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PATHOLOGY OF SPONTANEOUS NECROTIC ENTERITIS IN CAGE REARED COMMERCIAL LAYER CHICKEN

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Abstract: Necrotic enteritis (NE) caused by *C. perfringens*is one of the economically important disease in chicken raised in deep litter and rare in caged birds. However nowadays incidence of NE in cage reared layer chicken in Namakkal poultry belt of Tamil Nadu were noticed.Hence the current study on spontaneous necrotic enteritis among the gastrointestinal disordersin cage reared commercial layer chicken was investigated by cultural examination, necropsy, light microscopy and immunoperoxidase staining. Among the gastrointestinal disorders of commercial layer chicken necrotic enteritis contributed 28 % and it occurred as single (13 %) and combined (15 %) infection with coccidiosis (11 %), worm infestation (2%) and Newcastle disease (2%). Affected birds showed depression, anorexia, cyanotic comb, brownish diarrhea, dehydration, ruffled feathers and sudden death with a mortalityof 2.0% to 10%. The gross lesions of friable, thickened mucosa with firmly adhering yellowish brown diphtheritic membrane giving a dirty turkish towel appearance were noticed. The mean average intestinal lesion score in combined infections was higher (3.71) than the NE alone (3.47) affected birds. Microscopically intestinal mucosa showed diffuse degeneration, coagulative necrosis along with bacteria and infiltration of mononuclear cells. Indirect immunoperoxidase technique revealed the presence of dark golden brown color immunostain indicating clumps of *C. perfringens* organisms within and around the necrosed and desquamated cells of the intestinal mucosaand it was absent in viable tissue. Thus *C. perfringens* always appeared to be present in the lesions and to act locally causing a coagulative type of necrosis.

Keywords: Layer chicken, Necrotic enteritis, *C. perfringens,* Pathology, Spontaneous cases.

INTRODUCTION

Poultry egg and meat are a major source of protein for the global human population. Efficient and safe production of nutritious poultry food products will become increasingly important to meet growing consumer demand. A better understanding of the potential effects of changing environmental factors, emerging infectious diseases, and modern poultry-rearing practices on the efficiency and profitability of future poultry production will be essential for these processes to be realized. In this regard, necrotic enteritis has reemerged as a significant problem as a result of alteration in use of feed raw materials, climatic change and modern practices of high-density housing conditions (Van Immerseel *et al*., 2004; Williams, 2005).

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Necrotic enteritis (NE) is an acute or chronic enterotoxaemia caused by *Clostridium perfringens* (Van der Sluis 2003; Williams 2005). *C. perfringens* is a Gram-positive, obligate anaerobic, spore-forming bacterium readily found in soil, dust, used poultry litter and as a normal inhabitant of the gut microflora of healthy birds (Dahiya *et al*. 2006). Although *C. perfringens* is a normal inhabitant of the intestine, under certain circumstances it proliferates rapidly and may cause subclinical or clinical NE. The disease primarily affects broiler chickens (2-5 wk old) and turkeys (7-12 wk old) raised on deep litter but can also affect commercial layer pullets raised in cages (Broussard *et al*., 1986). Available literature suggest afew field based studies on spontaneous necrotic enteritis (Dhillon *et al*., 2004; Sawale *et al*., 2010; Malmurugan *et al*., 2012). Therefore the present paper describes the pathology of NE in commercial layer chicken above 20 weeks of age maintained entirely in raised cages.

MATERIALS AND METHODS

A study on the pathology of necrotic enteritis was performed on layer chicken housed commercial poultry farms located in Namakkal region of Tamil Nadu. The study period covered three consecutive years (October 2008 - September 2011). A total of 1000 layer chickens from 100 (10 per flock) commercial layer flocks with the history of increased mortality, symptoms and postmortem lesions suggestive of gastrointestinal tract disorders were utilized for this study. Impression smears from 100 samples prepared from jejunum of intestine were stained with Gram's staining and examined for the presence of *Clostridium* organisms. The flock diagnosis was established by necropsy and confirmed by bacteriological examinations.

