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Article

Occurrence of *Clostridium perfringens* in layer flocks of selected districts in Bangladesh: molecular typing, antimicrobial susceptibility

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Abstract: Necrotic enteritis (NE) is one of the important enteric diseases in poultry caused by *Clostridium perfringens* which leads to considerable economic losses for poultry raisers. The present research was carried out to estimate prevalence, risk factors and to confirm molecular pattern, antimicrobial susceptibility status of *Clostridium perfringens* in layer flocks in Mymensingh and Gazipur districts of Bangladesh, where a total of 287 samples were collected that comprised of water (30), workers' hand washing (30), and feed (30) as environmental pooled samples; cloacal swab (150) and post mortem sample (intestine) (47) as individual samples, and evaluated by basic (culture, biochemical test), and finally toxinotyping multiplex PCR assays. The flock-level data on potential risk factors were collected through semi-structured interviews with the farm owners and evaluated via univariable and multivariable logistic regression analyses where a *p*-value of <0.05 was considered as statistically significant. Overall flock-level prevalence of *Clostridium perfringens* was confirmed to be 6.3% (95% confidence interval [CI]: 3.8-9.7%). The occurrence of *C. perfringens* was found to be higher in the intestine samples (12.8%, 95% CI: 4.8- 25.7%) followed by feed (6.7%, 95% CI: 0.8-22.1%), cloacal swabs (6.0%, 95% CI: 2.8-11.1%) and workers' hand washing (3.3%, 95% CI: 0.1- 12.2%). History of immune-suppressive disease (like New castle disease/egg drop syndrome/infectious bursal disease) [Odds Ratio (OR) = 22.16, 95% CI: 1.40-349.36, P = 0.027] and flock size more than 3000 birds (OR = 11.16, 95% CI: 1.26-98.25, P = 0.029) were found to be associated with C. *perfringen*s infection status in the layer flocks. In this study, *C. perfringens* toxinotype A is circulating in layer flocks with 72.2% of isolates were established as multidrug-resistant as they demonstrated resistance between 3 to 6 antimicrobial agents. Evidence-based control measures to be taken namely use of probiotics, prebiotic, organic acids, and plant extracts as natural alternatives including control of immune-suppressive diseases are needed to minimize the NE infection and antimicrobial resistance in low biosecurity layer flocks.

Keywords: *Clostridium perfringens*; necrotic enteritis (NE); layer flocks; antimicrobial resistant; low-biosecurity; Bangladesh

1. Introduction

Clostridium perfringens is a gram-positive, spore-forming, rod-shaped anaerobic bacterium (Abdelrahim *et al*., 2019). This pathogen is distributed everywhere in the environments like as a usual component of soil, decaying vegetation, contaminated food, sediment or even gastrointestinal tract of poultry, and litter which could enable

horizontal transmission of this pathogen (Abdelrahim *et al*., 2019; Hibberd *et al*., 2011). In poultry, toxinotype A strains of *C. perfringens* is considered to be the causative agent of necrotic enteritis (NE), however, to a minor extent, toxinotype C is also accountable for this disease occurrence. The disease is characterized by the damage of tissue in the proximal part of the small intestine (Guran and Oksuztepe, 2013; Moore, 2015; Swayne *et al*., 2013). Usually, the number of *C. perfringens* in the intestinal content is low (approximately 10^4 CFU/g of ingesta). However, this disease may manifest when an influx of high bacterial load occurs due to the destruction of the intestinal mucosa (Al-Sheikhly and Truscott, 1977) to a concentration of microbial load which ranges from 10^7 to 10^9 CFU/g of ingesta responsible for toxin production (Kondo, 1988).

The toxinotype *C. perfringens* were classified into five groups namely A, B, C, D, and E; among them, A, C, and D have been identified as the causal agents of NE in chickens (Engström *et al*., 2003; Kalender *et al*., 2005). The production of numerous toxins like alpha (α), beta (β), epsilon (ε), and iota (ι) by *C. perfringens* is related with the pathogenicity of these organisms (Schlegel *et al.*, 2012; Yoo *et al.*, 1997). The α-toxin is produced by type 'A' strain whereas, α-toxin and β-toxin are produced by type 'C'. The α-toxin produced by *C. perfringens* is liable for the major virulence factor in the NE pathogenesis (Al-Sheikhly and Truscott, 1977). On the contrary, a few strains of *C. perfringens* produce NE B- like (NetB) toxin which also recognized as the significant role in the pathogenesis of NE (Keyburn *et al*., 2008, 2010).

Necrotic enteritis is a significant poultry disease that affects feed efficiency and growth rate (Miller *et al.*, 2010). The disease is measured as a paramount economic burden for the poultry industry; therefore, special measures are being directed on the necessity for control and prevention of NE at poultry production all over the world (Agunos *et al.*, 2013). A few situations may influence the occurrence of NE in chickens, viz. the presence of immunosuppressive disease, abrupt change in feed. Additionally, coccidiosis is a one of the important contributing factors for the occurrence of NE in poultry by damage of the intestinal endothelium that exposes the intestinal layer to the lumen. This situation offers a suitable environment for the propagation of *C. perfringens*, and thereby generates virulent toxins (Shojadoost *et al.*, 2012).

The actual burden of *C. perfringens* in laying flocks could not be estimated due to lack of representative studies and underreporting in the Bangladesh context (Hossain *et al*., 2012; Islam *et al.*, 2003). Therefore, the present study was conducted to isolate and identify of *C. perfringens* by molecular detection, and to confirm the flock level occurrence together with potential risk factors and antimicrobial susceptibility pattern in small-scale commercial layer flocks. The outcomes of this survey will generate baseline data for formulation of a control strategy of NE in layer birds as this is imperative for this economic important disease in low resource settings.

