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Toxinotyping of *Clostridium perfringens* in Poultry from Necrotic Enteritis Cases

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ABSTRACT

Clostridium perfringens is well known causative agent of necrotic enteritis in poultry and is mainly caused by Type A toxin. NetB toxin is found to be one of the newly emerging virulent toxin gene which is also responsible for necrotic enteritis. The present study was carried out to characterize and to type the different toxins associated with *C. perfringens* in NE cases of poultry. For the present study total 125 samples were collected from poultry birds, out of which 50 samples were of intestinal content from diseased birds, 50 cloacal swabs and 25 intestinal content from healthy birds. These samples were further processed for isolation, identification, and toxinotyping of *Clostridium perfringens* isolates. Onisolation of *C. perfringens* on blood agar total 43 isolates were found positive showing a pattern of double hemolysis on blood agar. The positive isolates of *C. perfringens* were further confirmed by using 16S rRNA species specific PCR. After confirmation isolates were processed for toxinotyping mainly targeting cpa, cpb and cpb2 toxins by using multiplex PCR. On toxinotyping it was found that NE in poultry birds were mainly caused by *C. perfringens* type A. On virulent gene detection of netB toxin, total 4 isolates were found positive for netB toxin. This study pointed out that *C. perfringens* type A is responsible for development of NE in poultry along with net B toxin which is a new key virulent factor. Further studies of netB toxoid and *C. perfringens* type A for vaccine production could minimize the clostridial problems in broiler farms.

Keywords: Necrotic enteritis, *Clostridium perfringens*, toxinotyping, poultry, netB, 16SrRNA.

Poultry production in India has taken a dramatic leap in the last four decades, emerging from an unscientific farming practice to commercial production system (Dar *et al*., 2017). The basic role of poultry production is turning feed stuffs into meat. Any slight alteration from the optimal condition is mostly accompanied by disruption of the growth process and all over performance. In the past, the use of antimicrobial growth promoters (AGP) in commercial poultry feed helped to control necrotic enteritis (NE) in poultry flocks. However, NE has reemerged as a significant problem in poultry production, likely as a result of national and international policies that ban or limit the use of AGP in poultry feeds. In addition, consumers preference had a large effect on bird production, and the push for poultry. Production with less medication in the diet has also had a significant effect on the increased incidence of NE in the past few years (Pavia and McElory, 2014), among these all enteric diseases are one of the most important problems in the poultry industry leading to production losses, high mortality and risk of contamination of poultry products for human consumption. Enteric disorders are frequently associated with an overgrowth of anaerobic bacteria named *Clostridium perfringens*.

Clostridium perfringens is a gram-positive rod and important anaerobic spore forming human and animal

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pathogen that can produce up to 17 different toxins and enzymes. The bacterium is commonly found in a wide variety of environments, from soil to the intestinal tracts of animals and human plays a major role as a pathogen (Shojadoost *et al*., 2010). *C. perfringens* strains are classified into five toxinotypes, A to E according to the production of four major extracellular toxins namely, alpha (α), beta (β), epsilon (ε) and iota (ι), while various strains can also produce other toxins including β2, theta(θ) perfringolysin (O), kappa (κ), delta (δ), mu (μ), enterotoxin, necrotic enteritis B-like toxin (NetB), TpeL (toxin *C. perfringens* large cytotoxin).Type A produces α toxin, type B produces α, β and ε toxins, type C produces α and β toxins, type D produces α and ε toxins and type E produces α and ι toxins (Dar *et al*., 2017).

The *C. perfringens* strains that infect avian species produce many extracellular toxins; however, toxins alpha (encoded by cpa), beta (cpb), beta-2 (cpb2), enterotoxin (cpe) and netB are the most significant. Toxins beta, beta-2 and netB are thought to be pore-forming in nature, causing increased permeabilities. Toxins beta-2 and netB may be found in several bacterial isolates and have been indicated as possible factors of pathogenesis, although incidence of these genes varies (Brady *et al*., 2010).

