

## EFFECT OF CARBOHYDRATE RICH DIET ON EXPERIMENTAL PATHOLOGY OF NECROTIC ENTERITIS IN BROILERS

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### ABSTRACT

The present research work was designed to know the effect of carbohydrate rich diet on the experimental pathology of necrotic enteritis in broilers. For experimental purpose, 15 birds of 14 days of age were grouped into 3 (A, B and C). Birds of group A were fed with 400gm maize/kg (40% increase) and group B were fed with 200gm maize/kg (20% increase) of normal feed from day 14 to day 27 and challenged from day 21 to 25 days with  $1 \times 10^8$  CFU/3ml of inoculums of *Cl. perfringens* consecutive 5 days. Group C was kept in control without additional maize. Birds of all groups were observed up to 27 days of age. The clinical signs diarrhea, ruffled feather were common in both group A and B, and less feed intake in group A than group B. There was no mortality in any groups. Birds of all groups were sacrificed at the day 28. Necrosis and hemorrhage in intestine, enlarged liver, and hemorrhage in the base of heart were found in group A (+++). In impression smear of intestines, the average 10-12 bacteria were found in group A and 8-10 bacteria were found in group B in higher magnification (100X). In histopathology, severe infiltration of inflammatory cells including heterophils and lymphocytes and sloughing off mucosal epithelium that changed by pink color cytoplasm with pyknotic nuclei were observed in group A (+++) while group B showed infiltration of inflammatory cells and sloughing off mucosal epithelium (++). The birds of group C were normal in all respects. Anaerobic bacteria counted from intestinal content of group A was  $2.8 \times 10^7$  CFU/ml, group B was  $2.2 \times 10^7$  CFU/ml and group C was  $1.0 \times 10^7$  CFU/ml. From the findings, it may be said that carbohydrate rich diet is a predisposing factor for necrotic enteritis in broilers.

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

### INTRODUCTION

The Poultry sector in Bangladesh is one of the fast growing agri-business in recent times. Outbreak of several devastating diseases is one of the major constraints causing economic loss and discouraging poultry rearing (Das *et al.*, 2005). Diseases are causing about 30% mortality of chickens per year (Miah *et al.*, 2011). 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 occurring in broilers from 2-6 weeks of age. It is caused by *Clostridium perfringens*. *Clostridium perfringens* is a gram positive, spore forming, anaerobic, large rod bacteria, which is present in the environment worldwide (Willis, 1969). Clinical signs of this disease include depression, decrease appetite, and reduce growth rate, diarrhoea and severe necrosis of the intestinal tract (Miah *et al.*, 2011). The incidence of necrotic enteritis (NE) diagnosed by post mortem examination in Mymensingh, Sylhet and Rajshahi district of Bangladesh are 0.52%-0.60% (Islam *et al.*, 1998; Talha *et al.*, 2001), 0.91% (Hossain *et al.*, 2002) and 0.44% (Islam *et al.*, 2003), respectively. The organism produces various toxins but alpha toxin is important for causing necrotic enteritis. Age, diet and coccidiosis are the predisposing factors of necrotic enteritis. Mortality due to necrotic enteritis is higher if they are feed on high concentration of wheat, rye, barley and protein (Al-Sheikhly and Truscott, 1977; Ferdoush *et al.*, 2013). The effect of coccidiosis (Shane *et al.*, 1985; Asaduzzaman *et al.*, 2011) and high protein diet (Ferdoush *et al.*, 2013) in the production of necrotic enteritis have been investigated in Bangladesh. To our knowledge, the effect of high carbohydrate diet in the production of necrotic enteritis has not been investigated. Therefore, the investigation has been undertaken to observe the effect of carbohydrate 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, Mymensingh during July 2013 to November 2013.

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### **Isolation and identification of *Clostridium perfringens***

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/ml**

Nutrient agar media was used in order to determine the CFU/ml of nutrient broth for inoculum. In dilution of 1:10 the colony was not countable and in 1:1000 dilution the colony was not detected. So, the colonies from 1:100 dilutions were counted (Fig. 6). Each of the bird of experimental groups was drenched with 3 ml of broth that contained  $1 \times 10^8$  CFU (Olkowski *et al.*, 2006).

