Original Article

Characterization of *β*-lactamase and quinolone resistant *Clostridium perfringens* recovered from broiler chickens with necrotic enteritis in Bangladesh

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Abstract

Background: *Clostridium perfringens* causes necrotic enteritis (NE) and is considered a major economic burden in the broiler industry and a significant foodborne pathogen, worldwide. **Aims:** *Clostridium perfringens* isolated from NE affected broiler chickens was aimed to characterize and the presence of *β*-lactamase and quinolone resistant genes were also investigated in the isolates. **Methods:** A total of 224 intestinal and caecal specimens were collected from NE affected broiler chickens and cultured to isolate *C. perfringens*. The toxicogenic characterization of *C. perfringens* was appraised using polymerase chain reaction (PCR) and antibiotic susceptibility testing (disc diffusion method). The selected *C. perfringens* isolates were characterized for β-lactamase and quinolone encoding genes by PCR analysis. **Results:** All isolates were cultured positive for *C. perfringens* and the toxin-encoding genes of *C. perfringens* (*α*-, *β*-, *β2*-, *ε*-, *ι*-, and enterotoxin) were also identified. About 65.6% of isolates had a multi-drug resistant (MDR) profile but none of these isolates were resistant or susceptible to all screened antibiotics. A subset of isolates, 160 and 98 were analyzed for β-lactamase and quinolone genes, respectively, and recognized *bla*$_{TEM}$, *bla*$_{SHV}$, and *bla*$_{OXA}$ in 64 (40%; CI: 32.35-48.03%; P<0.001) isolates, and *qnrB* and *qnrS* in 28 (28.57%; CI: 19.90-38.58%; P<0.001) isolates except *qnrA*. **Conclusion:** Therefore, the isolates of *C. perfringens* were toxicogenic and carried β-lactamase, and quinolone resistance genes. Nowadays, the rational use of antibiotics and safe production of broiler chickens are the major concern to save public health.

Key words: Broiler, β-lactamase, *Clostridium perfringens*, Antibiotic resistance, Quinolone

Introduction

Necrotic enteritis (NE) is a major interest for the broiler industry due to it being a major economic burden. Necrotic enteritis infection can reduce body weight by 12.0% and increase feed conversion ratio (FCR) by 10.9% compared to healthy birds. As a result, NE causes a loss of 878.19$ to 1480.52$ per flock in the USA and USD 2 to 6 billion worldwide per annum (Skinner *et al*., 2010; Wade and Keyburn, 2015). Broiler chickens need maximum utilization of feeds by maximum digestion, absorption, and metabolism of nutrients. Necrotic enteritis disrupts the intestinal mucosa lining of chicken that decreased nutrient absorption resulting in reduced feed efficiency or FCR which is the principal parameter of good nutrition (Yang *et al*., 2019). The major cause of NE is *Clostridium perfringens*, a Gram-positive, spore-forming, rod shaped, anaerobic bacteria that is ubiquitously present in the environment and normal gut flora (Miller *et al*., 2010; Freedman *et al*., 2015). It could become pathogenic when the gut microbiome is changed from the dynamic balance of the gut environment by stress, starvation, sudden change of feed, the therapeutic use of antibiotics or anthelmintic resulting in NE in poultry, enteritis in ruminants, non-ruminants, and humans (Prescott, 2016).

Clostridium perfringens has significant importance in public health as it can cause foodborne illness and has been reported as the second most common bacterial foodborne illness in the USA (Scallan *et al*., 2011).

The concerning point of *C. perfringens* is the ability to produce potent exotoxins which are responsible for developing specific enterotoxemia in animals and humans. It can encode for up to 17 exotoxins (Freedman *et al*., 2015) and based on the production of four major lethal exotoxins (*α*: alpha, *β*: beta, *ε*: epsilon, and *ι*: toxins), *C. perfringens* is classified into five classes from type A to type E which are responsible for specific diseases (Yoo *et al*., 1997). Both type A and B can produce *α* toxin, in addition to β toxin, that is highly necrotizing and lethal and responsible for severe intestinal necrosis (Cooper and Songer, 2010). The β toxin is encoded by plasmid-mediated *cpb* gene (Gkiourtzidis *et al*., 2001). Type B and D both can