2. Materials and Methods

2.1. Ethical statement

The study was accepted by the Animal Welfare and Experimentation Ethics Committee (AWEEC) of Bangladesh Agricultural University (AWEEC/BAU/2021(12)). The study farms were selected after consultation with the upazila (sub-district) livestock officers of respective sub-districts. Verbal consent was acquired from each of the layer farmers during interview data collection and following poultry sampling as the majority of them could not read and write.

2.2. Study design and selection of layer farms

A cross-sectional survey was conducted in two sub-districts (Mymensingh Sadar, Gazipur Sadar) of Mymensingh and Gazipur districts of Bangladesh (Figure 1). The farms were selected based on inclusion standards which included flock size ≥ 3000 birds under sector three poultry production system of the FAO classification (Dolberg, 2008) with a minimum / low-level of biosecurity practices. The low level of biosecurity parameters involved with inadequate provision of perimeter fencing and netting of the poultry farm/shade, quarantine facilities, footbath, dedicated shoes, and clothing of the poultry attendants during working at a poultry farm, waste management including cleaning and disinfection practices (C&D) (FAO, 2008). Initially, 34 farms were randomly chosen, however, four farms declined to participate field survey. Thus, the total number of layer farms stood at thirty (30) which comprised 16 farms from Mymensingh district and 14 farms from Gazipur district (Figure 1).

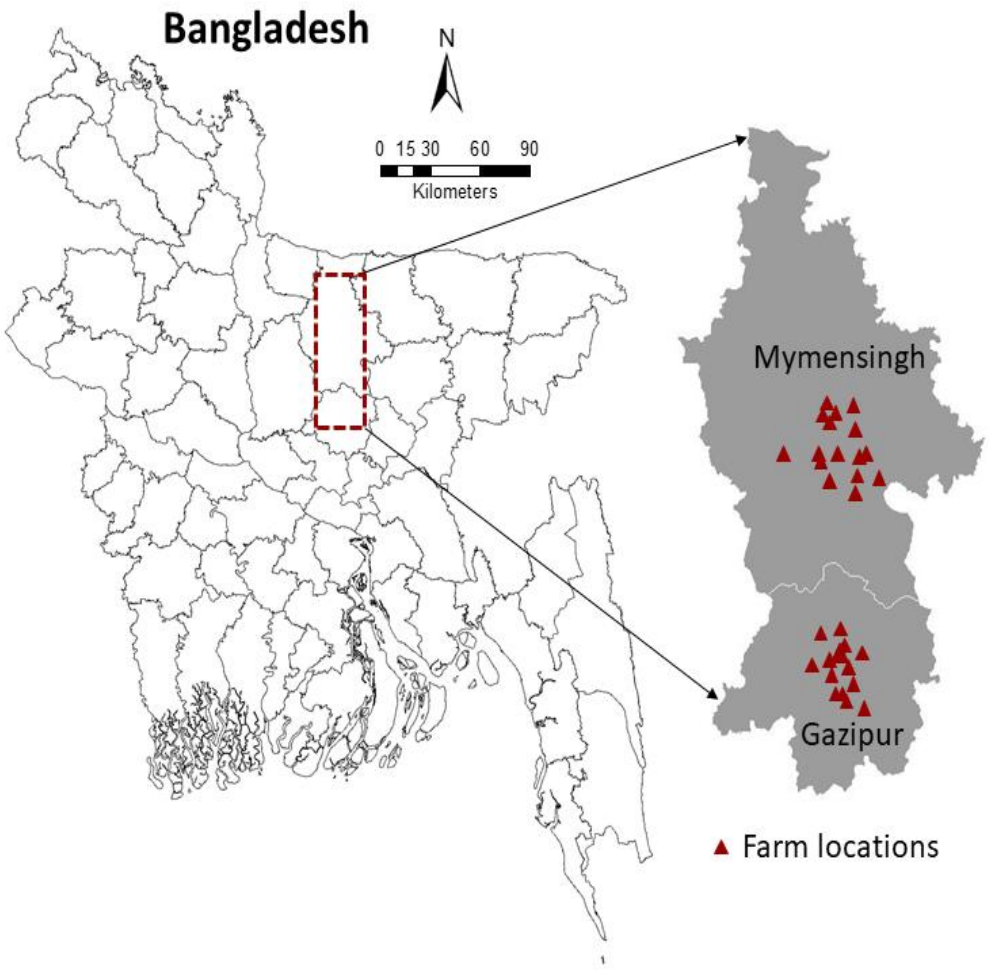

Figure 1. Location of commercial layers farms in Mymensingh and Gazipur districts of Bangladesh. All farms are not clearly visualized in the map as geo-spatial location of the some of the farms closely presented.

2.3. Sample collection, dispatch and processing

Two hundred eighty–seven environmental and poultry samples were randomly collected from 30 layer farms spanning from July 2019 to June 2020. A total of 8 samples (equal number) were collected from each farm which included water (n = 1), worker's handwashing (n = 1) and feed (n = 1) as environmental samples, and cloacal swab (n = 5) as poultry samples and recorded in a sample collection checklist. In case of the environmental sample, three sub-samples were collected and pooled together as an environmental sample (water, feed and workers' hand washing) for the respective category from each farm. The amount of samples was taken as 200 mL water, 100 g feed, 200 mL workers handwashing, 1 mL or mg cloacal swab. However, intestinal samples (n = 47) were collected from suspected dead birds during a postmortem examination of the enrolled farms. The normal saline was used for the preservation of swab samples. All samples were contained in sterile zipper bags and a unique identification code was used for labeling of the individual sample. The samples were dispatched to the laboratory at the Department of Microbiology and Hygiene, Bangladesh Agricultural University with following a cold chain (4-6°C) immediate after collection. A flow-chart of research methodology which included sample collection, culture and genotyping with confirmation of antimicrobial susceptibility pattern is presented in Figure 2.

