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REGULAR ARTICLE

Preliminary screening for toxin genes amongst stock cultures of *Clostridium perfringens* strains isolated from dogs and calves

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ABSTRACT

The spectrum of *Clostridium perfringens* infections ranges from food toxinosis to myonecrosis. In the current study, whole cell protein and toxin gene types were profiled in 12 randomly selected *C. perfringens* veterinary stock cultures from the University of Wisconsin, Madison to determine epidemiological similarity, or diversity amongst strains of animal origin. Whole cell protein analysis was done by SDS-PAGE while toxin gene typing was achieved by extracting DNA by boiling, DNA concentration and purity was determined by spectrophotometer and nanodrop while separation was carried out by checking it on gel electrophoresis. Multiplex PCR was used to identify the toxigenic gene-type. *C. perfringens* B and *C. perfringens* EE with established profiles were used as control strains. Isolates typed included strains cp 296, 309, 12872 (from dogs) and 304, 305, 306, 341, 342, 10754, 12218-2, 12218-3, 12473 (from calves). All 12 strains possess the *cpa* gene, 4 strains have cpb_2, 3 strains *etx*, 2 strains positive for *cpe* and 1 for *cpb*. None of the strains carries the *itx* gene. Two strains have only *cpa* gene however no strains has more than two toxin gene types, with *cpa*-cpb_2 combination being more frequent. *C. perfringens* 305 (*etx* and *cpa*) and 342 (*cpe* and *cpa*) shared the same protein profile but belong to different toxinotype. It is evident that the *cpa* gene is a marker for all *C. perfringens* strains, and similarity in protein profile is not sine qua non for toxin gene type.

1. Introduction

Clostridium perfringens is an anaerobic Gram positive spore forming bacillus with the characteristic box car appearance. The bacilli produce lecithinase (alpha toxin) and on blood agar a double zone of hemolysis. The spectrum of *C. perfringens* diseases is the consequence of the secretion of extracellular toxins. Five major types of *C. perfringens* (A, B, C, D and E) have been described based on toxin production with types B, C and D associated most with disease in domestic animals (Songer and Miskimins, 2005; Hendriksen et al., 2006; Ferrarezi et al., 2008). Illnesses and death losses in baby calves caused by *C. perfringens* are significant problems for producers raising calves in beef or dairy operations (Daly and Rotert, 2007). Cattle ranching are usually free range in Nigeria and the nomadic herd's men travel distance with their flocks. Death from these is usually due to fatigue or from trypa-

nosomiasis for which vaccination cover is mandatory. However not frequently reported is enterotoxaemia associated with *C. perfringens* infection (Itodo and Ike, 1990; Itodo et al., 2009). Consequently, not much has been done with respect to characterization of the toxin genes with the aid to facilitate diagnosis which through conventional methods is cumbersome.

Alpha toxin gene (*cpa*) which encodes the CPA toxin is present in all *C. perfringens* strains and is chromosomally located unlike other major toxin genes of *C. perfringens* which are plasmid borne (Cole and Canard, 1997; Li et al., 2007). The *cpb* gene which is carried on a virulent plasmid encodes CPB toxin. CPB is produced by types B and C of *C. perfringens*. Both types B and C animal disease are often accompanied by sudden death or acute neurological signs (McClane et al., 2004; Uzal, 2004). The gene *etx* is located on a conjugative plasmid and encodes ETX; an aerolysin-like, pore-forming toxin considered the major virulence factor of *C. perfringens* types B and D (Sayeed et al., 2007). ETX producing *C. perfringens* type D strains are the most common cause of colitis in sheep and goats (Uzal and Kelly, 1997; Uzal 2004).

Iota toxin (ITX) of *C. perfringens* toxinotype E has been implicated in hemorrhagic enteritis and sudden death in calves (Songer and Miskimins, 2005), but disease in sheep and goat is rare (Songer, 1998). The cpb_2 toxin gene is the next most frequent after *cpa* in *C. perfringens* (Fisher et al., 2006). The cpb_2 gene is present on *C. perfringens* type D isolates and may be borne on the same plasmid carrying the *etx* gene or on a different plasmid (Sayeed et al., 2007). However, in type E isolates the cpb_2 gene is always present on different plasmid carrying the *itx* gene (Li et al., 2007). CPB2 has been implicated in more animal disease both domestic and wild including swine, cattle, horses, sheep, goats, deer and bears (Dray, 2004; Hendriksen et al., 2006; Ferrarezi et al., 2008; Jores et al., 2008).