### **Experimental design**

For experiment a total number of 15 broiler birds of 14 days of age were collected from Sutiakhali poultry farm, Mymensingh and grouped into three (A, B and C). Each group contained five birds. The birds were maintained for 2 weeks with overall experimental optimal rearing condition. The feed and water supply was *ad libitum*. Generally carbohydrate concentrate in broiler feed is 70% (Reddy *et al.*, 2001). This experiment was performed with 40% and 20% increase level of maize and another group was given normal feed. Birds of group A were fed with 400gm maize/kg (40% increase of carbohydrate than normal) of normal feed from day 14 to day 20 and challenged on day 21 with  $1 \times 10^8$  CFU/3ml of inoculums for consecutive 5 days long term exposure trail (Olkowski *et al.*, 2006). The carbohydrate diet was also fed up to 28 days of age of the birds. Birds of group B were fed with 200gm maize/kg (20% increase of carbohydrate than normal) were given same amount of bacteria with same time schedule. The carbohydrate diet was also fed up to 28 days of age of the birds. Group C was kept as control with commercial normal pellet without *Clostridium perfringens* (Drew *et al.*, 2004). 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 27 (7 days) of post inoculation. In the experimental period the clinical signs, morbidity and mortality were recorded. Then birds were sacrificed on Day 28 (8 days post inoculation). Gross pathology recorded and impression smear from intestine were taken. For anaerobic bacteria count from intestinal content and study of microscopic pathology of different organs were performed.

### **Maintenance of stock culture**

Nutrient agar slants were used to maintain the stock culture for *Clostridium perfringens*. The *Clostridium perfringens* were inoculated in the slant by streaking and were incubated at 37°C for 24 hours. Finally, glycerol was overlaid and the culture was kept at -70°C for future use.

### **Observation of clinical signs**

All the birds of groups were observed for clinical signs up to 7 days (after oral administration of *Clostridium perfringens* in inoculated groups). The severity of clinical signs was graded as severe (+++), moderate (++) , mild (+) and almost normal (+/-).

### **Gross pathology**

Gross changes of all tissues at necropsy were carefully observed in birds of all groups. The changes of tissue were 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 of 2 birds from each group were prepared from jejunum on slide and fixed by methanol for 5 minutes. The prepared commercial Giemsa stain was poured on the slide and allowed to keep for 40 minutes. It was then washed with running tap water. The stained slides were examined at (100X) according to the procedure described by Rahman (1995).

### Anaerobic bacterial load in intestinal content

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

### Histopathology

The formalin fixed tissues (intestine, liver, heart and lung *etc.*) of all groups were trimmed, processed, sectioned and stained as per standard procedure (Luna, 1968).

## RESULTS

### Clinical signs

The clinical signs of necrotic enteritis in experimental birds of group A showed moderate (++) level of diarrhoea, somnolence and ruffled feather (Fig. 4). Group B showed the sings mild (+) diarrhoea, somnolence and ruffled feather. Birds of group C were normal. The table below shows the clinical signs (Table 1). There was no mortality in any groups of birds.

Table 1. Clinical signs of experimental birds

Group	No. of Birds	Type of exposure	Amount of exposure	Period of observation	Clinical signs
Group A	5	<i>Cl. perfringens</i> of $1 \times 10^8$ CFU/bird + 40%maize	400gm maize/kg (40% increase of carbohydrate than normal)+ $1 \times 10^8$ CFU/bird	1 week	Depression, ruffled feather and diarrhea. [Moderate level (++)]
Group B	5	<i>Cl. perfringens</i> of $1 \times 10^8$ CFU/bird + 20%maize	200gm maize/kg (20% increase of carbohydrate than normal)+ $1 \times 10^8$ CFU/bird	1 week	Depression, ruffled feather, diarrhoea. [Mild level (+)]
Group C	5	Normal feed	Normal feed Without organism	1week	Normal

### Incidence rate of infection

In this study the experimental birds were observed for 1 week from the initiation of exposure of consecutive 5 days oral administration of *Cl. perfringens*. The incidence rate of necrotic enteritis in birds of group A and B was 60% but the value was 0 in case of group C (Table 2). However the signs were moderate in group A, mild in group B and no signs in group C.

