

Original Research Article

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## Antibiotic Sensitivity and Histopathological Study of *Clostridium perfringens* Associated with Necrotic Enteritis in Poultry

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### ABSTRACT

Necrotic enteritis (NE) is sporadic disease of avian species which has been reported from most of the areas of world in which poultry are reared. The causative agent of necrotic enteritis is *Clostridium perfringens*, a gram-positive anaerobe. The aim of this study was isolation of *C. perfringens* from NE cases along with antibiotic sensitivity study of the isolates and histopathology of the NE suspected intestine. For the present study, total 50 samples were collected and inoculated in Robertson's cooked meat media for initial enrichment followed by streaking on clostridium agar with TSC supplements and on egg yolk agar. After anaerobic incubation for 24 hr, black coloured colonies were visualized on clostridial agar (with TSC supplement) and opalescent colonies were seen on egg yolk agar. Further identification of *C. perfringens* was done by microscopical appearance and biochemical characterization. Total 35 (70%) samples out of 50 samples were collected and tested for the presence of *C. perfringens*. These isolated and pure colonies of *C. perfringens* were further examined for antibiotic sensitivity test. Histopathological examination of intestine of NE affected bird revealed, necrosis of villi, infiltration of heterophils, macrophages and deposition of variable number of fibrinous shreds and amorphous connective tissue which is one of the indicative findings of NE in poultry. Further study concluded RCMM as one of the best medium for enrichment of *C. perfringens*. Antibiotic sensitivity study of *C. perfringens* isolates was found that amoxicillin was the drug of choice for NE in poultry. This paper also describes the gross and microscopical important features of the intestine of NE due to *C. perfringens*.

#### Keywords

Necrotic enteritis,  
Poultry,  
*Clostridium perfringens*,  
Antibiotic sensitivity test,  
Histopathology

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### Introduction

Poultry is one of the fastest growing and profitable segments of the agricultural sector in India with around eight percent growth rate per annum. India ranks 3rd in egg production and 7th in chicken meat production in the world (Watt Executive Guide, 2015).

*Clostridium perfringens* is one of the most important food-borne pathogen of humans and animals causing both histotoxic diseases and intestinal infections. It is Gram-positive, anaerobic, straight rod, spore former, found in the soil, dust, sewage, marine sediments and in the gastro-intestinal tract of humans and animals. Based on the production of four

major lethal toxins i.e. alpha ( $\alpha$ ) beta ( $\beta$ ), epsilon ( $\epsilon$ ) and iota (i), *C. perfringens* is divided into five major toxinotypes (A-E) (Yadav *et al.*, 2016). All *C. perfringens* strains are pathogenic for animals, only type A and C strains are found harmful to humans (Guran *et al.*, 2013). *C. perfringens* associated with NE mainly produce type A and type C toxinotype (Shojadoost *et al.*, 2010). Type A which mainly produces alpha toxin, has lecithinase activity and hydrolyzes lecithin into phosphorylcholine and diglyceride and causes pathologic changes in tissues (Kalender *et al.*, 2005).

Necrotic enteritis is an important clinical disease produced by *C. perfringens* that affects the poultry industry worldwide causing serious economic loss, about of two-dollar billions/year. This disease is characterized by severe necrosis of the small intestine mucosa in the proximal jejunum region and it is associated with high mortality rates. On the other hand, subclinical disease leads to a decreased performance, due to the extensive mucosal damage (Llanco *et al.*, 2012). In this study, the isolation, antimicrobial susceptibility and histopathology of *C. perfringens* strains isolated from chickens with necrotic enteritis were determinate.

## **Materials and Methods**

A total of 50 intestinal samples which showed signs of enteritis were collected aseptically from poultry farm. 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. All samples were collected aseptically in a sterile container and quickly transported to the laboratory in ice-cooled containers. Processing of the collected samples was done as soon as samples received to the department (Ezatkah *et al.*, 2016).

## **Isolation of *C. perfringens***

All the samples were inoculated in Robertson's cooked meat media (RCMM) and in Fluid thioglycolate broth for initial enrichment. Inoculated broths 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 culture was further streaked onto selective agar and egg yolk agar plates for cultivation of *Clostridium perfringens*. Isolation of bacterial pathogens was done as per the standard methods described in Bergey's Manual of Systematic Bacteriology, 1986.

