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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 $1x10^8$ 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 $1x10^8$ 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 $1.51x10^7$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 $1.1x10^7$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 $0.8x10^7$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.

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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 *Closridium 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 $1x10^8$ 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 $1x10^8$ 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 $1x10^8$ 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 histpathological 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. Then1 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 1. Clinical signs of Necrotic enteritis in experimental birds

Group	No. of Birds	Type of exposure	Amount of exposure	Period of observation	Clinical signs
Group A	5	*Cl. perfringens* of $1x10^8$CFU/bird + 50%Fish meal	50% fish meal 500gm/kg feed +2.5ml of$1x10^8$CFU/bird	1week	Depression, ruffled feather, diarrhoea. Moderate level(++).
Group B	5	*Cl. perfringens* $1x10^8$CFU/bird + Normal feed	2.5 ml of broth of $1x10^8$CFU/bird + normal feed	1 week	Depression, ruffled feather, diarrhoea. Mild level(+).
Group C	5	Normal feed	Normal feed	1week	Normal (±)

Prevalence of experimental infection

In this study the experimental birds were observed for 1week. 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)

Group	No. of birds	Type of Exposure	No. of birds affected	Prevalence (%)
A	5	*Cl. Perfringens* $1x10^8$CFU/bird + 50%Fish meal	4	80
B	5	*Cl. Perfringens* $1x10^8$CFU/bird +Normal feed	2	40
C	5	Control group	-	-

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* $1x10^8$ 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* $1x10^8$ 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

Group	Type of exposure	Amount of exposure	Necropsy findings
A	50%fish meal 500gm/kg feed + *Cl. perfringens*	50% fish meal 500gm/kg feed + 2.5ml broth of $1x10^8$CFU/ bird	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 (+++).
B	*Cl. perfringens* +Normal feed	2.5ml broth of $1x10^8$CFU/ bird + Normal feed	Hemorrhage on the base of the heart, liver, intestine which were moderate (++).
C	Normal feed		Almost normal necropsy findings (± to +)

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

Group	Plate number	Number of colonies	CFU/ml	Average CFU/ml
A	1	120	$1.2x\ 10^7$	$1.51x10^7$
	2	184	$1.84x\ 10^7$	
	3	150	$1.5x\ 10^7$	
B	1	99	$0.99x10^7$	$1.1x10^7$
	2	109	$1.09x10^7$	
	3	153	$1.53x10^7$	
C	1	70	$0.7x10^7$	$0.8x10^7$
	2	88	$0.88x10^7$	
	3	91	$0.91x10^7$	

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

Group	Type of exposure	Amount of exposure	Histopathology
A	50% fish protein + *Cl. perfringens*	50% fish protein (500gm/kg feed) + 2.5ml broth of $1x10^8$CFU /bird	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, +++).
B	*Cl. Perfringens*+ normal feed	2.5ml broth of $1x10^8$CFU /bird +normal feed	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, ++)
C	Control	Normal feed	Normal findings (± to +).

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 occurance 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.

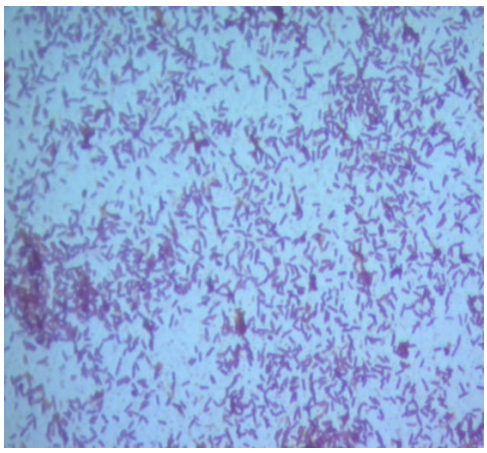

Figure 1. *Clostridium* in stab culture in Gram's staining shows rod, Gram positive (100x)

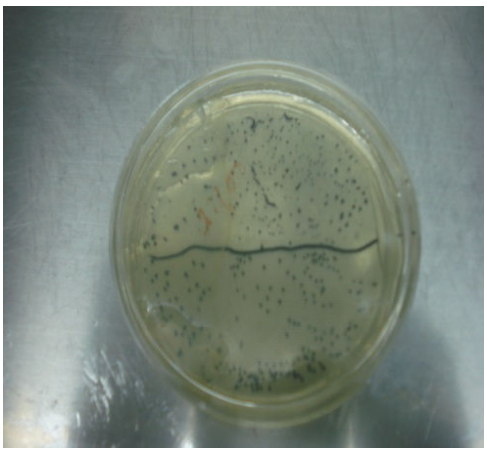

Figure 2. Colony counting for determination of CFU/ml in nutrient agar media with 5mm thickness sterile olive oil above the media