Clinical necrotic enteritis (NE) in young chickens is mainly caused by *C. perfringens* type A and, to a lesser extent, type C (Shojadoost *et al*., 2010). Toxin genotyping are of great importance in epidemiological studies of etiological agents and in detection of virulence factors of the pathogen

MATERIALS AND METHODS

A total of 125 samples comprising of intestinal and cloacal swabs (live birds) were collected aseptically from backyard and commercial poultry farm. Out of 125 samples collected, 75 were from healthy and 50 were from diseased bird showing signs of enteritis. Samples were collected from Pune and Satara district of Western Maharashtra. Intestinal samples were collected from suspected cases of birds with enteritis referred for post mortem cases at Department of Pathology, KNPCVS, Shirwal and from Omega laboratory, Lonand, Dist., Satara for this study. From diseased (enteritis suspected) birds, intestinal contents were collected and from healthy poultry birds, intestinal contents as well as cloacal swabs were collected. Samples comprising of cloacal swabs and intestinal contents were collected aseptically in a sterile container and quickly transported to the laboratory in ice-cooled containers. Processing of the collected samples were done as soon as samples received to the department.

Isolation of *C. perfringens*

All the samples received to the laboratory, were inoculated in Robertson's cooked meat media (RCMM). Inoculated RCMM were heated at 80°C for 10 minutes to destroy vegetative form of organisms, followed by anaerobic incubation using anaerobic jars with anaerogas pack at 37°C for 24-48 hours. After incubation, a loopful of broth cultures were further streaked onto 5% sheep blood agar for cultivation of *Clostridium perfringens* and for determination of hemolytic activity of the organism. The blood agar plates were incubated anaerobically for 24 hrs at 37° C (Balachandran *et al*., 2018).

Extraction of DNA from suspected colonies

Two to three suspected pure colonies from5% sheep blood agar plates, showing a double hemolysis pattern were suspended in 100µl of distilled water in a 1.5 ml microcentrifuge tube and further DNA was isolated by boiling method (Dar *et al*., 2017).

Confirmation of *C. perfringens* by using 16S rRNA species specific gene

The isolates were confirmed for detection of *C. perfringens* using species specific 16S rRNA gene sequences (Tonooka *et al*., 2005). After DNA extraction, the identity of the samples was confirmed as *C. perfringens* based on the amplification of specific 16S rRNA gene, using specific primers (Table 1). PCR was performed by preparing a final reaction volume of 20 µl in 0.2 ml thin walled PCR tubes. It was prepared by taking 3.3 µl master mix supplied with Taq DNA, MgCl2 and dNTPs; adding 2 µl each of forward and reverse primers, 2 µl template DNA and 10.7 µl nuclease free water. The reaction was carried out in a thermocycler as follows: initial denaturation of 94ºC for 2 min, followed by 35 cycles of 94ºC for 30 sec, 56ºC for 30 sec, 72ºC for 1 min and a final extension at 72ºC for 2 min.

Table 1: PCR primers used for detection of *C. perfringens* and its toxin cpa, cpb and cpb2 gene

Sl. No.	Targeted Gene	Oligonucleotide sequence	Amplicon size
1	16S rRNA	F: TAACCTGCCTCATAGAGT	481 bp
		R: TTTCACATCCCACTTAATC	
2	cpa	F: GCTAATGTTACTGCCGTTGA	324 bp
		R: CCTCTGATACATCGTGTAAG	
3	cpb	F: GCGAATATGCTGAATCATCA	198 bp
		R: GCAGGAACATTAGTATATCTTC	
4	cpb2	F: AAATATGATCCTAACCAACAA	548 bp
		R: CCAAATACTCTAATYGATGC	
5	NetB	F: GCTGGTGCTGGAATAAATGC	384 bp
		R: TCGCCATTGAGTAGTTTCCC	

Toxinotyping of *C. perfringens* by multiplex PCR

For typing, all the isolates of *C. perfringens* confirmed by species specific gene were subjected for multiplex PCR (m-PCR) using toxin genes α (cpa), β (cpb) and β2 (cpb-2) as referred previously by Brady *et al.* (2010) (cpa and cpb gene) and Dar *et al*., 2017 for cpb2. (Table 1). The multiplex PCR assays in this study were performed in 25 µl reaction volume in Mastercycler gradient (Eppendorf AG, Germany). Three pairs of specific primers corresponding to each toxin were used to determine the presence of cpa, cpb, and cpb2 toxin genes using a multiplex PCR technique for all species specific confirmed isolates. PCR was performed by preparing a final reaction volume of 25 µl in 0.2 ml thin walled PCR tubes. It was prepared by taking 3.5 µl master mix supplied with Taq DNA, $MgCl_2$ and dNTPs; adding 1.5 µl each of forward and reverse primers, 2 µl each template DNA and 10.5 µl nuclease free water. Detail about primers given in table 1. Samples were subjected to 35 PCR cycles, each consisting of Initial denaturation of 15 min at 95°C; 30 sec of denaturation at 94°C; 90 sec of annealing at 53°C, 90 sec of elongation at 72°C and final extension of 10min at 72°C.

