Molecullar Typing, Prevalence of netB and Antimicrobial Susceptibility among Clinical Isolates of *Clostridium perfringens* from Chickens in Southern China

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Abstract: *Clostridium perfringens* is an important pathogen of animals and humans and is the causative agent of Necrotic Enteritis (NE) in poultry. This study focused on the typing of intestinal *C. perfringens* isolates (n = 78, 54 from chickens with NE, 24 from healthy chickens) from China's Southern Guangdong province. All isolates were classified as type A using multiplex PCR assay. The recently described toxin gene associated with NE in chickens, netB was found in 21 (26.9%) of the isolates (18 from chickens with NE and 3 from unrelated chickens with no evidence of NE). The *in vitro* susceptibility results showed that ceftriaxone showed highest activity with lowest MIC_{50} values (0.25-0.5 μg mL^{-1}) followed by cefotaxime (0.25-4 μg mL^{-1}), penicilin (0.5-2 μg mL^{-1}). However, most of the isolates were resistant to lincomycin, tetracycline, metronidazole and neomycin as evident by the high MIC_{50}. Furthermore, 70.5% of the isolates (n = 55) were simultaneously resistant to >4 antibiotics and 30.8% of the isolates (n = 24) were resistant to all the antimicrobials tested. To the knowledge this is the first study of the prevalence of *C. perfringens* in broiler chickens in China and the first to examine the isolates for the recently identified novel toxin genes, *netB*.

Key words: China, *Clostridium perfringens*, molecullar typing, antimicrobial susceptibility, isolates, genes

INTRODUCTION

Clostridium perfringens (*C. perfringens*) is the most important clostridial pathogen of poultry, causing a panoply of diseases which include avian malignant disease, gizzard erosions and gangrenous dermatitis (Choi *et al.*, 2003; Piyankarage *et al.*, 1999; Thompson *et al.*, 2006). Necrotic Enteritis (NE) is a common and severe *C. perfringens*-induced disease and considered the most clinically dramatic bacterial enteric disease of poultry (Long *et al.*, 1974). In spite that the disease was first described almost 50 years ago, the virulence factors which lead to the development of NE have yet to be fully understood (Smyth and Martin, 2010). Pathogenicity of *C. perfringens* is associated with the production of four major toxins including alpha (α), beta (β), epsilon (ε) and iota (i). Cp strains are classified into five types (A-E) based on their ability to produce the above toxins (McClane, 2001; Petit *et al.*, 1999). *C. perfringens* type A is the most commonly recovered clostridial type from NE cases (Olkowski *et al.*, 2008). Some experimental studies indicate that alpha-toxin which was believed to be the major virulence factor in NE is not an essential factor in disease development (Keyburn *et al.*, 2006; Van Immerseel *et al.*, 2004).

Recently, Keyburn reported the discovery of a previously unidentified pore forming toxin of *C. perfringens* which they named NetB and the encoding gene was *netB*. The netB was only identified in strains recovered from chickens with NE. It was neither found in isolates from chickens which did not have NE (Keyburn *et al.*, 2008). Keyburn further reported that netB knockout mutants failed to produce NE in chickens. Results from subsequent studies showed that virulent netB-positive strains may be present in healthy birds at a low incidence and not all *C. perfringens* from chickens with NE were netB-positive (Chalmers *et al.*, 2008; Martin and Smyth, 2009; Nowell *et al.*, 2010), contradictory to the findings of Keyburn (Keyburn *et al.*, 2008).

Recent studies has focused on the role of widespread use of antimicrobials in growth promotion and therapy of infections in food-producing animals as a potential transfer route of antimicrobial-resistant bacteria or the genes encoding antimicrobial resistance into the human food chain (Piddock *et al.*, 2000). The varying, overuse and misuse of the tested antimicrobials in poultry industry has caused serious public health problems in many parts of the world (Khachatourians, 1998).

In China, there are no data on investigation of the new toxin gene (*netB*) and antimicrobial resistance of

C. perfringens isolated from chickens. This study was designed to determine the occurrence of netB among *C. perfringens* isolates from chickens with or without NE and determine its sensitivity to commonly used antimicrobials.

MATERIALS AND METHODS

Samples: About 103 broiler chickens with history of enteritis on 22 farms and 100 healthy chickens on 10 farms from Guangdong, China were examined. Jejunal swabs of the cecum and small intestine of all birds were collected for bacterial culture.

