Original Research

Isolation and Characterization of *Clostridium perfringens* from Suspected Cases of Necrotic Enteritis (NE) in Broiler Chicken

Hiramoni Sarmah*, Ritam Hazarika[1], Pankaj Deka and Rajeev Kumar Sharma

Department of Microbiology, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati, 781022, Assam, INDIA

[1]Department of Animal Biotechnology

***Corresponding author:** gk_sawale@yahoo.com

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Abstract

Considering necrotic enteritis (NE) as an economically important problem of broiler industry, a study was undertaken to establish the association of Clostridium perfringens with the disease. Screening of a total of 35 faecal samples/intestinal scrapping of broiler, either clinically affected or died due to suspected form of NE reveal 13 (37.14%) samples to be positive for C. perfringens, yielding an equal no. of isolates. All the isolates could exhibit cpa gene (α-toxin). Recovery of C. perfringens was found to be comparatively more from intestinal scrapings (10). Among the C. perfringens isolates, three were identified to be toxin type C, exhibiting cpb (β-toxin) along with cpa gene, while the rest 10 were identified as toxin type A. Profiling of the isolates for additional toxin gene (s) exhibited presence of netB and tpeL in only one of the C. perfringens of toxin type A. All the isolated C. perfringens, irrespective of toxin types could exhibit resistance towards different antimicrobial agents, of which eight were found to be resistant to more than one type of antimicrobial agents. Majority of the isolates were found to be resistant to ciprofloxacin and norfloxacin, while metronidazole, gatifloxacin, tigecycline, cefmetazole and clindamycin were found to be effective against the all the isolates. Release of toxins in the cell free supernatant was found to be comparatively higher, during the 6-8 hrs of incubation. Additional toxin genes made the C. perfringens toxin type A comparatively more pathogenic for mice.

Key words: Necrotic enteritis, *Clostridium perfringens toxin* type A, *cpa*, cpb, *netB*, *tpeL* gene

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Introduction

Poultry entrepreneurship is an emerging industry in India. Considering the growing demand for high value meat protein, broiler farming becomes a very lucrative among all categories of entrepreneurs. Necrotic enteritis (NE), a widespread disease in broilers was first described by Parish (1961), caused by *Clostridium*

perfringens. The toxigenic typesA are more frequently associated with NE in broiler, while type C is very rarely involved (Engstrom *et al.*, 2003). In addition to the conventional toxin associated genes (*cpa*) of *C. perfringens* type A, recent identification of new toxin, Beta2 (*cpb2*), NetB (*netB*), TpeL (*tpeL*) in recent years has proved the crucial association with induction of NE, as additional crucial factors. The recently identified essential virulence factor, the NetB has recently been described with clinical NE isolate of *C. perfringens* (Keyburn *et al.*, 2008). The *TpeL*, a member of large clostridial toxins (LCT) family could also be recorded in some *C. perfringens* type A NE isolates (Amimoto, *et al.*, 2007), Recent report on *netB*-positive strains of *C. perfringens* with additional *tpeL* gene has proved to cause more severe disease than strains lacking in *tpeL* (Coursodon, 2012).

Control of NE in poultry is most commonly practiced with variety of antimicrobial agents, typically administered in feed or water (Kulkarni *et al*., 2007), as well as growth promoter (Sarson *et al*., 2009). However, due to emerging concerns regarding antimicrobial resistance, the uses of growth-promoting antibiotics were being banned in the European Union and in Korea (Oakley *et al.*, 2011). Considering the update knowledge associated with C. *perfringens* and their association with NE in broiler, isolation and characterization of *C. perfringens* and their antimicrobial resistance are the first step in the control of bacterial disease. Considering the importance of the disease and the associated *C. perfringens* for broiler industry, the present study was designed to characterize the *C. perfringens* isolated from broiler with suspected form of NE, in respect to their toxin type, screening for additional virulent associated gene(s) and resistance pattern against commonly used antimicrobial agents.