The gene *cpe* encoding CPE can reside on either the chromosome or on plasmids (Collie and McClane, 1998). Most human food-poisoning isolates carry *cpe* on the chromosome (McClane et al., 2006), and are known to be divergent from other *C. perfringens* isolates (Deguchi et al., 2009). In contrast, *cpe*-positive isolates recovered from diseased animals or humans suffering from non-food-borne gastrointestinal disease carry *cpe* on large plasmids (Sparks et al., 2001).

C. perfringens is ubiquitous and causes diseases in humans, domestic and wild animals. Though some *C. perfringens* types are more adapted to certain animals, nevertheless, infections with same type in multiple animals have been described. Furthermore, the conventional diagnosis protocol for *C. perfringens* include isolation of the bacilli from disease site, detection of one or more toxin types and/or determination of the presence of toxin genes. The pitfalls in presence of bacilli in specimen as diagnostic and the technical details and cost of assays for toxin make isolation and toxin detection cumbersome approaches in epidemiological studies. The objective of the present study therefore was to use PCR and other molecular techniques to determine the toxin gene type of *C. perfringens* isolated from clinically sick or diseased animals in line with whole cell protein profile first as a marker for the toxin type elaborated by the bacilli in the disease process and secondly to assess epidemiological similarity or divergence in the animal isolates.

2. Materials and Methods

2.1. Clostridium perfringens strains

Twelve veterinary isolates of *C. perfringens* identified by conventional cultural, morphological and biochemical properties with strain numbers cp 296, 309, 12872 (from dogs) and 304, 305, 306, 341, 342, 10754, 12218-2, 12218-3, 12473 (from calves) and two reference strains of *C. perfringens* (strains Cp B and Cp EE) with known protein and gene profiles obtained from the stock culture collection of the Wisconsin Veterinary Diagnostics Laboratory of the Department of Pathobiological Sciences, University of Wisconsin were used in this study. Strains 309 from dog and strains 305 and 342 from calves were isolated from dead animals. The strains were supplied by Professor Okwumabua Ogi of the University of Wisconsin, Madison, USA and maintained at -8^{o}C. Before use, strains were sub-cultured on non-selective Brucella based blood agar plates and incubated in 5% and 10% CO_2 for 48 h and colonies Gram stained to test for purity. Preliminary screening tests including double zones of b-hemolysis, box-car shaped bacilli and positive lecithinase (alpha toxin) activity confirmed the isolates.

2.2. *DNA extraction*

Pure culture of *C. perfringens* on Blood agar plates were harvested into 1.5 ml tube containing 200 ul

of water. The tube was boiled at 100 ^{o}C for 20 min to release the DNA, cooled at -20 ^{o}C for 10 min and then centrifuged at 13000 rpm for 2 min to concentrate the DNA. The DNA concentration and purity were determined by spectrophotometer (Model, D-37520, Thermo Scientific, Waltham, Massachusetts, MA, USA) and nanodrop (Model, 2000, Thermo Scientific, Waltham, Massachusetts, MA, USA). The OD 260/280 reading of the purified DNA was ≥1.8. The concentration of DNA used was 50 ng/ul.

2.3. PCR primers and amplification

Primers for *C. perfringens* B and EE toxin genes *itx*, *cpa*, *cpb*, *cpb$_2$*, *cpe*, and *etx* were used for the amplification and together with dNTP (dATP, dTTP, dGTP, dCTP), amplitaq, PCR buffer, divalent cation (Mg^{2+}) and water constituted the master mix. PCR amplification was performed in a total volume of 50 µl made up of water (24.3 µl), 1X PCR buffer (5.0 µl), dNTP mix (2.4 µl of 0.48 µM), *itx* (1.3 µl of 0.52 µM), *cpa* (1.25 µl of 0.50 µM), *cpb* (0.9 µl of 0.36 µM), *cpb$_2$* (0.9 µl of 0.36 µM), *cpe* (0.85 µl of 0.34 µM), *etx* (1.1 µl of 0.44 µM), amplitaq (1.0 µl of 5 units), $MgCl_2$ (1.0 µl of 0.55 µM) and 10 µl of DNA. The negative control contains 10 µl of DNA free water in place of the test DNA or positive controls. A thermocycler (Gene Amp PCR System 9700; Applied Biosystems, Foster City, California, CA, USA) was used for the amplification. The PCR was set at 103 ^{o}C for a start run. The PCR was programmed to run at 94 ^{o}C for 5 min, and then a cycle of 35 runs comprising 94 ^{o}C for 1 min, 55 ^{o}C for 1min, 72 ^{o}C for 1 min followed by a run at 72 ^{o}C for 7 min. Storage of the mixture was at 4 ^{o}C until needed. The amplified PCR products were separated by check gel electrophoresis. The separated DNA fragments were visualized by staining the gel with ethidium bromide for 15 min and then de-stained in water for 15 min. The DNA bands were viewed by illumination with UV light and images photographed.