### Gross pathology

Table 3 shows the gross lesions in the experimental birds. At necropsy small intestine was found congested (Fig. 6) especially in group A. The lesion consisting of foul smelling brown fluid and bubble was present in the duodenum, jejunum and caecum; large amounts of hemorrhagic necrotic epithelial debris in the lumen of the bowel, enlarged liver (Fig. 5) and heart, hemorrhage on the base of the heart were seen. All these lesions were graded as severe (+++).Group B birds developed lesions with moderate severity (++) . Group C showed no significant lesions in the intestine.

### Staining characteristics in impression smear

Impression smear prepared from jejunum of 2 birds of group A showed numerous (10-12) shorts, thick, Gram-positive rods (100X) in each focus. These were suspected as *Clostridium perfringens* (Fig. 8). Samples from the birds of group B showed numerous (8-10) shorts, thick, Gram-positive rods (100X) in impression smears prepared from jejunum. In group C, no bacteria was found in impression smear (Fig. 7).

### Anaerobic bacteria count in intestinal content

Table 4 describes the anaerobic bacteria count in intestinal contents of experimental birds. In group A, challenged with *Clostridium perfringens*  $1 \times 10^8$  CFU/bird + 400gm maize/kg (40% increase of carbohydrate than normal) the bacterial load was  $2.8 \times 10^7$  /ml of intestinal fluid in 1:100 dilutions. In group B challenged with *Clostridium perfringens*  $1 \times 10^8$  CFU/bird+200gm maize/kg (20% increase of carbohydrate than normal) the bacterial load was  $2.2 \times 10^7$ /ml of intestinal fluid in 1:100 dilutions. In group C the bacterial load was  $1.0 \times 10^7$ /ml of intestinal fluid in 1:100 dilution.

### Histopathology

Table 5 shows the histopathological features of the affected organ of experimental birds. Birds of group A showed the sings of severe infiltration of inflammatory cells including heterophils and lymphocytes (+++) and sloughing off mucosal epithelium that changed by pink color cytoplasm with pyknotic nuclei (severe, +++) (Fig. 9). Focal necrosis in liver (Fig. 16) and myositis and patchy degenerative change of muscle fiber in heart (Fig. 12) were observed. Pneumonia was also found in lung (Fig. 14). The lesions were variable in birds. The severity of lesion was graded as severe (+++). Group B showed the sings of infiltration of inflammatory cells including heterophils and lymphocytes (++) and sloughing off mucosal epithelium that changed by pink color cytoplasm with pyknotic nuclei (++) (Fig. 10). Focal necrosis in liver and myositis and degenerative change of muscle fiber in heart were also observed but all of the lesions were moderate(++) in severity compare to the birds of group A. Birds of group C with normal diet appeared as normal histology in intestine, heart, lung and liver (Fig. 11, 13, 15 and 17).

Table 2. Incidence rate of NE in experimental broiler birds (Age of birds=14 days)

Group	No. of birds	Type of Exposure	No. of birds affected	Incidence rate (%)
A	5	<i>Cl. perfringens</i> $1 \times 10^8$ CFU/bird+400gm maize/kg (40% increase of carbohydrate than normal)	3	60
B	5	<i>Cl. perfringens</i> $1 \times 10^8$ CFU/bird + 200gm maize/kg (20% increase of carbohydrate than normal)	3	60
C	5	Control group	Nil	Nil

