EFFECT OF PROTEIN RICH DIET ON EXPERIMENTAL PATHOLOGY OF NECROTIC ENTERITIS IN BROILERS

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ABSTRACT

This study was designed to know the effect of protein rich diet (50% fish meal) on the experimental pathology of necrotic enteritis in broilers. The *Clostridium (Cl.) perfringens* was obtained from the Department of Pathology, Bangladesh Agricultural University. Reconfirmation and recharacterization of *Cl. perfringens* were performed by culture, microscopic examination, staining and biochemical tests. The experimental pathologic studies were performed with supplementation of protein rich diet and challenged with *Cl. perfringens* in broilers. The dose of the inoculum for experimental infection with *Cl. perfringens* was 1x10⁸ CFU/2.5ml. Fifteen birds of 21 days old were divided into 3 (A, B and C) groups each containing 5 birds. Birds of group A were fed with 50% fish meal at a rate of 500gm /kg of feed from day 21 to day 34 and challenged from day 28 to day 32 with 1x10⁸ CFU/2.5ml. Birds of group B were fed with normal feed and challenged on day 28 for consecutive five days. Group C was kept as control with commercial normal pellet without *Cl. perfringens*. Birds of all groups were observed up to 34 days of age for clinical signs. Eighty percent (4/5) of the birds of group A developed moderate clinical signs like diarrhoea, ruffled feather and less feed intake whereas 40% (2/5) birds of group B developed same clinical signs like group A but in mild form. There was no mortality in any groups. All the birds were sacrificed at Day 35. Severe necrosis and hemorrhage in intestine, enlarged liver and hemorrhage in the base of heart were noted in the birds of group A. On an average 2-5 bacteria were found in impression smear of intestines in higher magnification (100x), and anaerobic bacteria counted from intestinal content was 1.51x10⁷ CFU/ml. In histopathology, necrosis and reactive cells were found in liver, heart, lung and sloughing off intestinal epithelium was also found in intestines. On the other hand similar lesions like group A were observed in the birds of group B but in moderate form and no bacteria was found in impression smears of intestines. Anaerobic bacteria counted from intestinal content of this group was 1.1x10⁷ CFU/ml. In histopathology necrosis, reactive cells were found but less than group A. The birds of group C were normal in all parameters. However, anaerobic bacteria count from the intestinal content was 0.8x10⁷ CFU/ml. From this study, it may be concluded that protein rich diet is a predisposing factor for necrotic enteritis in broilers.

Key words: Protein rich diet, experimental pathology, necrotic enteritis, *Clostridium perfringens*, broilers

INTRODUCTION

Poultry industry is an emerging agribusiness started practically during 1980’s in Bangladesh (Huque, 2001). Infectious diseases are the major constraints of poultry rearing causing 30% mortality of chickens per year (Das et al., 2005). Among bacterial diseases, necrotic enteritis (NE) is one of the most important diseases in poultry that destroys the intestinal lining of the digestive tract. Outbreaks caused by *Clostridium perfringens* (CP) usually occur in broilers from 2-6 weeks of age. CP is a gram positive, spore-forming, anaerobic, large rod bacteria, which is present in the environment worldwide (Willis, 1969). Since 1961, NE is causing mortality, reduced feed conversion and growth rate (Hofshagen & Kaldhusdal, 1992). The predisposing factors of necrotic enteritis are diet, age and cocidiosis. The reported prevalence of NE based on post-mortem examination in Mymensingh, Sylhet and Rajshahi districts of Bangladesh are 0.52-0.60% (Islam et al., 1998; Talha et al., 2001) and 0.44% (Islam et al., 2003) respectively. At present NE in broilers is increasing in Bangladesh known from personal communication of poultry Veterinarians. The exact cause of this increase of NE is not known. Various risk factors are thought to be the cause of increase of NE in Bangladesh. Protein rich feed is known to be a risk factor of NE in poultry. However, no such investigations have been made in Bangladesh. This study describes the effect of protein rich diet on experimental pathology of necrotic enteritis in broilers.