produce ε-toxin that is encoded by plasmid-mediated *etx* gene and responsible for NE after cleavage by proteolytic enzyme (Chen *et al*., 2011). The type E can produce α-toxin and ι-toxin encoded by *cpa* and *iap* gene, responsible for gas gangrene, intestinal disorder, and diarrhea (Park *et al*., 2015). Enteric bacteria are able to acquire and exchange genetic materials via transposons, plasmids or through chromosomal exchange or mutation in DNA (Gyles and Boerlin, 2014). Several investigations revealed that *C. perfringens* is intrinsically resistant to several antimicrobial agents commonly used against Gram-positive bacterial infection and perform a role as multi-drug resistant (MDR) pathogens (Lee, 2016). This study also identified an MDR characteristic of *C. perfringens* as phenotypical and the responsible antibiotic resistant genes. The emergence and rapid spread of MDR *C. perfringens* poses a serious therapeutic challenge for simple foodborne illness by effective antimicrobial therapy, due to the scarcity of newer antimicrobial agents. Antibiotic resistance is an alarming issue now due to an emerging public health crisis and one of the top health challenges of the 21st century (Marshall and Levy, 2011; Ali *et al*., 2019). It is assumed that the antibiotic use in feed (e.g. bacitracin, tetracycline, avoparcin, vancomycin, virginiamycin erythromycin, tylosin, avilamycin, lincomycin, bambermycins, carbadox, etc) mainly focused on *C. perfringens* control as well as growth promoter called antibiotic growth promoters (AGPs) (Arnold *et al*., 2004; Marshall and Levy, 2011; Landers *et al*., 2012). Therefore, bacteria become resistant by adapting against repeated use of antibiotics with feeds and escape the mode of action of antibiotics by developing a resistance gene in their plasmid or chromosome (Diarra *et al*., 2007; Nhung *et al*., 2017).

There is a need to explore and elucidate the safe alternatives of antibiotics to control NE and to better understand the toxicogenic properties of *C. perfringens* isolated from NE affected broiler chickens with their antimicrobial resistance phenotypes and genotypes. There is a need to explore and elucidate the safe alternatives of antibiotics to control NE. In this context, the aim of the present work was to gain insights into the toxicogenic properties, antibiotic resistance patterns, presence of β-lactamase and quinolone encoded genes in *C. perfringens* isolated from broiler chickens with NE.

Materials and Methods

Sample collection and processing

A total of 224 clinically NE diagnosed Cobb500 broiler chickens between 7 and 30 days old came in Nourish Customer Service Laboratory, Cumilla and Rajshahi from 2017 to 2018. Chickens were inspected during the postmortem examination and were confirmed by observing standard NE lesions as previously described (Immerseel *et al*., 2004). Part of the small intestine and caecum were collected (2.5 cm of each) into a 10 ml tube containing 5.0 ml sterile phosphate buffered saline (PBS). The samples of intestine and caecum were homogenized by sterile pestle and mortar then clarified by centrifugation at 2147 × g for 10 min. The supernatant was collected and used as an inoculum.

Bacterial isolation

An inoculum of 1 ml sample was diluted into 9 ml of brain heart infusion (BHI) broth (Oxoid, UK) then incubated for 24 h at 37°C in an anaerobic vented jar with an appropriate amount of anaerobic gas generating from packs (Mitsubishi Gas Chemical America Inc., New York). Subsequently, 100 µL of pre-enriched BHI broth was inoculated into plates of tryptose sulfite cycloserine (TSC) agar base (Oxoid, UK) enriched with 5% egg yolk and supplemented with β D-cycloserine (Oxoid, UK). The plates were sealed tightly, incubated for 24 h at 37°C upsides down in an anaerobic condition. The black colonies on plates were examined for typical colonies of *C. perfringens* (Mwangi *et al*., 2018). Finally, the pure isolates were confirmed by the biochemical (lactose fermentation) and Gram-staining test.