2.4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

From the PCR results, three test strains (cp305, 342, 12218-2) were used to represent three genotype groups (*cpa* and *etx*, *cpa* and *cpb$_2$*, *cpa* and *cpe* respectively). The protein profiles of these were determined in parallel with *C. perfringens* B and EE strains and the run calibrated on a protein ladder (Rainbow Molecular Weight Markers). The SDS-PAGE was run with 1X SDS buffer at 30mA for 90 min. The protein molecular marker consisted of 10 min boiled mixture of 5 µl marker, 5 µl H_2O, 10 µl 2X SDS buffer. Protein was extracted from the bacterial strains by boiling. After running, the gel was stained in coomasie blue solution for 20 min and destained three times, rinsed in sterile water twice and left in sterile water for 24 h and dried with a slab gel dryer for band to be visible.

3. Results

All 12 strains possess the *cpa* gene while the *itx* gene was absent in all strains. Ten strains carry two toxin gene types while two strains *C. perfringens* 12473 and 12872 have the *cpa* gene only. The *cpb$_2$* gene is second in occurrence in the strains. Strains that carry the same toxin gene combination are 296, 305, and 341 (*etx* and *cpa*), 304, 306, 12218-2 and 12218-3 (*cpb$_2$* and *cpa*), 309 and 342 (*cpe* and *cpa*), 12473 and 12872 (*cpa* only), and 10754 (*cpb* and *cpa*) (Table 1). Plates 1 and 2 identified the various *C. perfringens* toxinotype as obtained by gel electrophoresis. Only two strains; 305 and 342 had similar protein profile (data not shown).

STRAINS	*etx*	*cpb$_2$*	*itx*	*cpa*	*cpe*	*cpb*
296	+	_	_	+	_	_
304	_	+	_	+	_	_
305	+	_	_	+	_	_
306	_	+	_	+	_	_
309	_	_	_	+	+	_
341	+	_	_	+	_	_
342	_	_	_	+	+	_
10754	_	_	_	+	_	+
12218-2	_	+	_	+	_	_
12218-3	_	+	_	+	_	_
12473	_	_	_	+	_	_
12872	_	_	_	+	_	_

Table 1: Toxin gene types in *C. perfringens*.
+; present
-; absent

4. Discussion

The twelve *C. perfringens* veterinary strains had the cpa gene and one of the other major toxin genes except *itx*. Two isolates however had *cpa* gene only (12473 from a calf and 12872 from a dog) which implied these produced only the CPA toxin. It was not clear whether the CPA of the *C. perfringens* was responsible for the disease in these animals more so that the symptoms as documented in the laboratory data file for the isolates were those of enterotoxemia or whether other factors including other *Clostridium* spp were involved. The role of CPA in animal diseases cannot be neglected as experimental evidences have implicated the CPA toxin in yellow lamb disease, enteritis, abomasitis and malignant edema in cattle, goat, pigs and sheep