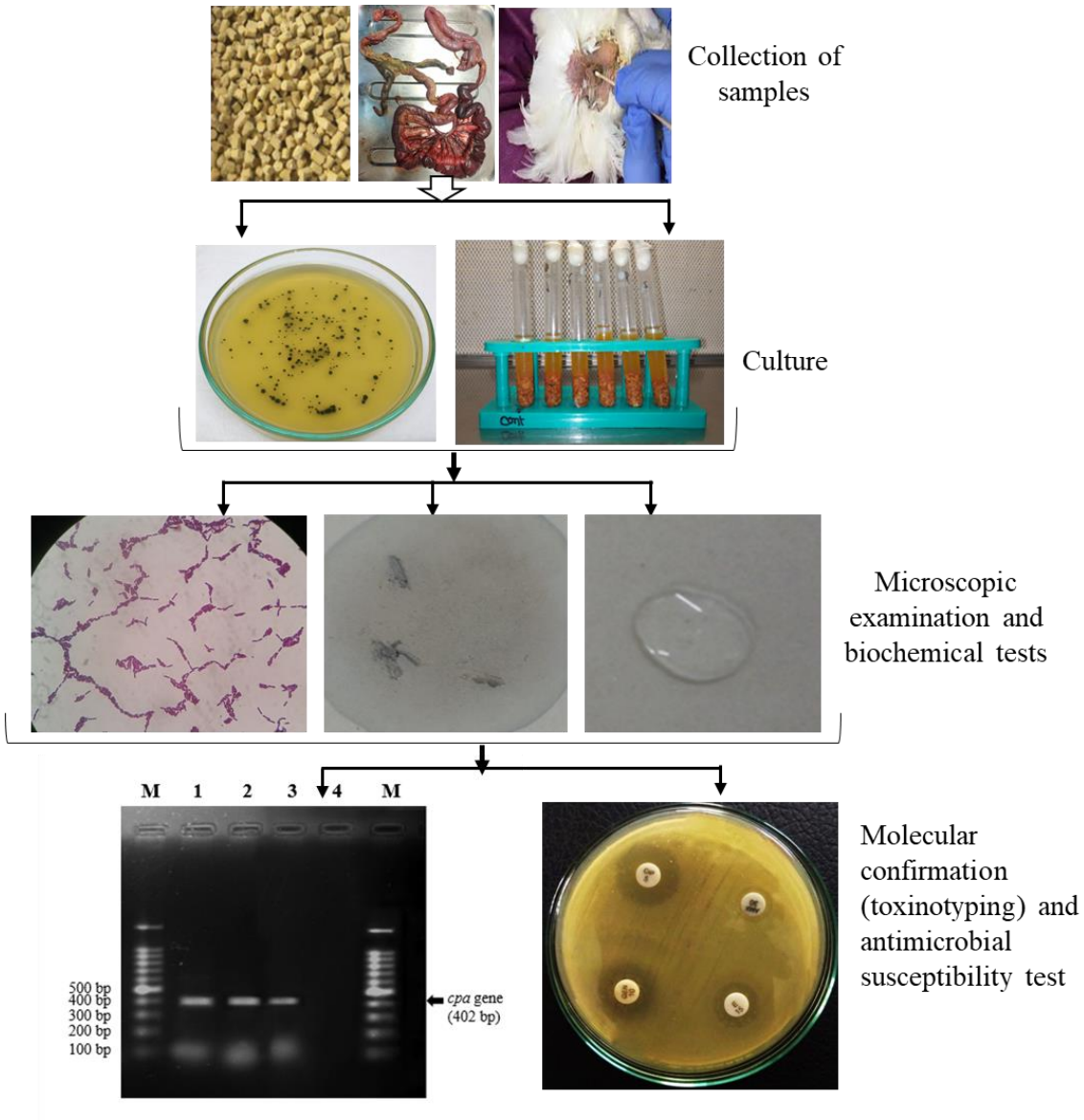

Figure 2. Flow chart of research methodology included sample collection, culture and genotyping including confirmation of antimicrobial susceptibility pattern.

2.4. Detection of *C. perfringens*

2.4.1. Culture and biochemical assays

Standard methods were employed for processing samples immediately after arrival at the laboratory (Ezatkhah *et al.*, 2016). An earlier described protocol was used for the isolation and identification of *C. perfringens* (Shelke *et al.*, 2018). In this method, Robertson's Cooked meat media (RCM broth), (HiMedia, Mumbai, India) was used for initial enrichment of the processed sample, and afterward, anaerobic incubation was done at 37°C for 24 h via candle jar method. The presence of turbidity in the incubated broth media indicates the existence of anaerobic bacteria. From the positive culture of a broth sample, a loopful of culture was streaked on perfringens agar base (TSC; HiMedia, India) with selective supplement (HiMedia, India), then incubation was done at 37°C for 24 h in the anaerobic jar. Continually, cultured plates were revealed after the growth of *C. perfringens*. The colony characteristics were checked and documented concurrently. In this primary evaluation, Gram's staining, motility test via hanging drop technique, and biochemical tests, viz. oxidase and catalase tests were done as per standard protocol (Agarwal *et al.*, 2009). Therefore, pure colonies were obtained and utilized for further molecular assessment.