Polymerase chain reaction (PCR) test for *C. perfringens* toxin genes screening

The DNA extraction of isolated *C. perfringens* was done by using the direct lysis method as described by Kim *et al*. (2011). Screening the presence of six major toxin genes (*α/cpa*, *β/cpb*, *B2/cpb2*, *ε/etx*, *ι/ipa*, and enterotoxin/*cpe*) by using six pairs of primers shown in Table 1. Total 25 µL of PCR amplification reaction mix were prepared using GoTaq® Green Master Mix (2×) (Promega, Madison, WI), 0.4 mM of each oligonucleotide primers, 6 µL of template DNA, and dH_2O. The PCR assay was performed in a thermal cycler (MiniAmp Plus Thermal Cycler, Applied Biosystems, USA) with amplification conditions as following: denaturation for 30 s at 94°C; primer annealing for 30 s at the respective temperature as 46°C for *cpa* gene, *etx* gene and *ipa* gene, 39°C for *cpb* gene, 48°C for *cpb2* gene, and 52°C for *cpe* gene; finally the chain extension for 30 s at 72°C. Then PCR products were separated by electrophoresis through 1% agarose gel stained with 0.5 µg/ml ethidium bromide at 95 V for 40 min. Then the amplification fragments were visualized under ultraviolet light at 254 nm wavelength (UVP, Inc., Upland, CA) (Fig. 1).

Antimicrobial susceptibility assay

Antibiotic susceptibility test was carried out for all 224 isolates by disc diffusion method on Muller Hinton agar (MHA) (Oxoid, UK) according to Clinical and Laboratory Standards Institute guideline (CLSI, 2012). The antibiotic discs were selected based on available antibiotics used in poultry and human medicine in Bangladesh including β-lactam (amoxicillin/clavulanic acid 30 µg; penicillin G 10 µg), quinolones (ciprofloxacin 5 µg; norfloxacin 5 µg), macrolides (erythromycin 15 µg), sulfonamides (co-trimoxazole 25 µg), aminoglycosides (gentamycin 30 µg; streptomycin 10 µg; amikacin 30 µg), cephalosporin (ceftriaxone 5 µg), and tetracyclines (tetracycline 30 µg) (Oxoid, UK).

Table 1: Oligonucleotide primer sequences used for *C. perfringens* toxin genes *cpa* (α-toxin), *cpb* (β-toxin), *cpb2* (β2-toxin), *cpe* (enterotoxin), *etx* (ε-toxin), and *iap* (ι-toxin)

Toxin/gene	Primer	Oligonucleotide sequence	Fragment length (bp)	References
α/cpa	CPALPHATOX1-F CPALPHATOX1-R	AAGATTTGTAAGGCGCTT ATTTCCTGAAATCCACTC	1167 bp	Gkiourtzidis *et al*. (2001)
β/cpb	CPBETATOX1-F CPBETATOX1-R	AGGAGGTTTTTTTATGAAG TCTAAATAGCTGTTACTTTGTG	1025 bp	
β2/cpb2	P319BETA2 P320BETA2	GAAAGGTAATGGAGAATTATCTTAATGC GCAGAATCAGGATTTTGACCATATACC	573 bp	
ε/etx	CPETOXIN1-F CPETOXIN1-R	AAGTTTAGCAATCGCATC TATTCCTGGTGCCTTAATT	961 bp	
Enterotoxin/*cpe*	CPENT1-F CPENT1-R	TAACAATTTAAATCCAATGG ATTGAATAAGGGTAATTTCC	933 bp	
ι/iap	CPIOTA1-F CPIOTA1-R	AATGCCATATCAAAAAATAA TTAGCAAATGCACTCATATT	821 bp	

Table 2: PCR primer sequences for β-lactamase and quinolone resistance genes detection