Gross pathology: The birds were inspected for gross lesions before collection of specimens. The entire intestine (except specimens collected for further studies) was opened for inspection of the mucosal surface. Intestinal and extra-intestinal lesions were recorded. Gross appearance of the jejunal and ileum lesions, is used as criteria for assigning NE scores. Lesion scoring of the NE was done following the methods of Williams *et al.*(2003) as follows 0 = no gross lesions, normal intestinal appearance; 1 = thin walled or friable and few whitish plaques in the serosal surface (mild); 2 = thin-walled, focal necrosis or ulceration, small amounts of gas production (moderate); 3 = thin-walled, large patches of necrosis, gas-filled intestine, small flecks of blood (marked or severe); 4 = severe extensive necrosis, marked haemorrhage, excessive amounts of gas in the intestine (very severe). Following postmortem examination, if the score was ≥1, then a piece of intestinal tissue (1.5 to 2.0 cm long) with the

gross NE lesion was collected in neutral buffered formalin solution for histopathological examination.

Light microscopy: The tissues were fixed in 10% neutral buffered formalin, dehydrated, cleared and embedded in paraffin wax in routine manual processing.Sections were cut approximately 5µm thick, mounted on glass slides, stained with haematoxylin and eosin, and covered with cover slips for histopathological examinations. Scoring of histopathology was not carried out because it has considerable potential for bias, since lesions are usually pre-selected for histopathological examination.

Immunohistochemistry: Immunoperoxidase staining was performed on formalin-fixed, paraffin-embedded specimens as described by Kaldhusdal *et al*. (1995) with some modifications. The indirect immunoperoxidase technique was applied on sections from the intestine with lesions using chicken polyclonal anti-*C. perfringens* type A antibody as a primary antibody and horse radish peroxidase conjugated anti-chicken IgG (Sigma, USA) as a secondary antibody. Sera from NE negative flocks were used as a negative control for anti-*C. perfringens* type A antibodies.

Bacterial Isolation: For isolation of the organism, causing necrotic enteritis, pooled intestinal contents and scrapping were collected from those flocks which are showing gross lesions suggestive of necrotic enteritis and clostridium organism on direct microscopic examination. Sterile saline was added to the collected materials and heated at 80^{o}C for 20 min in water bath. Then the processed intestinal contents were inoculated into thioglycollate broth, Robertson cooked meat medium with brain heart infusion broth and sterile liquid paraffin was poured to make a layer over the medium. Inoculated medium was incubated anaerobically at 37^{o}C for 24h. The presence of *C. perfringens* in the inoculated sample is indicated by turbidity in both the media. The positive cultures were streaked onto clostridial agar and perfringens agar with supplements. The plates were incubated in the anaerobic jar at 37^{o}C for 48h. The plates were observed for the growth of characteristic colonies of *C. perfringens*. The positive colonies obtained on clostridial and perfringens agar were inoculated on milk medium with reducing agentand sheep blood agar and tested for their ability to ferment glucose, maltose, lactose, sucrose, and mannitol. The isolates were also subjected to oxidase, catalase and gelatin liquification tests as described previously by Barrow and Feltham (1993).

RESULT

Among the 100 flocks examined for gastrointestinal lesions, 28 intestinal scrapings showed the presence of large gram- positive rods by Gram's staining suggestive of *C. perfringens* organisms under direct microscopic examination. Inoculation of 28 pooled processed intestinal contents in thioglycollate broth produced turbidity and saccharolytic reaction in Robertson cooked meat medium with brain heart infusion broth. Growth on the clostridial agar were obtained on the initial streak from the culture. The selective streaking of these colonies on perfringens agar with supplements revealed rough and black colonies with sulphate reduction, characteristics of *C. perfringens* organisms. Grey, flat, round, glistening and double haemolytic colonies were noticed on sheep blood agar. In milk medium, the classical stormy clot or stormy reaction was indicated as shreds of milk curds in sides of the tubes with excessive gas formation. The isolates were non motile in hanging drop method. No color change was produced by the isolates on filter paper containing oxidase reagent and proved oxidase negative. On catalase test, all the isolates did not produce effervescence of foams and hence proved catalase negative. All the isolates liquefied gelatin, fermented glucose, maltose, lactose and sucrose except mannitoland negative for oxidase and catalase. Based on the results obtained from the above said tests, the isolates were identified as *C. perfringens*.

Microscopic examination of intestinal scraping of 11 out of 28 NE affected flocks revealed coccidial oocysts along with *Clostridium sp*., on direct microscopic examination. From oocyst morphology and location, the species was confirmed as *Eimeria maxima*. However, no gross lesions suggesting coccidiosis were observed at the time of postmortem. In two flocks intestine showed the presence of *Ascardiagalli* along with the necrotic enteritis lesions. Lesions suggestive of Newcastle disease and necrotic enteritis was noticed in two flocks and the disease was confirmed by heamagglutination test.