Materials and Methods

Samples

A total of 35 samples comprising of faecal samples (25) from broiler chicken with diarrhea, and intestinal scrapping (10) from dead birds with gross intestinal lesions suggestive of necrotic enteritis (NE) were collected in sterile container and screened for *Clostridium perfringens.*

Isolation and Identification of *C. perfringens*

Collected samples were inoculated into respective tubes of Brain Heart Infusion (BHI) (Hi-Media) for enrichment and incubated at 37^0 C in anaerobic environment with anaero gas pack system (Hi-Media) for 24 hrs. Subsequently, 0.5ml of broth culture was inoculated into 5.0% v/v Blood Agar (BA) and further incubated anaerobically at 37^0 C for 24 hrs. Isolated colonies with double zone of heamolysis were studied for presumptive identification as *C. perfringens*, based on colony morphology, staining characteristics and

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cell morphology. Tentatively identified isolates of *C. perfringens* were confirmed by molecular detection of *C. perfringens* specific (*cpa*) gene in the isolates.

Molecular Characterization of the *C. perfringens* Isolates

All suspected *C. perfringens* isolates were explored for major toxin genes (*cpa*, *cpb*, *etx*, *iA*), by simplex polymerase chain reaction (PCR), following the method described by Titball *et al.* (1989). Presence of additional virulence associated genes, *netB* and *tpeL* in the isolates was investigated by simplex PCR with thermocycling condition, reported by Bailey *et al.* (2013) and with little modification in respect to the holding period at different steps. Template DNA was prepared from suspensions of respective isolates of *C. perfringens* in 100 µl of sterile nuclease free water. Details of the gene sequence of the primers used for molecular characterization of the isolates were depicted in Table 1.

Table 1: Gene sequence of the different primers used for detection of toxin genes

Toxin Genes	Gene Sequence	bp	References
cpa	5-GCTAATGTTACTGCCGTTGA-3	324	Titball *et al.* (1989)
	5-CCTCTGATACATCGTGTAAG-3		
cpb	5-GCGAATATGCTGAATCATCTA-3	180	Hunter *et al.* (1993)
	5-GCAGGAACATTAGTATATCTTC-3		
etx	5-GCGGTGATATCCATCTATTC-3	655	Hunter *et al.* (1992)
	5-CCACTTACTTGTCCTACTAAC-3		
iA	5-ACTACTCTCAGACAAGACAG-3	446	Perelle *et al.* (1993)
	5-CTTTCCTTCTATTACTATACG-3		
netB	5-CGCTTCACATAAAGGTTGGAAGGC-3	316	Matthew *et al.* (2013)
	5-TCCAGCACCAGCAGTTTTTCCT-3		
tpeL	5-ATATAGAGTCAAGCAGTGGAG-3	466	Amimoto *et al.* (2007)
	5-GGAATACCACTTGATATACCTG-3		

Amplification of target genes in the isolates of *C. perfringens* was carried out in 25 µl reaction vol. containing 12.5 µl of 2X PCR master mix (Thermo Scientific), 0.5 µM primers and 3µl of template DNA (100-150 ng) with an additional vol. of 0.5 µl of 25 mM $MgCl_2$ and making the final vol. to 25 µl with nuclease free water. The PCR reactions were performed in a thermocycler (Applied Biosystems) with the PCR conditions mentioned in Table 2. Confirmation of the amplified products were visualized under UV light of Gel Doc System (BioRad, USA), following electrophoresis. Amplified products, suggestive of *tpe*L gene (466bp) and *net*B gene (466bp) were confirmed and validated by performing a sequence alignment with *tpe*L and *net*B sequences in GenBank using genetic analysis software.

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Table 2: Thermal cycling condition for amplification of toxin gene(s) of *C. perfringens* isolates

Target gene	Temperature	Time	Purpose	No. of Cycles
cpa/cpb/etx/iA/tpeL	94^0C	4 min	Initial denaturation	1
	94^0C	1 min	Denaturation	35
	55^0C	1 min	Annealing	
	72^0C	1 min	Extension	
	72^0C	10 min	Final extension	1
netB	95^0C	5 min	Initial denaturation	1
	94^0C	30 sec	Denaturation	40
	55^0C	90 sec	Annealing	
	72^0C	90 sec	Extension	
	72^0C	10 min	Final extension	1