2.4.2. Molecular confirmation

The boiling method was applied for the extraction of DNA from the pure bacterial culture as previously described (Nyrah *et al*., 2017; Osman *et al.*, 2012). A single loopful of bacterial colonies were taken from the culture media and diluted in 1.5 mL microcentrifuge tubes with 200 µL distilled water through gentle vortexing, and then centrifuged at 14,000×g for 10 min. Thus, the pellet was separated and suspended in 200 µL of TE buffer via gentle vortexing. The microcentrifuge tubes were then boiled at 100°C for 15 min and directly chilled on ice for 10 min. For the DNA template of PCR, an aliquot of 50 µL of the supernatant was preserved. The toxin typing (alpha, beta, epsilon, and iota toxin) genes of *Clostridium perfringens* were detected through multiplex polymerase chain reaction (PCR). In this study, targeted oligonucleotide primer sequences parallel to toxin genes (alpha, beta, epsilon, and iota) of *C. perfringens* were applied in this evaluation (Yoo *et al.*, 1997). The primers including thermal conditions used in PCR assays are presented in Table 1.

A thermal cycler (Thermocycler, ASTEC, Japan) was used for the PCR assay as per previously described protocols (Merati *et al.*, 2017). A total volume of 50 µL was made in a PCR tube for amplification by adding each multiplex PCR reaction mix (alpha, beta, epsilon, and iota toxins) that included 8 µL of extracted DNA template from bacterial cultures, 25 µL PCR master mix (Promega, USA), 1 µL of each alpha, beta, epsilon and iota forward and reverse primers (20 pmol/µL), and 9 µL of PCR-grade water.

The products of PCR were then passed through gel electrophoresis (2% agarose, Invitrogen, Carlsbad, CA, USA), and further, stained with ethidium bromide (0.5 g/mL) and de-stained with distilled water, each step for 10 min, using a UV transilluminator (Biometra, Göttingen, Germany), and finally PCR images were captured.

Table 1. The list of primers and thermal condition employed in this study.

Target gene	Primer name	Primer sequence (5'-3')	Amplicon size (bp)	PCR condition for 35 cycle	Reference
cpa (Alpha toxin)	Cpa-F	GTTGATAGCGCAGGACATGTTAAG	402	Denature: 94°C, 30 s, annealing 59°C, 45 s and extension 72°C, 45 s	Kalender *et al*., 2005
	Cpa-R	CATGTAGTCATCTGTTCCAGCATC			
cpb (Beta toxin)	Cpb-F	ACTATACAGACAGATCATTCAACC	236	Denature: 94°C, 30 s, annealing 59°C, 45 s and extension 72°C, 45 s	
	Cpb-R	TTAGGAGCAGTTAGAACTACAGAC			
etx (Epsilon toxin)	Etx-F	ACTGCAACTACTACTCATACTGTG	541	Denature: 94°C, 30 s, annealing 59°C, 45 s and extension 72°C, 45 s	
	Etx-R	CTGGTGCCTTAATAGAAAGACTCC			
ia (Iota toxin)	Cpi-F	GCGATGAAAAGCCTACACCACTAC	317	Denature: 94°C, 30 s, annealing 59°C, 45 s and extension 72°C, 45 s	
	Cpi-R	GGTATATCCTCCACGCATATAGTC			

2.5. Antimicrobial susceptibility testing

Commercially available eight antimicrobial agents viz. amoxicillin (30 µg), ciprofloxacin (5 µg), azithromycin (30 µg), gentamicin (10 µg), erythromycin (30 µg), tetracycline (30 µg), streptomycin (10 µg), and norfloxacin (10 µg) (HiMedia, Mumbai, India) were used for the evaluation of the antimicrobial status of *Clostridium perfringens* strains via disk diffusion method. The zones of growth inhibition associated with the zone diameter were measured in accordance with the standard criteria of the Clinical and Laboratory Standard Institute (CLSI) (CLSI, 2018), and thus, established as susceptible (S), intermediate resistant (I), or resistant (R) against tested antimicrobial agents. In this assay, *E. coli* strain ATCC 25922 was used as a quality control organism. All evaluations were validated by accomplishment of at best two duplicates of the same test.

2.6. Data collection

The data on potential risk factors were collected from the layer farmers through pre-tested structured questionnaires. The questionnaire was administered which included 12 potential questions namely farm location (upazila and district), flock size, breed/line, age (wk), history of coccidia, source of water, waste disposal distance (m), history of immunosuppressive diseases (Newcastle disease/Egg drop syndrome/Infectious bursal disease), provision of footbath, feeder and drinker cleaning, and isolation of sick birds. The questionnaire was transformed into a local language for a better understanding of the layer farmers. The first author himself and two trained enumerators were engaged at the face-to-face interview data collection process.

2.7. Data management and statistical analyses

The data from both sources (laboratory evaluations and interview data) were captured in hard copies, and further preserved in Microsoft Excel® spreadsheets. Statistical analysis of the data was done by SPSS software (Version 22.0) to accomplish all analyses in this study. The categorical dichotomous data were presented as frequencies and proportion, and 95% binomial confidence intervals (CI).

2.7.1 Bivariable analysis

Primarily, bivariable logistic regression model was used to confirm the relationship between each independent variable and *C. perfringens* positivity status where a P-value of ≤ 0.20 in this analysis was taken further at the multivariable logistic regression model.

