

DETECTION OF BOVINE *Clostridium perfringens* BY POLYMERASE CHAIN REACTION

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ABSTRACT. A polymerase chain reaction (PCR) assay to detect *Clostridium perfringens* alpha toxin gene (cpa) was used to identify eighty-nine *C. perfringens* strains obtained from bovine clinical material. The strains were biochemically characterized as *C. perfringens*. The isolated strains were cultured on plates containing brain heart infusion agar with 5% sheep blood under anaerobic conditions. DNA extraction was performed by boiling. The 324 bp amplification product of cpa was observed in all isolates. *C. sordellii*, *C. botulinum*, *C. novyi*, and *C. septicum* were also tested but did not produce any alpha toxin gene amplification. These findings suggest that PCR is a useful assay in identifying *C. perfringens* toxin types.

KEY WORDS: *Clostridium perfringens*, PCR, alpha toxin, polymerase chain reaction.

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INTRODUCTION

Clostridium perfringens is a widely distributed pathogen known to cause many human and animal diseases. Domestic animals are known to be sources of human food poisoning; to decrease or eliminate this risk, strategies must be developed to prevent infected animals from entering the food chain (6,10,16).

C. perfringens produces a variety of exotoxins; four of these - alpha, beta, epsilon, and iota - are considered the major toxins and are used to group the bacteria into five types A, B, C, D, and E. Alpha toxin is produced by all strains and is involved in disease pathogenesis (15).

Organism typing is accomplished by culture filtrates with type-specific antisera; these are difficult to find, extremely expensive, time consuming, and ethically unacceptable for tests in experimental animals (12,13). The use of polymerase chain reaction (PCR) can avoid these problems and may be used to differentiate *C. perfringens* into its five toxin types (17,20).

In this study, 89 *C. perfringens* isolates that produced animal disease were submitted to PCR assay using primer sets to detect the presence of genes encoding alpha toxin.

MATERIALS AND METHODS

Strains

Samples obtained in the *post-mortem* examination of 144 bovines clinically suspected of having enterotoxaemia were analyzed for the presence of anaerobic microorganisms. This led to the isolation of 89 strains of *Clostridium* spp, biochemically identified as *C. perfringens* (1). We also used type A *C. perfringens* standard strain from the American Type Culture Collection (ATCC), ATCC 3624 as positive control, and *C. sordellii*, *C. botulinum*, *C. novyi*, and *C. septicum* strains to show reaction specificity.

A 3.0 µL aliquot from the positive control and each one of the isolated strains was streaked in a plate containing Brain Heart Infusion (BHI) agar with 5% sheep blood and incubated under anaerobiosis in McIntosh & Fields jars at 37°C for 18 to 24 hours.

After incubation, colonies were observed in relation to aspect, color, presence and type of hemolysis, and bacterial morphology, which was microscopically assessed in Gram-stained smears. Five colonies from each plate that were shiny, presented double hemolysis straight edges, and were identified as Gram-positive bacilli; they were collected in physiological saline solution to be analyzed.

Sample preparation

One to five *C. perfringens* colonies obtained from BHI blood agar cultures were centrifuged at 13,000Xg for 5 min, and the pellet was suspended in 50 µl of 10mM Tris- HCl, pH 9.0, 50 mM KCl, 2% Triton X-100. The mixture was vortexed, boiled for 10 min for cell lysis, centrifuged at 13,000 X g for 2 min, and 5 µl of the supernatant was used as a template.

Polymerase chain reaction – PCR

Alpha toxin gene (cpa) oligonucleotide primers were selected from published sequences (19): 5' GCT AAT GTT ACT GCC GTT GACC 3' and 3' TCT GAT ACA TCG TGT AAG 5'. PCR reactions were performed at a final volume of 50 µL with the following reagents: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 50 mM MgCl₂, 200 µM dNTP, 10 pmol of each primer, 2U *Taq* DNA polymerase, and 5 µl of template DNA. Amplification mixture was incubated in a thermalcycler PTC-200 DNA Engine. Samples were denatured at 95°C for 5 minutes and then submitted to 35 cycles of 1 min at 94°C, 1 min at 53°C, and 1 min at 72°C, with a further extension cycle of 10 min at 72°C. *C. perfringens* type A ATCC 3624 was used as positive control and water as negative control.