### DISCUSSION

The birds of group A showed moderate (++) depression, ruffled feathers and diarrhoea and the incidence rate was 60%. The birds of group B showed mild (+) clinical signs and the incidence rate was also 60%. The similar findings were reported by Williams (2005). In this study, carbohydrate supplemented group of birds (A and B groups) showed clinical signs like natural cases of NE. From this finding it is highly likely that maize 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 Bernier *et al.*, (1999); Samad (2005); Wilkie *et al.*, (2005). Gross lesions in the small intestine in birds of group A were distended with gas and brown fluid in the jejunum; enlarged liver and hemorrhage on the base of the heart (+++). Birds of Group B produced lesion of necrotic enteritis but moderate (++) in severity compare to the experimental birds of group A. Group C (control) showed no significant sign or lesion. Almost similar gross lesions were supported by others (Al-Sheikhly and Truscott, 1977; Bernier *et al.*, 1999; Wilkie *et al.*, 2005).

Table 3. Showing gross lesions in experimentally infected with necrotic enteritis

Group of bird	Type of exposure	Amount of exposure	Necropsy findings
A	<i>Cl. perfringens</i> of 1x10 <sup>8</sup> CFU/bird + 40%maize	400gm maize/kg (40% increase of carbohydrate than normal)+ 1x10 <sup>8</sup> CFU/ bird	Intestinal content consists of foul smelling brown fluid and bubble in the duodenum, jejunum and caecum; enlarged liver, hemorrhage on the base of the heart which were severe (+++).
B	<i>Cl. perfringens</i> of 1x10 <sup>8</sup> CFU/bird + 20%maize	200gm maize/kg (20% increase of carbohydrate than normal)+ 1x10 <sup>8</sup> CFU/ bird	Hemorrhage on the base of the heart, liver, intestine which were moderate (++)
C	Normal feed		Normal necropsy findings

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

Group of bird	Number of plate	Number of colonies	CFU/ml	Total average CFU/ml
A	1	320	3.20x 10 <sup>7</sup>	2.8x10 <sup>7</sup>
	2	280	2.80x 10 <sup>7</sup>	
	3	245	2.45x 10 <sup>7</sup>	
B	1	255	2.55x10 <sup>7</sup>	2.2x10 <sup>7</sup>
	2	220	2.20x10 <sup>7</sup>	
	3	190	1.90x10 <sup>7</sup>	
C	1	135	1.35x10 <sup>7</sup>	1.0x10 <sup>7</sup>
	2	90	0.90x10 <sup>7</sup>	
	3	75	0.75x10 <sup>7</sup>	

Table 5. Microscopic lesions

Group	Type of exposure	Amount of exposure	Histopathology
A	40% maize + <i>Cl. perfringens</i>	400gm maize/kg (40% increase of carbohydrate than normal)+ 1x10 <sup>8</sup> CFU /bird	In intestine, severe infiltration of inflammatory cells including heterophils and lymphocytes (+++) and sloughing off mucosal epithelium with pyknotic nuclei (severe, +++). Focal necrosis in liver. Myositis and patchy degenerative change of muscle fibers in heart. Pneumonia was also found in lung. The severity of lesion was graded as severe (+++).
B	20% maize + <i>Cl. perfringens</i>	200gm maize/kg (20% increase of carbohydrate than normal)+ 1x10 <sup>8</sup> CFU /bird	In intestine, infiltration of inflammatory cells including heterophils and lymphocytes (++) and sloughing off mucosal epithelium (++) . Focal necrosis in liver. Myositis and degenerative change of muscle fibers in heart. Pneumonia was also found in lung but all of the lesions were moderate (++) in severity.
C	Control	Normal feed	Normal findings (±).

In impression smear from intestinal lumen showed 10-12 short, thick and gram-positive rod shaped bacteria in birds of group A. In the birds of group B, the impression smear from intestinal lumen showed 8-10 short, thick

and gram-positive rod shaped bacteria and in group C, no *Clostridium* was found in impression smear. The finding was almost similar with that of Miah *et al.* (2011).