Antimicrobial Resistance Pattern of *C. perfringens* Isolates

All the *C. perfringens* isolates harboring different virulence gene(s) were screened for their resistance pattern to a group of commonly used antimicrobials by Epsilometric test (E-test) strips (Hi-Media). Cell suspensions ($1.5x10^8$cfu/ml) were made from pure broth culture of respective *C. perfringens* and inoculated into BHI agar plate by swabbing. The E-strips were gently pressed on the surface of the BHI agar and incubated anaerobically at 37ºC for 24 hr. The MIC was determined by reading the specific antibiotic concentration at the junction of the inhibition and full lawn growth. Resistance pattern of the isolates was determined by comparing the standards on MIC fixed for different antimicrobials by the Clinical and Laboratory Standards Institute (Spigaglia *et al*., 2011; Keessen *et al*., 2013).

Influence of Incubation Period on Release of *C. perfringens* Toxins

Pure isolated colonies of selected strain of *C. perfringens* toxin types A with presence of additional virulence gene (*net*B & *tpe*L) was grown in 10.0 ml BHI broth. 1ml overnight anaerobic broth culture was transferred to 30.0ml fresh BHI broth and further incubated anaerobically for different incubation periods, *i.e.*, 2, 4, 6, 8, 10 and 24hrs. Culture supernatants, extracted at different incubation periods were concentrated with trichloroacetic acid (TCA) technique (Koontz, 2014) and protein concentrations were estimated (Lowry *et al.*, 1951). The release of toxins by the selected *C. perfringens* isolate in the culture supernatants at different incubation period was evaluated by their protein profile. Protein profiling was done by Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) in 12% gel (Laemmli, 1970), using a standard protein mol wt marker (Puregene, Genetix, India).

Mouse Pathogenicity of Culture Supernatant of *C. perfringens*

Pathogenicity of culture supernatants of randomly selected isolates, representing different *C. perfringens* toxin types were evaluated in groups of mice as per the method of Fisher *et al.* (2006). Groups of six mice (17 to 20 g), irrespective of sex were injected through intravenous (i/v) route (tail vein) with 0.5ml of 8 hrs

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culture supernatants of selected *C. perfringens* toxin types in thioglycolate broth. Injected mice were observed for 72hr for physiological/ neurological changes, as well as mortality. Dead and clinically affected mice following injection were subjected to post mortem (PM) for gross changes in the internal organs as a marker for pathogenicity of selected *C. perfringens* toxin types.

Results and Discussion

Isolation and Identification of *C. perfringens*

Screening of 35 samples of broiler chicken with suspected from of NE revealed isolation of *C. perfringens* from 13 (37.14%) samples. All the isolates were confirmed to be *C. perfringens* by detection of *cpa* gene of 324bp size (Fig. 1).

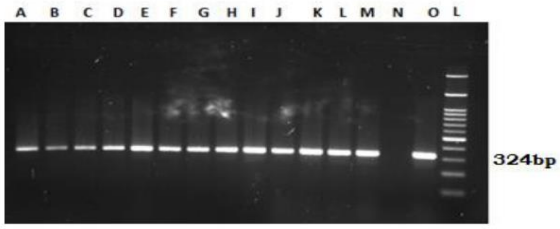

Fig.1 PCR amplified *cpa* gene (324 bp) of *C. perfringens*
L = 100bp ladder,
A-M = Amplified products field isolates
N = Negative control (Non-template)
O = Positive control ATCC-13124

The frequency of isolation was recorded to be more (10) from intestinal scrapings of dead birds with gross necrotic lesions, while only three feacal samples of diarrheic birds could reveal *C. perfringens* (Table 3).

Table 3: Screening of faecal samples and intestinal scraping of clinically affected broiler chicken for *Clostridium perfringens*

Sample Type	No. of sample screened	No. of sample morphologically +ve for *C. perfringens*	No. of *C. perfringens* isolates +ve for virulence gene						Toxin type
			cpa	*cpb*	*etx*	*iA*	*netB*	*tpeL*	
Faecal samples	25	3	3	--	--	--	--	--	A
Intestinal scrapings	10	10	10	3	--	--	1	1	A (7) C (3)
Total	35	13 (37.14)	13	3	--	--	1	1	A (10) C (3)

Figures in parenthesis indicate percentages

Among the isolated *C. perfringens*, 10 were identified as toxin type A, bearing *cpa*(324bp) gene alone,while the rest three isolates of intestinal scrapings could be recognized as type C, exhibiting both *cpb*(180bp) and *cpa* gene (Fig. 1 and 2).

