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Short Communication

Genotyping Toxins of *Clostridium perfringens* Strains of Rabbit and Other Animal Origins

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ABSTRACT

Clostridium perfringens is a pathogen of great economic importance, commonly known for toxin production that causes several diseases of animals and humans. Strain identification is necessary for accurate diagnosis of associated diseases caused by C. *perfringens* and the development of management strategies; hence toxin genotyping is more dependable than the traditional toxinotyping. Therefore, the present study was aimed at genotyping toxins of *C. perfringens* strains isolated from rabbit and other animals. A total of 60 strains of *C. perfringens* (19 from rabbit and 41 from cattle, sheep, goat and others animals) were isolated from infected spleens, livers and kidneys or intestinal contents. The isolates were identified using cultural and biochemical tests. Strain confirmation was done with a molecular biological approach. Multiplex and duplex PCR strategies were employed for genotyping *C. perfringens* strains by targeting the *cpa* and *cpb2* genes, respectively. The results of toxin-genotyping and pathological profile distribution indicated that out of the 19 strains isolated from rabbit, the Type A genotype of *C. perfringens*, expressing the *cpa* gene was 63.16 %, while the *cpa+cpb2* genotype was 36.84%. A similar finding was made for avian, ovine, and caprine isolates, with 90.90 % positive for the *cpa* gene and 9.10 % of the strains having the *cpa+cpb2* genotype. Based on the findings, an effective management technique, *viz a viz* genotype style distribution of the *C. perfringens cpa* gene in the ecosystem, and strain genotyping is recommended.

***Keyword*:** *Clostridium perfringens*, Genotype, PCR, Strain, Toxin.

Introduction

Clostridium perfringens is a ubiquitous pathogen known for the production of several toxins and hydrolytic enzymes.[1] Strains of the pathogen are classified into 5 toxigenic types (A-E). This grouping was based on the major toxin production (ε, α, ι, and β1) and the fatal effect, following intraperitoneal administration and specific seroprotection with neutralizing antibodies in lethality test carried out on mouse.[1] *C. perfringens* of toxin Type A is proposed to be the most wide spread in the environment. It is responsible for gangrene in humans, abomasitis in calf, hemorrhagic enterotoxemia and necrosis in ruminants, extensive enteritis in mammals and chickens, as well as the cause of food poisoning in humans.[2] The enteropathogenicity of toxin Type A is generally mediated by toxin Type α, which is encoded by the localized gene *cpa* in a variable area on the chromosome, close to the origin of replication region. Human enteritis induced by food poisoning in which toxin Type A is mediated by a toxin (enterotoxin), produced during sporulation of *C. perfringens*, encoded by the gene *cpe* and is located on the chromosome.
The gene on the plasmid is the same one that encodes inflammation of the intestine of non-nutritional origin for other animals and humans.[3]

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There are reports linking toxins Types A-E produced by *C. perfringens* as causative agent of dysentery in a variety of animals, including poisoning of infected cattle, haemorrhagic intestinal illness, sheep dysentery, disease of soft kidney and a combined sudden death of lamb and veal[2] .Necrotic bleeding of the intestinal mucosa and edema of the parenchymes that characterize the pathogenesis of toxin Types B, C and D are mediated by the action of β1 and ε toxins, encoded by the *cpb1* and *cpetx*, respectively, genes located on the plasmids. The composition of Types β1 and ε toxins are associated with Type B toxin, and are individually expressed and the products bind to toxin Types C and D respectively.[3]
Recently, Janneke *et al*.[4] described a new β2 toxin, which is encoded by *cpb*2 gene, located on the plasmid. β2 toxin is distinguishable from β1 toxin, which can be noticed through a C strain reference *perfringens* Type B and from a field strain Type C that can be isolated from necrotizing enterocolitis piglet. Recent works have demonstrated a wide spread of *β*2 toxinogenic *C. perfringens* isolated from ruminants, carnivores, chicken and also from healthy animals.[5] Due to the locations of these toxigenic factors on a variable section of the chromosome or/and the extrachromosomal elements, numerous strains have evolved, resulting to the causative agents of specific disease.[1]
After a recent spread of epizootic rabbit enteropathy (ERE), several authors,[6,7] investigated the role of *C. perfringens*. Although the etiology of the ERE remains unknown. *C. perfringens* genotypes cpb2, cpe, and cpa+ have been found in naturally occurring ERE as well as in experiments using the polymerase chain reaction PCR technique. This procedure has been widely accepted and developed into practical and reliable technique that increases accuracy and complete determination of phenotypes and pathological effects of *C. perfringens* in relation to genotyping of toxins.[1] For a precise diagnosis of pathologies that are associated with diseases caused by *C. perfringens* and the development of an effective strategy to control them, strain identification is required. To achieve these, toxin genotyping gives

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more accurate results than the conventional toxinotyping.[1] Therefore, the aim of this research was to gain an insight into identification of C. *perfringens* toxins by genotyping strains isolated from rabbit and other animals.

