Isolation and PCR-based detection of anaerobic *Clostridium bifermentans* isolated from slaughterhouse soil

Clostridium bifermentans is an obligate, anaerobic, spore-forming bacillus that usually stains Gram-positive in the early stages of growth[1]. They are ancient organisms that live in virtually all of the anaerobic habitats of nature where organic compounds are present, including soils, aquatic sediments and the intestinal tracts of animals[2]. *C. bifermentans* was first isolated from putrefying butcher's meat in 1902 by Tissier and Martelly[3]. It causes a variety of human infections, including soft-tissue infections and bloodstream invasion[4], gas-gangerene in association with enteritis and enterotoxaemia in animals, septic arthritis[5], necrotizing pneumonia, empyema[6] and osteomyelitis[7]. The characteristic that makes *C. bifermentans* strains significant is 7α-dehydroxylation property that converts cholic acid and cheno deoxycholic acid to deoxycholic acid and lithocholic acid respectively, in the human intestine[8]. *Clostridium* comprises approximately 200 closely related species. Due to maximum similarity, it is difficult to differentiate them from each other on the basis of biochemical and morphological characteristics. The technique of molecular biology may thus be an useful tool for differentiation and correct identification of the species. In the present study we used soil samples from a slaughterhouse for isolation of *C. bifermentans*. Identification of the bacterium was carried out by duplex polymerase chain reaction (duplex-PCR) using two newly designed oligonucleotides for: (1) the triose phosphate isomerase gene (*tpi)*, and (2) the phospholipase C genes, which encode for the lecithinase toxin of *C. bifermentans*. The designed primers were specific to *C. bifermentans* species and gave positive results, whereas negative results were obtained for the same genes in other *Clostridium* species. On the basis of morphological and biochemical studies, lecithinase-positive colonies were selected for DNA extraction and PCR amplification. Phospholipase C and *tpi* genes were amplified in 10 isolates out of the selected strains and gave the desired prominent band with both primers.

Selective medium was used to isolate *C. bifermentans.* One gram of soil sample (n = 6) collected from different locations of the slaughterhouse was serially diluted in sterile, anaerobically prepared phosphate buffer saline (PBS). Each dilution was inoculated in 30 ml serum vial of anaerobically prepared tryptone peptone glucose yeast extract broth (TPYG; pH 7.2), thoroughly mixed and incubated at 37°C for 5–7 days under anaerobic conditions (N_2 – 80%, CO_2 – 15%, H_2 – 5%). The culture was plated on egg yolk agar (EYA) plate followed by 3 days incubation at 37°C under anaerobic condition in an anaerobic jar. The lecithinase-positive colonies were picked and inoculated in TPYG broth medium. After three days of growth Gram staining and morphological studies were carried out. For biochemical characterization, the cultures were grown in TPYG medium at 37°C and the tests were performed as described by Holdman *et al.*[9]. The genomic DNA was extracted from selected isolates using Qiagen kit (USA) according to the procedure provided by the manufacturer.

Two new gene-specific primers were designed and synthesized by Fermentas (USA) to target the gene of interest in *C. bifermentans* isolates. Oligonucleotides were designed on published DNA sequences of *C. bifermentans*. The primers were selected from the unique genomic regions of the species (Table 1).

The primer sequences for the corresponding target gene are shown in Table 1. Both specific primers were determined using the Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov/BLAST/) program against similar sequences of microbial genomes. By the above study it was ensured that no homology was observed with known gene sequences of other *Clostridium* species. The cross-reactivity of each primer set was also evaluated by testing the DNA of non-target *Clostridium* species, which were closely related to *C. bifermentans*.

A duplex PCR was performed (to detect fragments of *tpi* and phospholipase C genes) in 25 µl reaction mixture. The mixture contained 10× PCR buffer, 10 mM dNTPs mixture, 2.5 mM $MgCl_2$, 10 pmol of each primer (MBl, fermentas, USA), 0.5 unit *Taq* DNA polymerase and 1 µl of template DNA. PCR was performed in a thermal cycler (Biometra, Germany) programed by initial denaturation step at 94°C for 5 min followed by 35 cycles of amplification at 94°C for 1 min, 55°C for 1 min and 72°C for 2 min, and then final extension at 72°C for 10 min in order to allow the completion of DNA extension. The standard strain ATCC 638 of *C. bifermentans* was used as positive control in PCR amplification.