MATERIALS AND METHODS

The research work was carried out in the Department of Pathology, Faculty of Veterinary Science, Bangladesh Agricultural University (BAU), Mymensingh during January 2013 to May 2013.

Collection of *Clostridium perfringens*  
*Clostridium perfringens* were obtained directly from TSI media that were preserved in Department of Pathology, BAU, Mymensingh.
Reisolation and identification of organisms
The organism was cultured in nutrient agar and nutrient broth. The organism was stained with Gram’s stain for morphological study. Biochemical tests for *Clostridium perfringens* were performed following routine standard procedures (Miah, 2011).

Determination of CFU for inoculums
Nutrient broth was used in order to determine the CFU of *Clostridium perfringens*. In dilution of 1:10 the colony was not countable and in 1:1000 dilutions the colony was not detected. So, the colonies from 1:100 dilutions were counted. Each of the bird of experimental group was drenched with 2.5 ml of broth that contained $1 \times 10^8$ CFU (Olkowski et al., 2006).

Collection of birds and feeding with protein rich diet
Fifteen broiler birds of 21 days old were collected from poultry farm of the Bangladesh Agricultural University. The birds were maintained for a total period of 2 weeks with optimal rearing condition. The feed and water supply was ad libitum. The small sized dry fishes were purchased from local KR market. The dry fishes were ground with grinder and mixed with appropriate amount with commercial broiler feed pellets.

Experimental design
A total of 15 birds of 21 days of age were divided into three groups (A, B, and C) each containing five birds. Birds of group A were fed with 500gm fish meal/kg of feed from day 21 to day 27 and challenged on day 28 with $1 \times 10^8$ CFU/2.5ml of inoculums for next consecutive 4 days as long term exposure trail (Olkowski et al., 2006). The protein diet i.e. 50% fish meal @ 500gm/kg was also fed up to sacrifice of the birds. Birds of group B were fed with normal feed and challenged on day 28 with $1 \times 10^8$ CFU/2.5ml of inoculums and for consecutive 4 days. Group C was kept in control with commercial normal pellet without *Cl. perfringens*. The feed and water supply was ad libitum in all groups of birds. Experimentally inoculated birds were observed for every 24 hours interval up to day 34 (for 7 days). In the experimental period the clinical signs, morbidity, mortality were recorded. The birds were sacrificed at day 35. The gross pathology, impression smear from intestine, anaerobic bacteria count and microscopic pathology of sacrificed birds were recorded (Olkowski et al., 2006).

Clinical signs
Birds of all groups were observed for clinical signs for 7 days. The severity of clinical signs was graded as moderate (++) and mild (+).

Gross pathology
Gross tissue changes at necropsy were carefully observed in birds of all groups. The change of tissue was recorded and representative tissue samples (intestine, liver, heart, lung, kidney and spleen) were preserved in 10% neutral buffered formalin for histopathological studies. The severity of lesions was graded as severe (+++), moderate (++), mild (+) and almost normal (+/-).

Impression smear
Impression smears were prepared from jejunum on slides from 2 birds from each group and fixed by absolute methanol for 5 minutes. The slides were stained with Giemsa stain for 40 minutes. It was then washed with running tape water. The stained slides were examined at high magnification (100x) according to the procedure described by Rahman (1995).

Histopathology
The formalin fixed tissues (intestine, liver, heart, lung, kidney and spleen) from birds of all 3 groups were trimmed, processed, sectioned and stained as per standard procedure (Luna , 1968).

Anaerobic bacteria count in intestinal content
Table 4 describes the anaerobic bacteria count in intestinal contents of experimental birds. The average bacterial load in group A was higher than those in group B and group C. Severity of histopathological lesions in different group of birds was graded as severe (+++) that indicates presence of reactive cells, congestion, necrosis and sloughing off luminal epithelia. The moderate (+) that indicates moderate presence of reactive cells, congestion, necrosis and sloughing off luminal epithelia. The mild (+) that indicates the mild presence of reactive cells, congestion and necrosis. Almost normal (+/-) that indicates normal structure.
**Anaerobic bacterial load in intestinal content**

For counting of anaerobic bacteria load in intestinal content of experimental bird, the 1:100 dilution of intestinal content was used. About 1 µl of intestinal fluid from 2 sacrificed birds of each group along with 9 µl of PBS were taken in Eppendorf tube. Then 1 µl of this solution was poured on nutrient agar media with micropipette and was spread with ladder. Three Petri dishes were used for one bird. Subsequently, sterilized olive oil was poured on the media and incubated at 37° C for overnight in a candle jar for colony counting (Olkowski et al., 2006).