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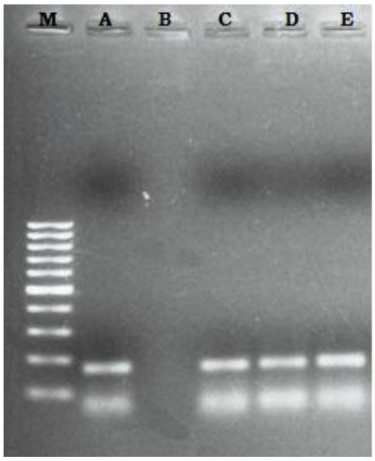

Fig. 2 PCR amplified *cpb* **gene (180bp) of** *C. perfringens*
M = 100bp ladder,
A = Positive control (Strain.200302)
B = Negative (Non-template) control
C-D=Sample DNA

Isolates were negative for *etx* (ε-toxin) and *iA* (ι-toxin) genes. One of the toxin type A isolates of intestinal scraping was found to possess additional virulent genes, *netB* and *tpeL* with 316 bp and 466 bp size, respectively (Table 3, Fig. 3 & 4).

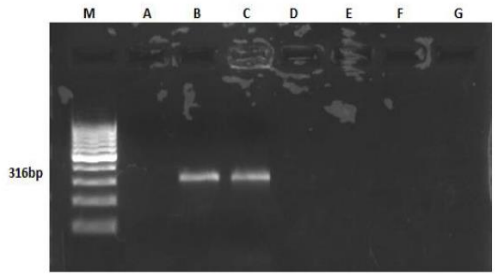

Fig.3 PCR amplified *netB* **gene (316bp) of** *C. perfringens*
M = 100bp ladder,
A = Negative control (Non-template)
B = Positive control strain (Strain.200302)
C-G= Sample DNA

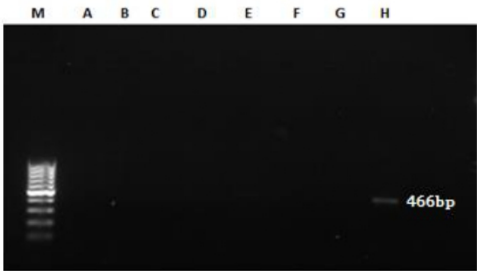

Fig. 4 PCR amplified ***tpeL*** **gene (466bp) of** *C. perfringens*
M = 100bp ladder,
A = Negative control (Non-template)
B-G = Negative sample DNA
H = Sample DNA+ve for *tpeL* gene

Sequences obtained from PCR products of both *net*B and *tpe*L matched the target sequence (at least 99% max identity) with significantly low *E*-values after performing a BLAST search (Table 4). Toxin type A of *C. perfringens* was previously recorded as the most common toxin type than other types that induce necrotic enteritis in poultry (Park *et al.*, 2015).

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Table 4: Detail of BLAST output for the sequenced PCR products

Target Gene	Sequence Source	Description of the Top Result	Gen Bank Accession	Max Identity (%)	*E*-value
*net*B	H-B09	*Clostridium perfringens* strain 200302-1-1-Ba necrotic enteritis *toxin B (netB) gene, complete cds*	GU433338.1	99	1e-134
tpeL	H-B09	*Clostridium perfringens* gene for TpeL, complete cds	AB262081.1	99	0

They recorded 100 percent isolation of *C. perfringens* from cases of necrotic enteritis with gross lesions. However, there are lot of contradictory reports on association of NetB toxin as an additional virulence factor of *C. perfringen*s and its association with necrotic enteritis in poultry.

