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Characteristics of *Clostridium perfringens* Isolated from Chicken in Korea

Young Ju Lee
College of Veterinary Medicine, Kyungpook National University, 702-701 Daegu, Republic of Korea

Abstract: *Clostridium perfringens* (*C. perfringens*) is major cause of Necrotic Enteritidis (NE) in poultry. *C. perfringens* types A and C are the only types linked to the disease observed in avian species and toxins alpha (encoded by cpa), beta (cpb), beta-2 (cpb2), enterotoxin (cpe) and netB are the most significant in disease. Researchers report here the toxinotyping of NE-producing and commensal isolates of *C. perfringens* from chickens in Korea. All isolates recovered from NE lesion or normal flora were type A (cpa positive, cpb negative, etx negative, cpIo negative). The cpb2 and luxS showed no significant difference between NE-producing and commensal isolates but of the 27 netB positive isolates, 25 (36.2%) had been recovered from 69 chickens with NE while only 2 (4.9%) isolates were recovered from 41 chickens with normal flora.

Key words: *Clostridium perfringens*, toxinotyping, flora, lesion, chickens

INTRODUCTION

Clostridium perfringens (*C. perfringens*) are spore-forming bacteria that can be found in the soil, litter, feed, intestine of all animals but can cause Necrotic Enteritidis (NE) in poultry. The presence of intestinal damege produced by coccidia are considered predisposing factors that increses *C. perfringens* proliferation and the incidence of NE (Al-Sheikhly and Al-Saieg, 1980; Annett *et al*., 2002).

C. perfringens types A, B, C, D and E are currently recognized as the major pathogenic contributors. Types A and C are the only types linked to the disease observed in avian species (Tschirdewahn *et al*., 1991; Cooper and Songer, 2009). The *C. perfringens* strains that infect avian species produce many extracellular toxins however, toxins alpha (encoded by cpa), beta (cpb), beta-2 (cpb2), enterotoxin (cpe) and netB are the most significant (Engstrom *et al*., 2003; Martin and Smyth, 2009).

Researchers report here the toxinotyping of NE-producing and commensal isolates of *C. perfringens* from chickens in Korea. In addition, researchers have investigated these toxin-encoding genes to genotype *C. perfringens* isolated from healthy or disease chickens in order to identify an association between the possession of toxin encoding genes and disease.

MATERIALS AND METHODS

Bacterial isolates and growth conditions: Isolates for the present study had been obtained from chickens by swabbing of small intestine of 110 birds which died during rear on broiler breeder farm or commercial broiler farms. Sixty nine isolates were recovered from the NE-producing chickens. These birds has originated from 1 broiler breeder farm and 3 broiler farms which were experiencing an increased mortality rate and showed clinical NE characterized by confluent mucosal necrosis of large part of the small intestine, covered with a yellow-brown or bile-stained pseudomembrane or depression in the mucosal surface with discolored, amorphous material adhering to the mucosal surface. Forty one chickens originated 4 broiler farms showed no evidence of NE but 41 isolates were subsequently recovered for examination based on the comparison the toxinotype between NE-producing and commensal isolates. All swabs were streaked onto 5% sheep blood agar and incubated overnight at 37°C under anaerobic conditions. Resultant colonies which exhibited a double zone of hemolysis and which failed to grow under aerobic conditions were tentatively identified as *C. perfringens*. Isolates were preserved in preservation media (Brucella broth; BBL, Mississauga, Canada) and 50% buffered glycerol (1 M $MgSO_4$, 1 M Tris pH 8 and H_2O) and were kept at -80°C until use.

Preparation of DNA: Template DNAs for PCR were extracted using a QIAamp DNA Mini kit (Qiagen, France) according to manufacturer's directions. The DNA concentration was measured spectrophotometrically at A260.

Toxinotyping by polymerase chain reaction: All isolates which had been tentatively identified as *C. perfringens*

Corresponding Author: Young Ju Lee, College of Veterinary Medicine, Kyungpook National University, 702-701 Daegu, Republic of Korea

Table 1: Primers used for toxinotyping in this study

Target genes	Primer	Sequence (5'→3')	Amplicon size (bp)	Annealing temperature	References
cpa	Forward	GTTGATAGCGCAGGACATGTTAAG	324	55	Garmory *et al.* (2000)
	Reverse	CATGTAGTCATCTGTTCCAGCATC			
cpb	Forward	ACTATACAGACAGATCATTCAACC	195	55	Garmory *et al.* (2000)
	Reverse	TTAGGAGCAGTTAGAACTACAGAC			
cpb2	Forward	AGATTTTAAATATGATCCTAAC	567	53	Bueschel *et al.* (2003)
	Reverse	CCAATACCCTTCACCAAATACTC			
cpe	Forward	GGAGATGGTTGGATATTAGG	233	53	Bueschel *et al.* (2003)
	Reverse	GGACCAGCAGTTGTAGATA			
etx	Forward	GCGGTGATATCCATCTATTC	655	53	Garmory *et al.* (2000)
	Reverse	CCACTTACTTGTCCTACTAAC			
cpIo	Forward	ACTACTCTCAGACAAGACAG	446	53	Garmory *et al.* (2000)
	Reverse	CTTTCCTTCTATTACTATACG			
luxS	Forward	ACAAAGGTTAAGGCACCATATGT	385	53	Ohtani *et al.* (2002)
	Reverse	ACCTGTTTTGCATGACTCTTAGCT			
netB	Forward	GCTGGTGCTGGAATAAATGC	384	53	Keyburn *et al.* (2008)
	Reverse	TCGCCATTGAGTAGTTTCCC			

were tested for the presence of cpa, cpb, etx, cpIo, cpb2, cpe and luxS which encode, respectively for alpha toxin, beta toxin, epsilon, Iota, beta-2 toxin, enterotoxin and *AI-2* and *netB* gene. All primers are shown in Table 1.

