Original Research

Molecular Characterization and Virulent Gene Detection of *Clostridium perfringens* from Necrotic Enteritis Cases in Kadaknath Fowl

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Abstract

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. Necrotic enteritis (NE) is one of the most clinically associated bacterial enteric disease of poultry caused by Clostridium perfringens, which is an anaerobic, gram-positive bacteria. In present study, 08 Kadaknath birds, which showed signs of inappetence and diarrhea, were presented for necropsy. Gross examination of the fowls revealed necrotic to ulcerative lesions in intestine. Clostridium perfringens organisms were isolated from intestine after anaerobic incubation. Initial enrichment of intestinal samples were done in Robertson's cooked meat media (RCMM) and streaked on perfringens agar base with T.S.C supplements and for further confirmation, these colonies were streaked on egg yolk agar for lecithinase activity. The isolates were confirmed by 16S rRNA gene specific PCR and further processed for necrotic enteritis B like toxin (NetB) gene. Out of 08 intestinal samples processed, 04 were found positive for C. perfringens by phenotypic and genotypic confirmation. All confirmed C. perfringens isolates were processed for NetB gene and out of 4 samples, 01 was positive for NetB gene. This study further pointed out that C. perfringens NetB toxin is a one of the new key virulent factor for the development of NE in poultry birds.

Key words: *Clostridium perfringens*, Kadaknath**,** Necrotic Enteritis, Virulent Gene

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Introduction

Clostridium perfringens is the normal bacterial flora of gastrointestinal tract in both human and animals. It has also been shown to cause a number of diseases in humans and animals and reported to be a causal agent of necrotic avian enteritis throughout the world. In the past, NE has been controlled in poultry flocks with

antimicrobial growth promoters in commercial poultry feed (Williams, R.B. 2005). However, since the ban of these supplementations due to policy changes, NE has re-emerged as a costly disease in poultry industry (Cooper, K.K. and Songer, J.G. 2009)

Clostridium perfringens is classified into five toxin types (A to E) based on differential production of the four major toxins, alpha, beta, epsilon, and iota (Petit L *et.al* 1999). NE is caused primarily by *C. perfringens* type A and, to a lesser extent, type C strains (Agarwal *et al*., 2009, Cooper, K. K. 2009 and Van Immerseel, 2009). *Clostridium perfringens*, is a rod shaped, gram positive, anaerobic, spore forming bacterium and is wide spread in broilers having a significant economic burden on the poultry industry worldwide (Dahiya *et al*., 2006). NE is an acute, often fatal, disease of chickens characterized by depression, loss of appetite and sudden death. It occurs both as an acute clinical or a subclinical disease with necrosis in the intestines or as *C. perfringens*-associated hepatitic change (CPH) or fibrinoid necrosis in the liver (Nyrah *et al*., 2017).

The virulence of *C. perfringens* is attributable to at least 17 different toxins, while individual strains produce only a subset of these toxins. *C. perfringens* does not invade healthy cells, but produce various toxins and enzymes that are responsible for associated lesions and symptoms. The toxin production depends on the *C. perfringens* strain involved and each type of toxin induces a specific syndrome. The correct identification of pathovar is critical for epidemiological studies and development of effective preventive measures, including vaccination (Petit *et al*., 1999).

Materials and Method

Intestines were collected from 08 suspected cases of NE referred for post mortem cases at Department of Pathology, KNPCVS, Shirwal and from Omega laboratory, Lonand, Dist., Satara for this study. Intestinal contents were collected aseptically in a sterile container and quickly transported to the laboratory in ice-cooled containers. Processing of the collected samples was carried as soon as samples were received to the department.

Isolation of C. perfringens

All the intestinal 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 in anaerobic jars with anaero gaspack at 37°C for 24-48 hours. After incubation, a loopful of broth cultures was streaked onto perfringens agar with TSC supplements and on egg yolk agar for cultivation of *Clostridium perfringens* and for determination of lecithinase activity of the organism, respectively. The agar plates were incubated anaerobically for 24 hrs at 37° C. Identification of bacterial pathogens was done as per the standard methods described in Bergey's Manual of Systematic Bacteriology, 1986.

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Extraction of DNA from Suspected Colonies

Purification and extraction of bacterial genomic DNA was performed with phenol chloroform method as described by Sambrook *et al*. (1989) with slight modification. The genomic DNA thus purified was used as a template DNA.