Detection of virulent gene netB of *C. perfringens*

For the detection of virulent gene netB all the positive isolates were subjected to Uniplex PCR (Ezatkhah *et al*., 2016). Details of the primers are given in Table 1. The Uniplex PCR assays in this study were performed in 25 µl reaction volume in Mastercycler gradient (Eppendorf AG, Germany). PCR was performed by preparing a final reaction volume of 25 µl in 0.2 ml thin walled PCR tubes. It was prepared by taking 12.5 µl master mix supplied with Taq DNA, $MgCl_2$ and dNTPs; adding 2 µl each of forward and reverse primers, 3 µl template DNA and 5.5 µl nuclease free water. PCR conditions were 95°C for 10 min, 35 cycles of 94°C for 30 sec, 55°C for 30 s and 72°C for 1 min followed by 72°C for 10 min.

RESULTS AND DISCUSSION

Phenotypic identification of *C. perfringens*

The aim of present study was toxinotyping of *C. perfringens* associated with necrotic enteritis (NE) in poultry. Out of 50 samples of NE suspected birds and 75 samples of healthy birds, 35 isolates from NE suspected birds and 08 isolates from healthy birds respectively (total = 43), were characterize phenotypically as *C. perfringens*. For phenotypic characterization all the samples were inoculated in RCM, which showed heavy turbidity along with gas production in RCMM after 24-48 hrs. of incubation,turning the meat particles pink in colour along with foul odor.

RCMM along with Brain heart infusion (BHI) broth was found to be the best medium for initial isolation of *C. perfringens* as described by Malmurugan *et al.* (2012). Das *et al.* (2008) and EI-Jakee *et al*. (2013) used RCMM for initial isolation of *C. perfringens* from necrotic enteritis cases of poultry.

After enrichment, samples were streaked on 5% sheep blood agar. On 5% sheep blood agar after incubation, grey, glistening, smooth and round colonies of 1-2 mm diameter surrounded by double zone of haemolysis, were visualized, which is a characteristic feature of *C. perfringens*. Similar types of colonies were observed by Salah-Eldin *et al*. (2015) and Keyburn *et al*. (2010) of *C. perfringens* on blood agar. For morphological characteristics the culture smears were prepared from the pure colonies and stained by Gram's method which revealed Gram- positive, short and plumpy rods with blunt ends which correlated well with the reports of Hafez *et al*. (2011) and Khairy *et al*. (2013).

Genotypic Identification of *C. perfringens* by using species specific 16S rRNA gene

All the total 43 phenotypically confirmed isolates were further subjected to species specific 16S rRNA gene PCR, which showed 100 percent positive towards *C. perfringens* by species specific PCR. Molecular characterization revealed that all 43 isolates were positive for *C. perfringens* by species specific PCR showing amplicon size of 481 bp on 1.5% agarose gel electrophoresis (Fig. 1).

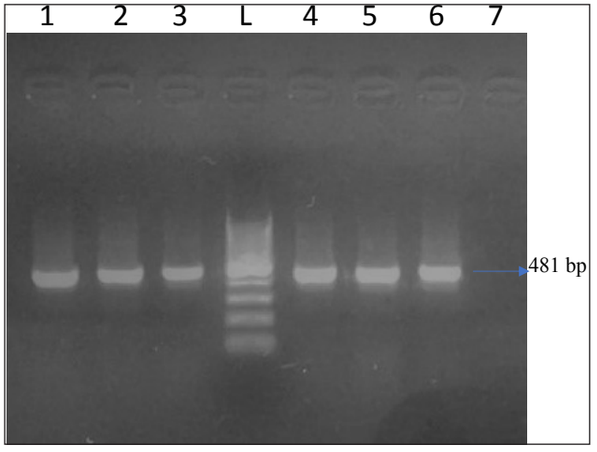

Fig. 1: Agarose gel showing 481 bp amplicon of *C. perfringens* (Species specific PCR); Lane 1,2,3,4,5,6 – Positive samples; Lane 7 – Negative samples; L – 100 bp DNA Ladder

These present findings were in close association with the results of Nyrah *et al*. (2017) who recorded 66 isolates from poultry and further confirmed by 16S rRNA species specific PCR. Similar findings were reported by Dar *et al*. (2017) who confirmed all the isolates of *C. perfringens* by species specific PCR. Nazki *et al*. (2017) recorded 125 (70.62%) isolates were positive for *C. perfringens*, by using species specific primers.