Necrotic enteritis affected flocks showed depression, anorexia, reluctance to move, somnolence, cyanotic comb, dehydration and ruffled feathers. Some birds also revealed soiled vent with brownish fecal material adhering to cloaca. Sudden death after showing clinical illness for shorter duration was observed in many flocks. Mortalityranges from 2.0 to 10%.

On necropsy examination majority of the dead birds were in good body condition. Dehydration was usually present, as indicated by dark, dry pectoral musculature and pale kidneys with prominent lobular outlines. The liver was congested and the gall bladder dilated.

The intestines are thin walled, friable, dilated and filled with gas (Fig 1). The mesenteric vessels were engorged with blood. On opening, the intestinal lumen contained bile-stained fluid and granular debris. The mucosa was covered with firmly adhering yellowish brown diphtheritic membrane giving a dirty Turkish towel appearance to the mucosa (Fig. 2). These lesions were usually confined to the small intestine, primarily jejunum and ileum. Few affected birds also revealed these lesions in the colon, caecum and rectal regions. In some cases, a dry core of granular debris in the ileum was found together with a dilated and thin-walled jejunum containing bile -tinged, flocculent fluid. Intestinal lesion score in spontaneous necrotic enteritis affected layer chicken was presented in Table 1. The mean average lesion score observed in NE, NE and coccidiosis, NE and worm infestation and NE and Newcastle disease were 3.47, 3.71, 3.65 and 3.60 respectively.

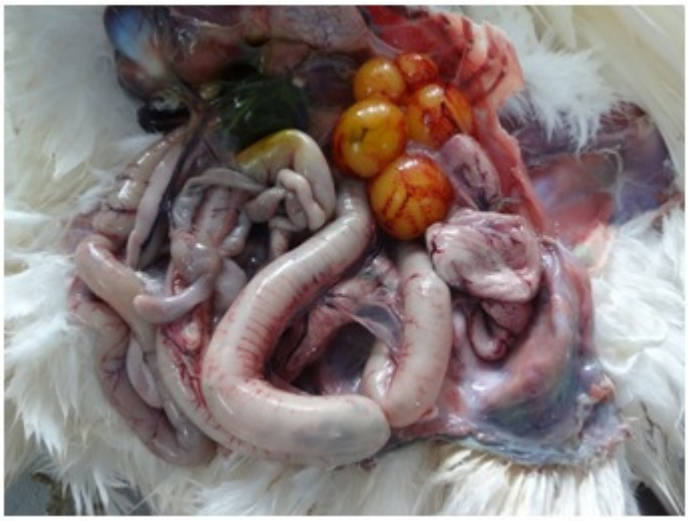

Fig 1. NE affected bird showing the ballooning of intestine due to dilation by gas.

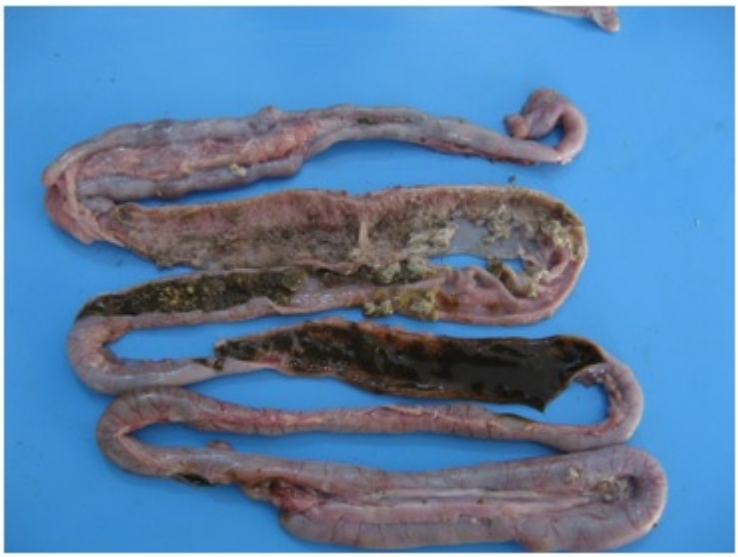

Fig 2. NE affected birds showing pseudomembrane formation in the jejunum and ileum.