The number of anaerobic bacteria was  $2.8 \times 10^7$  CFU/ ml of intestinal fluid in birds of group A and  $2.2 \times 10^7$  CFU/ ml in birds of group B while  $1.0 \times 10^7$  CFU/ ml in birds of group C. The similar findings were reported by Ferdoush *et al.* (2013). The histopathological lesions in intestine, liver, lung and heart in birds of group A were severe (++ to +++) than the birds of group B and C ( $\pm$  to +). The lesions described above in the present study corresponded with the findings of other investigators (Al-Sheikhly and Truscott, 1977; Fukata *et al.*, 1988; Hutchison and Ridell, 1990; Rhodehamel *et al.*, 1998; Shamimuzzaman, 1999; Samad, 2005; Keyburn *et al.*, 2008).

This study did not cover the pathogenesis of changing of micro ecology in intestine produced by maize in broilers. However, reports from published data describe that high level of carbohydrate in diet like maize, wheat increase the risk of occurrence of necrotic enteritis. This increased level of carbohydrate may be triggering factor for over growth of *Clostridium perfringens* and production of clinical NE (Drew *et al.*, 2004). From the findings of the present investigation, it may be said that carbohydrate rich diet may be the predisposing factor for necrotic enteritis in broilers.

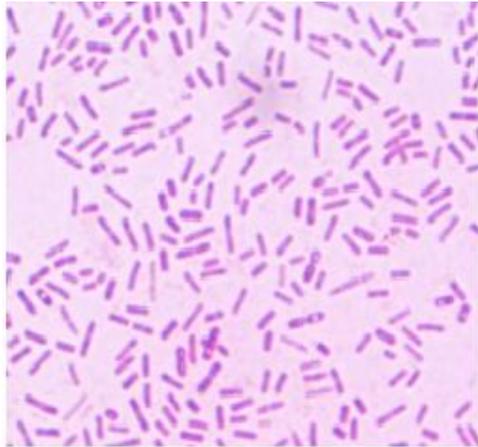


Fig. 1. *Clostridium* in Gram's staining shows rod, Gram positive (100X)



Fig. 2. Culture of *Clostridium* in blood

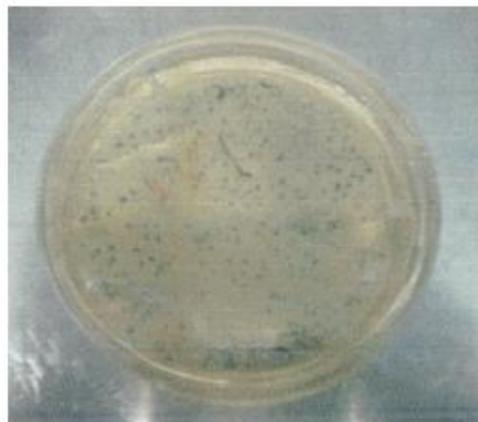


Fig. 3. Colony counting for determination of



Fig. 4. Bird of group A shows diarrhoea after



Fig. 5. Bird of group A (40% increase of carbohydrate than normal) shows enlarged and hemorrhagic liver



Fig. 6. Bird of group A (40% increase of carbohydrate than normal) shows hemorrhage in intestine (severe, +++)

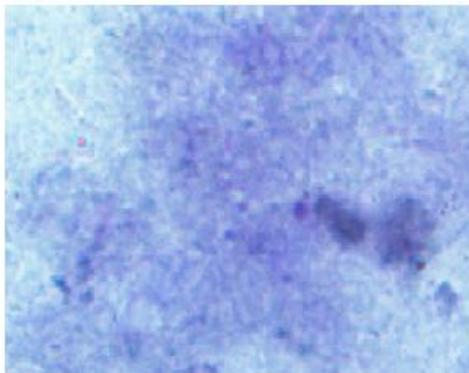


Fig. 7. *Clostridium* not found in bird of group C in Giemsa staining of impression smear(100X)