Toxinotyping of *C. perfringens*

Total 43 isolates which were confirmed by 16S rRNA were further processed for toxinotyping of cpa, cpb and cpb2 toxin by multiplex PCR (Fig. 2 and 3).

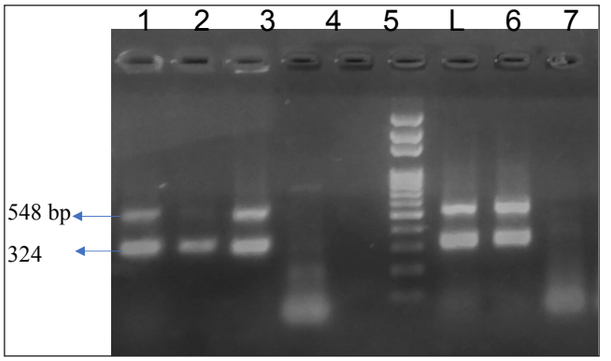

Fig. 2: Agarose gel showing 324 bp amplicon of cpa toxin and 548 bp of cpb2 toxin

Lane 1,2,3,6,7- Positive samples for cpa and cpb2; Lane 4,5,8- Negative control; L - 100 bp DNA Ladder

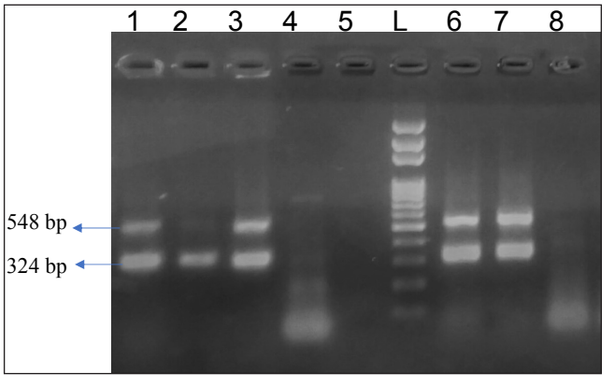

Fig. 3: Agarose gel showing 324 bp amplicon of cpa toxin and 548 bp of cpb2 toxin.

Lane 1,2,3,6,7- Positive samples for cpa and cpb2; Lane 4,5,8- Negative control; L - 100 bp DNA Ladder

Out of total 35 isolates from NE suspected birds, 17 (48.57%) isolates showed cpa toxin, 12 (34.28%) isolates

showed cpb toxin, 06 (13.95%) isolates were positive for cpb2 toxin. Among this, 06 (13.95%) isolates carried both the gene cpa and cpb2. Total 08 isolates from healthy birds were found positive for *C. perfringens*, out of which cpa toxin was detected in 50 percent of isolates while, cpb and cpb2 were found negative. On toxinotyping of the isolates in different types, it was seen that, out of 43 isolates, 27(62.79%) isolates belonged to *C. perfringens* type A and 12 (27.90%) isolates belonged to *C. perfringens* type C. Toxin cpa (alpha) has been indicated as a main virulence factor for the development of NE in poultry and this toxin hydrolyses phospholipids and promotes cellular membrane disorganization which leads to destruction of intestinal mucosa. Nyrah *et al*. (2017) detected total 66 isolates of *C. perfringens* and showed 100 percent positivity towards cpa toxin while 13 isolates (19.69%) showed positivity for cpb2 toxins. Recently, it was speculated that cpb2 positive *C. perfringens* may cause focal duodenal necrosis in egg-laying chickens (França *et al*., 2016). The results of cpb2 in their study was in agreement with the present findings with similar agreement. Park *et al*. (2015) and Balchandran *et al*. (2018) recorded 100 percent prevalence of cpa toxin in the isolates of *C. perfringens* from NE cases while Rasool *et al*. (2017) reported 86.4 percent prevalence of cpa gene, but the prevalence of cpa gene in the present study was recorded less as compared to other studies. The possibility of the same may be due to the presence of other toxin gene which were prominently expressed and reduced the expression of the cpa gene.

Isolates carrying both the gene i.e. cpa and cpb2 were recorded in the present study, the findings of which was similar to the findings of Guran *et al*. (2013) and Datta *et al.* (2014) who found cpa and cpb2 with 2.1 percent and 33.3 percent prevalence rate, respectively. Toxin cpb (betatoxin) is a highly trypsin-sensitive protein that causes mucosal necrosis and possibly central nervous signs observed in domestic animals. It is responsible for the diseases caused by *C. perfringens* type C which causes necrotic enteritis in fowls.