Materials and Methods

Sources of Clostridium perfringens strains

In this study, two collections of *C. perfringens* strains were obtained. In the first group, 19 strains were isolated from blind sick rabbits that were affected with enteropathy. The second group involved 41 strains isolated from the spleens, livers and kidneys of cattle, sheep, goat, chicken, pigs, dogs, cat and mouse. The primary isolation was carried out on 5% sheep blood agar plates after incubation at 37ºC for 24 h. Colonies suspected to have *C. perfringens* were subcultured anaerobically on medium thioglycolate broth or Tryptone Glucose Yeast Extract (TGY) broth (3% tryptone, 2% yeast extract, 0.1% glucose, and 0.1% L-cysteine) at 37ºC for 24–48 h. The broth cultures were centrifuged and the pellets were stored at -20ºC and all of the isolates were lyophilized in skim milk and stored at +4ºC for collection.[3]

Ethical consideration

The protocol for the use of experimental animals was approved by the Department of Biology Ethics committee, College of Science, Mustansiriyah University, Baghdad, Iraq.

Pathological formes

The pathological characteristics of rabbits affected with enteropathy have been classified based on inflammatory character (enterocolitis) and non-inflammatory (liquid content, constipation of the blind, mucoid content). The pathological features found in the other animals were grouped based on the main pathological picture, within the category of enteropathy (inflammatory lesions or degenerative) and other pathology (trauma, hemorrhagic or congestive syndrome, diseased generative or post-mortem autolysis).

Isolation and identification of Clostridium perfringens

In 2 mL of sterilized water, 0.5 g of infected spleens, livers and kidneys or intestinal content was suspended and shaken vigorously. One mL of the suspension was inoculated onto thioglycollate (BioMérieux SA, Marcy l'Etoile, France) broth and incubated at 37°C for 24 h. An aliquot of 1 mL of the culture was taken with a sterilized pipette and inoculated on blood agar medium (BioMérieux, SA) and the resulting cultures were incubated anaerobically at 37°C for 24 h. Suspected colonies of *C. perfringens* were sub-cultured on fresh blood agar, incubated anaerobically and the pure colonies obtained were analyzed by Gram staining, catalase and oxidase tests. Biochemical identification of pure colonies was done using the commercial API20A kits (BioMérieux, SA).[3]

PCRs for molecular identification and genotyping of Clostridium perfringens strains

DNA was extracted from five colonies, each from the *C. perfringens* strains using the Promega Wizard Genomic DNA Isolation Kit (USA), following the manufacturer's instructions. Molecular confirmation of the strains was done following the method described by Ravinder *et al*,[8] using PCR-amplification of the 16S rDNA gene, followed by bioinformatic analysis. Also, toxin genes were assessed by employing PCR strategy according to the method described.[4,9,10] A multiplex PCR was used for detecting the presence of *cpa* gene, while *cpb2* gene was detected by a duplex PCR. A mixture of 25 µL reaction volume containing $MgCl_2$ (2.5 mM), dNTPs (250 µM), Taq polymerase (0.05 U/µl) and 10 µM of the selected primers were utilized for the multiplex PCR. Duplex PCR was made in a 25 µl reaction volume containing $MgCl_2$ (2.5 mM), dNTPs (250 µM), Taq polymerase (0.05 U/µL) and 10 µM of the selected primers (Table 1). The amplification program used for simplex and multiplex PCR started with an initial denaturation at 94ºC for 5 min, followed by 35 cycles of 1 min at 94ºC, 1 min at 60 and 54ºC respectively, 1 min at 72ºC, and a final extension step of 10 min at 72ºC. The PCR products were electrophoresed on 1.5% agarose gel and visualized by Biorad Gel DocXR Imagine system (USA).

