Histopathological changes indicating the severe susceptibility period of coccidiosis affected chicken

Coccidia of the genus *Eimeria* are members of the family Eimeriidae, which belongs to the subphylum Apicomplexa. Sporulation of oocysts occurs outside the host. During this process oocysts, which are the end result of sexual reproduction, sporulate to form an oocyst containing four sporocysts, each containing two sporozoites. When sporulated oocysts are ingested by a susceptible host, the infective cycle begins. After ingestion, the sporulated oocyst undergoes excystation, which releases individual sporozoites. The sporozoites invade the intestinal epithelium and round up to form trophozoites, followed by nuclear divisions to form immature meronts (schizonts). The meronts reproduce asexually by multiple fission to form a number of merozoites. The mature meronts and the host cells in which they are contained then rupture, releasing the merozoites, which undergo one to several additional generations of merogony. Eventually, merozoites invade cells and develop into
either microgamonts or macrogamonts. The former undergo multiple divisions, resulting in the formation of numerous microgametes, which are flagellated and motile. The latter give rise to a single macrogamete. Upon release, the microgametes invade cells containing macrogametes, and fertilization occurs. After fertilization, an oocyst wall forms and the oocysts mature; the host cell then ruptures, releasing the oocysts into the intestinal lumen, where they are discharged in the feces. The process of sporulation then takes place starting a new infective cycle.

The pathology of coccidial infection varies with the species of *Eimeria* and the host, but some features are common to all infections. Clinical signs include lethargy, depression, decreased food and water intake, and a decrease in normal grooming behavior. Decreases in weight gain or actual weight loss may be apparent. The water and mucus content of fecal material is increased, and diarrhea may be present. Blood may also be present in the feces. Gross pathological lesions may include grey or white nodules or striations on the luminal surface of the intestine, and hemorrhagic enteritis may be noted. Microscopically, a pericryptal infiltrate of mononuclear cells and granulocytes is often seen, accompanied by edema and a thickening of the mucosa. In chickens, a large percentage of the cellular infiltrate is composed of CD81 lymphocytes, which are sometimes visible as large aggregates in the crypts.

*Eimeria tenella*, the most pathogenic strain of coccidia in chicken, is usually located in cecum and leading to cecal coccidiosis (Augustine, 2001 and Allen et al, 1998). The disease is manifested by severe symptoms such as diarrhea, dehydration, and death in young animals (Levine, 1985). The economic loss is made up of the mortality, the growth and development retardation of the affected bird and the diminution of their body masses and rates of the oviposition, deterioration of the meat quality and so on. Although it is generally accepted that acquired immunity is parasite-host species-specific, Coccidiosis was considered an important disease because endogenous stages of the parasites and a high number of oocysts in feces were associated with intestinal lesions. Though, it is well known that the coccidian infected birds were susceptible histologically but
lack of information regarding the proper susceptible post infection period and their changes during this period is the main driving force for the present study. Therefore, the study was conducted for examination of the histopathological changes
Material and methods

Experimental protocol

Twenty four coccidia-free Rhode Island Red chicks were used in the present study. One-day-old coccidia free chicks (45 ± 3.5 g) were collected from a local poultry farm, reared for three days to acclimatize in standard poultry cages (2.5 ft²), providing proper environmental condition (stress free) and given non-medicated feed and water. After three days, the birds were randomly distributed into treatment (TB) and control (CB) groups at the rate of sixteen birds per cage and reared in cage maintaining the same environmental condition. Four chicks of both groups were sacrificed at twenty-four, seventy-two and one hundred and forty four hours of post infection period.

Collection, preparation and inoculation of oocysts

Pools of feces were collected from young and mature birds of local coccidian infected poultry farm. Samples were diluted into 2.5% aqueous potassium dichromate (K₂Cr₂O₇) and kept in petri dishes for complete sporulation at room temperature for three days (Goldova, 1998). After sporulation, oocysts were recovered by centrifugation with saturated sugar solution as described by Duszynski and Wilber (1997) and used in subsequent analysis.