Bacterial isolation and identification: All swabs were streaked onto 5% sheep blood agar and incubated overnight at 37°C under anaerobic conditions. Isolates were presumptively identified by gram staining and lecithinase production and confirmed biochemically as *C. perfringens* based on the following tests, lactose and inositol fermentation, stormy fermentation in litmus milk, nitrate reduction, gelatinase production and motility tests (FDA, 1998). 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 stored at -80°C until further testing.

All tentatively identified *C. perfringens* were examined to determine their genotypes by multiplex PCR according to a previously described protocol (Gharaibeh *et al.*, 2010).

Detection of netB: All isolates were examined for the presence of the *netB* gene as described by Nowell *et al.* (2010).

Determination of Minimal Inhibitory Concentrations (MICs) of antimicrobials against *Clostridium perfringens* isolates: A total of 10 antimicrobials shown in Table 1 were used to test their MIC against the isolate confirmed to be *C. perfringens* by PCR. Prior to antimicrobial susceptibility test, isolates were subcultured twice on Brucella Blood Agar plate (BBA). Colonies from

Table 1: Antimicrobials used for MICS testing with their origins

Antimicrobial	Sources
Lincomycin	Sigma
Penicillin	Sigma
Ceftriaxone	Roche
Cefotaxim sodium salt	Sigma
Erythromycin	Sigma
Tetracycline	Sigma
Neomycin sulfate	Amresco
Metronidazole	Biolab
Tylosin tartrate	Sigma
Ofloxacin	Sigma

48 h BBA were suspended in enriched thioglycollate medium without indicator (Difco), to a turbidity equivalent to that of a 0.5 McFarland standard. An overnight fresh culture of each isolate in Schaedler broth (Difco) that contained approximately 10^6 CFU mL^{-1} was used in MIC testing. MIC was performed in duplicates in flat sterile 96 well plates. Final antimicrobial concentration ranged from 0.125-256 mg mL^{-1}. *C. perfringens* type A (CVCC52, from China Institue of Veterinary Drug Control) was run together with the samples as a reference strain. In each run, both sterility and growth control were included. MIC was read as the lowest antimicrobial concentration that inhibits visible bacterial growth after anaerobic incubation for 18-24 h.

RESULTS AND DISCUSSION

***Clostridium perfringens* recovery, genotyping and toxinotyping by PCR:** A total of 54 *C. perfringens* isolates were recovered from 103 broiler chickens with history of enteritis and retarded growth on 22 farms and 24 isolates were obtained from 100 healthy broiler chickens on 10 farms. All of the 78 isolates were confirmed as type A by positive PCR reaction for only alpha toxin gene (Fig. 1). Using the netB-specific primers of Nowell *et al.* (2010), a netB PCR product of the expected 383 bp in length was obtained in 21 out of the 78 isolates (26.9%) (Fig. 2). It was found in normal chickens at a low incidence (12.5%, n = 3) while a significantly higher incidence (33.3%, n = 18) was found in chickens with NE.

Minimal Inhibitory Concentrations (MICs) of antimicrobials against clostridium perfringens isolates: A total of 78 *C. perfringens* isolates were tested for their susceptibility to 10 antimicrobials and *C. perfringens* CVCC52 was used as a quality control strain in this study.
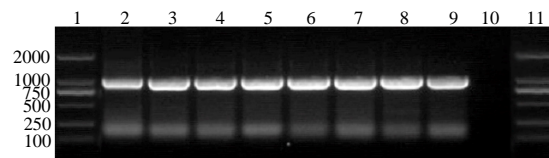

Fig. 1: PCR for alpha gene (900 bp) to confirm isolates to be *Clostridium perfringens*. Lanes 1 and 11: DNA marker, Lane 2: *Clostridium perfringens* type A reference strain (CVCC52), Lanes 3-9: Confirmed *Clostridium perfringens* isolates and Lane 10: Negative control
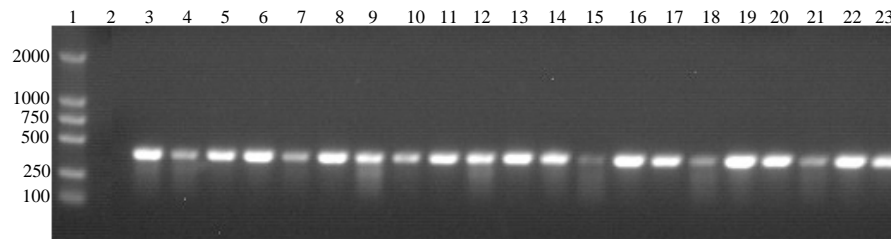
Fig. 2: PCR for *netB* gene (383 bp) from confirmed *Clostridium perfringens* type A isolates. Lanes 1: DNA marker, Lane 2: Negative control; Lanes 3-9: netB-positive isolates