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

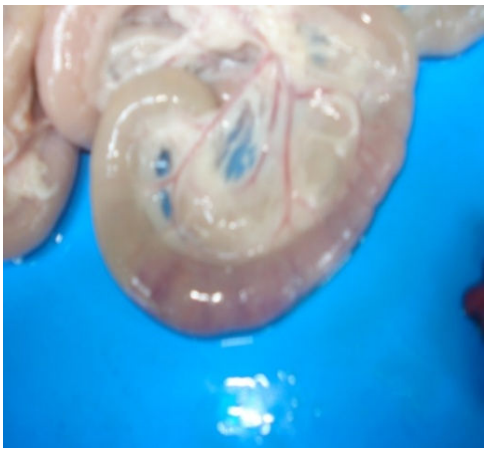

Figure 4. Bird of group A shows lesions hemorrhagic enteritis (*Clostridium perfringens* $1x10^8$ CFU/bird plus high protein diet)

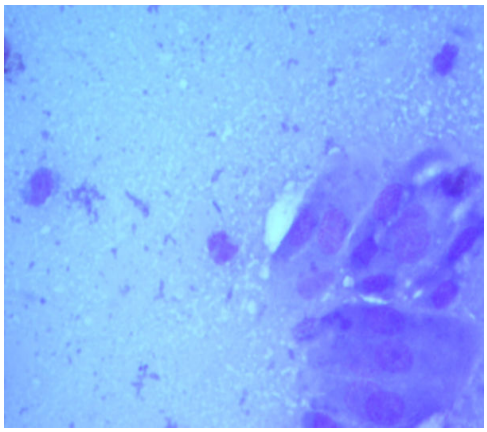

Figure 5. *Clostridium* found in impression smear of intestine in birds of group A bird. (Giemsa Stain 100 X)

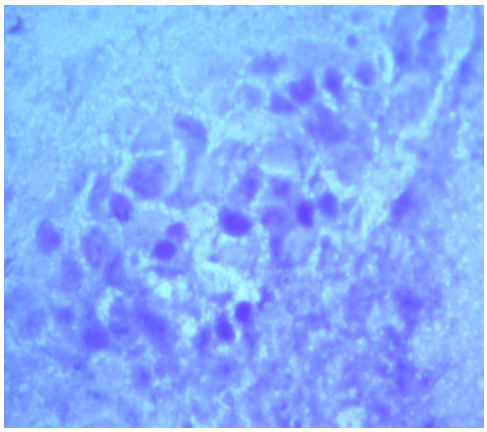

Figure 6. *Clostridium* not found in intestine of group C bird. (Giemsa Stain 100 X)

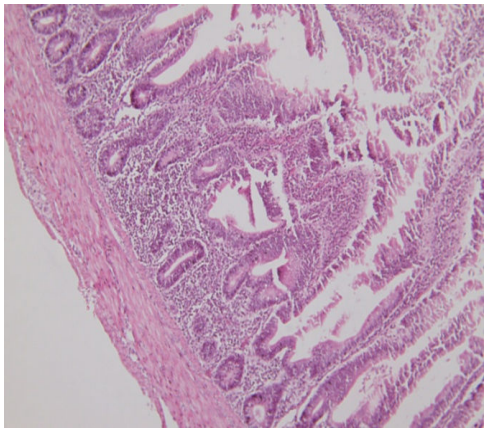

Figure 7: Group A birds show hemorrhage, congestion in sub mucosa of small intestine and sloughing off epithelium (severe,+++) (H&E,10 X)

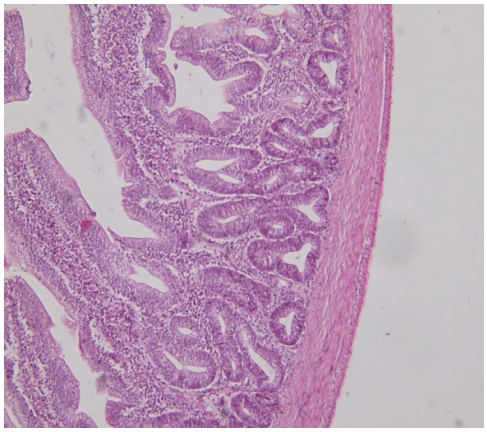

Figure 8. Group C birds show normal histology in sub mucosa of small intestine (± to+)(H&E,10 X)