A phase contrast microscope (Olympus, CX41) and an Olympus microscope with digital camera (Model no. C-5060, 4X wide zoom lens) was used for oocyst identification. The number of oocysts per gram of feces (O/g) was determined according to the technique described by Menezes and Lopes (1995).
Mc Master method of counting oocyst:

The graduated bottle is filled with water up to the 42 cc mark and glass beads are added. Three grams of faeces is weighed out, stirred well and placed in the bottle. The stopper is fitted to the bottle and it is then shaken well until the faecal matter is broken down. The mixture of water and faeces is poured through a screen, liquid matter is collected and the debris is discarded. 45 cc of saturated common salt solution is added to the strained fluid, mixed well and immediately withdrawn to fill the chambers of the Mc Master slide. All the oocysts are counted in the two separate centimeter squares.

Chicks of the treatment group were orally inoculated by isolated *Eimeria tenella* at the rate of $10^6$ oocyst per bird, while the control group was inoculated with inoculum buffer only.

**Examination procedure**

**Pathological examination**

WBC and RBC were counted by routine laboratory method and hemoglobin percentage of blood recorded using the Hemoglobin meter (Digital Hb meter 185, Systronics).

Total count of Red blood corpuscles

1. The apparatus is cleaned and dried and rinsed with 1% solution of sodium citrate to avoid coagulation of blood in the pipette.
2. The pricking needle is sterilized by heating it over a flame.
3. The intravenous blood sample is taken.
4. The tip of the RBC pipette is put in the blood and sucked upto 0.5 mark. No air bubble should enter the pipette i.e. the stream of blood should be a continuous one.
5. RBC diluting fluid is sucked into the pipette upto 101 mark. The blood and the diluting fluid is mixed thoroughly by rotating it for 3 to 4 minutes. Now the dilution of blood is 1 part blood : 200 parts diluting fluid i.e. 200 times.
6. A drop of diluted blood is put from the bulb of the pipette on the surface of the haemocytometer slide.

7. A coverslip is placed on the two platforms over the counting chambers taking care that no air bubbles get in.

8. After five minutes, when the blood cells settle down, counting is done.

9. Counting is to be done in 5 of the 25 small squares. For sake of convenience, 4 small squares at the four corners and the central small squares are selected for counting red blood corpuscles. This means that counting of the red blood cells is done in 5x16=80 smaller squares in which volume of blood is 1/4000 cu.mm. Then the average number of red blood cells per square is calculated

Total count of white blood corpuscles:

1. WBC pipette is used instead of RBC pipette and the blood is sucked upto 0.5 mark

2. WBC diluting fluid is sucked upto 11 mark. The dilution of blood is 1 part blood: 20 parts diluting fluid

3. Counting of WBC is done in two of the 4 counting chambers. Each of these 4 counting chambers has an area of 1 sq. mm, and this area is divided into 16 small squares. Each small square has an area of 1/16 sq.mm. So, the volume of blood in each small square is 1/16x10 = 1/160 cu.mm.

4. Therefore, volume of blood in each of the 4 WBC counting chambers having 16 small squares is 1/160x16=1/10 cu.mm. But the blood has been diluted 20 times. So, the volume of blood in each of the four WBC counting chambers is 1/10x1/20= 1/200 cu.mm.

5. Counting of WBC is done in 4 small corner squares of any two counting chambers i.e. in 8 small squares in total
**Histological Examination**

Sections of two cm were excised from small intestine, and caecum and fixed in 10% buffered formalin, processed and stained by hematoxylin-Eosin according to Behmer *et al.* (1976) for histological examination. The tissue is fixed in 10% formalin overnight and dehydrated in up grades of alcohol. Subsequently, the tissue is cleaned in xylol and block is prepared in paraffin medium. Sections of 6 µm are cut in the microtome. Sections are then stained in haematoxylin and eosin.

The phase contrast microscope (Olympus, CX41) and an Olympus microscope with digital camera (Model no. C-5060, 4X wide zoom lens) was used for sequential examination of different histological sections. Pictures were made using the above digital camera.

*Lesion index (the maximum is 4) was examined using the following scheme:*

**Assessment 1** - scarce petechial haemorrhages on the mucosal surface and slight thickening of intestinal mucosa.

**Assessment 2** - a small number of haemorrhages up to pinhead size on the mucosal surface, oedema and thickening of the intestinal mucosa.