Table 2: Frequency distribution of minimal inhibitory concentration of 10 antimicrobials against 78 *Clostridium perfringens* isolates

	Number of *C. perfringens* isolates with MIC values ($\mu g\ mL^{-1}$)														
Antimicrobial	≤0.125	0.25	0.5	1	2	4	8	16	32	64	128	≥256	Range	MIC_{50}	MIC_{90}
Lincomycin	0	0	0	0	0	0	0	1	2	0	0	75	16 to ≥256	≥256.00	≥256
Penicillin	25	15	0	8	11	0	0	0	0	0	0	19	0.125 to ≥256	0.25	128
Ceftriaxone	34	16	8	4	2	1	0	0	0	0	0	13	0.125 to ≥256	0.25	64
Cefotaxim	29	21	4	5	7	2	0	0	0	0	0	10	0.125 to ≥256	0.50	128
Erythromycin	0	0	0	0	0	0	0	8	8	12	21	29	16 to ≥256	128.00	≥256
Tetracycline	0	2	0	2	7	11	0	0	0	1	0	55	0.25 to ≥256	≥256.00	≥256
Neomycin	0	0	0	0	3	0	7	0	0	4	14	48	2 to ≥256	128.00	≥256
Metronidazole	0	0	0	2	0	5	0	0	0	0	0	71	1 to ≥256	≥256.00	≥256
Tylosin	0	0	0	1	2	0	0	37	24	1	0	13	1 to ≥256	64.00	≥256
Ofloxacin	0	0	1	0	0	0	29	32	1	1	0	14	0.5 to ≥256	32.00	≥256

Table 2 shows the MIC values and number of resistance of *C. perfringens* isolated from chickens with NE. *C. perfringens* isolates showed various resistant to antimicrobials tested in the present study. Most of the isolates showed resistant to lincomycin, tetracycline, metronidazole and neomycin as evident by the high MIC_{50}. Ceftriaxone showed highest activity with lowest MIC_{50} values (0.25-0.5 $\mu g\ mL^{-1}$) followed by cefotaxime (0.25-4 $\mu g\ mL^{-1}$) and penicilin (0.5-2 $\mu g\ mL^{-1}$). MIC_{50} was relatively high for ofloxacin (8-16 $\mu g\ mL^{-1}$), tylosin tartrate, (16-32 $\mu g\ mL^{-1}$) and erythromycin (16-64 $\mu g\ mL^{-1}$) and high for tetracycline (64-128 $\mu g\ mL^{-1}$), neomycin sulfate (64-256 $\mu g\ mL^{-1}$), metronidazole (≥256 $\mu g\ mL^{-1}$) and lincomycin (≥256 $\mu g\ mL^{-1}$). MIC_{90} was the lowest for ceftriaxone (64 $\mu g\ mL^{-1}$), penicillin (128 $\mu g\ mL^{-1}$) and cefotaxim sodium salt (128 $\mu g\ mL^{-1}$). All of the other antimicrobials tested had an MIC_{90} ≥256 $\mu g\ mL^{-1}$. About 70.5% of the isolates (n = 55) were simultaneously resistant to >4 antibiotics and 30.8% of the isolates (n = 24) were resistant to all of the antimicrobials tested. Ceftriaxone had the highest activity with overall MIC_{50} and MIC_{90} of 0.25 and 64 $\mu g\ mL^{-1}$, respectively. MIC_{50} and MIC_{90} for each antimicrobial are shown in Table 2.

NE is primarily caused by *C. perfringens* type A and to a lesser extent type C strains (Thompson *et al.*, 2006). It is worthwhile to note that the *C. perfringens* type A was the primary cause of the CPE-mediated food poisoning in the industrialized countries and poses significant public health concerns (Fisher *et al.*, 2005). In the present study, typing of *C. perfringens* isolates by multiplex PCR revealed that all isolates were only positive to alpha toxin gene of the four major lethal toxin genes indicating that all *C. perfringens* isolates represented type A. In Korea, only *C. perfringens* type A was present in poultry (Yoo *et al.*, 1997). More recently and similar to the present study, only *C. perfringens* type A from poultry was reported in Finland (Heikinheimo and Korkeala, 2005) and Sweden (Engstrom *et al.*, 2003).