Target gene	Primer	Sequence (5´-3´)	Amplicon size (bp)	References
bla$_{TEM}$	MultiTSO-T_F MultiTSO-T_R	CATTTCCGTGTCGCCCTTATTC CGTTCATCCATAGTTGCCTGAC	800	Dallenne *et al*. (2010)
bla$_{SHV}$	MultiTSO-S_F MultiTSO-S_R	AGCCGCTTGAGCAAATTAAAC ACCCGCAGATAAATCACCAC	713	
bla$_{OXA}$	MultiTSO-O_F MultiTSO-O_R	GGCACCAGATTCAACTTTCAAG GACCCCAAGTTTCCTGTAAGTG	564	
qnrA	QnrAm-F QnrAm-R	AGAGGATTTCTCACGCCAGG TGCCAGGCACAGATCTTGAC	580	Cattoir *et al*. (2007a)
qnrB	QnrBm-F QnrBm-R	GGMATHGAAATTCGCCACTG TTTGCYGYYCGCCAGTCGAA	264	Cattoir *et al*. (2007b)
qnrS	QnrSm-F QnrSm-R	GCAAGTTCATTGAACAGGGT TCTAAACCGTCGAGTTCGGCG	428	Cattoir *et al*. (2007a)

PCR: Polymerase chain reaction

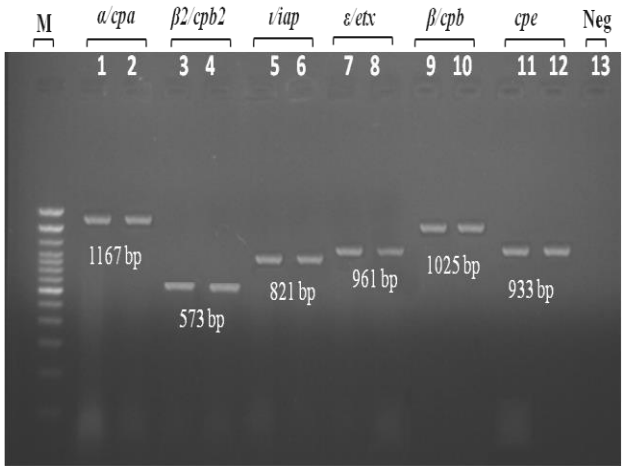

Fig. 1: Detection of seven toxin genes of *C. perfringens* by PCR. Lane M: Marker (DNA ladder, 100 bp), Lanes 1-2: *α/cpa*, Lanes 3-4: *β2/cpb2*, Lanes 5-6: *ι/ipa*, Lanes 7-8: *ε/etx*, Lanes 9-10: *β/cpb*, Lanes 11-12: *cpe*, and Lane 13: Negative control

The 0.5 McFarland standard inoculum of pure colonies was streaked homogenously on MHA using a sterile cotton swab and incubated at 37°C for 12 h. Then inhibition zone was measured and categorized as susceptible, resistant, and intermediate according to CLSI (2012) guideline.

Antibiotic resistance gene identification

The isolates identified as resistant against amoxicillin/clavulanic acid and penicillin G were considered for β-lactamase encoding genes (*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{OXA}$) screening. Quinolone resistant encoding genes (*qnrA*, *qnrB*, *qnrS*) were screened for those isolates containing phenotypic resistance against ciprofloxacin and norfloxacin. Total of 5 µL DNA was subjected to each multiplex PCR in a 25 µL reaction mixture containing 1 × PCR buffer (0.025U/µL Taq DNA polymerase, reaction buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP (dATP, dCTP, dGTP, and dTTP) (Thermo Scientific PCR Master Mix 2×) and a variable concentration of specific primers (Table 2). β-lactamase genes amplification was carried out as follows: initial denaturation at 94°C for 10 min, 30 cycles of 94°C for 40 s, 60°C for 40 s, and 72°C for 1 min and, a final

elongation step at 72°C for 7 min. Quinolone resistant genes (*qnrA*, *qnrB*, and *qnrS*) amplification was carried out at 10 min at 95°C and 35 cycles of amplification consisting of 1 min at 95°C, 1 min at 54°C and 1 min at 72°C and 10 min at 72°C for the final extension.

Statistical analysis

The data from sample collection and laboratory results were recorded as coding into Microsoft Excel spreadsheet 2010 (Microsoft Corporation, WA, USA) and analyzed by exporting to STATA version 25.0 (Stata Corp. College Station, TX, USA) (IBM Corp, 2017). Descriptive cross tabulation statistics and frequency statistics were used for prevalence data analysis. The Chi-square test (χ^2 value) was used for calculating statistical significance level. The confidence levels at 95% (95% CI) were calculated and probability value of $P \leq 0.05$ was considered for statistical significance level.

