Molecular typing of *Clostridium perfringens* toxins recovered from Central Saudi Arabia

Ihab M. Moussa, MD, PhD, *Ashgan M. Hessan,* MD, PhD.

ABSTRACT

الأهداف: عمل مقارنة بين استخدام التفاعل التسلسلي المبلمر، وأساليب التشخيص التقليدية المستخدمة من أجل تعيين أنماط السموم المعزولة من البكتيريا المطثية الحاطمة (*Clostridium perfringens*) والتي جُمعت من مصادر مختلفة في الرياض، المملكة العربية السعودية.

الطريقة: أُجريت هذه الدراسة في مركز التمييز البحثي في التقنية الحيوية، جامعة الملك سعود، الرياض، المملكة العربية السعودية، وقد استمرت الدراسة خلال الفترة من أبريل إلى سبتمبر 2009م. لقد جُمعت عينات البراز من 150 حيواناً ظهرت عليهم أعراض تسمم الدم المعوي المنشأ، ومن مزارع مختلفة من مدينة الرياض، المملكة العربية السعودية. وقد استخدمت الطرق التقليدية من أجل تعيين 27 سلالة بكتيرية مولدة للسموم من المجموع 150، والتي جُمعت من عينات البراز والمحتوى المعوي للحيوانات. وبالمقابل فقد قمنا بتحليل كافة السلالات بواسطة التفاعل التسلسلي المبلمر، وباستخدام بادئات معينة لكل من جينات السموم التالية: ألفا، وبيتا، وأبسلون، وأيوتا.

النتائج: أشارت نتائج الدراسة إلى وجود جين سم ألفا في 22 سلالة بكتيرية من النمط أ (81.5%)، بينما كشفت الطريقة التقليدية عن ظهور 20 سلالة فقط من النمط أ(74.1%). كما تم تصنيف النمط الجزيئي لسلالة واحدة (3.7%) من النمط ج، و3 سلالات (11.1%) من النمط د باستخدام التفاعل التسلسي المبلمر، بالإضافة إلى تأكيد نتائج التفاعل التسلسلي المبلمر للطريقة التقليدية في تصنيف سلالة واحدة من النمط ب (3.7%). ولقد تمكنت طريقة التفاعل التسلسلي المبلمر من الكشف المباشر لسلالتين أخرتين من النمط أ والتي تم عزلها من عينات البراز والمحتوى المعوي لحيوانات الدجاج التي أُجريت عليها الدراسة، وقد كانت نتائج تحليل هذه العينات سلبية في الاختبارات البكتيرية التقليدية.

خاتمة: أثبتت الدراسة إمكانية استخدام التفاعل التسلسلي المبلمر كطريقة بديلة لطرق التشخيص التقليدية وذلك من أجل تحديد وتعيين أنماط السموم المعزولة من البكتيريا المطثية الحاطمة.

Objectives: This 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 were identified and typed by conventional methods. All the strains were analyzed by PCR using specific primers for alpha, beta, epsilon and iota toxin genes. The experimental work was carried out at the Center of Excellence in Biotechnology, King Saud University, Riyadh, Kingdom of Saudi Arabia.

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. One strain (3.7%) was identified as type C and 3 strains (11.1%) were identified as D by PCR typing. Moreover, PCR results confirmed the traditional methods in typing one strain as type B (3.7%). Also, PCR method can detect 2 other strains of type A directly from the feces and intestinal contents of the examined chicken, which provide negative results in bacteriological examination.

Conclusion: Polymerase chain reaction technique can be used as an alternative diagnostic method for detection and typing of *Clostridium perfringens*.

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From the Center of Excellence in Biotechnology Research (Moussa), and the College of Applied Studies and Community Service (Hessan), King Saud University, Riyadh, Kingdom of Saudi Arabia.

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Address correspondence and reprint request to: Prof. Dr. Ihab-Mohamed I. Moussa, Center of Excellence in Biotechnology Research, King Saud University, PO Box 2455, Riyadh, Kingdom of Saudi Arabia. Fax. +966 (1) 4678456. Tel. +966 560749553. E-mail: imoussa1@ksu.edu.sa/moussaihab@gmail.com

Clostridium perfringens (*C. perfringens)* is an important pathogenic agent causing, among other diseases, enteritis in humans and enterotoxemia in domestic animals.[1-5] 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.[6,7] 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.[8] Types B, C and D primarily occur in the intestine of animals and only occur occasionally in humans. The strains of these types have also been isolated from soil in areas where enteritis by the organisms was affecting a significant number of animals and humans.[9] The strains of types B and D are the causative agents of enterotoxemia in domestic animals such as calves, lambs, and piglets. Type C strains cause enteritis necroticans in humans and enterotoxemia in animals. The pathogenicity of E strains is not clear and has seldom been isolated.[8,9] Typing of an organism is accomplished with the culture filtrate, type-specific antisera and experimental animals such as mice and guinea pigs.[10] The most commonly used test to detect the toxin in clinical specimens is the mouse neutralization test. However, it requires large number of mice, time consuming, and non-specific toxicity caused by other substances can falsify the interpretation.[11] This study reports a comparison between polymerase chain reaction (PCR) and conventional diagnostic methods for typing of the *C. perfringens* toxins collected from Riyadh, Saudi Arabia.