It has recently been reported by Keyburn that netB, a newly discovered pore forming toxin is critical to the development of NE in chickens (Keyburn *et al.*, 2008). They also reported that the isolated netB-positive strains only came from chickens with NE (Keyburn *et al.*, 2008). The definitive discovery of the toxin which enables *C. perfringens* to produce NE obviously would represent a major breakthrough in the understanding of the pathogenesis of this serious and economically important disease. Therefore, results from subsequent studies showed that virulent netB-positive strains may be present in healthy birds at a low incidence and not all *C. perfringens* from chickens with NE were netB-positive (Chalmers *et al.*, 2008; Martin and Smyth, 2009; Nowell *et al.*, 2010). The present investigation revealed a prevalence of netB positive *C. perfringens* isolates of 12.5 and 33.3% in diseased and healthy broilers, respectively.

This result differs slightly from the investigations performed in Australia, Canada, the United States and Denmark (Chalmers *et al.*, 2008; Keyburn *et al.*, 2008;

Martin and Smyth, 2009; Nowell *et al.*, 2010) where the prevalence of the *netB* gene in *C. perfringens* isolates obtained from broilers suffering from NE were generally higher (52-100%). The US investigations (Martin and Smyth, 2009) also revealed netB-positive *C. perfringens* in healthy broilers with low prevalence (8.8%) (Martin and Smyth, 2009). The Canadian and Danish investigations revealed higher prevalence (35 and 61%, respectively) than that of the present study (Chalmers *et al.*, 2008; Nowell *et al.*, 2010). It is worth noting that a study reported that the incidence of the *netB* gene in *C. perfringens* isolates obtained from healthy broilers was higher than that in isolates from chickens suffering from NE (Abildgaard *et al.*, 2010). This finding is not consistent with the hypothesis that netB is the cause of NE (Keyburn *et al.*, 2008) suggesting that either netB is not essential to the development of NE in all cases or that netB is readily lost by the organism. Experimental studies in a disease model are necessary to investigate the disease producing capabilities of both the netB negative strains recovered from cases of NE and also the netB positive strains recovered from normal chickens.

Minimal inhibitory concentration results revealed that isolates had various susceptibilities to these antimicrobials. In this study, the three antimicrobials that were highly active against *C. perfringens* had the MIC_{50} of 0.25 µg mL^{-1} for both ceftriaxone and penicilin and 0.5 µg mL^{-1} for cefotaxime. Lincomycin, metronidazole and tetracycline were historically known to be among the antimicrobials of choice to treat *C. perfringens* infections in China, their MIC_{50} was ≥256 µg mL^{-1}. Similar results were presented from United States, Jordan and Belgium where MIC_{50} were very high for these antimicrobials (Gharaibeh *et al.*, 2010; Martel *et al.*, 2004; Yoo *et al.*, 1997). These results indicated marked resistance development. Differences in MICs profiles between antimicrobials may reflect the varying, overuse and misuse of the tested antimicrobials in poultry industry in China. Researchers recommend using ceftriaxone, cefotaxime and penicilin for treatment of *C. perfringens* infection in China. Researchers also recommend stopping the use of the popular feed additives that contains lincomycin, metronidazole and tetracycline to control *C. perfringens* infections.

This study also revealed a high prevalence of antimicrobial-resistant *C. perfringens* strains. About 70.5% of the isolates (n = 55) were simultaneously resistant to more than four antibiotics and 30.8% of the isolates (n = 24) were resistant to all of the antimicrobials tested. This may reflect the widespread or inappropriate use of antimicrobials in chickens resulting in the emergence of multiple-drug resistant strains. They correlate with the amounts and types of antimicrobials consumed by these populations and changes in resistance can be considered as an early warning system for resistance to be expected in potentially pathogenic bacteria (Van den Bogaard *et al.*, 2000). In food-producing animals, a low prevalence and low degree of antimicrobial resistance in the intestinal flora should be considered a distinguishing quality and safety marker.

CONCLUSION

To the knowledge this is the first study of the prevalence of *C. perfringens* in broiler chickens in China and the first to examine the isolates for the recently identified novel toxin genes, *netB*. However, the role of netB in the induction of necrotic enteritis needs to be further investigated by determining the disease producing capability of both netB positive strains recovered from normal chickens and netB negative strains recovered from chickens with NE.

ACKNOWLEDGEMENTS

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