Study reports on comparisons between polymerase chain reaction (PCR) and conventional diagnostic methods for typing *Clostridium perfringens* toxins collected from Central Saudi Arabia.

Methods: Fecal samples from 150 animals showing signs of enterotoxemia were collected from 24 April 2009 to 25 September 2009, from different farms located in Riyadh, Kingdom of Saudi Arabia. Twenty-seven toxigenic strains of *Clostridium perfringens* were recovered from 150 fecal and intestinal content samples.

Results: The results revealed alpha toxin gene *Clostridium perfringens* type A in 22 (81.5%) strains out of 27 toxigenic strains, however, only 20 (74.1%) of them were identified previously as type A by classical method.

Conclusion: Polymerase chain reaction technique can be used as an alternative diagnostic method for detection and typing of *Clostridium perfringens*. The study concluded that the PCR technique can be a valuable tool for identifying and typing *Clostridium perfringens* toxins in fecal samples from animals in Central Saudi Arabia.
Clostridium perfringens (C. perfringens) is an important pathogenic agent causing, among other diseases, enteritis in humans and enterotoxemia in domestic animals. The pathogenicity of this organism is associated with several toxins. The alpha, beta, epsilon and iota toxin are the major lethal toxins produced by the organism and are closely related to its virulence. Usually, C. perfringens has been classified into 5 toxigenic types (A-E) on the basis of its ability to produce the major lethal toxins. Type A strains are the most commonly encountered and produce food poisoning and gas gangrene in humans and animals, and necrotizing colitis and enterotoxemia in horses.

Methods. Fecal samples from 150 animals, 47 diarrheic calves (age ranged from 1-4 months) showed signs of enterotoxemia, 34 adult sheep and 34 lambs (age from 1-12 weeks) showed signs of diarrhea and enterotoxemia were collected from different farms at different localities in Riyadh, Kingdom of Saudi Arabia. As well as 35 samples from intestine of broiler chickens exhibited diarrhea and showed clinical signs of necrotic enteritis were collected from 24 April 2009 to 25 September 2009. The samples from sick and freshly dead animals were collected in plastic bags and were transported refrigerated to the laboratory where they were processed within 4 hours of collection.

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incubation, one of each culture was Centrifuge at 5000xg, 5min, then the sediment was washed 5 times with sterilized phosphate buffered saline, pH 7.2 (PBS) and finally suspended in 500 µl of sterilized PBS. The suspension was kept at 95°C for 15 minutes, and after centrifugation at 15,000 rpm for 5 minutes, 10 µl of the supernatant was directly used for PCR. The extracted DNA of the standard strains and of the bacterial isolates yielded from bacteriological examination was tested by PCR using each primer set. Concurrently, the crude DNA extracted from each fecal sample tested by the same primer pairs. All reactions were carried out in a final volume of 50 µl in micro-amplification tube (PCR tubes). The reaction mixture was adjusted according to Garmory et al.\textsuperscript{11} The samples were subjected to 35 PCR cycles, each consisting of 30 second of denaturation at 94ºC, 30 second of annealing at temperature according to the type of toxin and 1.5 minute of extension at 72ºC. Final extension was carried out at 72ºC for 10 minutes, and the PCR products were stored in thermal cycler at 4ºC until they were collected. The PCR products were visualized by agarose gel electrophoresis, according to Moussa and Shibli.\textsuperscript{16} Calculation of the fragment size was performed at the National Center of Biotechnology Information website using NCBI BLAST software.\textsuperscript{19}

**Results.** Bacteriological examination and typing of toxigenic *Clostridium perfringens* isolates of 150 fecal using mice neutralization test and dermonecrotic test in guinea pigs are summarized in Table 1. Detection toxin producing *Clostridium perfringens* strains depending on Nagler’s reaction and pathogenicity in guinea pigs indicates that 27 *C. perfringens* strains (87.1%) out of the 31 tested isolates were toxigenic and 4 (12.9%) were non-toxigenic (Table 1).

In the present investigation, types of *Clostridium perfringens* isolates recovered from feces and intestinal contents of different sources by PCR using alpha, beta, epsilon and iota were undertaken. Also, attempts to use this technique to detect these genes in intestinal contents and feces were directly described.

Firstly, the specificity of the oligonucleotide primers was confirmed by the positive amplification of only toxin genes from the extracted DNA of *C. perfringens* without non-specific amplification of other standard enteric bacterial strains. In order to compare the traditional typing and PCR, fecal and intestinal contents samples were examined using PCR for the presence of alpha, beta, epsilon and iota toxins genes. Polymerase chain reaction methods may detect all the bacteriologically positive samples for *C. perfringens* (n=27 [100%]). Moreover, this methods may also detect the alpha toxin gene in other 2 samples (1.33%) previously revealed negative isolation of *C. perfringens* by bacteriological examination. The 27 *C. perfringens* isolates were recovered from the bacteriological examination of feces and intestinal contents of different isolated and were typed using PCR for the presence of alpha, beta, epsilon and iota toxins genes.

