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EXPERIMENTAL PRODUCTION OF NECROTIC ENTERITIS IN BROILER CHICKENS

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ABSTRACT

The present study was conducted to isolate and identify the *Clostridium perfringens*, the etiology of necrotic enteritis (NE) from broiler chickens and experimental production of NE with this isolate. A total of 50 samples were collected from jejunum of necropsied birds for isolation and identification of *Cl. perfringens*. Out of 50 samples, only 4 isolates of *Cl. perfringens* were isolated and identified (prevalence 8%). In experimental NE, the birds of group A (orally administered with only 0.1 ml ($1x10^3$ sporulated *Eimeria acervulina* oocysts/bird) showed dullness, ruffled feather, vent feather soiled with bloody faeces after 1 week of coccidial challenge. The birds of group B (orally administered $1x10^3$ sporulated *Eimeria acervulina* oocysts/bird and 1 ml of 2 days old broth culture of *Cl. perfringens*) showed severe depression, ruffled feathers, bloody faeces with fibrinous cast with 80% prevalence rate and 30% mortality in experimental NE. The birds of group C (orally inoculated with 1 ml of 2 days old broth culture of *Cl. perfringens*) showed no striking clinical, gross and histopathological lesions. Postmortem changes in small intestine (duodenum) were congestion and haemorrhages specially for birds of group A. The most severe gross lesions comprised of ascites, enlarged liver and heart, intestinal distension, profuse haemorrhage, fibrinous cast, fragile intestinal wall and gas bubble formation in the small intestine (duodenum, jejunum and ileum) of birds of group B. Histologically, birds of group B showed hemorrhage and congestion in liver, heart and intestine, desquamation of intestinal epithelium and intense leukocytic infiltration in intestine, liver and heart. The findings obtained from this study showed that simultaneous coccidial infection enhanced the pathological lesions of NE.

Key words: *Cl. perfringens*, culture, sporulated coccidia, pathogenesis.

INTRODUCTION

In Bangladesh, among different constrains of poultry industries, outbreak of several devastating diseases is one of the major constraints causing economic loss and discouraging people for poultry rearing (Das *et al*., 2005; Islam, 2005). Diseases are causing about 30% mortality of chickens per year. Incidence of the diseases varies depending on the geo-climatic condition, season, breed and age of birds. A survey report on both breeding flocks of commercial broiler and layer in major poultry raising belt in and around Dhaka and Gazipur districts in Bangladesh was conducted by Saleque *et.al.*(2003) and reported bacterial, viral, mycoplasmal, protozoal, parasitic, fungal and non-infectious diseases as 45%, 17%, 12.4%, 6.6%, 4.5%, 1.5% and 12.4%, respectively.

Among bacterial diseases, NE is one of the most important diseases in poultry that destroys the intestinal lining cells of the digestive tract, occurring outbreaks in broilers from 2-5 weeks of age. It is caused by *Cl. perfringens,* which is an important pathogen of a wide spectrum of veterinary diseases (McClane *et al.,* 1992). It is considered to be most widely occurring pathogenic bacterium. Clinical signs of this disease include depression, decreased appetite, reduced growth rates, diarrhoea, and severe necrosis of the intestinal tract. Under normal conditions, the bacteria live harmlessly in the gut but when gut microecology is drastically altered *Cl. perfringens* can proliferate. In its most acute form, NE causes sudden death of many birds within a few hours, without showing any clinical signs of the disease. Mortality is usually between 2-10% but can be as high as 40-50% resembling the symptoms of coccidiosis and may be mis-diagnosed. Birds with a milder, sub clinical form of NE may actually cause the cost of the producer more money. These birds may suffer from diarrhoea, appear as depressed and have decreased feed intake. This adversely affects growth rate, feed conversion and uniformity. A diseased bird may be received 30% less energy and 7% less protein than a healthy bird (Calnek *et al.,* 1991)

The incidence of necrotic enteritis in Mymensingh district of Bangladesh is 0.60% (Islam *et. al.,* 1998) and 0.52% (Talha *et. al.,* 2001). The incidence of necrotic enteritis in Sylhet and Rajshahi Region of Bangladesh is 0.44% (Islam *et. al.,* 2003) and 0.91% (Hossain *et. al.,* 2002), respectively.