PCR for Detection of C. perfringens by using 16S rRNA Species Specific Gene

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 (Tonooka *et al*., 2005) (Table 1). PCR was performed by preparing final reaction volume of 20µl in 0.2 ml thin walled PCR tubes. It was prepared by taking 12µl master mix supplied with Taq DNA, $MgCl_2$ and dNTPs; adding 1µM each of forward and reverse primers, 100ng template DNA and 5µl nuclease free. Samples were subjected to 35 PCR cycles, each consisting of initial denaturation for 02 min at 94°C; 30 sec of denaturation at 94°C; 30 sec of annealing at 56°C, and 1 min of elongation at 72°C. The final amplified PCR products were subjected to electrophoresis in 1.5% agarose gel after staining with ethidium bromide (5µl /100 ml).

Table 1: PCR primers used for detection of 16S rRNA gene of *C. perfringens*

Gene	Primer and Oligonucleotide Sequence	Amplicon Size	Reference
16S rRNA	F-TAACCTGCCTCATAGAGT	481bp	Tonooka *et al*. (2005)
	R-TTTCACATCCCACTTAATC		

Detection of Virulent Gene netB of C. perfringens

For the detection of virulent gene netB, all the 16s RNA gene positive isolates were subjected to uniplex PCR (Brady *et al*., 2010). Details of the primers are given in Table 2. The uniplex PCR assays in this study were performed in 25µl reaction volume in 0.2 ml thin walled PCR tubes. It was prepared by taking 12.5µl master mix supplied with Taq DNA, $MgCl_2$ and dNTPs, adding 1µM each of forward and reverse primers, 100ng template DNA and 5.5µl nuclease free water. The amplification programme was subjected to 35 PCR cycles, each consisting of initial denaturation of 10 min at 95°C; 30 sec of denaturation at 94°C; 30 sec of annealing at 55°C, and 1 min of elongation at 72°C. The final amplified PCR products were subjected to electrophoresis in 1.5% agarose gel after staining with ethidium bromide (5µl /100 ml).

Table 2: PCR primers used for detection of netB virulent gene of *C. perfringens*

Gene	Primer and Oligonucleotide Sequence	Amplicon Size	Reference
netB	F: GCTGGTGCTGGAATAAATGC	384bp	Ezatkhah *et al*. (2016)
	R: TCGCCATTGAGTAGTTTCCC		

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Result and Discussion

Phenotypic Identification of C. perfringens

The aim of present study was to confirm the presence of *C. perfringens*, which is associated with necrotic enteritis (NE) in poultry, and detection of virulent gene netB in these bacterial isolates. Out of 08 intestinal samples from NE suspected birds, 04 revealed the presence of *C. perfringens.* For isolation and phenotypic characterization of *C. perfringens* all the samples were inoculated in RCMM, which after 24-48 hrs of incubation showed heavy turbidity along with gas production and pink discoloration of meat particles 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 Rasool *et al*. (2017) used RCMM for initial isolation of *C. perfringens* from necrotic enteritis cases of poultry. After enrichment, samples were streaked on perfringens base agar with TSC supplements and on egg yolk agar. On perfringens base agar after incubation, rough and black color colonies with sulphite reduction were visualized (Figure no. 1). Pure colonies from perfringens agar plates were selected and further streaked on egg yolk agar medium, which showed characteristic diffused opalescence due to lecithinase activity of alpha toxin. Similar types of colonies of *C. perfringens* were observed by Keyburn *et al*. (2010) and Salah-Eldin *et al*. (2015) on egg yolk agar and clostridial agar with TSC supplement.

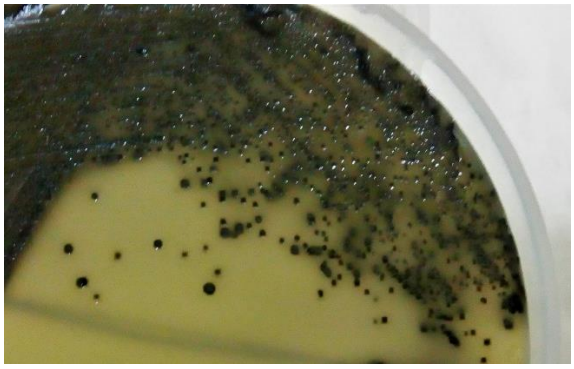

Fig. 1: Black colored colonies on clostridial agar with TSC supplements

Molecular Identification of C. perfringens by using Species Specific 16S rRNA Gene

All the 04 phenotypically confirmed isolates of *C. perfringens* were further subjected to species-specific 16S rRNA gene PCR, which showed 100 percent (Fig. 2). Similar findings were reported by Dar *et al*. (2017) who confirmed all the isolates of *C. perfringens* by species specific PCR. 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.