**RESULTS**

**Clinical signs**

The clinical signs of NE in group A birds were moderate (+++) level of diarrhoea (Figure 3), somnolence and ruffled feather. Whereas the clinical signs in Group B birds were mild (+) diarrhoea, somnolence and ruffled feather. Birds of group C were normal (Table 1). There was no mortality in any group of birds.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Birds</th>
<th>Type of exposure</th>
<th>Amount of exposure</th>
<th>Period of observation</th>
<th>Clinical signs</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5</td>
<td>Cl. perfringens of 1x10^8 CFU/bird + 50% fish meal</td>
<td>50% fish meal 500gm/kg feed +2.5ml of1x10^8 CFU/bird</td>
<td>1 week</td>
<td>Depression, ruffled feather, diarrhoea. Moderate level(++)</td>
</tr>
<tr>
<td>B</td>
<td>5</td>
<td>Cl. perfringens 1x10^8 CFU/bird + normal feed</td>
<td>2.5 ml of broth of 1x10^8 CFU/bird + normal feed</td>
<td>1 week</td>
<td>Depression, ruffled feather, diarrhoea. Mild level(+)</td>
</tr>
<tr>
<td>C</td>
<td>5</td>
<td>Normal feed</td>
<td>Normal feed</td>
<td>1 week</td>
<td>Normal (+)</td>
</tr>
</tbody>
</table>

**Prevalence of experimental infection**

In this study the experimental birds were observed for 1 week. Eighty percent birds of group A showed moderate (+++) clinical signs while 40% birds of group B showed mild (+) clinical signs. Birds of group C were normal (Table 2).

**Gross pathology**

At necropsy the small intestine (duodenum) was found congested specially in group A. The foul smelling brown fluid and bubble was present in the duodenum, jejunum (Figure 4) and caecum, large amounts of hemorrhagic necrotic epithelial debris in the lumen of the bowel, enlarged liver and heart, hemorrhage on the base of the heart. All these lesions were graded as severe (+++). Birds of group B developed similar lesion as group A birds but with moderate (+++) severity. Birds of group C showed no sign in the intestine (Table 3).

**Staining characteristics in impression smear**

Impression smear prepared from intestine of 2 birds of group A showed numerous (2-5) short, thick, Gram-positive rods (100 X) (Figure 5). In smears prepared from infected tissues bacteria was also observed, arranged in single, pair and group. These were suspected as *Clostridium perfringens*. Samples from the birds of group B showed no bacteria in impression smears prepared from jejunum. In birds of group C bacteria was not found in impression smear (Figure 6).

**Histopathology**

The histopathological features of the affected organs of experimental birds are shown in Table 5. Birds of Group A showed the sings of hemorrhage, congestion in sub mucosa of small intestine (duodenum, jejunum and ileum) and sloughing off epithelium in small intestine (Figure 7). Focal necrosis in liver (hepatitis) (Figure 13), hemorrhage and accumulation of reactive cells were also found in epicardium of heart (epicarditis) (Figure 9). Pneumonic lesion was also found in lung. *Aspergillus* nodule was noted in the lungs of one bird (Figure 11). The lesions in group A birds were graded as severe (+++). Group B showed the sings of hemorrhage and congestion in sub mucosa of small intestine (duodenum, jejunum and ileum).
Hemorrhage and accumulation of reactive cells were also found in liver (Figure 14), heart and lung but all of the lesions were moderate(++) in severity in compare to the birds of group A. Birds of group C appeared almost normal histopathology (± to +) in different organs (Figure 8, Figure 10, Figure 12).