Results

Isolation and identification of *C. perfringens*

Out of 224 clinically suspected NE samples, *C. perfringens* were identified in 100% samples by the conventional cultural characteristics method. Finally, all isolates were confirmed for *C. perfringens* by identifying presence of six toxin genes including *cpa*, *cpb*, *cpb2*, *etx*, *ipa*, and *cpe* among which *cpa* gene was present in all isolates.

Toxinotyping of isolated *C. perfringens*

The toxin typing of *C. perfringens* were done by PCR assay in which 100% (n=224) isolates were positive for *cpa*, while individually only 32.59% (73/224; CI: 26.50-39.15%) isolates carried *cpa* gene. 8.04% (18/224; CI: 4.83-12.40%) of isolates were identified positive for both *cpa* and *cpe* genes; moreover, 24.01% (54/224; CI: 18.66-30.25%) isolates were *cpa*+ and *cpb2*+ classified as *C. perfringens* type A. Type C was identified 11.61% in which *cpa*+, *cpb*+ and *cpb2*+ genes were 7.59% (17/224; CI: 4.48-11.87%), and *cpa*+, *cpb*+, *cpb2*+ and *cpe* genes were 4.02% (9/224; CI: 1.85-7.49%). Type D was identified 18.64% in which *cpa*+ and *etx*+ genes were 7.59% (17/224; CI: 4.48-11.87%), and *cpa*+, *cpb2*+ and *etx*+ genes were found 12.05% (27/224; CI: 8.10-17.05%). Furthermore, type E was recognized 4.02% (9/224; CI: 1.85-7.49%) (with *cpa*+ and *iap* genes) (Table 3). Type B however was not found in any isolates. This results were shown statistically significant relation ($\chi^2 = 154$; $P<0.001$).

Antimicrobial susceptibility test

Among the isolates, about 147 (65.62%) were found resistant to three or more antibiotic classes termed as MDR. While none of them were resistant or susceptible to all tested antibiotics. Among the tested isolates, most resistance observed against co-trimoxazole 96.42% (216/224) and streptomycin 92.85% (208/224), followed by tetracycline 75% (168/224), gentamicin 70.98% (159/224), penicillin G 68.75% (154/224), norfloxacin 39.73% (89/224); erythromycin 34.82% (78/224); amoxicillin/clavulanic acid 30% (67/224); ciprofloxacin 17.85% (40/224); and ceftriaxone 2.67% (6/224) (Table 4). However, the highest susceptible antibiotics were

Table 3: Distribution of the different *C. perfringens* types and their toxin genes isolated from intestine and caecal samples of necrotic enteritis in broiler chicken

C. perfringens types	*C. perfringens* toxin genes	Number of isolates (%)	95% CI	χ^2 value	P-value
A	*cpa*	73 (32.59)	26.50-39.15	154	0.001
A	*cpa*, *cpe*	18 (8.04)	4.83-12.40		
C	*cpa*, *cpb*, *cpb2*	17 (7.59)	4.48-11.87		
C	*cpa*, *cpb*, *cpb2*, *cpe*	9 (4.02)	1.85-7.49		
D	*cpa*, *etx*	17 (7.59)	4.48-11.87		
D	*cpa*, *cpb2*, *etx*	27 (12.05)	8.10-17.05		
A	*cpa*, *cpb2*	54 (24.11)	18.66-30.25		
E	*cpa*, *iap*	9 (4.02)	1.85-7.49		
Total: 224					

Table 4: Antibiotic susceptibility patterns of *C. perfringens* isolates (n=224) recovered from broiler chickens affected by necrotic enteritis

Antibiotics	Susceptible % (n)	Resistant % (n)	Intermediate % (n)	Non-susceptibility (resistant+intermdiate) % (n)
Amoxicillin/clavulanic acid	50% (112)	30% (67)	20% (45)	54.46% (122)
Penicillin G	5.8% (13)	68.75% (154)	25.44% (57)	94.19% (211)
Ciprofloxacin	71.87% (161)	17.85% (40)	10.26% (23)	28.12% (63)
Norfloxacin	44.64% (100)	39.73% (89)	15.62% (35)	55.35% (124)
Erythromycin	46.87% (105)	34.82% (78)	18.3% (41)	53.12% (119)
Co-trimoxazole	3.57% (8)	96.42% (216)	0	96.93% (216)
Ceftriaxone	96.42% (216)	2.67% (6)	0.9% (2)	3.57% (8)
Amikacin	97.76% (219)	0	2.23% (5)	0.89% (2)
Gentamycin	16.96% (38)	70.98% (159)	12% (27)	83.03% (186)
Streptomycin	7.14% (16)	92.85% (208)	0	92.85% (208)
Tetracycline	15.17% (34)	75% (168)	9.82% (22)	84.82% (190)
P=0.85				