Electrophoresis

For the detection of the PCR products, 10 µL sample of amplified DNA was examined by electrophoresis in 2 % agarose gel with TBE 0.5 X (0.045M Tris-borate and 1mM EDTA, pH 8.0) running buffer. Gel was stained with 0.5 µg/mL ethidium bromide. Molecular sizes were determined based on a 100 bp ladder molecular mass marker. The reaction products were analyzed and photographed under UV light.

PCR specificity

Total genomic DNA of *C. botulinum*, *C. sordellii*, *C. septicum*, and *C. novyi* were used in PCR reactions as described above.

RESULTS

A total of 89 *C. perfringens* isolates were genotyped using PCR. Figure 1 shows the 324 bp-amplified fragment in agarose gel electrophoresis. The gene encoding alpha-toxin (cpa) was detected in the reference strain (type A ATCC 3624) and in all isolated field strains. None of the PCR assays using DNA from *C. botulinum*, *C. sordelli*, *C. septicum*, and *C. novyi* produced this 324 bp fragment, which is uniquely associated with *C. perfringens*.

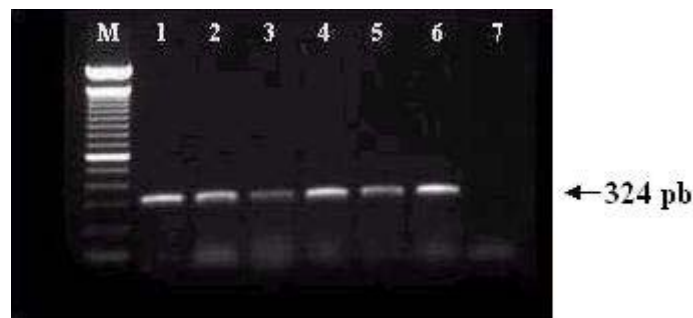


Figure 1. Detection of *C. perfringens* alpha toxin gene amplified by PCR. M= 100 bp molecular weight marker ladder; 1-5= clinical samples; 6= *C. perfringens* ATCC 3624 (Type A); 7= negative control.

DISCUSSION

Enterotoxaemia usually occurs in bovine when prophylactic measures are not observed. The presence of *C. perfringens* is also a public health problem since humans can be intoxicated by ingesting contaminated meat. *C. perfringens* toxin A is a powerful toxin and infection may result in myonecrosis, hemolysis, and increased vascular permeability. The major lethal effects associated with this toxin are gas gangrene in humans and necrotic enteritis and enterotoxaemia in animals (4,9,14).

It has been identified by seroneutralization in laboratory animals, using specific antisera. This toxin-typing technique requires continuous supply of laboratory animals and use of monovalent diagnostic sera, which are increasingly difficult to find and extremely expensive. Moreover, toxin-typing results are obtained in less than 24 or even 48 h observation (7,18). The use of PCR as a diagnostic assay for detection of toxins producing *C. perfringens* may offer considerable advantage over the above techniques (3,5,8,11).

In this study, alpha toxin gene was found in *C. perfringens* reference strain type A (ATCC 3624) and in all field isolates tested. No other *Clostridium* spp gave rise to any amplification of the alpha toxin gene. These results confirm observations made by other authors (2) using PCR amplification to show that alpha toxin gene is specific to *C. perfringens* species. Thus, the alpha toxin gene PCR is suggested as a diagnostic method for confirmation of *C. perfringens* species, providing a good alternative to the time-consuming and specific mouse neutralization test normally used in laboratory routine.

Prophylaxis of enterotoxaemia in animals is achieved by vaccination; PCR technique can thus become a first-choice tool for identification and typing of *C. perfringens* strains that cause disease. In turn, this would simplify the development of vaccines according to epidemiological situation (7).

In this trial, *C. perfringens* field isolates were not random and, therefore, may not accurately represent the distribution of toxin types in animals or disease conditions. However, the predominance of alpha toxin isolates suggests the importance of this agent in animal disease in Brazil. Additional studies must be performed with specific primers to detect other toxins to know the real prevalence of *C. perfringens* types.

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