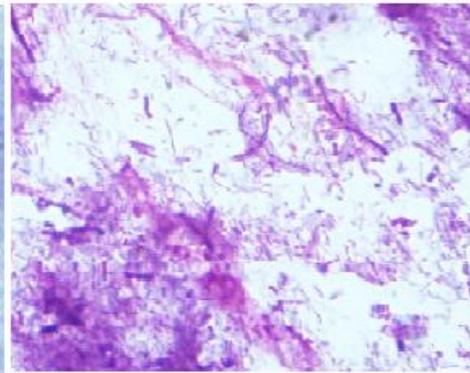


Fig. 8. *Clostridium* found in impression smear in birds of group A (challenged with 40% increase of carbohydrate than normal + *Clostridium perfringens*  $1 \times 10^8$  CFU/bird) (100X)

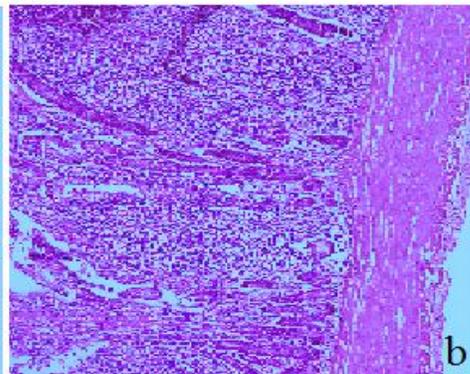
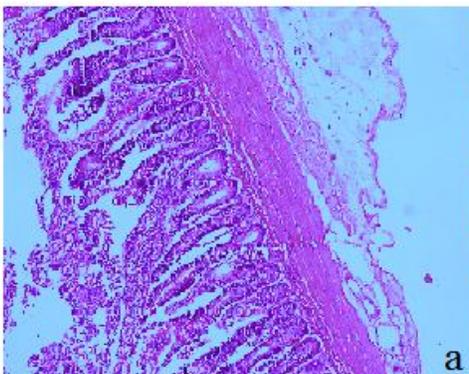


Fig. 9. Bird of group A shows severe infiltration of inflammatory cells including heterophils and lymphocytes (+++) and sloughing off mucosal epithelium that changed by pink color cytoplasm with pyknotic nuclei (severe, +++)(H&E, 10X).

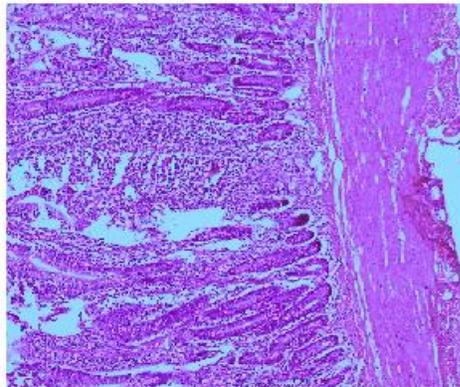


Fig. 10. Bird of group B shows severe infiltration of inflammatory cells including heterophils and lymphocytes (++) and sloughing off mucosal epithelium that changed by pink color cytoplasm with pyknotic nuclei (++) (H&E, 10X)

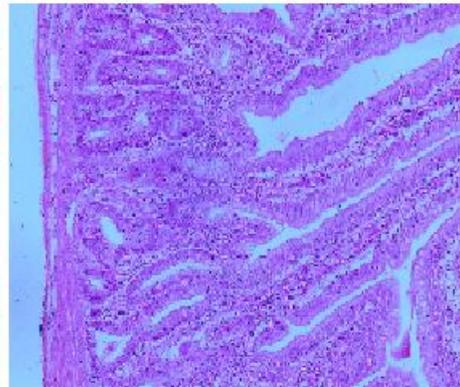


Fig. 11. Bird of group C (kept in control) shows histology in sub mucosa of small intestine (+/-to+) (H&E, 10X)

