Alpha toxin specific PCR for detection of toxigenic strains of *Clostridium perfringens* in Poultry

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Abstract

Aim : Isolation of *Clostridium perfringens* from necrotic enteritis cases in poultry and confirmation by alpha toxin specific PCR

Materials and methods: Robertson cooked meat medium with Brain Heart Infusion broth was used for isolation of *C. perfringens* from intestinal contents of necrotic enteritis suspected birds. Positive cultures from perfringens agar were further confirmed by biochemical tests and subjected to alpha toxin specific PCR.

Results and Discussion: Twenty *Clostridium perfringens* isolates were isolated from intestinal contents of thirty five NE suspected birds. Out of the twenty isolates, fourteen were isolated from commercial broilers of 2 to 6 wk of age and six from commercial layers of 9 to 15 wk of age. Frequency of isolation of *C. perfringens* was more with Robertson cooked meat medium with BHI broth than thioglycollate broth alone. When positive cultures were streaked on to clostridial agar appreciable luxuriant growths were obtained and the selective streaking of these colonies on perfringens agar with supplements revealed rough and black colonies with sulphate reduction. The isolates produced rough and black colonies with sulphate reduction on perfringens agar, double zone haemolysis on sheep blood agar, stormy clot fermentation on milk medium and opalescence on egg yolk medium. The isolates were found negative for oxidase, catalase, liquefied gelatin, fermented glucose, maltose, lactose and sucrose except mannitol. All the fourteen isolates obtained from commercial broilers proved the alpha toxin producing strains of *C. perfringens* when they were subjected to alpha toxin specific PCR.

Conclusion : This study revealed alpha toxin specific PCR is highly useful for detection of toxigenic strains of *Clostridium perfringens in* poultry

Key words: Alpha toxin, *Clostridium perfringens*, Necrotic enteritis, PCR, Poultry

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Introduction

Clostridium perfringens type A and to lesser extent type C causes necrotic enteritits (NE) in poultry. Normally, the number of *C. perfringens* in the intestine is low (about 10^4 cfu/g of digesta). The disease occurs when high numbers of bacteria coincide with a damaged intestinal mucousa [1]. The disturbances in normal intestinal microfolora may cause rapid proliferation of C. *perfringens*, increasing bacterial numbers the range from 10^7 to 10^9 colony forming units (cfu)/g of digesta resulting in toxin production [2]. So far, over 800 serotypes of *C. perfringens* are known and 17 different toxic fractions have been isolated [3]. The *C. perfringens* strains were classified into five toxinotypes (A, B, C, D and E) based on the production of four major toxins viz., alpha, beta, epsilon and iota [4]. *C. perfringens* type A strains produce the chromosomal encoded toxin, while C. *perfringens* type C strains produce toxin together with toxin [5].

However, the chromosomal encoded alpha toxin is considered as the main virulence factor for NE in poultry because birds are about 200 times more susceptible to alpha toxin than to beta or epsilon toxin [6].

The alpha toxin has been implicated inseveral diseases including NE in chickens [7]. The toxin destruction of mucosal tissue manifests as macroscopic lesions that are usually seen in jejunum and ileum but can also appear in duodenum [8]. It is a potent toxin with haemolytic, lethal, dermonecrotic, vascular permeabilization and platelet aggregating properties [9] and it has direct effects on host metabolism

including inhibition of neutrophil chemotaxis, vaso-constriction, haemolysis of erythrocytes and necrosis of other body cells and modulation of cell metabolism by activating the arachidonic acid cascade and protein kinase C [10]. The polymerase chain reaction assay (PCR) was used for detection of alpha toxigenic strains of *C.perfringens* [11-14].

In India, NE was first reported by Chakraborty, *et.al.*, [15]. Now, the NE is emerged as a worldwide problem [16] and it is a common disease found in all poultry growing areas of the world.

Aim of the study was Isolation of clostridium perfirngens from necrotic enteritis cases in poultry and confirmation by alpha toxin specific PCR.

Materials and Methods

For isolation of the organism causing necrotic enteritis, thirty five birds suspected for NE were collected from poultry diagnostic and research centers of M/s. Suguna poultry farm, M/s. Venkateshwara Hatcheries Limited, Palladam, M/s. Pioneer Hatcheries, Namakkal. Apart from that, ten commercial farms in and around Namakkal and Udumalpet area from both broiler and layer farms, where, NE cases were reported.

For isolation of the organism causing necrotic enteritis, sterile saline (v/v) was added to the collected specimens consisting of intestinal contents and scrapings then heated at 80°C for 20 min in water bath. The processed Intestinal contents were inoculated into thioglycollate broth, Robertson cooked meat medium with brain heart infusion broth and sterile liquid paraffin was poured to make a layer over the medium. Inoculated medium was incubated at 37° C for 24 hr. The presence of *C. perfringens* in the inoculated sample is indicated by turbidity in both of the media. The positive cultures were streaked on to clostridial agar and perfringens agar with supplements. The plates were incubated in the anaerobic jar at 37° C for 48h.