Detection of virulent gene netB of *C. perfringens*

Recently, *C. perfringens* strains producing netB toxin have been considered as the definitive cause of NE in chickens (To *et al.,* 2017). In present study, total 04 (11.42%) isolates showed positivity towards netB out of 35 isolates from diseased birds (Fig. 4). None of isolates were positive for netB from healthy birds. NetB showed overall 9.30 percent prevalence from 43 isolates. These findings were in correlation with Lyhs *et al*. (2013) reported that the netB was identified in 6.6 percent from *C. perfringens* strains which were isolated from NE cases in turkeys. Tolooe *et al*. (2011) who reported, 52.8 percent prevalence of netB from diseased birds and none from healthy birds.

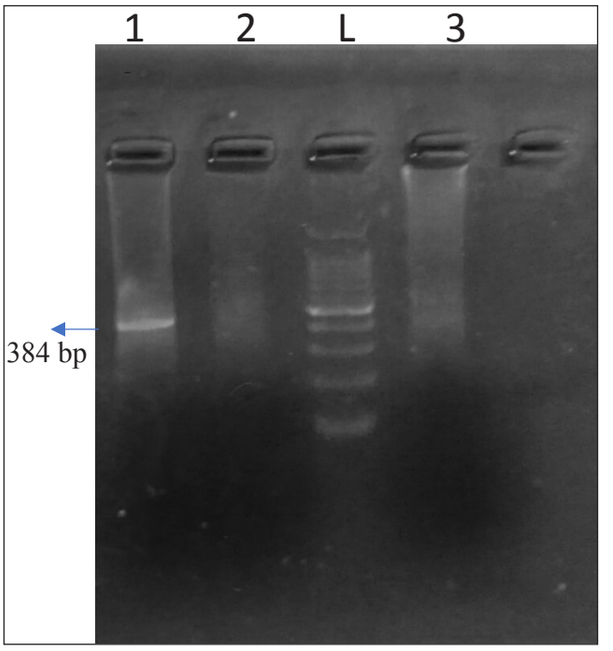

Fig. 4. Agarose gel showing 384 bp amplicon of netB gene

Lane 1 – Positive samples; L- 100 bp DNA Ladder

Ezatkhah and associates (2016) reported netB gene for the first time at a low incidence (7.77%) in chickens with NE in organic broiler farms and detected 17.98 percent prevalence by PCR. Keyburn *et al*. (2009) studied various *C. perfringens* isolates from NE- positive and NE- negative flocks from three continents and found 70 percent and 3.6 percent netB positive isolates, respectively. Further studies suggest that, potentially virulent toxins cpb2 along with netB could contribute to disease effects in *C. perfringens* isolates (Keyburn *et al*. 2008). Gaucher *et al*. (2017) reported an increase in the proportion of *C. perfringens* strains harboring the cpb2 gene and the netB

gene. A possible explanation could be that netB is easily transferred between several *C. perfringens* genotypes, thus increasing their virulence (Johansson *et al*., 2009). NetB is involved in virulence and is a secreted protein that is readily accessible to the host immune system, and this represents a promising target for vaccine development (Keyburn *et al*. 2010). Confirmation of the role of netB in disease came from the finding that most necrotic enteritis outbreak strains carry the netB gene, whereas nonnecrotic enteritis derived *C. perfringens* isolates lack this gene (Keyburn *et al*., 2008; Keyburn *et al*., 2010). Present study focused on targeting the netB toxin which is one of the virulent gene detected in NE disease from poultry. Toxin netB is produced only when the *C. perfringens* concentration is high and sufficient damage is caused to host cell (Timbermont *et al*. 2011).

CONCLUSION

Isolation and identification of *Clostridium perfringens* were done from NE cases as well as from healthy birds. The overall incidence of necrotic enteritis due to *C. perfringens* was found to be 34.4 percent. 16S rRNA PCR can be used as one of the confirmatory tests for detection of *C. perfringens* from NE cases. Most of the isolates of *C. perfringens* carried the toxin gene cpa (alpha), followed by cpb(beta) and cpb2. Theoccurrence of *C. perfringens* type A more predominant than *C. perfringens* type C among the NE cases of poultry birds. In the present study, netB which is one of the key virulent pore forming toxin of *C. perfringens*, was detected in 9.3 percent among the NE affected poultry birds. Further studies shall be designed to know the role of the netB toxin as well as the different toxins associated with necrotic enteritis and which could be useful in future for development of vaccines.

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