## **Selective plating for growth of *C. perfringens***

For isolation of *C. perfringens*, a loopful culture of RCMM broth culture was streaked on perfringens agar base (T.S.C Supplement) plate and incubated anaerobically in jar with anaerogas pack at 37°C for 24 - 48 hours.

Plates were observed for growth of *Clostridium perfringens*. After incubation the shape, size and type of colonies on agar were recorded.

Further confirmation of the isolates was done by streaking on egg yolk agar for lecithinase production followed by Gram's staining of the isolates. Pure cultures thus obtained were further processed for conventional biochemical characterization.

## **Identification of *C. perfringens***

After isolation, identification of *C. perfringens* was done by using Biochemical test such as catalase test, oxidase test and motility test (Hanging drop test).

### **Antibiotic sensitivity test of bacterial isolates**

Each of the bacterial isolate obtained from the samples of intestine of necrotic enteritis from suspected poultry birds were subjected to *In Vitro* antibiotic sensitivity testing by disc diffusion technique as per the method of Bauer *et al.*, (1966). The commercially available antibiotic discs and interpretative chart of zone of inhibition were used from HiMedia Laboratories Ltd., India. The antibiotics and its concentrations used for this test were amoxicillin (10 µg), ampicillin (10 µg), amikacin (30 µg), cloxacillin (1 mcg), enrofloxacin (10 µg), gentamycin (10 mcg), oxytetracycline (30 mcg), penicillin (10 units), streptomycin (10 µg).

### **Histopathology of NE suspected intestine**

The tissue samples of intestine were collected in 10 percent Neutral buffered formalin (NBF) and processed by routine paraffin embedding method described by Chauhan (1995). The tissue section of 4-6 microns were stained with routine Hematoxylin (H) and Eosin (E) method suggested by Chauhan (1995).

## **Results and Discussion**

### **Isolation and identification of *C. perfringens* from poultry**

The *C. perfringens* isolated from NE suspected intestine were identified based on morphological, cultural and biochemical characteristics. After enrichment, total 35 samples showed heavy turbidity with gas production in RCMM, turning the meat particles pink in color along with foul odor, while FTG broth did not show any growth (Plate No.1). The present study showed RCMM under anaerobic condition was the best medium for isolation of *C. perfringens* from NE suspected samples.

RCMM was the found to be best medium for enrichment of *C. perfringens* as described by Das *et al.*, (2008) and Nyrah *et al.*, (2017) used RCMM for initial enrichment of *C. perfringens* from necrotic enteritis suspected broiler chicken. Agarwal *et al.*, (2009) studied on *C. perfringens* and for the same they used thioglycolate enrichment medium for initial isolation.

### **Identification of *C. perfringens***

All the 35 positive broths which were streaked on perfringens agar with tryptose sulphite cycloserine (TSC) supplements, showed rough and black color colonies with sulphite reduction (Figure 2). Pure colonies from perfringens agar plates were selected and streaked on egg yolk agar medium, which showed characteristic diffused opalescence due to lecithinase activity of alpha toxins (Figure 3). Similar types of colonies were observed by Salah-Eldin *et al.*, (2015) and Keyburn *et al.*, (2010) of *C. perfringens* on egg yolk agar and clostridial agar with TSC supplement. Further morphological examination revealed Gram- positive, short and plumpy rods with blunt ends (Figure 1) which correlated well with the reports of Hafez *et al.*, (2011) and Khairy *et al.*, (2013).

On biochemical examination, the organisms failed to produce purple color on filter paper containing oxidase reagent and proved that the organisms were oxidase negative. The catalase test revealed none of the isolates produced effervescence and hence were catalase negative. On examination of isolates for motility by hanging drop preparation, it was proved that all the organisms were non-motile. Effat *et al.*, (2007) and Mostafa *et al.*, (2016) had reported *C. perfringens* isolates were found to be negative for oxidase and catalase.

In all total 35(70%) samples were found positive for *C. perfringens* from 50 diseased

(NE suspected) samples which were collected from NE suspected poultry birds.