Table 5: Antibiotic resistance gene prevalence of *C. perfringens* isolates (β-lactamase resistant isolates= 160; quinolones resistant isolates= 98) recovered from broiler chickens affected by necrotic enteritis

Resistant genes		Number of isolates (%)	95% CI	χ^2 value	P-value
β-lactamase	bla_{TEM}	22 (13.75)	8.82-20.07	54.6	0.001
	bla_{SHV}	17 (10.62)	6.31-16.47		
	bla_{OXA}	25 (15.63)	10.37-22.20		
	Sub-total	64/160 (40.00)	32.35-48.03		
Quinolone	*qnrA*	0	-	35.03	0.001
	qnrB	18 (18.37)	11.26-27.47		
	qnrS	10 (10.20)	5.00-17.97		
	Sub-total	28/98 (28.57)	19.90-38.58		

shown in amikacin 97.76% (219/224), ceftriaxone 96.42% (216/224), and ciprofloxacin 71.87% (161/224) in comparison with the other antibiotics. But no significant correlation (P=0.85) was found among antibiotics.

Identification of *β*-lactamase and quinolone encoded genes

A total of 160 isolates was shown resistant against amoxicillin/clavulanic acid and penicillin G that was considered screening for three β-lactamase genes including bla_{TEM}, bla_{SHV} and bla_{OXA}. The β-lactamase encoding genes were identified in 64 (n=160; 40%; CI: 32.35-48.03%) isolates and the prevalence was found 13.75% (22/160; CI: 8.82-20.07%), 10.62% (17/160; CI: 6.31-16.47%), and 15.63% (25/160; CI: 10.37-22.20%) for bla_{TEM}, bla_{SHV} and bla_{OXA}, respectively. Contrarily, total of 98 isolates was identified as resistant against ciprofloxacin and norfloxacin encoding genes. Whereas, quinolone encoding genes were identified in 28 (n=98; 40%; CI: 19.90-38.58%) isolates including 18.37% (18/98; CI: 11.26-27.47%) *qnrB* gene and 10.20% (10/98; CI: 5.00-17.97%) *qnrS* gene, but no *qnrA* gene was found. The results of both β-lactamase and quinolone encoding genes prevalence were shown statistically significant (P<0.001) relation (Table 5).

Discussion

The broiler industries all over the world are facing significant challenges with NE caused by *C. perfringens. Clostridium perfringens* can directly affect the feed digestion and nutrient absorption as well as affecting feed conversion ratios (FCR) (Mwangi *et al*., 2018). The standard clinical signs of NE like roughened intestinal surfaces, low absorption, and retardation of growth were the sampling criteria of this study (Cooper and Songer, 2010). *Clostridium perfringens* type A is the most prevalent type of this bacterium in poultry that was found about 64.73% (145/224), herein (Yoo *et al*., 1997). Besides, type A has public health importance and is responsible for gas gangrene and food poisoning in humans (Uzal *et al*., 2014). Enterotoxigenic *C. perfringens* is the major pathogen of foodborne diseases to humans where the main sources of this organism are poultry and meat products (Cooper *et al*., 2013). It was estimated that about one million people are affected by *C. perfringens* per year and it has become the second highest foodborne pathogen in the USA (Scallan *et al*., 2011).