Positive amplification of 1167 bp fragments of alpha toxin gene was observed in 22 (81.48%) strains and identified as type A using the PCR method as shown in Table 2 and Figures 1 & 2; however, 20 strains (74.07%) were previously identified as type A by conventional methods. None of the isolates were found to be iota producers, one strain (5.56%) was identified as B and showing positive amplification of 1025 bp fragment of beta toxin, 961 bp fragment of epsilon toxin gene and 1167 bp fragment of alpha toxin gene by PCR typing, which were consistent with conventional typing by animal test as shown in Figure 1. Moreover, only 1 strain (3.7%) was identified as C and 3 strains (11.11%) were identified as type D by PCR typing as shown in Figure 2.

**Discussion.** *Clostridium perfringens* has been identified as an important agent of different diseases including gas gangrene, food poisoning, and diarrhea as well as enteritis and fatal enterotoxemias in domestic animals and humans.\textsuperscript{19} Bacteriological examination of fecal and intestinal contents samples of diseases and freshly dead animals was 20.7%. The bacteriological

<table>
<thead>
<tr>
<th>Sources and number of samples</th>
<th>Positive samples</th>
<th>Toxigenic isolates</th>
<th>Types of toxigenic isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Type A</td>
</tr>
<tr>
<td>Calves (n=47)</td>
<td>11 (23.4)</td>
<td>9 (29.0)</td>
<td>8 (88.9)</td>
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<tr>
<td>Adult sheep (n=34)</td>
<td>9 (26.5)</td>
<td>8 (25.8)</td>
<td>4 (50.0)</td>
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<tr>
<td>Lambs (n=34)</td>
<td>5 (14.7)</td>
<td>5 (16.1)</td>
<td>4 (80.0)</td>
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<tr>
<td>Broiler chickens (n=35)</td>
<td>6 (17.1)</td>
<td>5 (16.1)</td>
<td>4 (80.0)</td>
</tr>
<tr>
<td>Total (n=150)</td>
<td>31 (20.0)</td>
<td>27 (87.1)</td>
<td>20 (74.1)</td>
</tr>
</tbody>
</table>

Data are expressed as number and percentage (%).
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Table 2 - Comparison between the conventional methods and PCR for detection and typing of Clostridium perfringens.

<table>
<thead>
<tr>
<th>Methods for typing</th>
<th>Toxigenic positive samples</th>
<th>Types of toxigenic isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type A</td>
<td>Type B</td>
</tr>
<tr>
<td>Conventional methods</td>
<td>27</td>
<td>20 (74.2)</td>
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<td>Polymerase chain reaction</td>
<td>27</td>
<td>22 (81.5)</td>
</tr>
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</table>

Data are expressed as number and percentage (%)

Figure 1 - Agarose gel electrophoresis showing amplification of 961 base pair fragment of epsilon toxin gene from the extracted DNA of Clostridium perfringens isolates type D (Lanes 1, 2, and 7) while Lanes 3 and 4 showing amplification of 1025 base pair fragment of beta toxin gene from the extracted DNA of Clostridium perfringens isolates type C. Lane 5 showing 100 base pair ladder (100, 200 to 1500) and Lane 6 showing amplification of 1167 base pair fragment of alpha toxin gene from extracted DNA of Clostridium perfringens isolates type A.

Figure 2 - Agarose gel electrophoresis showing amplification of 1167 base pair fragment of alpha toxin gene in Lanes 6, 7, & 9. Lanes 1, 2, 3, and 10 showing amplification of 961 base pair fragment of epsilon toxin gene from the extracted DNA of Clostridium perfringens type D isolates. Lane 8 showing the 100 base pair (100, 200 to 1,500 base pair). DNA ladder while lanes 4 & 5 showing no amplification of 961 fragment of epsilon toxin gene from the extracted DNA of Clostridium perfringens isolates.