2.7.2 Multivariable analysis

At last, a multivariable logistic regression model was done to confirm the association between the variables and *C. perfringens* infection. To exclude non-significant factors (p> 0.05) from the model a backward selection procedure was employed. The association between the variables were estimated by this model. The Hosmer-Lemeshow test was directed to assess the goodness-of-fit of the logistic regression model. In this final model with a P value of ≤ 0.05 were considered as risk factors for C. *perfringens* infection in flock-level.

3. Results

3.1. Sample and flock-level prevalence of C. perfringens

The survey confirmed an overall prevalence of *C. perfringens* as 6.3% (95% CI: 3.8-9.7) together with all sample- categories through basic (culture, biochemical tests), and finally toxinotyping PCR assays. Amongst the different samples, intestinal sample was documented to be highly contaminated (12.8%, 95% CI: 4.8-25.7) followed by feed (6.7%, 95% CI: 0.8-22.1), cloacal swab (6.0%, 95% CI: 2.8-11.1) and workers' handwashing (3.3%, 95% CI: 0.1-17.2). The distribution of prevalence in different samples was found to be statistically non-significant compared to each other (P= 0.21) (Table 2). Among the 30 farms, 16 were confirmed as positive with *C. perfringens*, thus, a farm-level prevalence of 53.3% (95% CI: 34.3-71.7) was obtained in this study.

Table 2. Prevalence of *C. perfringens* in samples collected from 30 layer farms in Mymensingh and Gazipur districts of Bangladesh.

Sample type	Number of samples tested (n[1])	Number of positive samples	Prevalence (%[2])	95% Confidence Interval [CI[3]]	Chi-square *p*-value
Water	30	0	0.0	0.0-11.6	0.21
Workers' hand washing	30	1	3.3	0.1-17.2	
Feed	30	2	6.7	0.8-22.1	
Cloacal swab	150	9	6.0	2.8-11.1	
Intestine (post mortem samples)	47	6	12.8	4.8-25.7	
Total (N[4])	287	18	6.3	3.8-9.7	

[1]n= number of samples
[2]%= Percentage
[3]CI= Confidence Interval
[4]N= Total number of samples

3.2. Molecular confirmation

Eighteen isolates were confirmed through toxinotyping *cpa, cpb, etx,* and *cpi* gene-based multiplex PCR assay and found to be positive for *cpa* gene. Therefore, all isolates were established as toxinotype A of *C. perfringens*. As none of the isolates carried *cpb, etx,* or *cpi* genes indicating the absence of toxinotype B, C, D, or E among the positive isolates of *C. perfringens* (n = 18) screened under this study.

3.3. Risk factors assessment

3.3.1. Location

Among 30 farms, 46.7% (n = 14) from Gazipur district and remaining 53.3% (n = 16) from Mymensingh district were taken under this study. Fifty percent farms (n = 8) from Mymensingh district and 53.3% (n = 8) farms

from Gazipur district were found to be positive with *C. perfringens*. However, no significant association was found among the locations (district) of the farms and farm level infection status (Table 3).

3.3.2. Breed/line

Of surveyed farms, 36.7% (n = 11) and 63.3% (n = 19) flocks were Hisex brown and ISA brown breed/line, respectively. Among them, 54.5% (n = 6) and 52.6% (n = 10) flocks were found to be positive under Hisex brown and ISA brown breed/lines, respectively. However, there was no significant association was found among these breeds/lines and flock level positive status (Table 3).

Table 3. Bivariable analyses between potential determinants and flock level positive status in 30 layer farms from Mymensingh and Gazipur districts of Bangladesh.

Variables[1]	No (%) of positive farms	OR[2]	95% CI [3]	P-value
Location (District)				
Gazipur (n=14)	8 (57.1)	0.75	0.17- 3.17	0.98
Mymensingh (n=16)	8 (50)	Ref.		
Breed/Line				
Hisex brown (n=11)	6 (54.5)	1.08	0.24-4.79	0.91
ISA brown (n=19)	10 (52.6)	Ref.		
Flock size				
< 3000) (n=17)	6 (35.2)	Ref.		
(≥ 3000 (n=13)	10 (77)	6.11	1.19- 31.16	0.058
History of coccidiosis in the flock				
With history (n=22)	10 (45.5)	Ref.		
Without history(8)	6(75)	3.6	0.59-21.93	0.30
History of immune suppressive disease				
With history (n=8)	7 (87.5)	10.11	1.05-97.0	0.02
Without history (n=22)	9 (41)	Ref.		
Feeder and drinker clean				
Yes (n=27)	14 (51.8)	Ref.		
No (n=3)	2(66.7)	0.53	0.04-6.66	0.90
Provision of foot bath				
Yes (n=7)	2 (28.6)	Ref.		
No (n=23)	14 (60.8)	0.25	0.04-1.52	0.13
Provision for isolation of sick birds				
Yes (n=16)	6 (37.5)	Ref.		
No (n=14)	10 (71.4)	0.24	0.05-1.12	0.13
Waste disposal distance	10 (71.4)	4.17	0.92-18.77	0.063
<10 m (n=14)	6(37.5)	Ref.		
>10 m (n=16)				

[1]n=number of layer farms corresponding to the each category
[2]OR= Odds ratio
[3]CI= Confidence Interval

3.3.3. Flock size

Of surveyed farms, 56.7% (n = 17) farms were included with a flock size ≥ 3000 birds, among them 35.2% farms (n = 6) confirmed with *C. perfringens* infection. On the other hand, 43.3% of farms (n = 13) were included with a flock size <3000 birds, of them, 77% (n = 10) farms were confirmed with the infection. There was a significant association confirmed between flock size and flock level *C. perfringens* occurrence status (OR= 11.16, 95 CI: 1.26-98.25, P=0.029) (Tables 3 and 4).