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Necrotic enteritis is reported by postmortem examination in our country (Islam *et. al.,* 1998, Talha *et. al.,* 2001, Hossain *et. al.,* 2002 and Islam *et. al.,* 2003) but isolation and identification of causal agent in Bangladesh have not been performed. Therefore, the study was undertaken for the isolation and identification of *Cl. perfringens* from dead or sick bids and experimental pathogenesis study with this local isolate of *Cl. perfringens.*

MATERIALS AND METHODS

Collection of samples

Fifty samples were collected from jejunum of chickens suffering from diarrhoea as well as showing typical postmortem lesions of enteritis from S.K Diagnostic centre, Mymensingh. Intestinal contents from jejunum were collected aseptically in nutrient broth and transported in ice box.

Isolation of *Cl. perfringens*

Sample was first inoculated into nutrient agar stab. For maintenance of anaerobic condition candle jar was used and 1 to 2 cm layer of sterilized oil (Olive oil) was poured on the surface of the medium (Eyre, 2009). The culture was incubated for 24 hrs at 37^0 C. For determination of hemolytic activity of the organism, the samples from stab culture were spread on blood agar media (sheep blood) and were incubated anaerobically for 24 hrs at 37^0 C. Colonies showing a typical double zone of haemolysis formed in each plate and that was considered as *Clostridium* organism. For lecithinase test egg yolk emulsion (0.5 ml) was mixed with nutrient broth (10 ml) and 1% NaCl was added for clearance of media. The inoculating loop was thrusted with sample to the egg yolk media and was incubated anaerobically for 5 days at 37^0 C. A typical opalescent was produced in the media that considered as positive lecithinase test for *Cl. perfringens*. After that colonies of each isolate were inoculated into TSI (triple sugar iron) agar stab. TSI agar was used to indicate whether H_2S had been produced due to the reaction of sulphur containing compounds. Hydrogen sulphide reacted with the ferrous ion of the medium producing ferric sulphide, a black precipitate indicated positive TSI reaction for H_2S producing bacteria (Eyre, 2009).

Characterization of *Cl. perfringens*

The isolated organisms were identified by their colony morphology, microscopic examination, motility study, enzymatic activity and relevant biochemical tests according to standard laboratory methods (Eyre, 2009)**.**

Maintenance of stock culture

Nutrient agar slants were used to maintain the stock culture for each of the bacterial isolate. The *Cl. perfriengens* were inoculated in the slant by streaking and were incubated at 37^0 C for 24 hrs. Finally, glycerol was overlaid and the culture was kept at -70^0 C (Eyre, 2009).

Cleaning of oocysts

The duodenal content was collected sacrificing infected broiler chicken. The content was homogenized using stirrer after being collected in 2% potassium dichromate and kept it for 1 week at room temperature (RT) for sporulation. Then 40 ml of medium was taken in a sterile Falcon tube and centrifuged at 1000 rpm for 15 minutes and supernatant was discarded. After that sediment was washed with PBS (pH 7.4) by centrifugation for three times at 1000 rpm for 15 minutes. Finally, the inoculums were stored at RT (Al-Sheikhly and Al-Saieg, 1980; Baba *et al*., 1992; Williams *et. al*., 2003).