Detection of *netB* and *tpeL* gene in one of the *C. perfringens* toxin type A from suspected cases of NE in the present study is in agreement with Bailey *et al.* (2013). They reviewed the strains of *C. perfringens* bearing both the *netB* and *tpeL* gene to be more virulent than strains without or only with *netB* gene along with major toxin gene(s). Although association of *C. perfringens* with necrotic enteritis was established by Gornatti-Churria *et al.* (2014), they could not observe *netB* gene. The prevalence of *C. perfringens* type A and C with or without additional virulence gene(s) in cases of necrotic enteritis in broiler chicken could be ascertained from the present study.

Antimicrobial Resistance Pattern of *C. perfringens* Isolates

Irrespective of toxin types, all *C. perfringens* isolates showed resistance towards different antimicrobial agents. Highest resistance was observed against ciprofloxacin (76.92%), followed by norfloxacin (53.85%), tetracycline (46.15%), colistin (23.07%) and levofloxacin (15.38%). Clindamycin, metronidazole and tigecycline were found to be effective for all the. Among the resistant isolates, multiple resistances were exhibited by eight isolates towards ciprofloxacin, norfloxacin, levofloxacin and tetracycline in different combinations (Table 5). Diarra and Molouin (2014) opined that the use of tetracyclines, as growth promoters in poultry production, might lead to the development of tetracycline resistance among *C. perfringens* strains. Prevalence of resistance towards tetracycline was also recorded among 53.0 percent of *C. perfringens* isolates of NE affected broiler (Mwangi *et al.*, 2018).

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Table 5: Resistance pattern of *C. perfringens* isolates towards different antimicrobial agents

No. of Isolates	No. of isolates resistant to									
	CMZ	**CLI**	**LEV**	**TET**	**MTZ**	**GAT**	**CL**	**TGC**	**CPH**	**NOR**
13	0	0	2 (15.38)	6 (46.15)	0	0	3 (23.07)	0	10 (76.92)	7 (53.85)
	No. isolates showing multiple resistance to									
8	**CPH/ NOR/ LEV**	**CPH/ NOR/ TET**		**CPH/ NOR**		**CPH/ LEV**		**CPH/ TET**		
	1 (12.5)	3 (37.5)		2 (25.0)		1 (12.5)		1 (12.5)		

Figures in parenthesis indicate percentages; Cefmetazole (CMZ); Norfloxacin (NOR); Ciprofloxacin (CPH); Clindamycin (CLI); Levofloxacin (LEV); Tigecycline (TGC); Tetracycline (TET); Metronidazole (MTZ); Gatifloxacin (GAT); Colistin (CL)

Highly resistant *C. perfringens* isolates to tetracycline and levofloxacin was also recorded by Hmood *et al.* (2019). However, contrary to the present observations, Park *et al*. (2015) recorded strains of *C. perfringens* from NE cases to be intermediate resistant to tetracycline and clindamycin. However, more than 80.0 percent of *C. perfringens* isolates of the same study revealed susceptible to norfloxacin.

Influence of Incubation Period on the Release of *C. perfringens* Toxins

The present study revealed the influence of incubation period on release of toxins in *C. perfringens* culture supernatant. Protein conc. was recorded to be gradually increased in the cell free culture supernatant with increase in incubation period and reached the peak (8.0mg/ml), during 8 hrs anaerobic incubation. The protein conc was recorded to be 4.25mg/ml and 4.0mg/ml in the culture supernatant with 10 and 24 hr incubation, respectively. Detection of 13 no. of distinct protein bands within the range of 29 to 250kDa, including NetB (33kDa), alpha (43kDa), TpeL (180kDa) could be recorded in the supernatant, during 8 hrs of incubation. The clarity of the protein bands was found to be faint with increase incubation period (Fig. 5).