3.3.4. History of coccidiosis

Of 30 farms, nearly 73.3% (n = 22) farms were included with a history of coccidia infection, and 26.7% (n = 8) farms without a history of coccidia infection. However, 45.5% and 75% of farms were found to be positive

among these categories of farms, respectively. However, history of coccidia infection was found to be non-significant with the farm- level infection status of *C. perfringens* (Tables 3 and 4).

3.3.5. History of immune-suppressive disease

Of 30 farms, 26.7% (n = 8) farms were documented with a history of immune-suppressive disease (Newcastle disease/Egg drop syndrome/Infectious bursal disease), among them, 87.5% of farms were confirmed positive with *C. perfringens* infection. However, 73.3 % (n = 22) farms were found without a history of immune-suppressive disease, of them, 40.9% (n = 9) farms shown positive with *C. perfringens* infection. History of immune-suppressive disease was established to be associated with flock-level infection status (OR= 22.16, 95% CI: 1.40-394.36, P= 0.027) (Tables 3 and 4).

3.3.6. Feeder and drinker cleaning

Among the surveyed farms, the majority (90%, n = 27) of the farms practiced cleaning of feeder and drinker on daily-basis. Nearly half of them (51.8%, n = 14), were found to be positive with *C. perfringens*. On the contrary, 10% (n = 3) farms did not practice regular cleaning of feeder and drinker, among them, 66.7% (n = 2) of farms were confirmed as positive status with *C. perfringens*. However, there was no significant association between the cleaning status of feeder and drinker with flock level occurrence (Tables 3 and 4).

3.3.7. Provision of footbath

Among the surveyed farms, 23.3% (n = 7) farms were found with the provision of a footbath at the entry point of the farm/shed. Among them, 28.6% (n = 2) farms were confirmed with the infection. On the other hand, the majority of the farms (76.7%, n = 23) did not have with footbath, among them, 60.8% (n = 14) farms were found positive status for *C. perfringens*. However, there was no association between the provision of a footbath and flock level infection status (Tables 3 and 4).

3.3.8. Isolation of sick birds

Of 30 farms, 53.3 % (n = 16) were with the isolation facilities for sick birds. Among them 37.5% (n = 6) were found positive. However, 46.7% (n = 14) farms had no sick bird isolation facilities. Among them, 71.4% (n = 10) were confirmed positive status for flock-level infection. However, there had no association between sick bird keeping facilities and flock level positive status (Tables 3 and 4).

Table 4. Multivariable model of the risk factors for *Clostridium perfringens* infection in 30 layer farms from Dhaka and Mymensingh districts of Bangladesh.

Risk factors	Odds Ratio	95% CI	P-Value
Flock Size			
≥ 3000	11.16	1.26- 98.25	0.029
< 3000	Ref.		
Provision for isolation of sick birds			
Yes	Ref	0.49-29.15	0.201
No	3.78		
Provision of foot bath			
Yes	Ref.	0.34-116.45	0.216
No	6.29		
History of immune-suppressive disease			
With history	22.16	1.40- 349.36	0.027
Without history	Ref.		

3.3.9. Waste disposal distance

Of surveyed farms, 43.3% (n = 13) disposed of poultry waste within 10 m of the farm premises. Amongst them, 71.4% (n = 10) farms were confirmed positive. On the other hand, 53.3% (n = 16) farms disposed >10 m distance from a poultry farm, where flock level positive status was established as 37.5% (n=6). However, there was no association between the distance of poultry waste disposal and flock level positive status for *C. perfringens* (Table 3).

3.4. Antibiogram

3.4.1. Antimicrobial susceptibility pattern

In this study all strains (n = 18) were found to be fully susceptible (100%) to ceftriaxone, whereas 77.8% (n = 14) isolates were found to be susceptible to amoxicillin followed by chloramphenicol (50%, n = 9), and ciprofloxacin (38.89%, n = 7). In this study, all isolates (n = 18) were presented 0% susceptibility status to erythromycin and gentamicin.

Alarmingly, all isolates documented fully resistant (100%, n = 18) to gentamycin followed by 77.8%, 72.22 %, 38.9%, 27.8%, and 11.1% to oxytetracycline, erythromycin, ciprofloxacin, chloramphenicol, and amoxicillin, respectively (Table 5).

Table 5. Antimicrobial status of *C. perfringens* strains (n = 18) isolated from 30 layer farms.