Table 1. Lesion score in spontaneous necrotic enteritis affected commercial layer chicken

Flock No.	Age (Wk)	Flock Size	Mortality (%)	No. of birds examined	Diagnosis	Lesion score					Average lesion score
						0	1	2	3	4	
1	55	20000	2.25	10	NE	0	0	3	2	5	3.2
2	23	4500	3.90	10	NE	0	0	0	2	8	3.8
3	31	15000	2.20	10	NE	0	0	2	2	6	3.4
4	46	12000	2.90	10	NE	0	0	0	4	6	3.6
5	27	10000	2.00	10	NE	0	0	3	2	5	3.2
6	35	5000	2.41	10	NE	0	0	1	2	7	3.6
7	59	16000	4.55	10	NE	0	0	0	1	9	3.9
8	20	10000	2.65	10	NE	0	0	2	2	6	3.4
9	34	50000	2.00	10	NE	0	0	3	3	4	3.1
10	28	17000	2.70	10	NE	0	0	1	3	6	3.5
11	27	40000	2.75	10	NE	0	0	0	5	5	3.5
12	26	15000	2.25	10	NE	0	0	2	2	6	3.4
13	32	20000	2.80	10	NE	0	0	0	3	7	3.6
14	35	20000	4.50	10	NE +Cocci	0	0	0	0	10	4.0
15	26	12000	3.05	10	NE +Cocci	0	0	0	3	7	3.7
16	37	30000	1.70	10	NE +Cocci	0	0	2	3	5	3.3
17	43	12000	2.10	10	NE +Cocci	0	0	0	3	7	3.7
18	29	6500	2.40	10	NE +Cocci	0	0	0	2	8	3.8
19	39	25000	2.50	10	NE +Cocci	0	0	0	2	8	3.8
20	25	11000	2.00	10	NE +Cocci	0	0	0	3	7	3.7
21	61	30000	2.65	10	NE +Cocci	0	0	0	2	8	3.8
22	20	7000	2.00	10	NE +Cocci	0	0	0	4	6	3.6
23	31	9000	3.80	10	NE +Cocci	0	0	0	2	8	3.8
24	28	12000	1.74	10	NE +Cocci	0	0	0	4	6	3.6
25	26	10000	2.60	10	NE + Worm	0	0	1	3	6	3.4
26	34	8000	4.32	10	NE + Worm	0	0	0	1	9	3.9
27	55	10,000	2.58	10	NE +ND	0	0	1	3	6	3.5
28	43	15,000	3.72	10	NE + ND	0	0	0	3	7	3.7

Histopathological examination of intestine revealed diffuse degeneration and partial to complete coagulative necrosis of villi characterized by cytoplasmic vacuolation and eosinophilia, chromatin condensation, karyorrhexis and karyolysis(Fig. 3). Intestinal lumen contained masses of tissue fragments, necrotic cells, cell debris and numerous short plump rod like bacteria (Fig 4). The lamina propriashowed varying degrees of degeneration to necrotic changes with mononuclear cells infiltration (Fig. 5). In NE and coccidiosis affected birds along with the necrotic lesions, presence of several coccidial oocysts in the lumen of intestine sections were noticed, however very few oocysts were identified in the mucosal epithelium.

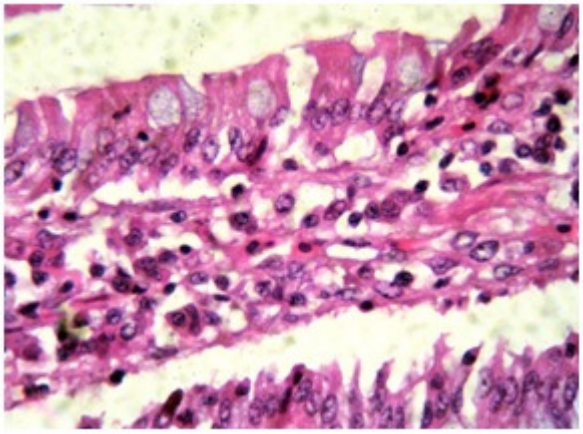

Fig 3. NE: Intestinal mucosa showing diffuse degeneration and necrotic changes. H&E x 400