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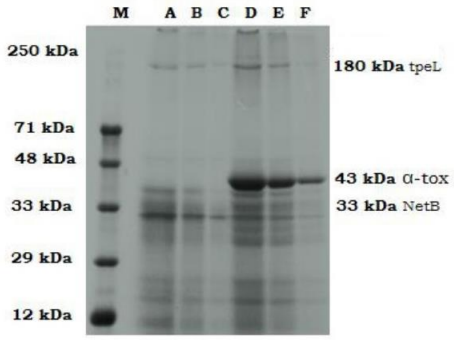

Fig. 5 SDS-PAGE profiles of *C. perfringens* culture supernatant at different incubation period.
M = Protein marker
A = Culture supernatant at 2 hrs incubation
B = Culture supernatant at 4 hrs incubation
C = Culture supernatant at 6 hrs incubation
D = Culture supernatant at 8 hrs incubation
E = Culture supernatant at 10 hrs incubation
F = Culture supernatant at 24 hrs incubation

The incubation period beyond the stationary growth phase was recorded to have a negative influence on detectable level of *C. perfringens* alpha toxin (Park and Mikolajcik, 1979). Based on western blot analyses, Chen and McClane (2015) could reveal high levels of TpeL toxin in culture supernatant of *C. perfringens*, during 6 to 12 hr of incubation. However, there have been discrepancies in the literature regarding the influence of incubation time on toxin release in the culture supernatant by *C. perfringens*.

Mouse Pathogenicity of Culture Supernatant of *C. perfringens*

All the four randomly selected isolates representing Type A and C were found to be pathogenic for mice with variable intensity. Following inoculation, the 8hrs culture supernatant of *C. perfringens* (type A), possessing *netB*and *tpeL*gene could produce mortality in two of the six mice, while the remaining toxin type A and C could not cause death in any of the inoculated mice. However, all the inoculated survived mice could exhibit physiological changes, *e.g.* reluctant to move and bending of tail at different time of observation, till 72 hrs post inoculation (Table 6). None of the animals of the control group could show any mortality and clinical symptoms. Gross changes, *viz.*, haemorrhage, congestion, and gas production in the intestine of few sacrificed affected mice, were the indications about the toxemic condition leading to physiological changes and death in mice.

Fisher *et al.* (2006) could also demonstrate the lethal activity of multiple toxins in the culture supernatants of *C. perfringens* type C in mice. They recorded a strong positive correlation between mouse lethality and the level of *C. perfringens* beta toxin. Intragastric (i/g) inoculation of *C. perfringens* type D (8 x 10^9cfu) was found to produce 100 percent lethality in mice within 4 hr of post inoculation with significant

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neurological signs, gross mild brain edema and dilation of the small intestines (Fernandez-Miyakawa *et al*., 2007).

Table 6: Pathogenicity of *Clostridium perfringens* culture supernatants in mice

Toxin Type	No. of isolate tested	Dose (ml)	Inoculum	No. of Mice inoculated	No. of Mice Died Within 48hr	No. of mice showed physiological changes		
						12 –24hr	24 –48hr	48 –72hr
A	1	0.5ml	8hrs culture supernatant	6	2	-	4	--
	2	0.5ml	8hrs culture supernatant	6	0		6	--
C	3	0.5ml	8hrs culture supernatant	6	0	-	6	
	4	0.5ml	8hrs culture supernatant	6	0		6	-
Control		0.5ml	Sterile broth	6	-	-	-	-
Total				**30**	**2**		**22**	**--**

However, no clinical disease or mortality could be observed in inoculated mice with washed cells of *C. perfringens* type A. Dose dependent clinical signs of depression, rough hair coat, respiratory distress, diarrhea, feeble heartbeats were recorded in rats, following inoculation of *C. perfringens* crude toxins (Miah*et al*., 2010).

Conclusion

The present study could establish the association of *Clostridium perfringens* type A with cases of Necrotic Enteritis (NE) in broiler chicken. Highest recovery of *C. perfringens*, irrespective of toxin types from intestinal scraping rather than from intestinal contents and faecal swabs may opine for selection of clinical samples from suspected case of NE for confirmed association of *C. perfringens.* The absence of additional virulence associated gene(s) in the toxin type C has proved their rare association with cases of NE in broiler chicken. Presence of additional toxin associated gene(s) in the *C. perfringens* type A isolates makes the strains comparatively more virulent. An incubation period of 6-8 hrs is confirmed to be suitable for release of virulent and immunogenic protein(s) by *C. perfringens* isolates. Based on the resistance patter of *C. perfringens* type A of NE affected broiler chickens throws a light on gradual increases in prevalence of antimicrobial anaerobic bacteria in broiler, which may create a great problem in control of NE.

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