On the basis of lecithinase-positive colonies, 30 isolates were selected for characterization. Under light microscope the isolates were found to be Gram-positive, straight or slightly curved rods in pairs or as single cells. All the strains grew between 20°C and 45°C with the optimum temperature for growth at 30–37°C. The optimum pH for growth was reported as 7.2. All 30 isolates were able to ferment many sugars like lactose, glucose, fructose, mannitol, inulin, mannose and sorbitol. None of the isolates fermented dextrose, galactose, maltose, adonitol, rhamnose and cellobiose, and only 60% and 30% isolates were able to ferment melibiose and rhamanose respectively. Only 13 isolates were positive for indole production and gelatin liquefaction

Table 1. Description of designed primers for PCR

Target gene (primers)	Primer sequence (5′–3′)	T_m (°C)	Location	Amplicon size (bp)
Phospholipase gene (*cbp*)	F-ATGAATGGGAAAAGGGGAAT	53.2	434–454	203
	R-CCCTGATGTAGTGTCGTTTCC	59.8	616–637	
Triosephosphate isomerase (*tpi* gene)	F-GCACTATGCAGATAATGGAGCCA	58.4	153–175	252
	R-GCTTTCTCAGTTTGCACTTTACAA	57.6	381–405	

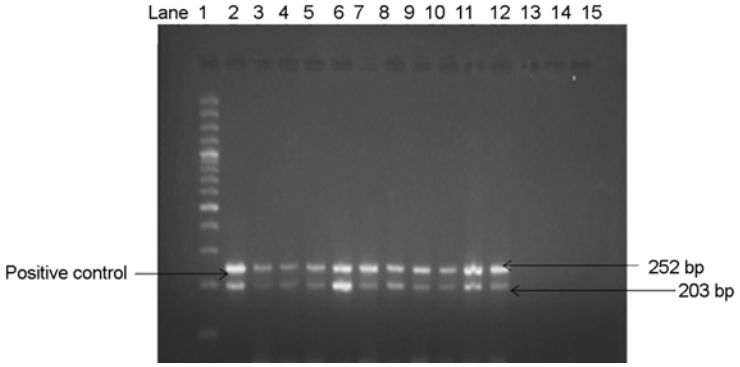

Figure 1. Duplex PCR products using DNA template of isolated *Clostridium bifermentans* with *dop* and *tpi* gene primers. Lane 1, 100 bp ladder; lane 2, Positive control and lanes 3–15, Isolated strains of *C. bifermentans.*

test, while they were negative for nitrate reduction and urease test. Using morphological and biochemical characterization we concluded that the 13 isolates gave maximum similarity with *C. bifermentans* when compared with standard culture, ATCC 638.

Duplex PCR assay was used for the rapid detection and confirmation of the strains. PCR is observed as a discriminatory technique for identification of *C. bifermentans*. The selected 13 isolates were subject for duplex PCR with gene-specific primers. Both gene-specific primers have shown specific amplification in PCR, and amplicons of expected size (203 and 252 bp) were obtained (Figure 1). Out of 13, only 10 isolates were found positive for *cbp* and *tpi* gene in the duplex PCR. The main purpose of this study is to develop an easy and reliable methodology for the diagnosis and differentiation of *C. bifermentans* species from the other species of *Clostridium.*

The entire Gram-positive, rod-shaped, strictly anaerobic bacteria isolated from soil samples of a slaughterhouse were identified as *C. bifermentans*. Most of the *Clostridium* species, i.e. *C. perfringens*, *C. novyi*, *C. septicum*, *C. histolyticum*, *C. botulinum* and *C. bifermentans* share biochemical and morphological characteristics and were also reported in the soil samples. It is difficult to differentiate *C. bifermentans* from the others on the basis of morphology and a biochemical test. Thus we used a molecular technique (PCR-based detection) to confirm the identity of the isolates. In the present study, specific primes (*cbp* and *tpi*) in one set have shown specific amplification with isolated DNA of isolates, and amplified products of expected sizes were obtained in duplex PCR. The size of the amplified genes, *cbp* and *tpi* was 203 and 252 bp respectively. The *cbp* gene confirmed lecithinase production, while *tpi* was reported as a housekeeping gene in *C. bifermentans* species.

Detection of microorganisms using traditional culture-based methods is laborious, less sensitive[10] and not able to differentiate closely related species by phenotypic traits. Nowadays, different molecular techniques are available for the diagnosis of many types of bacteria from different types of sample. PCR could be an interesting alternative to consider, not for identification of the bacteria, but also to know the toxigenic characteristics of the isolates. The major advantages of PCR are its rapidity, sensitivity and the possibility to detect any type of microorganism. Different PCR-based techniques have also been applied for the detection of other pathogenic Clostridia in animal faeces, such as *C. perfringens*[11–13] and *C. botulinum*[14,15]. However, to the best of our knowledge no such application of similar techniques to detect *C. bifermentans* in faecal, medical or environmental samples has been reported.

Gene-specific, primer-based PCR technique is popular for identification of bacterial species. This technique is highly reproducible and successfully differentiates the strains. By the above experimental study, we concluded that PCR-based detection using gene-specific primers is a sensitive and reliable method for detection of *C. bifermentans* species. The technique is also described as an advanced approach for identifying bacterial species of the same genus.

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