Counting of infective dose of oocysts

1 ml of coccidial sample was diluted in 9 ml saturated salt (NaCl) solution. A portion of the suspension was withdrawn with the help of plastic transfer pipette and each of the two chambers of McMaster slide was filled with 0.15 ml of suspension. After 3 to 5 minutes oocysts in two chambers were counted by using low power objective (10x). Total oocysts number was calculated by dividing the figure by 0.3 and multiplied by dilution factor 10. Then the concentration of sporulated oocysts was adjusted to $1X10^3$/0.1ml (Baba *et al*., 1992; Williams *et. al*., 2003).

Experimental design

For the experiment, a total number of 30 broiler birds 2-3 weeks of age (obtained from Trishal poultry farms) were reared for experimental study. They were divided into three groups (A, B and C) (Fig. 1). All group of birds were given commercial pillet feed, *adlibitum* water with 50% protein rich fish meal. Each group contained 10 birds. Group A was orally administered with coccidia *Eimeria acervulina* ($1x10^3$ sporulated oocysts/0.1ml), group B for coccidia *Eimeria acervulina* $1x10^3$ sporulated oocysts/0.1ml/bird + *Cl. perfringens* (1ml of 2 days old culture broth) and group C (inoculated with 1ml of 2 days old culture broth of *Cl. perfringens*). Experimentally inoculated birds were observed for every 24 hours interval for the observation of effects of different inoculum upto 2 weeks after drenching. In the experimental period mortality rate, necropsy and histopathological findings were recorded.