The bacteria isolated anaerobically from NE specimens showed the characteristic colony types of *C. perfringens*, were gram stained and confirmed to be *C. perfringens* by standard biochemical tests as described by Barrow and Feltham [17].

Identification of toxigenic strains by Polymerase Chain Reaction (PCR): To design the PCR, alpha toxin specific primers (CP – F- AGT CTA CGC TTG GGA TGG AA and CP – R- TTT CCT GGG TTG TCC ATT TC), which flanked 900 base pair DNA sequence, according to Baums *et al.*, [18] were used.

To perform the PCR, 2 ul template DNA, prepared by the heat lysis method of Baums *et al.*, 2004, was added to a 50 ul reaction mixture with the following reagents 1.25 U Taq DNA polymerase, 50 mM Pottassium chloride, 30 mM Tris-Hcl, 1.5 mM Mg^{2++}, 200 µM of each dNTP and 50 picomoles of each primer. The thermocycling (incubations for 1 min at 95°C, 55°C and 72°C respectively was 35 times) was preceded by incubation for 2min 30 seconds at 95°C. Six microlitre of the amplicons was separated on 1.5% agarose gel according to standard procedure.

Results and Discussion

Inoculation of processed intestinal contents in thioglycollate broth produced turbidity and saccharolytic reaction in Robertson cooked meat medium with brain heart infusion broth. Appreciable luxuriant growths on the clostridial agar were obtained on the initial streak from the culture. The selective streaking of these colonies on perfringens agar with supplements revealed rough and black colonies with sulphate reduction.

The isolates produced double zone haemolysis on sheep blood agar, stormy clot fermentation on milk medium and opalescence on egg yolk medium. The isolates were found negative for oxidase, catalase, liquefied gelatin, fermented glucose, maltose, lactose and sucrose except mannitol.

Based on the results obtained from the above said tests, and in consultation with Bergey's Manual of determinative bacteriology [19], the isolates were identified as *C. perfringens*. Thus twenty *Cl. perfringens* isolates were obtained from the intestinal content of thirty five NE suspected broilers. Out of the twenty isolates, fourteen were isolated from commercial broilers of 2 to 6 wk of age, six from commercial layers of 9 to 15 wk of age. These findings correlate with the reports of detection of *C. perfringens* in 2-6 wk broiler chickens [20] and isolation of *C. perfringens* from 7 to 16 wk commercial layer birds [21].

The primer combination used in this study was reliable and very specific in amplifying 900 bp fragment of the alpha toxin gene- cpa of *C. perfringens* but not other genes cpb, etx, iap, cpe and cpb2, encoding the , , , entero and 2 toxins of *C. perfringens* as proved by Baums, *et.al.*, [18]. All the twenty isolates produced the predicted amplification size of 900 bp, with the gene coding for alpha toxin production (Fig .1) hence all the isolates are proved as the alpha toxin producing strains of C. *perfringens.* Similar to present study, Engstrom *et al.* [12] analysed 53 isolates of *C. perfringens* from NE affected poultry from different parts of Sweden by PCR for toxin typing. They reported that all the isolates were

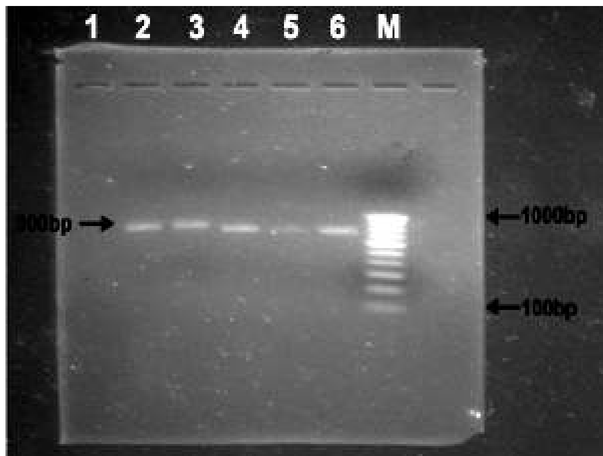

Figure-1. An agarose gel stained with ethidium bromide with PCR amplification products of C. perfringens isolates. Lane M: 100bp ladder; Lanes 2 to 6: Isolates

belonged to the toxin type A of *C. perfringens.* Several authors also noted that isolates from NE affected poultry were alpha toxin producing toxigenic strains of C. *perfringens* [11,13,14].

Based on the study, we concluded that twenty isolates toxigenic strain of *C. perfringens* were isolated from necrotic enteritis cases in poultry farms in TamilNadu, India. PCR-based assay was used for the detection of *C. perfringens*. This method is rapid, sensitive, and specific. Although limited numbers of clinical samples were used in the present study, it has been shown that PCR could be a convenient tool for detecting *C. perfringens* infection because it requires minimal laboratory facilities and is relatively simple and inexpensive to perform.

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Competing interests

The authors declare that they have no competing interests.

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