Name of antimicrobial agents	Symbol	Number of isolates (n = 18)			Sensitivity (%)	Resistant (%)
		R[1]	I[2]	S[3]		
Amoxicillin	AMX	2	2	14	77.8	11.1
Chloramphenicol	CHL	5	4	9	50.0	27.8
Ciprofloxacin	CIP	7	4	7	38.9	38.9
Erythromycin	ERY	13	5	0	-	72.2
Gentamicin	GEN	18	0	0	-	100.0
Oxytetracycline	OXT	14	2	2	11.1	77.8
Ceftriaxone	CRO	0	0	18	100.0	-

[1]R=Resistant, [2]I= Intermediate, [3]S= Sensitive

3.4.2. Distribution of multiple resistant status

In this evaluation, of 18 isolates 11.1% (n = 2), 16.7% (n = 3) isolates were shown resistant to one (GEN) and two (GEN-OXT) antimicrobial agents, respectively. On the contrary, 33.3% (n = 6) isolates were found resistant to 3 antimicrobial agents (E-GEN-OXT, n = 4 and CIP-ERY-GEN, n = 2), 16.7% isolates (n = 3) to four antimicrobial agents (CIP-ERY- GEN-OXT, n = 1 and CHL-ERY-GEN-OXT, n = 2), 16.7% (n = 3) isolates to five antimicrobial agents (CHL-CIP-ERY-GEN-OXT, n = 2 and AMX-CIP-ERY-GEN-OXT, n = 1). However, 5.5% isolates were presented resistant to six antimicrobial agents (AMX-CHL-CIP-ERY-GEN-OXT). However, 72.2% (n = 13) isolates were document resistant against ≥3 antimicrobial agents, thus, these traits classified them as multidrug resistant (MDR) organisms (Table 6).

Table 6. Antimicrobial resistant pattern of *C. perfringens* isolated from layer flocks. Antimicrobial agents Amoxicillin (30 µg), Chloramphenicol (30 µg), Ciprofloxacin (5 µg), Erythromycin (15 µg), Gentamicin (10 µg), Ceftriaxone (30 µg) and Oxytetracycline (30 µg), were used to confirm antimicrobial resistant pattern.

Number of antimicrobial agents	Antimicrobial resistance pattern [1]	*Clostridium perfringens* (n[1] = 18)	
		No. (%) of strains	Subtotal [No. (%)]
One to two antimicrobial agents			
1	GEN	2 (11.1)	2 (11.1)
2	GEN-OXT	3 (16.7)	3 (16.7)
Total against 1-2 antimicrobial agents			5 (17.8)
≥ 3 antimicrobial agents			
3	ERY-GEN-OXT	4 (22.2)	6 (33.3)
	CIP-ERY-GEN	2 (11.1)	
4	CIP-ERY-GEN-OXT	1 (5.5)	3 (16.7)
	CHL-ERY-GEN-OXT	2 (11.1)	
5	CHL-CIP-ERY-GEN-OXT	2 (11.1)	3 (16.7)
	AMX-CIP-ERY-GEN-OXT	1 (5.5)	
6	AMX-CHL-CIP-ERY-GEN-OXT	1 (5.5)	1 (5.5)
Total against ≥3 antimicrobial agents			13 (72.2)

[1]n= Number of isolates

[2]GEN= Gentamicin, OXT= Oxytetracycline, ERY= Erythromycin, CIP= Ciprofloxacin, CHL= Chloramphenicol, AMX= Amoxicillin

4. Discussion

In this study, we confirmed occurrence through molecular detection of toxinotyping gene including antimicrobial susceptibility status of *C. perfringens* strains isolated from small-scale commercial layer flocks of two major poultry rearing districts of Bangladesh. Further, the study evaluated potential risk factors for flock level occurrence of *C. perfringens*. In this study, α-toxin gene-targeted primers were used for the detection of *C. perfringens* in collected samples from the layer flocks. Therefore, 18 strains were validated for *cpa* gene of 402 bp that signifies *C. perfringens* type A in collected samples as per standard protocols (Merati *et al*., 2017; Nyrah *et al*., 2017).

More than fifty percent of farms (16/30) were found to be contaminated with *C. perfringens* where a sample level prevalence was established as 6.3% (95% CI: 3.8-9.7) in layer flocks. The findings of this study have supported that *C. perfringens* is living in the intestinal tract of animals and humans as a commensal pathogen (Miller *et al*., 2010). Usually, healthy chickens have *C. perfringens* in the intestinal tract with a concentration of $<10^5$ CFU/g ingesta. However, clinical disease of NE in healthy flock occurs when a number of organism increase between 10^7 to 10^9 CFU/g ingesta (McDevitt *et al*., 2006).

Our study finding is consistent with a related study that confirmed a prevalence of *C. perfringens* as 10.76% in poultry and livestock samples in Tamil Nadu, India (Anju *et al*., 2021). Numerous research confirmed the higher prevalence of 33.89% in poultry feed samples in Tamil Nadu, India (Udhayavel *et al*., 2017), 33.63% and 18.91% in broilers and layers poultry, respectively in Kashmir, India (Dar *et al*., 2017), and 53% in dead broilers in West Bengal, India (Sarkar *et al*., 2013). In this study, we confirmed comparatively a lower prevalence in layer flocks than earlier studies conducted in different geographical locations as 29.6% in Taiwan (Zhang *et al*., 2018); 48.82% (Xiu *et al*., 2020), 25.37% in Pakistan (Haider *et al*., 2022) and 23.1% (Fan *et al*., 2016) in China; and, 24.72% and 23.28% in Canada (Chalmers *et al*., 2008); 57.9% in Egypt (Osman *et al*., 2012). These study findings could not compare explicitly in country context due to the lack of substantial reference data on the prevalence rate of NE layer chicken in Bangladesh. However, a recent study confirmed a flock level prevalence of *C. perfringens* as 10.3% in broiler chicken (Tresha *et al*., 2021). Additionally, a few past studies established the incidence rate of *C. perfringens* that varies from 0.4 to 1% based on clinical findings and post mortem lesions (Hossain *et al*., 2012; Islam *et al*., 2003).