Clostridium perfringens is also called a commensal bacterium found in the gastrointestinal tract without showing pathogenicity except with high infective dose and stress (Miller *et al*., 2010; Cooper *et al*., 2013; Mwangi *et al*., 2018). Toxins are the major virulence factor of *C. perfringens* that plays a significant role in gut pathogenicity and public health concerns (Cooper *et al*., 2013). This study demonstrated that the isolated *C. perfringens* were capable to produce six types of toxins which should be seriously noticed in poultry enteric health and public health. The *cpb2* gene represents the β2 toxin the most lethal enterotoxin for poultry found in a maximum number of isolates (Gkiourtzidis *et al*., 2001). There is a lack of in-depth studies on *C. perfringens* and to the best of our knowledge, *C. perfringens* toxin typing is absence in Bangladesh although the prevalence of NE is demonstrated at 8% in broiler chicken (Miah *et al*., 2011).

Antimicrobial resistance is the burning issue in Bangladesh as well as globally (Ahmed *et al*., 2019). Antibiotic used in animal feeds as a growth promoter is also an issue in Bangladesh including the subcontinent. Since 2010 the government of Bangladesh made a decision to completely ban any antibiotics as growth promoter in animal feeds through the legislation of Fish and Animal Feed Act (2010). Using anticoccidial drugs and antibiotics in animal feeds to control coccidiosis and NE were the prime focus of animal feed manufacturers (M'Sadeq *et al*., 2015). The present study revealed that 100% of the *C. perfringens* isolates were resistant to at least one antibiotic and there was no single isolate susceptible to all tested antibiotics. It was also found that the isolates were resistant to amoxycillin/clavulanic acid, ciprofloxacin, and norfloxacin. The findings showed alarming signs to the veterinary and public health aspects due to the number and variation of human antibiotics used in veterinary practices. It was encouraging to Bangladesh that in May 2019 Bangladesh government prohibited using of human antibiotics to veterinary practice and at July 2019 High Court of Bangladesh gave a rule that only registered veterinarians have the right to prescribe antibiotics to animals (The Daily Star, 2019). The most antibiotic classes used in poultry feeds include glycolipids (bambermycin), ionophores (salinomycin), polypeptides (bacitracin), and β-lactams (penicillin) as

AGP (Singer *et al*., 2019). Although these antibiotics improve feed efficiency and increase growth, they modify intestinal flora and create a selective pressure to develop antibiotic resistance (Collier *et al*., 2003). Therefore, several European countries banned or restricted the use of AGP in the feed (Casewell *et al*., 2003). The β-lactam antibiotic like penicillin had been used in poultry feeds for growth promotion (Collier *et al*., 2003). We found β-lactamase encoding genes like *bla*$_{TEM}$, *bla*$_{SHV}$, and *bla*$_{OXA}$ in *C. perfringens* which indicated a major public health concern because they can be shed into the environment and share across other microflora resulting in superbugs in the future (Burki, 2018). Prevalence of extended spectrum beta-lactamase (ESBL) producing isolates was demonstrated in 37.8% isolates from Bangladesh and 42.3% isolates from India (Abrar *et al*., 2019).

The fluoroquinolone-resistant antibiotics can carry several classes of plasmid-mediated quinolone resistance genes like *qnrB*, *qnrC*, *qnrD*, *qnrS*, and *qnrVC* that can reduce the susceptibility to fluoroquinolone (Garoff *et al*., 2017). We found *qnrB* and *qnrS* genes in *C. perfringens* isolates that supported the evidence of the presence of *qnrB* and *qnrS* genes in *C. perfringens* isolated from poultry (Poirel *et al*., 2012).

Toxigenic *C. perfringens* plays an important role to develop NE in broiler chickens. Six different types of toxigenic encoding genes were identified from 224 *C. perfringens* isolates of NE diagnosed in broiler chickens. The *C. perfringens* isolates have developed resistance to more than one antibiotic. The irrespective use of β-lactamase and quinolone in the poultry ration or therapeutics can cause hazards for human health through intestinal microflora imbalance, antimicrobial resistance as well as impaired therapeutic efficacy. *Clostridium perfringens* is an important public health pathogen and monitoring should be taken into account the prevention and control from poultry feeds and products. Any ignorance can cause severe foodborne health hazards. Therefore, rationing use of any antimicrobials and strict legislation and enforcement to antimicrobial use in food animals along with maintaining withdrawal period is mandatory to combat antimicrobial resistance.

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Conflict of interest

None of the authors have any conflict of interest to declare.

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