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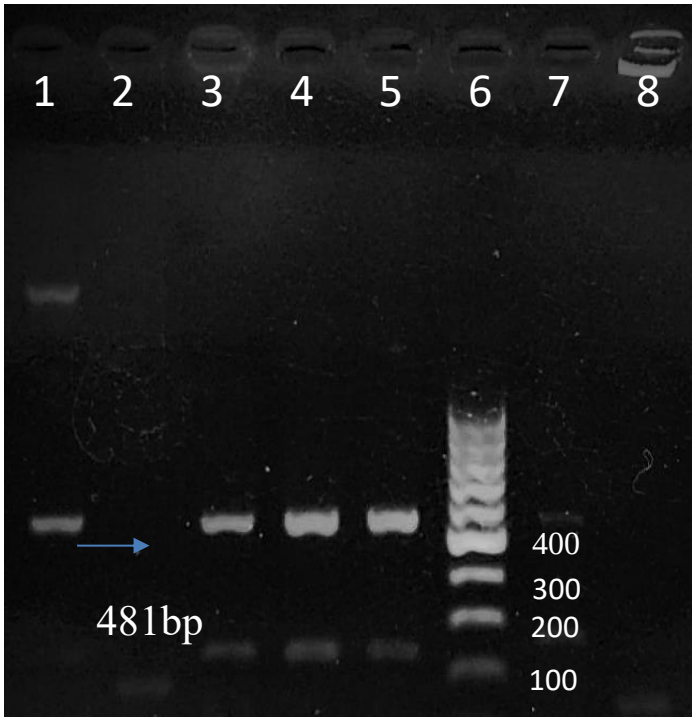

Lane 1,3,4,5- Positive samples; Lane 2,7,8- Negative control; Lane 6- 100 bp ladder

Fig. 2: Agarose gel showing 481bp amplicon of *C. perfringens*

Detection of Virulent Gene netB of C. perfringens

C. perfringens strains producing netB toxin have been considered as the definitive cause of NE in chickens (To *et al.*, 2017). In present study, 01 (25%) isolates, showed positivity towards netB, out of 04 isolates from diseased Kadkanath birds. (Fig. 3). These findings are in correlation with Ezatkhah and associates (2016), who 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. 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) reported 52.8 percent prevalence of netB from diseased birds and none from healthy birds. Virulence testing of numerous strains in a standard disease induction model showed that only strains producing NetB were capable of producing disease. NetB is an essential virulence factor in the pathogenesis of necrotic enteritis (Keyburn, A. *et al*., 2010). It secretes a protein that is readily accessible to the host immune system, and thus represents a promising target for vaccine development (Keyburn *et al*., 2010). Toxin netB is produced only when the *C. perfringens* concentration is high and sufficient damage is caused to host cell (Timbermont *et al*., 2011). Confirmation of the role of netB in disease came from the finding

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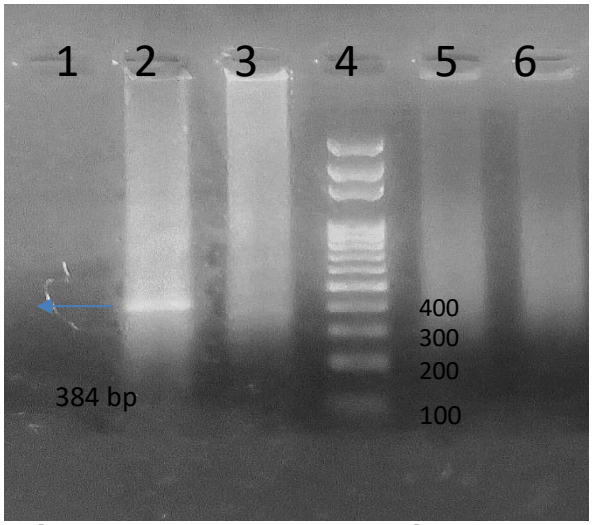
that most necrotic enteritis outbreak strains of *C. perfringens* carry the netB gene, whereas non-necrotic enteritis derived isolates lack this gene (Keyburn *et al*., 2008; Keyburn A *et al*., 2010a).



Lane 2- Positive sample; Lane 1,3,5,6- Negative samples; Lane 4- 100 bp ladder

Fig. 3: Agarose gel showing 384bp amplicon of netB gene of *C. perfringens*

Conclusion

The present study reveals the occurrence of virulent necrotizing Net B gene in the Kadaknath fowls. Further details study on pathogenesis can opens the significant opportunities for the development of novel vaccines against necrotic enteritis in poultry.

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