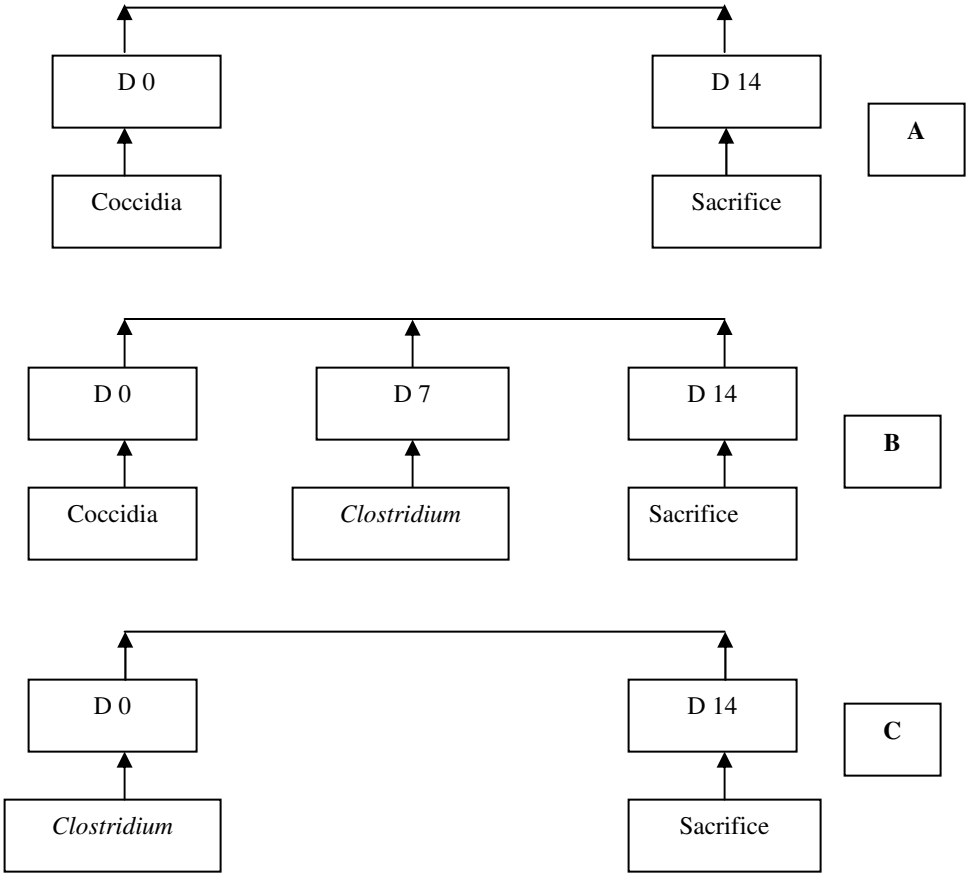

Fig. 1 shows the time schedule for different types of exposure

RESULTS AND DISCUSSION

Table 1 shows the clinical signs of experimental NE with local isolate of *Cl. perfringens*. In this study, the experimental birds were observed for 2 weeks. About 50% of birds of group A (drenched with only 0.1 ml ($1x10^3$ sporulated *Eimeria acervulina* oocysts/bird) showed dullness, ruffled feather, vent feather soiled with bloody faeces after 1 week of coccidial challenge. The clinical signs produced by coccidia recorded in the present investigation corresponded with the findings of others (Al-Sheikhly and Truscott, 1977; Al-Sheikhly and Al-Saieg, 1980; Baba *et al*., 1992; Williams *et. al*., 2003; Samad, 2005). A total 80% of birds of group B (coccidia+*Clostridium*) showed severe depression, ruffled feathers, bloody faeces with fibrinous cast and

ultimately died (30%) 3 days before sacrifice (Table 2). The clinical signs of present investigation reported in birds of group B drenched with *E. acervulina* (0.1 ml ($1x10^3$ sporulated oocysts/bird) plus *Cl. perfringens* (1ml of 2 days old broth) were almost similar by the findings of other investigators (Truscott and Al-sheikhly, 1977; Al-sheikhly and Al- saieg, 1980; Shane *et al.,* 1985; Cowen *et al*., 1987; Baba *et al.,* 1992; Williams *et al.,* 2003; Van *et al.,* 2004; Drew *et al.,* 2004 and Olkowski *et al.,* 2006). These authors described supply of protein rich feed and simultaneous infection with coccidia might act as predisposing factor for the production of NE. Birds of group C (inoculated with 1ml of 2 days old culture broth of *Cl. perfringens*) did not show any clinical signs.

Table 1. Clinical signs in experimental necrotic enteritis

Group	Type of exposure	Clinical signs
Group A	*E. acervulina*	Dullness, ruffled feather, vent feather soiled with bloody faeces
Group B	*E. acervulina* + *Cl. perfringens*	Dullness, ruffled feather, bloody diarrhea mixed with fibrinous cast
Group C	*Cl. perfringens*	No clinical signs

Prevalence and mortality

In experimental NE, the prevalence rate was 80% in group B birds (0.1 ml ($1x10^3$ sporulated *Eimeria acervulina* oocysts/bird) plus *Cl. perfringens* (1ml of 2 days old broth)(Table 2) which were relatively higher than the reports by other authors (Islam *et al.* 1998; Talha *et al.* 2001; Hossain *et al.* 2002; Islam *et al.* 2003). This is probably due to the fact that other authors diagnosed the diseases mostly based on postmortem examination. No striking clinical signs, mortality, gross and histological lesions were found in birds of group C (inoculated with *Clostridium* only).

Present study showed 10% mortality of birds in group A (drenched with only 0.1 ml ($1x10^3$ sporulated *Eimeria acervulina* oocysts/bird) while 30% mortality in birds of group B (0.1 ml ($1x10^3$ sporulated *Eimeria acervulina* oocysts/bird) plus *Cl. perfringens* 1ml of 2 days old broth) (Table 2*).* But Shane *et al*. (1985) observed 35% mortality (28/80) in birds which received an oral dose of *E. acervulina* simultaneously with a ration containing *Cl. Perfringens* but 41% mortality (33/80) of birds that were fed an inoculated ration two days after an oral dose of *E. acervulina* and 10% mortality which were fed an inoculated ration four days after an oral dose of *E. acervulina.*