DNA sequencing: All products obtained following PCR for netB were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The purified PCR products were sequenced then sequences were compared in the GenBank database using Basic Local Alignment Search Tool (BLAST) through the internet (http://www.ncbi.nlm.nih.gov/BLAST) with an Accession No. EU143239 (Keyburn *et al*., 2008).

RESULTS AND DISCUSSION

Toxinotyping: All preseumptive *C. perfringens* isolates were confirmed as *C. perfringens* by toxinotyping. All isolates recovered from NE lesion or normal flora were type A (cpa positive, cpb negative, etx negative, cpIo negative, Table 2).

Distribution of cpb2 among chicken isolates: Of the 82 cpb2 positive isolates from chickens, 53 (76.8%) isolates had been recovered from 69 chickens with NE while 26 (63.4%) isolates were recovered from 41 chickens which did not have evidence of NE at necropsy examination by experienced personnel.

Distribution of luxS among chicken isolates: Of the 110 luxS positive isolates, 68 (98.6%) and 40 (97.6%) isolates had been recovered from 69 chickens with NE and 41 chickens with normal flora, respectively.

Distribution of netB among chicken isolates: Of the 27 netB positive isolates, 25 (36.2%) had been recovered from 69 chickens with NE while only 2 (4.9%) isolates were recovered from 41 chickens with normal flora.

In this study, *C. perfringens* isolates from NE-producing or healthy chickens were genotype A, a genotype which has previously been associated with NE in chicken (Park *et al*., 1996). *C. perfringens* type A commonly occurs in North America and is one of two types associated with NE (Songer, 1996). Alpha toxin was found in all bacterial cultures from the field isolates, normal flora and post-challenge isolates. In the present study, alpha toxin was recurrently found to be the only major toxin pointing to *C. perfringens* type A as the main type noted, apart from a few exceptions that are described later in this manuscript.

Toxin genes for cpb, cpe and etx were absent in all field strains, normal flora and post-challenge isolates. Toxins cpb or etx are associated with *C. perfringens* types other than type A and would have changed the toxinotype noted. Toxin gene cpe is known to cause human food poisoning and may be found in type A and type C *C. perfringens* but is not commonly seen in poultry (Gholamiandekhordi *et al*., 2006; Songer, 1996; Tschirdewahn *et al*., 1991).

It has been suggested that potentially virulent toxins cpb2 and netB could contribute to disease effects in field isolates (Bueschel *et al*., 2003; Keyburn *et al*., 2008). In the present research, these genes were also found in varying degrees in normal flora. However, no obvious NE was noted in the birds used for normal flora samples despite the presence of at least one of these toxin genes in all of the isolates. The evidence of the level of virulence of these toxin genes remains inconclusive. The results agree with those of previous studies which suggested that cpb2 and netB did not necessarily have a direct correlation with the incidence of NE (Gholamiandekhordi *et al*., 2006; Martin and Smyth, 2009). Positive PCR results for both *netB* and *cpb2* toxin genes in the field isolates could play a role in pathogenesis and

Table 2: *C. perfringens* PCR toxinotyping profile between to necrotic enteritidis producing and normal small intestine

	Toxin genes							
Isolates origin	*cpa*	*cpb*	*cpb2*	*cpe*	*etx*	*cpIo*	*luxS*	*netB*
NE-producing chickens (n = 69)[a]	69 (100)[b]	0 (0)	53 (76.8)	0 (0)	0 (0)	0 (0)	68 (98.6)	25 (36.2)
Healthy chickens (n = 41)	41 (100)	0 (0)	26 (63.4)	0 (0)	0 (0)	0 (0)	40 (97.6)	2 (4.9)
Total	110 (100)	0 (0)	79 (71.8)	0 (0)	0 (0)	0 (0)	109 (99.1)	27 (24.5)

[a]n = number; [b]No. of isolates included (%)

Table 3: *C. perfringens* PCR toxinotyping patterns between necrotic enteritidis producing and normal small intestine origin

	No. of strains by origin (%)	
Patterns of toxin gene included	NE-producing chickens (n = 69)[a]	Healthy chickens (n = 41)
cpa-luxS-netB-cpb2	18 (26.1)	1 (2.4)
cpa-luxS-netB	6 (8.7)	1 (2.4)
cpa-luxS-cpb2	23 (33.3)	26 (63.4)

[a]n = number

may have enhanced necrosis in the intestine. However, some studies have shown that β-2 toxin may not be significantly associated with NE (Bueschel *et al*., 2003; Gholamiandekhordi *et al*., 2006; Keyburn *et al*., 2008). It should be noted that the occurrence of a gene does not necessarily determine phenotype of toxin production which may be a stronger determinant of virulence (Abildgaard *et al*., 2010) (Table 3).

Positive findings for luxS were expected based on the level of conservation of this gene between bacterial types (Bassler, 1999). As a quorum-sense-related gene, luxS is reliant on predisposing factors to increase colonization by causing a shift in the gut ecology and bacterial numbers before allowing toxin production. In the case of alpha toxin, it was found that luxS had only a slight stimulatory effect and was not completely dependent on luxS for toxin production (Ohtani *et al*., 2002). However, in the results using NE-producing strains there was no difference between luxS findings in normal flora versus NE-producing strains.

CONCLUSION

This study provides a baseline for prevalence of *C. perfringens* isolated from NE-producing or healthy chickens. Therefore, more research into much better correlation between gene expression of toxin and the pathogenic capacity of *C. perfringens* may be beneficial for a epidemiological approach of NE-producing farms.

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