *C. chauvoei* (Fig. 1). The number of rRNA

Fig. 2. (A) PCR amplification of a 509 bp fragment from total DNA purified from cultures. Lanes 1-6: *C. chauvoei* strains, lanes 7-10: *C. septicum* strains, lane 11: *C. novyi*, lane 12: *C. perfringens*, lane 13: *C. sordellii*, lane 14: *B. anthracis*, lane 15: *C. carnis*. PCR was done with the *C. chauvoei* specific primers IGSC4 and 23UPCH. (B) *Hind* III-digestion of the PCR products. Lane numbers correspond to organisms as labeled in the far-right column of Table 1.

operons has been completely determined in some eubacteria by Southern hybridization or PCR of the rRNA genes. For example, *Escherichia coli* has seven operons [15], *S. aureus* has 9 operons [8], *C. perfringens* [6] and *C. difficile* have 10 rRNA operons [7]. Therefore, it is possible for *C. chauvoei* to have more than three operons. We designed PCR primers specific to the region of the 16S-23S rDNA spacer region and 23S rDNA sequences of *C. chauvoei*. Our single-step PCR system was confirmed as distinguishing *C. chauvoei* from the causative microorganisms of 'malignant edema' and anthrax, such as *C. septicum*, *C. novyi*, *C. perfringens*, *C. sordellii* and *B. anthracis* without restriction digestion of the PCR products (Table 1 and Fig. 2). *C. chauvoei* is especially difficult to distinguish from *C. septicum*. It has been reported that *C. chauvoei* and *C. septicum* have various common antigens [10, 16, 20] and the similarity of the 16S rRNA gene sequence between *C. chauvoei* and *C. septicum* is 99.3% [13]. Moreover, there are problems in diagnosis, since disease caused by *C. septicum* shows very similar symptoms to blackleg. In this study, all of the *C. chauvoei* strains had the 509 bp PCR product, none of the *C. septicum* strains had a PCR product. This result suggests that the 16S-23S rDNA spacer region sequences might be less conserved than 16S

rRNA gene sequences between *C. chauvoei* and *C. septicum*, and that *C. chauvoei* can be distinguished from *C. septicum* by the difference in the 16S-23S rDNA spacer regions.

The isolation of *C. chauvoei* is very difficult, since *C. chauvoei* requires hard anaerobic conditions, and clinical specimens are often contaminated with other anaerobic bacteria including clostridia in soil, which grow faster than *C. chauvoei* in a culture medium. Indeed, in this study, the bacterial cells isolated from the infected muscle and blood in CLB broth did not have a 509 bp product, and the cells did not produce hemolytic zones on CLA medium (data not shown). Kuhnert *et al.* [14] also reported that the primary culture of samples on a blood agar plate was overgrown with the concomitantly isolated *C. septicum*, but gave a clear result for *C. chauvoei* in their PCR. Since blackleg is an acute infection, and most animals succumb to this disease due to acute septicemia with a high rate of mortality, rapid and direct detection systems of *C. chauvoei* without culture are desirable. Our single-step PCR system can be used as a method for identification of *C. chauvoei* (Table 1 and Fig. 2), and for direct detection of the bacteria in clinical materials from blackleg (Fig. 3B). Our study shows that the 16S-23S rDNA spacer regions of *C. chauvoei* have the potential to be

Fig. 3. (A) Detection of *C. chauvoei* DNA from mice experimentally infected with *C. chauvoei* by the single-step PCR system. Lane 1: culture of *C. chauvoei* ATCC10092^T (positive control), lane 2: sample from an uninfected mouse (negative control), lanes 3-5: samples from mice infected with *C. chauvoei*. (B) Detection of *C. chauvoei* DNA from clinical materials by the single-step PCR system. Lane 1: culture of *C. chauvoei* ATCC10092^T (positive control), lane 2: sample of muscle from an uninfected cow (negative control), lane 3: sample of blood from a cow infected with blackleg, lane 4: sample of muscle from the cow, lane 5: sample of liver from the cow, lane 6: culture of liver from the cow.

used as identification markers of *C. chauvoei*. Especially, *rrnA* is a fundamental 16S-23S rDNA spacer region in *C. chauvoei*, which could possibly be used for determining *C. chauvoei* DNA from clinical materials.

Comparison of the 16S-23S rDNA spacer region sequences of the causative clostridia of gas gangrene, such as *C. septicum*, *C. novyi*, *C. sordellii* and *C. perfringens*, enabled the design of species-specific oligonucleotide primers for use in PCR-based detection. This single-step PCR system may serve as an improved diagnostic method for *C. chauvoei* infection based on molecular genetics.

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