Results and Discussion

Genotypes of Clostridium perfringens strains isolated from rabbit

In this study, a PCR-based strategy was employed for toxin type identification from *C. perfringens* strains isolated from rabbit and other animals. To establish the ability of *C. perfringens* to cause fatal enterotoxaemia, a consideration of the related pathological pictures is appropriate. Our results confirm the widespread diffusion of toxin Type A among animal species. The *cpa* genotype (Type A) was characterized to be 73.3 % of all the examined strains (i.e. 63.2 % of rabbit origin, while 78.1 % from other animals). The most prominent result of our study was that *C. perfringens* toxin Type A strains were found in rabbit (19 strains), cattle (6 strains), sheep and goat (7 strains), pigs (5 strains), chickens (5 strains), dogs (13 strains), cat and mouse (each of them has 2 strain). Our finding is in agreement with a similar epidemiological and toxin-genotyping study on rabbits, which was reported by Italian authors.[3] They found out that of the 70 *C. perfringens* strains that were toxin-genotyped, the Type A strain was the most recovered. In our study, the other genotype of *C. perfringens* strain considered was the one expressing both the *cpa* and *cpb2* genes. For the *cpb2* genotype, 36.84 % of the strains was of the rabbit origin, while 21.95 % were strains that belong to species of other animals. There have been some studies which revealed an *in vivo* synergistic role of the *cpa* and *cpb2* gene products in the small intestine of bovine enterotoxaemia, leading to the production of necrotic and hemorrhagic lesions.[11]

Conversely, there have been some reports of *C. perfringens* strains of *cpb* and *cpa* genotypes being isolated from healthy animals, an observation which strengthens the predisposing factor. Research has shown that when the physiological equilibrium of the resident microflora is altered as a result of several factors which include overuse of antibiotics, existence of dietary factors that inactivate trypsin, or improper feed, allow the colonization of toxigenic Clostridia.[5] Fast growth of *C. perfringens* in the intestine can itself be considered as pathogenic factor of the bacterium to gain elements of the extra chromosomal factor that contains extra toxin-encoding genes.[1] The argument that may support this proposition relates to the function of *C. perfringens* in non-enteropathies of rabbit inflammatory effects. Pathological conditions associated with about 90 % of *C. perfringens* strains of rabbit origin that were tested in this study were compatible with ERE. It is probable that 46 % of the isolated strains of C. *perfringens* with the activity of necrosis should not be considered as the cause of primary fatal diseases such as traumatic disease syndrome, hemorrhagic and degenerative diseases of animals. In 1996 in Europe, a non–inflammatory, digestive syndrome called ERE appeared and became mortality in rabbit farming. The disease became very difficult to control and it was opined that the occurrence might be due to co-infection of *C. perfringens* with other rabbit pathogens.[12] For a very long time, the ERE etiology remains unknown. Recent study carried out by Licois Marlier,[13] suggested that the disease may involve an anaerobic bacterium that produces an unknown toxin, effective in the first phase of infection. The *C. perfringens* certainly would have a contributory function in cases of high mortality observed during spontaneous ERE.

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Table 1: Nucleotide sequences of primers used for PCR and amplicon lengths

Gene	Direction	Nucleotide sequence (5´-3´)	Amplicon length (bp)
cpa	Forward	AGT CTA CGC TTG GGA TGG AA	900
	Reverse	TTT CCT GGG TTG TCC ATT TC	
cpb2	Forward	CAA GCA ATT GGG GGA GTT TA	200
	Reverse	GCA GAA TCA GGA TTT TGA CCA	

Table 2: Pathological profiles and toxin genotypes of *Clostridium perfringens* strains isolated from rabbit

Pathological profile	Genotype (n)		Total
	cpa	***Cpa + cpb2***	
Enterocolitis	1	1	2
Abdominal swelling, fluidization of the contents of the blind, absence of intestinal inflammation	5	3	8
Dilatation of the small intestine, constipation of the blind, mucoid content in the colon, absence of intestinal inflammation	6	3	9
Total	12	7	19

Table 3: Pathology types and toxin genotypes of *Clostridium perfringens* strains isolated from animals of autoptic activity

Profile of pathological alteration	Animals involved	Genotype		Total
		cpa	***cpa+cpb2***	
Hemorrhagic syndrome	Cattle, dog, chickens sheep, goat, mouse	12	6	18
Congestion, necrosis	Cattle, dog, chickens, sheep	4	0	4
Traumatic death	Dog, sheep	3	1	4
Congestive hemorrhagic	Dog, pig	3	1	4
Syndrome-degenerative disease or postmortem autolysis	Dog, goat, chickens, cat, cattle	10	1	11
Total		32	9	41

Conclusion

The results obtained in this study confirm the widespread occurrence of Type A genotype of *C. perfringens* strains expressing the *cpe* gene in the environment. For effective management strategy, a determination of strain genotype is recommended. Considering that the characterized strains of *C. perfringens* were mainly the result of severe clinical cases, they represent the main strains that concern the diagnostic activity and therefore represent those that most need to be subjected to control and eradication.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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