**Assessment 3** - many haemorrhages up to pinhead size on the mucosal surface, oedema and thickening of the intestinal mucosa, degenerative changes in the mucosal epithelium, caecums contain necrotic cheese-like debris

**Assessment 4** - many haemorrhages up to pinhead size on the mucosal surface, oedema and pronounced thickening of the intestinal mucosa, strong degenerative changes in the mucosal epithelium, caecums are full of necrotic cheese-like debris containing many oocysts and blood traces.

**Statistical analysis**

All data were presented as mean ± S.E. and statistically analyzed by one-way analysis of variance (ANOVA). If the main effect was found significant, the ANOVA was followed by a least significance difference (LSD) test. All statistical tests were considered significant at 5% probability level using statistical package M-STAT.

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Clinical signs and gross lesions

No remarkable clinical signs were observed in both treated and control birds at the period of twenty four hours. Weakness, lack of appetite, ruffled feathers, uncoordinated movements, loss of weight were apparent in treated birds at seventy two hours which showed increasing trend in the birds of one hundred and forty four hours. Production of fecal matter gradually decreased in treated birds than that of control one with time. Liquid bloody feces (bloody diarrhea) was found at one hundred and forty four hours in the treated bird, whereas normal feces produced by control bird. Upper small intestine showed small blood spots in the one hundred and forty four hours treated bird. The external appearance of the caecum was bloodish red in colour in this case (Figure 9a, b).

Pathological changes

The mean hemoglobin content of blood samples of treated birds differed markedly from the control one (ANOVA, P < 0.05). The concentration ranged from 14.2 to 17.8 and 12.4 to 14.0 mg per 100ml in control and treated birds, respectively (Figure 10a). Total mean count of RBC and WBC in treated birds showed low value than that of the control one. RBC and WBC counts ranged from 2.441 to 2.721 x 106 per cu mm and 6788 to 6850 per cu mm, respectively in control bird and 1.825 to 2.511 x 106 per cu mm and 4955 to 6763 per cu mm, respectively in treated one (Figure 10b, c). Hemoglobin, RBC and WBC of treated bird reduced by twelve to twenty-one percent, twelve to twenty five percent and five to twenty-seven percent than that of control one, respectively. Minimum values of hemoglobin, RBC and WBC were found in the bird of one hundred and forty four hours post infection period.
Histological observations

Histological sections of the small intestine and caecum showed clear structural changes, whereas no changes were observed in liver and kidney of treated bird. All sections of control bird showed a normal structure. Spots of blood (lesions) were observed in the small intestine of the treated birds. The villi of small intestine were blunt in shape and the length of villi gradually decreased in treated bird as post infection period progressed (Figure 11a, b and c).
Figure 9: Photographs showing the difference between the caeca of infected (a) and control birds.
Figure 10: Responses of Hemoglobin concentration (a), RBC count (b) and WBC count (c) in the birds of increasing post infection period.
Figure 11: Figure showing the difference between the intestinal villi of infected (a) and control (b) birds and showing the shorter villi in treated bird (c).
Conclusion

Results obtained from the present study demonstrated a clear pathological, histological and biochemical alterations in the *Eimeria tenella* infected birds. Furthermore, the data of the experiment revealed that the alterations were clear in different post infection periods (i.e., twenty four, seventy two and one hundred and forty four hours) and more prominent in the birds of higher post infection periods (one hundred and forty four hours) (Bandyopadhyay et al. 2006d).

Hemoglobin content, RBC and WBC count were twelve to twenty-one percent, twelve to twenty five percent and five to twenty-seven percent reduced values in treated bird which indicated that *Eimeria tenella* is responsible for creating such condition in which Hemoglobin, RBC and WBC produces from their origin in low rate. *Eimeria tenella* causes pathogenic changes in chicken (Augustine 2001; Allen 1998). Lesions were observed in the small intestine of the treated bird. The villi of intestine showed blunt shape and the length of villi gradually decreased in treated birds as post infection period progressed. All these histological changes occurred in the small intestine only due to infection of *Eimeria tenella*. These observations resemble those described by Tsunoda and Muraki (1971); Norton and Pierce (1971); Tsutsumi (1972) not only because of the site of infection, but also morphology was similar. All these changes were more distinct in the birds of one hundred forty four hours of post infection.

Therefore, it can be concluded that the birds were more susceptible at one hundred and forty four hours period of post infection exhibiting the high intensity of pathological and histological alterations by *Eimeria tenella*. 