Table 2. Prevalence of NE in experimental broiler birds (age of bird=28 days)

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of birds</th>
<th>Type of Exposure</th>
<th>No. of birds affected</th>
<th>Prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5</td>
<td><em>Cl. Perfringens</em> 1x10⁸ CFU/bird + 50% Fish meal</td>
<td>4</td>
<td>80</td>
</tr>
<tr>
<td>B</td>
<td>5</td>
<td><em>Cl. Perfringens</em> 1x10⁸ CFU/bird + Normal feed</td>
<td>2</td>
<td>40</td>
</tr>
<tr>
<td>C</td>
<td>5</td>
<td>Control group</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

DISCUSSION

This study was undertaken to know the effect of protein rich diet on the pathology of necrotic enteritis (NE) caused by *Clostridium perfringens* in broilers. The birds of group A (challenged with *Clostridium perfringens* 1x10⁸ CFU/bird following feeding with 50% fish meal at a rate of 500gm/kg) showed moderate (+++) depression, ruffled feathers and diarrhea and the prevalence of infection was 80%. The birds of group B (challenged with *Clostridium perfringens* 1x10⁸ CFU/bird with normal feed) showed mild (+) clinical signs and the prevalence of infection was 40%. The similar findings were also reported by Miah *et al.* (2011). In this study, fish meal supplemented group of birds (A group) showed clinical signs like natural cases of NE. From this finding it is highly likely that fish meal developed an anaerobic environment in the lumen of intestine influencing the growth of *Clostridium perfringens*. Almost similar clinical signs in experimental NE cases corresponded with the findings of other authors (Bernier *et al.*, 1999; Samad, 2005; Wilkie *et al.*, 2005).

Table 3. Gross lesions in broiler experimentally infected with necrotic enteritis

<table>
<thead>
<tr>
<th>Group</th>
<th>Type of exposure</th>
<th>Amount of exposure</th>
<th>Necropsy findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>50% Fish meal 500gm/kg feed + <em>Cl. perfringens</em></td>
<td>50% Fish meal 500gm/kg feed + 2.5ml broth of 1x10⁸ CFU/bird</td>
<td>Intestinal content consists of foul smelling brown fluid and bubble was present in the duodenum, jejunum and caecum, enlarged liver, hemorrhage on the base of the heart which were severe (+++).</td>
</tr>
<tr>
<td>B</td>
<td><em>Cl. perfringens</em> + Normal feed</td>
<td>2.5ml broth of 1x10⁸ CFU/bird + Normal feed</td>
<td>Hemorrhage on the base of the heart, liver, intestine which were moderate (++).</td>
</tr>
<tr>
<td>C</td>
<td>Normal feed</td>
<td></td>
<td>Almost normal necropsy findings (± to +)</td>
</tr>
</tbody>
</table>

Table 4. Anaerobic bacteria count in intestinal content of experimental bird

<table>
<thead>
<tr>
<th>Group</th>
<th>Plate number</th>
<th>Number of colonies</th>
<th>CFU/ml</th>
<th>Average CFU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>120</td>
<td>1.2x10⁷</td>
<td>1.51x10⁷</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>184</td>
<td>1.84x10⁷</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>150</td>
<td>1.5x10⁷</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>99</td>
<td>0.99x10⁷</td>
<td>1.1x10⁷</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>109</td>
<td>1.09x10⁷</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>153</td>
<td>1.53x10⁷</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>1</td>
<td>70</td>
<td>0.7x10⁷</td>
<td>0.8x10⁷</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>88</td>
<td>0.88x10⁷</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>91</td>
<td>0.91x10⁷</td>
<td></td>
</tr>
</tbody>
</table>
Experimental pathology of necrotic enteritis in broiler

Gross lesions in birds of group A as observed in this study corroborate the results of others (Al-Sheikhly and Truscott, 1977; Bernier et al., 1999; Wilkie et al., 2005). The impression smear from intestinal lumen showed 2-5 short, thick, and gram-positive rod shaped bacteria found in birds of groups A. In the birds of groups B and C Clostridium was not found in impression smear. The finding was almost similar to that observed by Miah et al. (2011).