Table 2. Prevalence and mortality of birds in experimental necrotic enteritis

Group	Type	Age (wks)	No. of birds	Type of exposure	No. of birds clinically ill	Prevalence rate (%)	Mortality rate (%)
Group A	Broiler	2 to 3	10	*E. acervulina*	05	50	10
Group B			10	*E. acervulina* + *Clostridium*	08	80	30
Group C			10	*Clostridium*	00	00	00

Pathology

Table 3 and 4 describe the gross and microscopic lesions of experimental NE in broiler birds. The birds of group A (drenched with only 0.1 ml ($1x10^3$ sporulated *Eimeria acervulina* oocysts/bird) showed severe hemorrhage in the duodenum and jejunum (Fig. 1) while microscopically, showed glandular proliferation in duodenum and jejunum and presence of coccidial merozoites in intestinal epithelium and lamina propria (Fig.s 7-8).

In present experimental study, the resulted lesions in birds of group B(0.1 ml ($1x10^3$ sporulated *Eimeria acervulina* oocysts/bird) plus *Cl. perfringens* (1ml of 2 days old broth) were confined to the small intestine (duodenum, jejunum and ileum) which were distended with gas and often friable (Fig.s 3-4). Hemorrhage was also found in small intestine. The main lesion of fibrino-necrotic enteritis always localized in the small intestine either in its entire length or just a segment (duodenum, jejunum or ileum) and characterized by the disappearance of the surface epithelium and necrosis of the villi. Ascites, enlarged liver with accumulation of leukocytes around the central veins (Fig.s 5-6), dilated left ventricle of heart and hemorrhage on all of the heart were found occasionaly. In histopathological study, large numbers of clostridia were seen among the sloughed cells, congestion and hemorrhage were found in lamina propria and foci of coagulation necrosis of the tips of the villi (Fig.s 9-10).

The birds of group C (inoculated with 1ml of 2 days old culture broth of *Cl. perfringens*) did not show any striking lesions grossly (Fig. 2) and microscopically. The gross and histopathological lesions described above in different groups of birds, in the present study, corresponded with the findings of other investigators (Al-Sheikhly and Truscott, 1977; Fukata *et al*., 1988; Hutchison and Riddell, (1990); Samad, 2005; Keyburn *et al*., 2008).

Table 3. Gross lesions in experimental necrotic enterits in broilers

Group	Type of exposure	Necropsy findings
Group A	*E. acervulina*	Severe hemorrhage in the duodenum and jejunum
Group B	*E. acervulina* + *Cl. perfringens*	Ascites, severe hemorrhage in the duodenum and jejunum, blood mixed with fibrinous mass, gas bubble present in the duodenum, jejunum and caecum, enlarged liver, hemorrhage on the base of the heart.
Group C	*Cl. perfrigens*	Normal necropsy findings

Table 4. Microscopic lesions in experimental necrotic enterits in broilers

Group	Type of exposure	Histopathology
Group A	*E. acervulina*	Glandular proliferation in duodenum and jejunum. Presence of coccidial merozoites in intestinal epithelium and lamina propria.
Group B	*E. acervulina* + *Cl. perfringens*	Hemorrhage and congestion in sub mucosa of small intestine (duodenum, jejunum and ileum). Sloughing of epithelium in small intestine. Hemorrhage and accumulation of reactive cells also in liver and heart
Group C (Control)	*Cl. perfringens*	Normal findings

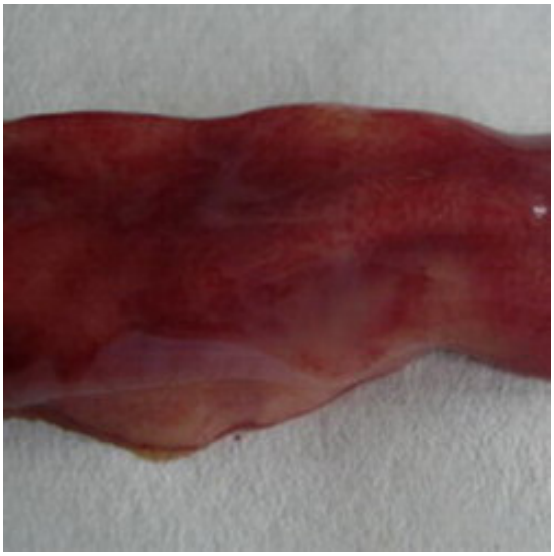

Fig.1 Orally administered group A (coccidia) shows congestion and hemorrhage in the duodenum.