LANE M: DNA Marker

LANE B: +VE Control

LANE EE: +VE Control

LANE 1: *C perf* 304

LANE 2: *C perf* 305

LANE 3: *C perf* 306

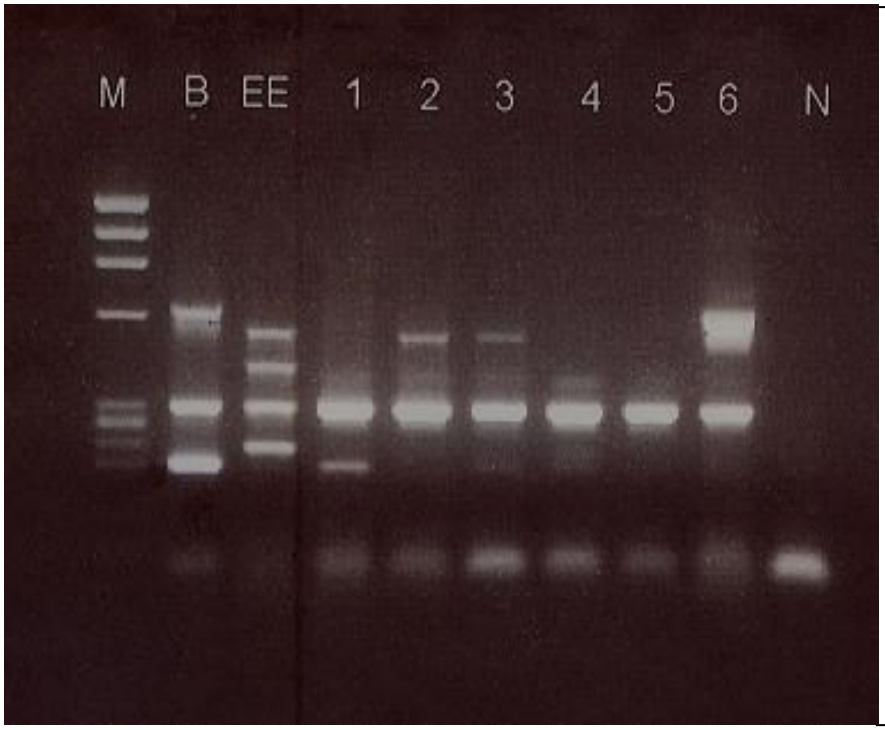
LANE 4: *C perf* 309

LANE 5: *C perf* 341

LANE 6: *C perf* 342

LANE N:-VE Control

Plate 1: PCR gel electrophoresis of *C. perfringens* toxin genes. Lanes M, B and EE represent the DNA marker, *C. perfringens* B and EE respectively. The negative control is lane N while the test strains are in lanes 1-6. Lanes 1-6 (all test strains) is positive for *cpa* (alpha, 324 bps). Lanes 2 and 5 (305 and 341 respectively) are positive for *etx* (epsilon, 655bps). Lanes 1 and 3 (304 and 306) are positive for *cpb*$_2$ (beta$_2$, 567bps). Lanes 4 and 6 (309 and 342 respectively) are positive for *cpe* (enterotoxin, 233bps).



LANE M: DNA Marker

LANE B: +VE Control

LANE EE: +VE Control

LANE 1:*C perf* 10754

LANE 2:*C perf* 12218-2

LANE 3:*C perf* 12218-3

LANE 4:*C perf* 12473

LANE 5:*C perf* 12872

LANE 6:*C perf* 296

LANE N:-VE Control

Plate 2: PCR gel electrophoresis of *C. perfringens* toxin genes. Lanes M, B and EE represent the DNA marker, *C. perfringens* B and EE respectively. The negative control is lane N while the test strains are in lanes 1-6. Lanes 1-6 (all test strains) positive for *cpa* (alpha, 324 bps), Lane 6 (296) is positive for *etx* (epsilon, 655bps), Lane 2 and 3 (12218-2 and 12218-3 respectively) are positive for *cpb*$_2$ (beta$_2$, 567bps). Lane 1 (10754) is positive for *cpb* (beta, 196).

(Roeder et al., 1988; Choi et al., 2003; Fernandez and Uzal, 2005).

Considering that the cpa gene was present in all *C. perfringens* the presence of another toxin gene as detected in other isolates may be indicative of their role in the veterinary diseases or death of the animals. The above is significant; though *cpa* roles in gas gangrene in humans and yellow lamb disease in sheep are well established, its involvement in diseases in other animals remains subjective (Awad et al., 2001; Uzal et al., 2010). Nine of the 12 isolates were from sick animals while three were isolated from dead dog and calves. The isolates from the decease animals were 309 (dog), 305 and 342 (calves). *C. perfringens* 309 had the *cpe* gene that codes for toxin E which has been implicated in recurrent diarrhea in dogs (Weese et al., 2001).

The isolates from dog carry the same gene types with some of the cattle's. For instance strain 296 from dog has same toxin gene as strains 305 and 341 from cattle. Similarly, strain 309 (dog) and 342 (cattle) have genotype combination of *cpa-cpe*. The epidemiological significance of this was not readily obvious except that cattle and dog may constitute pool of the *cpe* gene from which humans may acquire infection. This may be the case for non-food-borne gastroenteritis due to type A *C. perfringens* enterotoxin (CPE) producer which also occur in animals especially dogs (Collie and McClane, 1998; Sparks et al., 2001).

The multiplex PCR analysis of toxin genes makes it easier to detect toxinotypes amongst *C. perfringens* isolates. This occludes the need for elaborate toxin assays which may involve the use of animals or cell lines. From this study it was easy to predict toxin group of the isolates without necessarily assaying for toxin. Consequently, isolates 296, 305 and 341 may belong to either type B or D, isolates 309, 342, 12473 and 12872 are of type A. Isolate 10754 is either type B or C, however isolates producing cpb_2 may not be easily placed as additional investigations are required. Of importance is that presumptive assessment of toxinotype can be determined by toxin gene detection.

5. Conclusion

This study was done with stock cultures of *C. perfringens* with the only available information of source and clinical conditions of the animals. The study had aptly shown that PCR determination of toxin gene type in *C. perfringens* may be predictive of the toxin responsible for disease in animals.

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