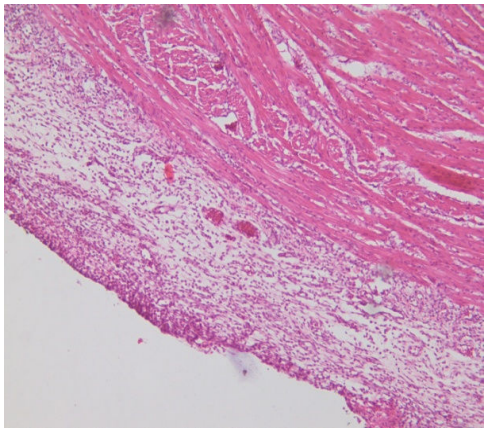

Figure 9. Group A birds show epicarditis, patchy degenerative change of muscle fiber (+++) (H&E,10X)

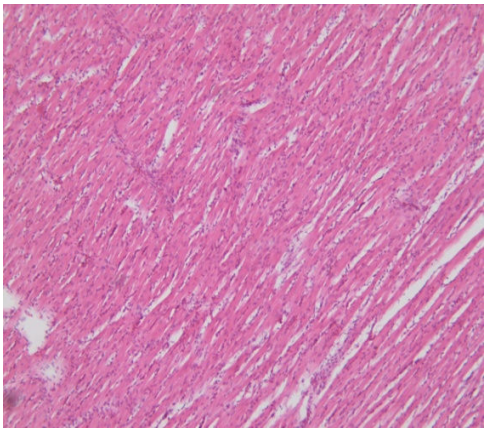

Figure 10. Group C birds show normal histology in heart muscle (± to +) (H&E,10 X)

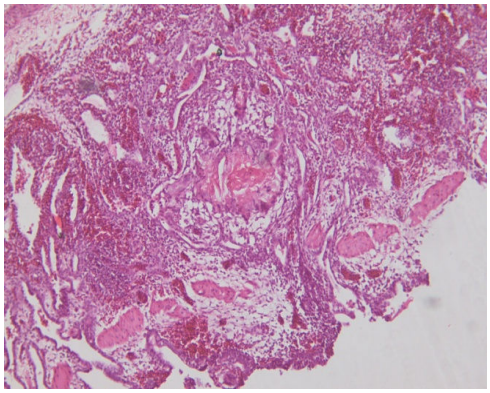

Figure 11. Group A birds show pneumonia, filling of lung alveoli by heterophil, erythrocyte, exudates, network of fibrin and presence of *Aspergillus* nodule(+++) (H&E,10 X)

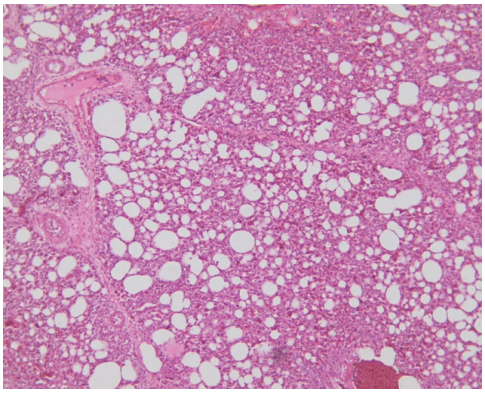

Figure 12 : Group C birds show slight pneumonia(+/- to+) in lung (H&E,10 X)

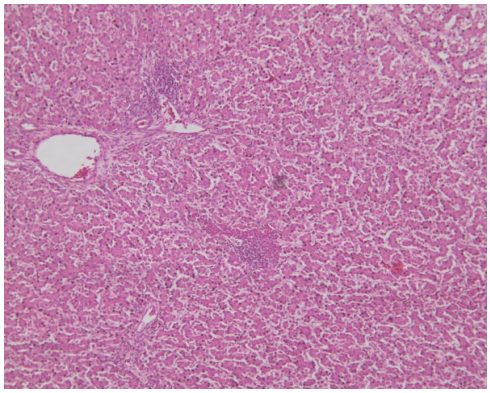

Figure 13. Group A birds show focal necrosis, presence of cells in liver (+++) (H&E,10 X)

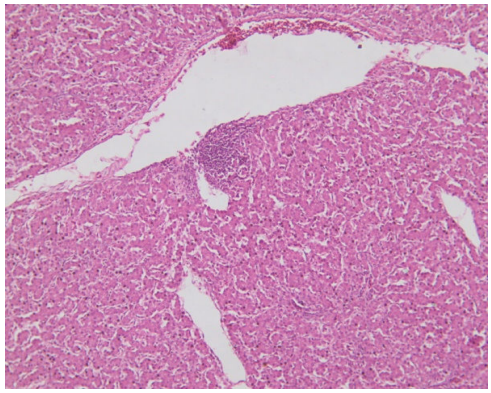

Figure 14. Group B birds show focal necrosis, presence of reactive cells in liver (++) (H&E,10 X)