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. *Clostridium perfringens* was isolated by the procedure of Quinn et al.[12] Typical colonies were identified as described by Murray et al,[13] depending on characteristic colonial morphology, hemolysis activity, Gram staining, and biochemical test. Determination of toxigenic isolates of *C. perfringens* by Nagler's test was carried out according to Baldassi et al.[14] While, the pathogenicity to guinea pigs was carried out according to Quinn et al.[12] For typing toxigenic isolates of *C. perfringens* isolates, neutralization test in mice was carried out according to Baldassi et al.[14] Dermonecrotic test in guinea pigs was performed according to Sterne and Batty.[15] General procedures for animal care and housing were in accordance with the United States Department of Agriculture, through the Animal Welfare Act (7USC 2131) 1985 and Animal Welfare Standards incorporated into Title 9 of the Code of Federal Regulations, Part 3, 1991. For determination of primers specificity, three strains of *C. perfringens* types A, B and D (Animal health Research institute, Dokki, Egypt) were used as a positive control. As well as reference strains of enteric bacteria including *Salmonella Typhimurium* ATCC 11511, *Staphylococcus aureus* ATCC 29737, *Salmonella Enteritidis* ATCC 13076 and *Escherichia coli* serotype 0157:H7 ATCC 35150 were used as negative controls. Four sets of primer pairs were used; the first pair was designed with reference to sequence published for alpha toxin by Osman et al,[9] α/cpa F- 5'-AAG ATT TGT AAG GCG CTT-3' and α/cpaR, 5'-ATT TCC TGA AAT CCA CTC-3' specific for the alpha toxin (α/cpa) gene with an annealing temperature of 56ºC and amplify 1167bp fragments. The second pair was α/cpb F- 5'-AGG AGG TTT TTT TAT GAA G-3' and α/cpb R, 5'-TCT AAA TAG CTG TTA CTT TGT G-3' specific for the beta toxin (α/cpb) gene with an annealing temperature 49ºC and amplify 1025 bp fragments.[9] The third pair was α/etx F 5'-AAG TTT AGC AAT CGC ATC-3 and α/etx R 5'-TAT TCC TGG TGC CTT AAT-3 'specific for the epsilon toxin (α/etx) gene with an annealing temperature of 46ºC and amplify 961 bp fragments according to Osman et al.[9] The fourth pair was iota F 5`- TTT TAA CTA GTT CAT TTC CTA GTTA-3`and iota R5`- TTT TTG TAT TCT TTT TCT CTA GATT-3` specific for the iota toxin gene with an annealing temperature of 45ºC and amplify 298 bp fragments according to Songer and Meer.[18] The DNA of the standards strains and of the other bacterial isolates yielded from bacteriological examination was extracted by hexadecyltrimethyl ammonium bromide (CTAB), according to Moussa and Shibl.[16] Meanwhile, the extractions of DNA from fecal samples were carried out according to Uzal[17] with few modifications as follows: Broth enrichment of fecal samples was carried out on thioglycolate broth at 37°C under anaerobic conditions. After overnight

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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.[11] The samples were subjected to 35 PCR cycles, each consisting of 30 second of denaturation at 94º C, 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 Shibl.[16] Calculation of the fragment size was performed at the National Center of Biotechnology Information website using NCBI BLAST software.[19]

Results. Bacteriological examination and typing of toxigenic *C. perfringens* isolates of 150 fecal using mice neutralization test and dermonecrotic test in guinea pigs are summarized in **Table 1**. Detection toxin producing *C. 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 *C. 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 type 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.[19] Bacteriological examination of fecal and intestinal contents samples of diseases and freshly dead animals was 20.7%. The bacteriological

Table 1 - Bacteriological examination and typing of toxigenic *Clostridium perfringens* isolates by using mice neutralization test and dermonecrotic test in guinea pigs.