Overall antibiotic sensitivity profiling of the 35 confirmed *C. perfringens* isolates, indicated that, amoxicillin was most effective against 30 (85.71%) isolates of *C. perfringens* tested followed by ampicillin 25 (71.42%) and oxytetracycline 20 (42.85%). Total 18 (51.42%) isolates were intermediate sensitive to enrofloxacin followed by gentamycin and streptomycin which were intermediate sensitive against 15(42.85%) and 10 (28.37%) isolates respectively. Out of 35 isolates, 28 (80%) were found resistant to penicillin, followed by 25 (71.42%) to cloxacillin, 22 (62.85%) to amikacin (Figure 4).

These findings were closely similar to the Llanco *et al.*, (2012) found that the antibiotic like amoxicillin, amoxicillin-clavulanic acid, cefoxitin, chloramphenicol, enrofloxacin, metronidazole and penicillin streptomycin showed susceptibility towards isolates of *C. perfringens*. Martel *et al.*, (2004) reported that the antibiotic avilamycin and amoxicillin showed good activity, while all isolates were resistant to high concentrations of flavomycin. Along with this Agarwal *et al.*, (2009) found highest sensitivity to fluroquinolones Viz. ciprofloxacin (93.3%) and enrofloxacin (86.6%) followed by chloramphenicol (76.6%), oxytetracycline (70%), co-trimoxazole (63.3%) cefatoxime (40.0%), gentamicin (36.6%), ampicillin (33.3%) and colistin (13.3%) and all the

isolates showed highly resistant to streptomycin and neomycin (100%). Tansuphasiri *et al.*, (2005) and Singh *et al.*, (2013) observed that *C. perfringens* isolates which were obtained from different sources showed resistance to tetracycline (28-78%), ceftriaxone (1-2.5%), chloramphenicol (3%) and penicillin (9%). The variation in the antimicrobial pattern might be due to indiscriminate use of these antibiotics as feed additive and prophylaxis as well as therapeutic agent in poultry industry in India.

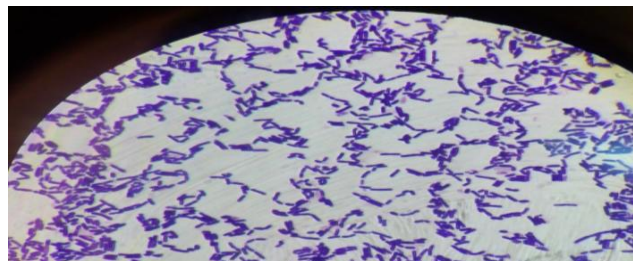
### **Histopathology of NE suspected intestine gross lesions**

It was observed that the intestine was having necrotic lesions of variable size, shape and consistency. These were small pin point dark in color, some were uneven shaped with irregular margins and covered with tenacious diptheric membranes, slightly elevated above the mucosa of intestines. Some of the lesions were blackish in color.

### **Histopathological examination**

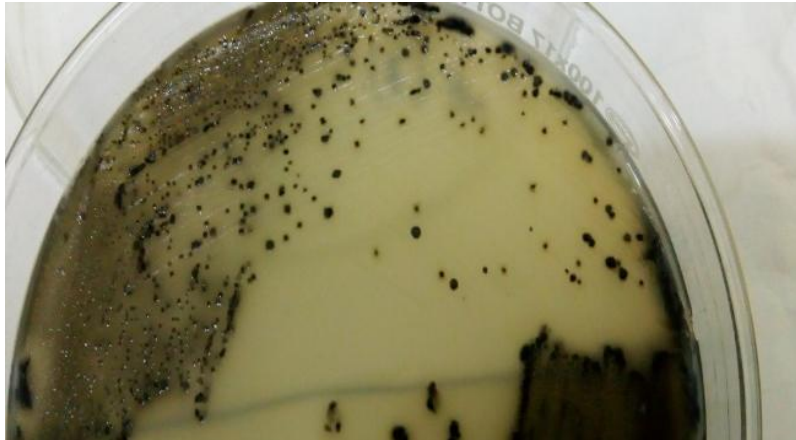
Histopathological examination revealed necrosis of villi, infiltration of heterophils, macrophages and deposition of variable number of fibrinous shreds and amorphous connective tissue. Amorphous material also includes dead cells and debris present around the mucosa and submucosa, in some of the cases it was extended up to muscularis layer (Figure 5).