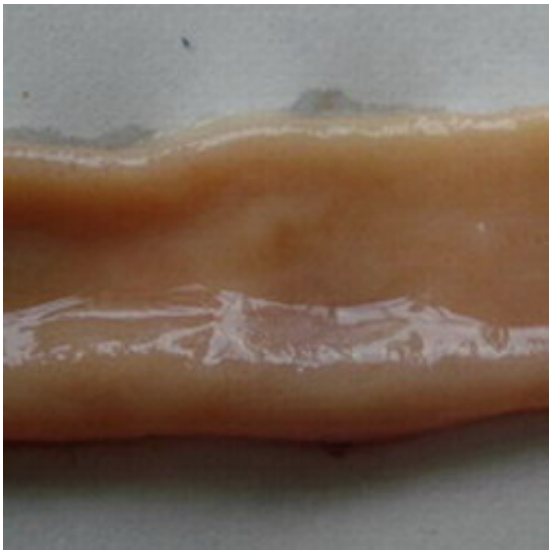

Fig.2 Orally administered group C (only *Cl. perfringens*) shows the normal duodenum.

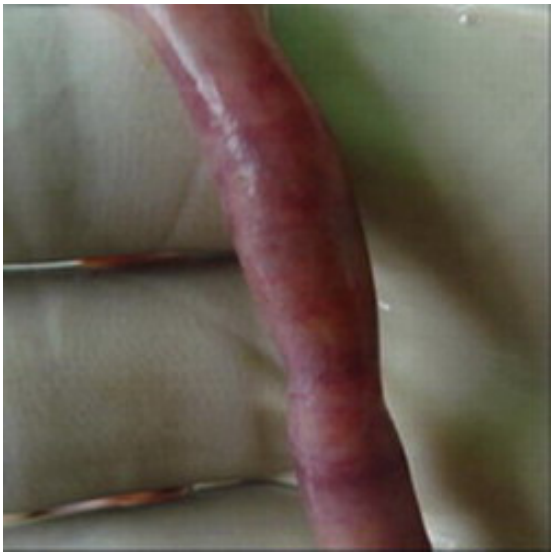

Fig. 3. Orally administered group B (coccidia+*Clostridium*) shows congestion and distended ileum.

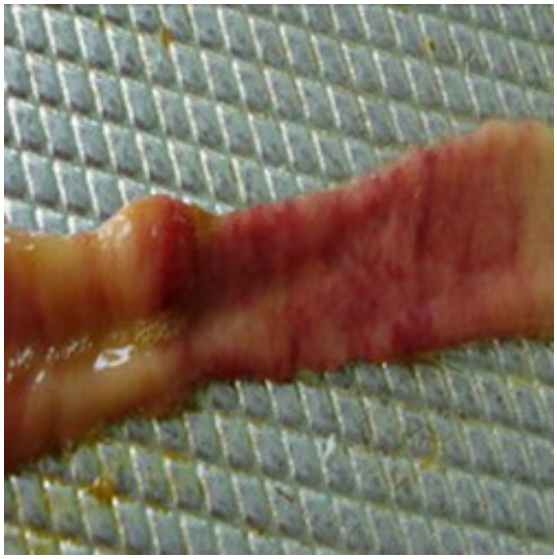

Fig.4. Orally administered group B (coccidia+*Clostridium*) shows hemorrhage and fibrino-necrotic area in ileum.