Sources and number of samples	Positive samples	Toxigenic isolates	Types of toxigenic isolates				
			Type A	Type B	Type C	Type D	Type E
Calves (n=47)	11 (23.4)	9 (29.0)	8 (88.9)	0	0	1 (11.1)	0
Adult sheep (n=34)	9 (26.5)	8 (25.8)	4 (50.0)	1 (12.5)	1 (12.5)	2 (25.0)	0
Lambs (n=34)	5 (14.7)	5 (16.1)	4 (80.0)	0	0	1 (20.0)	0
Broiler chickens (n=35)	6 (17.1)	5 (16.1)	4 (80.0)	0	1 (20.0)	0	0
Total (n=150)	**31 (20.0)**	**27 (87.1)**	**20 (74.1)**	**1 (3.7)**	**2 (7.4)**	**4 (14.8)**	**0**

Data are expressed as number and percentage (%).

Table 2 - Comparison between the conventional methods and PCR for detection and typing of *Clostridium perfringens*.

Methods for typing	Toxigenic positive samples	Types of toxigenic isolates								
		Type A		Type B		Type C		Type D		Type E
Conventional methods	27	20	(74.2)	1	(3.7)	2	(7.4)	4	(14.8)	0
Polymerase chain reaction	27	22	(81.5)	1	(3.7)	1	(3.7)	3	(11.1)	0

Data are expressed as number and percentage (%)

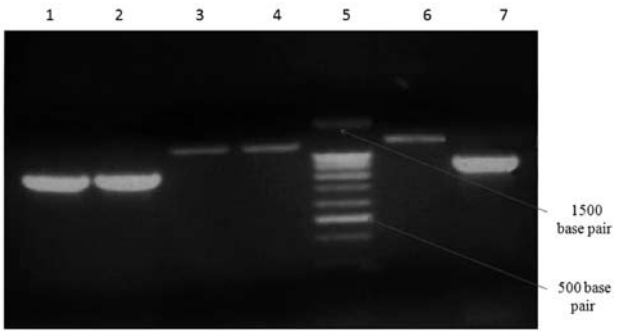

Figure 1 - Agarose gel electrophoresis showing amplification of 961 base pair fragment of epsilon toxin gene from the extracted DNA of *Clostridium perfringens* isolates type D (Lanes 1, 2, and 7) while Lanes 3 and 4 showing amplification of 1025 base pair fragment of beta toxin gene from the extracted DNS 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.

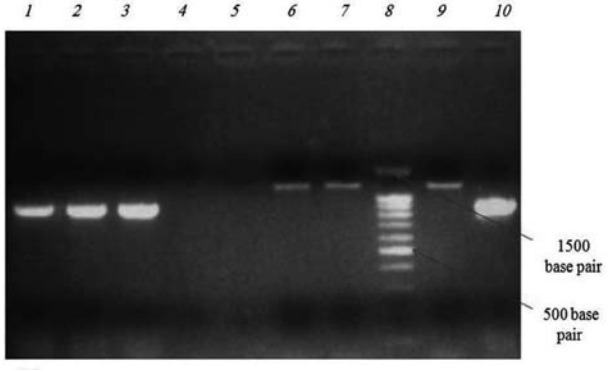

Figure 2 - Agarose gel electrophoresis showing amplification of 1167 base pair fragment of alpha toxin gene in Lanes 6, 7, & 9). Lanes 1, 2, 3, and 10 showing amplification of 961 base pair fragment of epsilon toxin gene from the extracted DNA of *Clostridium perfringens* type D isolates. Lane 8 showing 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[20-22] that *C. perfringens* was the most prevalent isolates in cases of gas gangrene and enterotoxemia in sheep and lambs with similar incidence rates. Furthermore, the incidence of *C. perfringens* in fecal and intestinal contents samples of chicken were ranging from 0-22%, as previously reported.[22-24]

Typing of toxigenic *C. perfringens* isolates recovered from calves, adult sheep, lambs, and chickens depending on neutralization test in mice and dermonecrotic test in guinea pigs (**Table 1**). The results of the present study indicate that *C. perfringens* type A is the most prevalent type in calves, sheep, lambs, and broiler chicken. These results were also similar to those found in previous studies.[20,21,26-29] *Clostridium perfringens* type A and to a lesser extent type C in broiler chicken have been reported as a cause of necrotic enteritis worldwide.[28,30-32] However, Hunter et al[19] reported that type B strains of *C. perfringens* was identified as a causative agent of 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.[15,29] 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[33,34] and disfavor on humanitarian grounds.[6] In addition, this method may not detect the non- or poorly-toxigenic variants found within all types on *C. perfringens*.[17] The isolation of pathogenic *C. perfringens* in gas gangarene 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.[22] 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.[31,33] 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.[9] 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.[34] A procedure to extract the DNA in order to overcome these hindrances was used according to Uzal et al[17] 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[29] 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.[29] 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.

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