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REFERENCES

1. Al-Sheikhly F, Truscott RB (1977). The pathology of necrotic enteritis of chickens following infusion of broth cultures of *Clostridium perfringens* into the duodenum. *Avian Disease* 21: 230-240.
2. Bernier G, Pnaneuf JB and Filion R (1999). Necrotic enteritis in broiler Chickens: Study of the factors favouring the multiplication of *Clostridium perfringens* and the experimental transmission of the disease. *Canadian Journal of Comparative Medicine* 41(1): 112-16.
3. Das A, Mazumder Y, Dutta BK, Shome BR, Bujarbaruah KM and Kumar A (2008). *Clostridium perfringens* Type A from broiler chickens with necrotic enteritis. *International Journal of Poultry Science* 7(6): 601-609.
4. Drew MD, Syed NA, Goldade BC, Laarveld B and Kessel AG (2004). Effects of dietary protein source and level on intestinal populations of *Clostridium perfringens* in broiler chickens. *Infection and Immunity* 83(3): 414-420.
5. Hofshagen M and Kaldhusdal M (1992). Barley inclusion and avoparcin supplementation in broiler diets.Effect on small intestinal bacterial flora and performance. *Poultry Science* 71: 959-969.
6. Huque QME (2001). Poultry industry in Bangladesh and strategies for its improvement. Proceedings of 2nd International Poultry Show and Seminar. February 2001, IDB Bhaban, Dhaka, Bangladesh. pp 34-39.
7. Islam MR, Das BC, Hossain K, Lucky NS and Mostafa MG (2003). A Study on the Occurrence of Poultry Diseases in Sylhet Region of Bangladesh. *International Journal of Poultry Science* 2(5): 354-356,
8. Islam MR, Khan MAHNN, Das PM and Bari ASM (1998). Poultry diseases diagnosed at necropsy in 1997and 1998 in the Department of Pathology, Bangladesh agricultural University, Mymensingh. Proceedings of 5th BSVER Annual Scientific Conference 3-4 Dec. 1998, Bangladesh agricultural University, Mymensingh.
9. Keyburn AL, Boyce JD, Vaz P, Bannam TL, Ford ME, Parker D, Di Rubbo A, Rood JI and Moore RJ (2008). NetB, a new toxin that is associated with avian necrotic enteritis caused by *Clostridium perfringens*. *PLOS Pathogens* 4(2): 26.
10. Luna LG (1968). *Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology*. 3rd edition. McGraw Hill Book Co., New York, USA. pp. 134-140.
11. Miah MS, Asaduzzaman M, Sufian MA and Hossain MM (2011). Isolation of *Clostridium perfringens*, Causal agents of necrotic enteritis in chickens. *Journal of the Bangladesh Agricultural University* 9(1): 97-102.
12. Olkowski AA, Wojnarowicz C, MChirino-Trejo and Drew MD (2006). Responses of broiler chickens orally challenged with *Clostridium perfringens* isolated from field cases of necrotic enteritis. *Research in Veterinary Science* 81: 99-108.
13. Rahman MM (1995). *Bacteriology*. Luman-Sakib Prokasoni. Bangladesh Agricultural University, Mymensigh-2202, Bangladesh.

14. Rahman MS (1997). Investigation of an outbreak of enterotoxaemia in cattle of Mymensigh. M. S. Thesis. Department of Veterinary Medicine, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensigh-2202, Bangladesh.
15. Rhodehamel G, Bruce HL, Hunter DB, Parreira VR, Kulkarni RR, Jiang YF, Prescott JF and Boertin P (1998). Muhi locus sequence typing analysis of *Clostridium perfringens* isolates from necrotic enteritis outbreaks in broiler chicken populations. PhD thesis. Department of Pathobiology, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada
16. Samad MA (2005). *Poultry Science and Medicine.* LEP publication No. 10, Lyric-Epic Prokashoni Bangladesh Agricultural University Campus, Mymensingh. pp. 537-538.
17. Shamimuzzaman AHM (1999). Characterization of *Clostridium perfriengens* isolated from goat and its antibiotic sensitivity. MS Thesis. Department of Microbiology and Hygiene, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensigh-2202, Bangladesh
18. Talha AFSM, Hossain MM, Chowdhury EH, Ban ASM, Islam MR and Das PM (2001). Poultry Diseases occurring in Mymensingh District of Bangladesh. *The Bangladesh Veterinary Journal* 18: 20-23.
19. Wilkie DC, Kessel, White LJ, Laarveld B and Drew MD (2005). Dietary amino acids affect intestinal *Clostridium perfringens* populations in broiler chickens. *Canadian Journal of Animal Science* 85(2): 185-193.
20. Willis TA (1969). *Clostridia of Wound Infection*. Butterworth and Co., London. pp. 135-139.