**Figure.1** Microscopic appearance of *C. perfringens* in Gram's stain





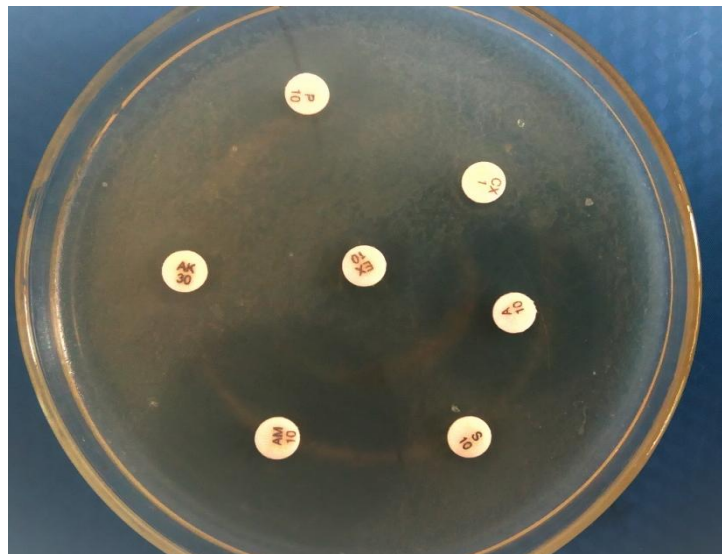
**Figure.2** Black colonies of *C. perfringens* on clostridial agar with TSC supplement



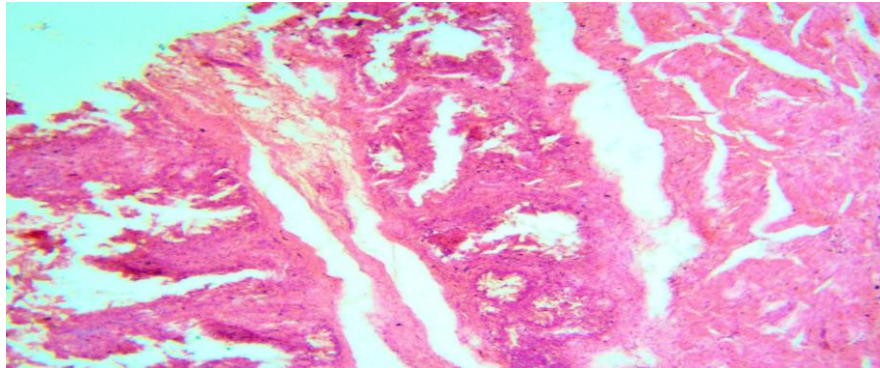
**Figure.3** Opalescent colonies of *C. perfringens* on egg yolk agar



**Figure.4** Antibiotic sensitivity pattern of *C. perfringens* isolates



**Figure.5** Histopathological examination showed destruction of villi



These findings were in agreement with some authors like Balachandran *et al.*, (2018) and Brady *et al.*, (2010) reported that on necropsy the mesenteric vessels were engorged with blood and intestinal lumen contained bile stained fluid and granular debris. The mucosa was covered with firmly adhering yellowish brown diphtheritic membrane giving a dirty Turkish towel appearance. On histopathology of the duodenal and jejunal sections showed necrosis of villous tips or entire villi, down to the crypts, with many large bacilli in this necrotic and cellular debris. Lyhs *et al.*, (2012) recorded that the histopathology confirmed a severe necrosis by the affecting the tips of the villi or the entire villi, together with a demarcation zone of inflammatory cells, degenerated epithelial cells, fibrin, and colonies of clostridia-like, rod-shaped bacteria. However, the crypts were usually intact.

In conclusion, the overall incidence of necrotic enteritis due to *C. perfringens* was found to be 70 percent. RCMM was found to be the best medium than FTG for enrichment of *C. perfringens*. *C. perfringens* isolates was found sensitive to Amoxicillin, Ampicillin, Oxytetracycline followed by Enrofloxacin and higher percentage of resistant was found towards Penicillin, Cloxacillin and Amikacin. Histopathological examination of the intestine from NE suspected birds, confirmed the occurrence of NE in poultry. This paper

describes the gross and microscopical important features of the intestine of NE due to *C. perfringens*.

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