Among the risk factors, flock size (≥ 3000 birds/ flock) and immune-suppressive disease were documented as potential risk factors for flock level occurrence of *C. perfringens* in the studied layer flocks. The study confirmed flock size (≥ 3000 birds/ flock) 11.16 (95% CI: 1.26- 98.25) times more likely to be positive with *C. perfringens* compare to those farms with < 3000 birds. A large flock could transmit the infectious agents due to movement of high density of birds in a poultry house. However, in larger flocks, the infection can spread spontaneously among the whole flocks as a larger volume of water, feed, and litter are needed which may contaminated previously.

The study confirmed of a history of immune-suppressive disease (New castle disease/egg drop syndrome/infectious bursal disease) was significantly associated with the flock level occurrence of *C. perfringens* in layer poultry. Another study confirmed that infection with viruses like infectious bursal disease virus, Marek's disease virus, and chicken anemia virus could have an immunosuppressive impact (Hoerr, 2010), and thus increase the severity of NE (Moore, 2016). Several studies indicated that these diseases are considered to be as risk factors in NE disease transmission dynamics (Stringfellow *et al*., 2009).

The study showed that *C. perfringens* isolates were 100% sensitive to ceftriaxone, followed by amoxicillin (77.8%) and chloramphenicol, (50%) and ciprofloxacin (38.9%), Oxytetracycline (11.1%). These findings are partially corroborated by other studies conducted in different geographical locations (Agarwal *et al*., 2009; Algammal and Elfeil, 2015; Mehtaz *et al*., 2013, Haider *et al*., 2022). However, the study established that a few antimicrobial agents, like erythromycin, gentamicin presented susceptible to 0.0% isolates which is contradictory to a research as *C. perfringens* strains were found as 100% sensitive to gentamicin (Udhayavel *et al*., 2017). This finding confers our hypothesis that particular antibiotics are being used exponentially like erythromycin, gentamicin, oxytetracycline, and ciprofloxacin in poultry rearing in Bangladesh as growth promotors or feed additives (Alam *et al*., 2020).

The study confirmed 72.2% (n = 13) isolates as multidrug-resistant as they showed resistance between 3 to 6 antimicrobial agents. Our study findings are not consistent compared to different studies on *C. perfringens* antibiotic resistance conducted in different locations: Japan (Benno *et al*., 1998), Canada (Slavić *et al*., 2011), the USA (Watkins *et al*., 1997), Belgium (Lanckriet *et al*., 2010), Bulgaria (Marina *et al*., 2009), and Sweden (Johansson, 2006). However, this finding is narrowly supported by another study of Korea as most of the strains of *C. perfringens* isolated from NE outbreaks are presented resistant to common antimicrobial agents like gentamicin and streptomycin (Park *et al*., 2015). The main reason for the development of multidrug resistance is

the widely use of antibiotics as growth promoters and feed additives in poultry feed for the prevention of *C. perfringens* including other gram-positive bacteria (Diarra and Malouin, 2014).

In this study, 33.3% (n = 6) strains present resistance to 3 antimicrobial agents (ERY-GEN-OXT and CIP-ERY-GEN), 16.7% (n = 3) separately for each of the two groups to four antimicrobial agents (CIP-ERY-GEN-OXT and CHL-ERY-GEN-OXT), and five antimicrobial agents (CHL-CIP-ERY-GEN-OXT, AMX-CIP-ERY-GEN-OXT), respectively. Alarmingly, 5.5% of isolates (n = 1) were documented resistant to six antimicrobial agents (AMX-CHL-CIP-ERY-GEN-OXT). Large number of poultry farmers in Bangladesh have a financial burden and they mostly rely on feed/drug sellers and chick suppliers for financial support as credit. Therefore, farmers collect antimicrobial agents usually from them (Imam *et al*., 2020) that facilities imprudent use of antimicrobial agents in poultry rearing and causes antimicrobial resistance.

A variety of potential therapeutic and prophylactic measures are being dynamically developed to address the NE burden in poultry nowadays throughout the world. Alternatives like the use of prebiotics (Vidanarachchi *et al.*, 2013), probiotics (Cao *et al*., 2012), organic acid (Geier *et al*., 2010), and plant extracts (Lee *et al*., 2013) as well as vaccines would be more suitable tools in poultry production in the present time (Tsiouris, 2016) considering food safety and public health aspects.

5. Conclusions

The study confirms the presence of multidrug resistance *C. perfringens* toxinotype A in layer flocks, which intensifies the burden linked with treatment failures in layer flocks including public health implications. Thus, proof-based control measures that are appropriate to use of antimicrobials alternatives namely prebiotic, probiotic, organic acids, plant extract including vaccines could adapt at low biosecurity conditions. Control of immune-suppressive diseases in layer flocks is critically needed to block the infection of NE in lager flocks is imperative. Participatory awareness creation and motivational activities on the appropriate use of antimicrobial agents under an antimicrobial stewardship intervention is also demanding.

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Data availability

The data that support the findings of this study are included in the manuscript. However, the reader will touch to corresponding author for any data inquiry.

Conflict of interest

None to declare.

Authors' contribution

Mohammad Arif: Methodology, Formal analysis, Writing-original draft; Nigar Sultana: Investigation, Methodology; Sk Shaheenur Islam: Data curation, Software, Visualization, Writing-review & editing; Arunima Oarin Tresha: Investigation, Visualization; Sayed Abdullah-Al-Mamun: Investigation, Formal analysis; Md. Ashiquen Nobi: Writing-review & editing; Mohammad Ferdousur Rahman Khan: Writing-review & editing; S. M. Lutful Kabir: Conceptualization, Funding acquisition, Supervision. All authors have read and approved the final manuscript.

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