examination revealed in feces and intestinal contents of diseased and freshly dead calves (23.4%), adult sheep (14.7%), lambs (26.5%) and broiler chickens (17.1%). The differentiation between toxigenic and non-toxigenic C. perfringens isolates depending on Nagler’s reaction and pathogenicity in guinea pigs as shown in Table 1, indicates that out of the tested C. perfringens isolates, 27 (87.1%) were toxigenic and 4 (12.9%) were non-toxigenic. These results are similar with several other studies that C. perfringens was the most prevalent isolates in cases of gas gangrene and enterotoxemia in foals, lambs, sheep, and goats. The variations in the prevalence of diseased cases among literatures could be explained on the basis of epidemiological predisposing factors that could affect the animal farms. Characterization of C. perfringens and its toxins is well established, although few data are available in Saudi literature about its prevalence related to animal diseases with special reference to enterotoxemia in lambs and calves. In traditional procedures, C. perfringens was first isolated from the samples under investigation and then the toxigenicity of the isolates was tested for the detection of toxigenic C. perfringens. Until now, the toxin has been identified by sero-neutralization in laboratory animals (mouse or guinea pig) using specific antisera. This toxin-typing requires a continuous supply of laboratory animals and the use of monovalent diagnostic sera which are increasingly difficult to find and are extremely expensive. Moreover, the result of the toxin-typing cannot be obtained until 24 or even 48 hours observation. It also has the inaccuracy of biological assays, such as variation in individual animal
sensitivity, non-specific toxicity from other substances that may be present in intestinal contents and disfavor on humanitarian grounds. In addition, this method may not detect the non- or poorly-toxigenic variants found within all types on C. perfringens. The isolation of pathogenic C. perfringens in gas gangrene and enterotoxemia is very difficult, since the clostridia must be cultured under strict anaerobic conditions, and affected specimen are frequently contaminated with other anaerobic bacteria which outgrow more than the pathogenic clostridia. Therefore, rapid and direct detection systems for pathogenic C. perfringens, without the need for culture, are desirable. Enterotoxemia, a disease which mainly affects sheep, is a toxic infection originating in the digestive system. It can lead to serious losses if prophylactic measures are not strictly applied. The prophylaxis of enterotoxemia in animals is achieved by rapid diagnosis and vaccination: the PCR technique can thus become a first-choice tool for the identification and typing of the C. perfringens strains which initiate these diseases. In turn, this would simplify the development of vaccines adapted the epidemiological situation. In order to compare between the traditional typing and PCR, fecal and intestinal contents samples were examined using PCR. This method may detect all the bacteriologically positive samples for C. perfringens (n=27 [100%]). Moreover, it may detect the alpha toxin gene in other 2 samples (1.33%) that previously revealed a negative isolation of C. perfringens by using the bacteriological examination. This indicate the higher sensitivity of PCR in comparison with the conventional methods. The higher sensitivity of PCR could be explained that toxin gene in the PCR might not be able to produce this toxin due to mutation in the gene or in the genes regulating the transcription/expression. On the other hand, the in vitro conditions might hamper toxin production resulting in a difference in outcome of the 2 tests. However, direct testing of fecal samples by PCR may be hampered due to inhibition of DNA polymerase by substances present in specimens. A procedure to extract the DNA in order to overcome these hindrances was used according to Uzal et al. in this investigation. The results presented in Table 2 and Figure 1 revealed 22 (81.48%) strains and were identified as type A by the PCR; however, only 20 strains (74.07%) were previously identified as type A by classical tests. None of the isolates were found to be iota producers, one strain (5.56%) was identified as B and showing positive amplification of 1025 bp fragment of beta toxin. Moreover, only 1 strain (3.7%) was identified as type C and 3 strains (11.1%) was identified as type D by PCR typing as shown in Figure 2. These results confirm the conclusion of Heier et al. who stated that the PCR method has proved efficacious. The specificity and sensitivity are excellent and superior to those of the classical methods. The study showed that PCR is a rapid and useful method for genotyping of C. perfringens and suggested as a diagnostic method for confirmation of C. perfringens species. On the other hand, toxin gene typing by PCR has advantage to be practicable directly from primary culture colonies and hence is able to detect toxin genes which are unstable maintained, such as beta toxin gene and iota gene, which might be lost during the cultivation process needed for the biological method. Moreover, toxin gene detection is able to measure the presence of virulence factors that are tightly regulated and specifically expressed during infection and hence remain undetected by phenotypic methods in culture. The non-specific amplification due to contamination with other DNA was the main limitation of this study.

In conclusion, the PCR toxin gene typing method is well applicable and has shown to be a rapid and efficient method and recommended for epidemiological investigations of clostridial disease of animals in Saudi Arabia. However, further studies with multiplex PCR for simultaneous detection of the major toxins of C. perfringens were suggested.

References

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18. Songer JG, Meer RR. Genotyping of *Clostridium perfringens* by Polymerase chain reaction is a useful adjunct to diagnosis of clostridial enteric disease in animals. *Anaerobe* 1996; 2: 197-203.