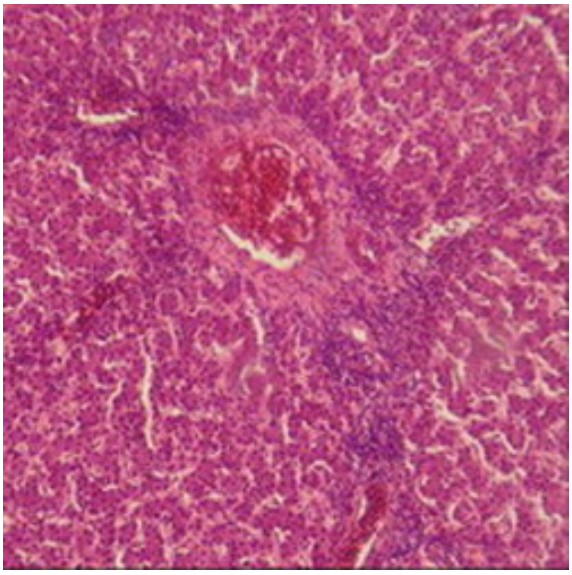

Fig. 5. Orally administered Group B (coccidia + *Clostridium*) shows congested blood vessels and accumulation of heterophils and lymphocytes in liver (H&E, x82.5).

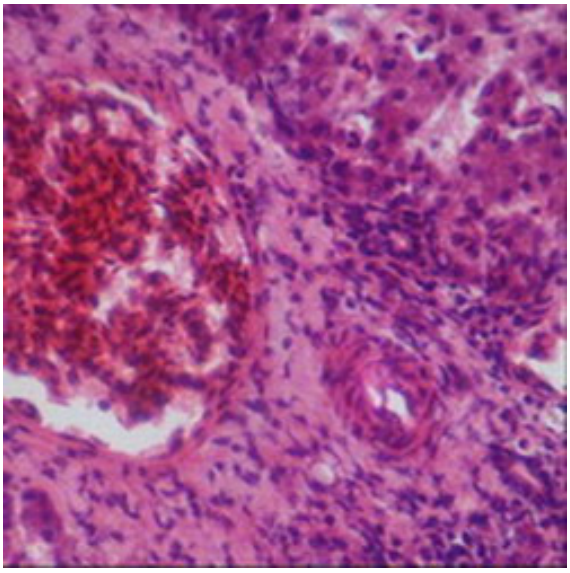

Fig.6. Orally administered Group B (coccidia + *Clostridium*) shows congested blood vessels and accumulation of heterophils and lymphocytes in liver (H&E, x330).

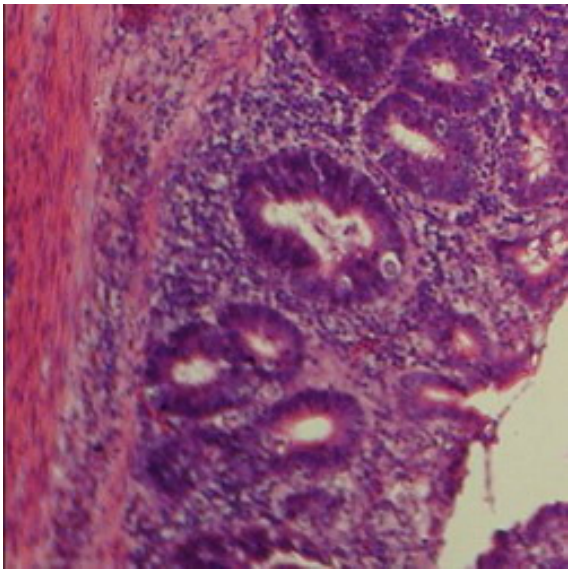

Fig. 7.Orally administered group A (coccidia) shows schizonts (arrow) containing merozoites in jejunal glandular epithelium (H&E, x330).