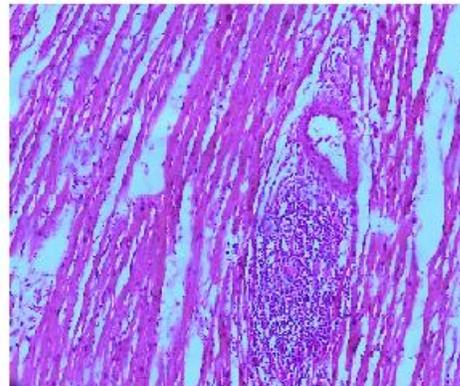


Fig. 12. Bird of group A bird shows myositis, patchy degenerative change of muscle fiber in heart (++) (H&E, 10X)

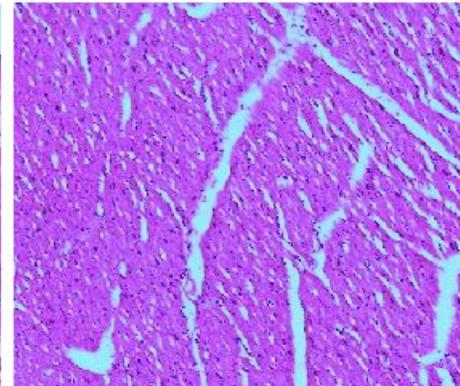


Fig. 13. Bird of group C (kept in control) shows normal histology in heart muscle ( $\pm$ ) (H&E, 10X)

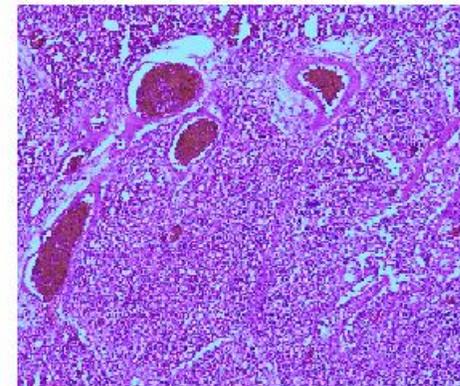


Fig. 14. Bird of group A bird shows pneumonia, filling of lung alveoli by heterophil, erythrocyte and exudates (+++) (H&E, 10X)

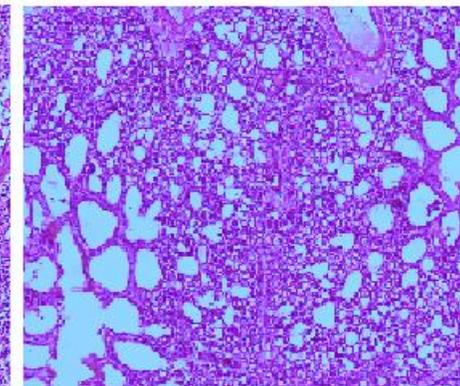


Fig. 15. Bird of group C (kept in control) shows normal structure in lung (H&E, 10X)

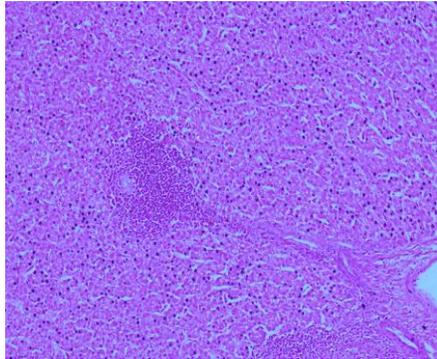


Fig. 16. Bird of group A shows focal necrosis in liver (+ to ++)(H&E, 10X)

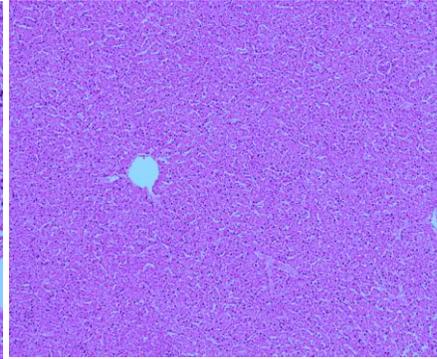


Fig. 17. Bird of group C (kept in control) shows normal histology of liver (H&E, 10X)

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