Table 5: Microscopic lesions of necrotic enteritis experimentally produced in broiler birds

<table>
<thead>
<tr>
<th>Group</th>
<th>Type of exposure</th>
<th>Amount of exposure</th>
<th>Histopathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>50% fish protein + Cl. perfringens</td>
<td>50% fish protein (500gm/kg feed) + 2.5ml broth of 1x10^8 CFU /bird</td>
<td>Hemorrhage and congestion in sub mucosa of small intestine (duodenum, jejunum and ileum). Sloughing of epithelium in small intestine. Focal necrosis in liver, hemorrhage and accumulation of reactive cells were also found in liver, heart and spleen. Pneumonia in lung (severe, +++).</td>
</tr>
<tr>
<td>B</td>
<td>Cl. Perfringens+ normal feed</td>
<td>2.5ml broth of 1x10^8 CFU /bird +normal feed</td>
<td>Slight hemorrhage and glandular proliferation of the duodenum and jejunum. Focal necrosis in liver, hemorrhage and accumulation of reactive cells were also found in liver and heart but moderate than bird of group A (Moderate, ++)</td>
</tr>
<tr>
<td>C</td>
<td>Control</td>
<td>Normal feed</td>
<td>Normal findings (+ to +).</td>
</tr>
</tbody>
</table>

The anaerobic bacteria counting in different groups of birds can not be compared with the findings of others due to lack of available data. The histopathological lesions described in this study corresponded with the findings of other investigators (Shamimuzzaman, 1999; Samad, 2005; Keyburn et al., 2008).

This study did not cover the pathogenesis of changing of microecology in intestine produced by fish meal in broilers. However, reports from published data describe that high level of animal protein in diet like fish meal increase the risk of occurrence of necrotic enteritis by causing high concentration of glycine and methionine levels in lower small intestine. This increased level may act as a triggering factor for over growth of Clostridium perfringens and clinical NE (Drew et al., 2004).

In this study, orally inoculated Clostridium perfringens produced disease but the severity was moderate. But, feeding of excess fish meal in association with Clostridium perfringens caused the more pathological lesions than only Clostridium treated group of birds. These results confirmed that the excess dietary protein has effect on the pathology of necrotic enteritis in broilers.
Figure 1. *Clostridium* in stab culture in Gram’s staining shows rod, Gram positive (100x)

Figure 2. Colony counting for determination of CFU/ml in nutrient agar media with 5mm thickness sterile olive oil above the media

Figure 3. Shows diarrhoea after drenching of *Clostridium perfringens* 1x10^8 CFU/bird plus high protein diet (group A)

Figure 4. Bird of group A shows lesions hemorrhagic enteritis (*Clostridium perfringens* 1x10^8 CFU/bird plus high protein diet)

Figure 5. *Clostridium* found in impression smear of intestine in birds of group A bird. (Giemsa Stain 100 X)

Figure 6. *Clostridium* not found in intestine of group C bird. (Giemsa Stain 100 X)
Experimental pathology of necrotic enteritis in broiler

Figure 7: Group A birds show hemorrhage, congestion in sub mucosa of small intestine and sloughing off epithelium (severe, ++++) (H&E, 10 X)

Figure 8. Group C birds show normal histology in sub mucosa of small intestine (± to +) (H&E, 10 X)

Figure 9. Group A birds show epicarditis, patchy degenerative change of muscle fiber (++) (H&E, 10X)

Figure 10. Group C birds show normal histology in heart muscle (± to +) (H&E, 10 X)

Figure 11. Group A birds show pneumonia, filling of lung alveoli by heterophil, erythrocyte, exudates, network of fibrin and presence of Aspergillus nodule (+++) (H&E, 10 X)

Figure 12. Group C birds show slight pneumonia(+/− to +) in lung (H&E, 10 X)
ACKNOWLEDGEMENT

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