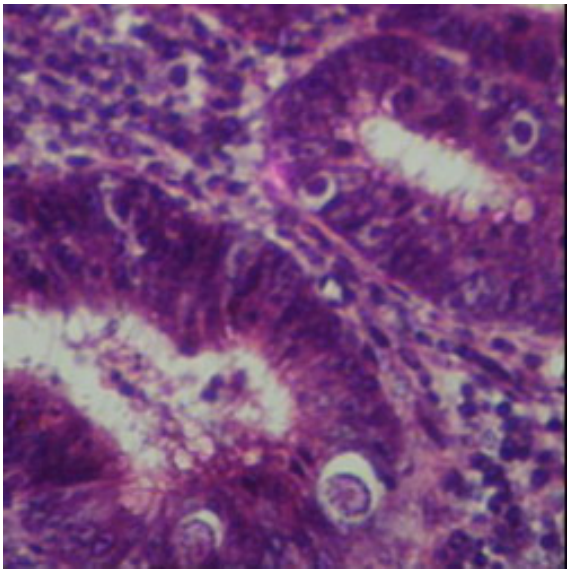

Fig. 8.Orally administered group A (coccidia) shows schizonts (arrow) containing merozoites in jejunal glandular epithelium (H&E, x825).

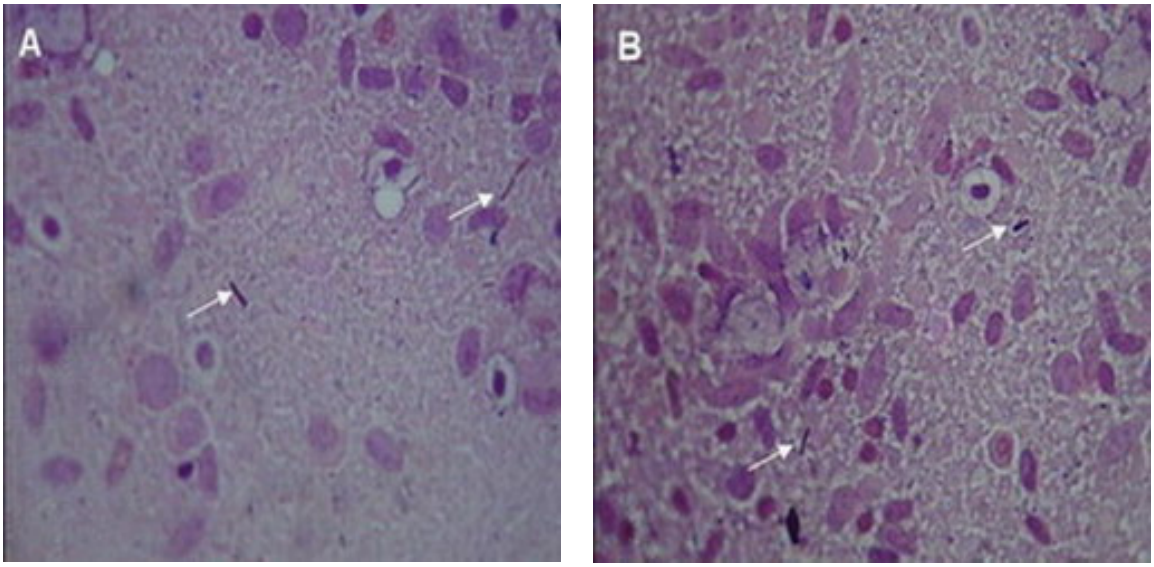

Fig.9. (left) and Fig. 10. (right) Gram's staining of impression smear prepared from jejunum of group B (coccidia+*Clostridium*) shows Gram positive short rods (arrows, A & B) of *Clostridium* sp. (x825)

Clostridium perfringens like organism were successfully isolated and identified from clinically suspected cases of necrotic enteritis. The disease was reproducible in experimentally inoculated broiler when birds were infected with coccidia with one week ahead of experimental clostridial infection.

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