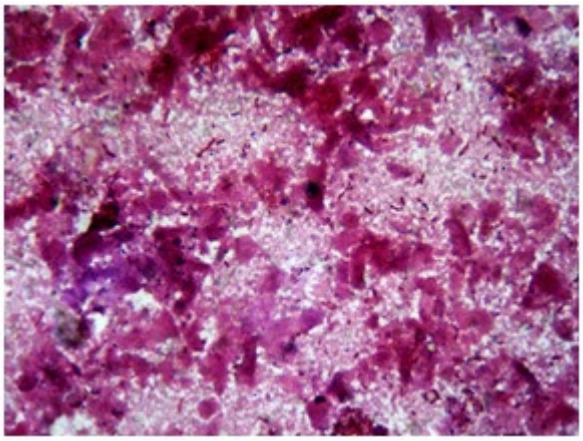

Fig 4. NE: Intestine lumen containing short plump rods along with the desquamated epithelial cells. H&E x 1000

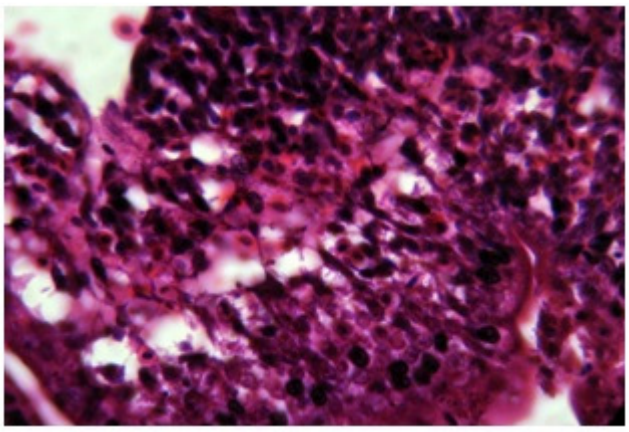

Fig 5. NE: Intestine showing infiltration of mononuclear cells in the lamina propria H&E x 400

Dark golden brown colourimmunostain indicating clumps of *C. perfringens* organisms (Fig. 6) was observed in indirect immunoperoxidase staining. The reaction was noticed within and around the necrosed and desquamated cells of the intestinal mucosa. No specific staining was

noticed in viable cells. Positive reaction was confined mainly to the jejunal region of the small intestine. Duodenum and ileum regions showed mild reaction in very few cases.

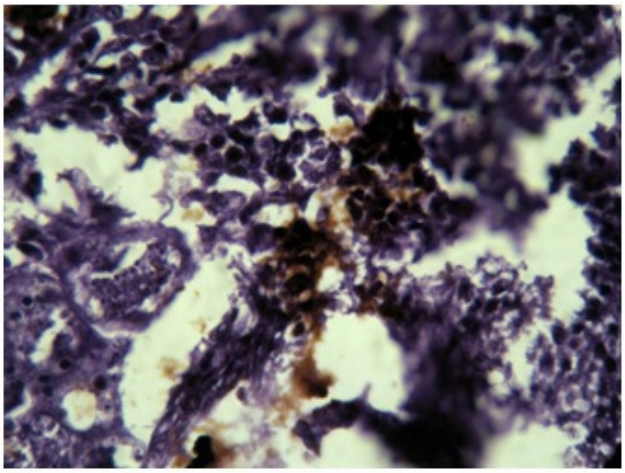

Fig 6. NE –IPT: Intestine showing golden brown color indicating clumps of *Clostridium Perfringens* organisms among the dead cells. IP&H x 400.

DISCUSSION

In the present investigation, 28 out of 100 flocks with gastrointestinal disorders showed the lesions of necrotic enteritis and the intestinal scrapings revealed the presence of large gram-positive rods by Gram's staining suggestive of *C. perfringens* and it was confirmed by morphological and biochemical characters which was in agreement with earlier work on the isolation (Craven *et al*., 2001; Malmarugan *et al*., 2012). The present study showed an intimate association of *C. perfringens* with the characteristic lesions of necrotic enteritis, supporting a causative role for the bacteria in the development of the disease.

Necrotic enteritis was recorded as single (13.0 per cent) and as combined (15 0 per cent) infections. Of note, chickens in 11 of the 28 flocks had concurrent infection of coccidiosis. The intestinal damage caused by coccidia is an essential predisposing factor for NE (Al- Sheikhly and Al-Saieg, 1980; Rodgers et al., 2015; Williams, 2005), allowing *C. perfringens* overgrowth and production of toxins (Van Immerseel *et al*., 2008). Intestinal damage during *Eimeria* infection will result in leakage of plasma proteins into the lumen of the intestinal tract, which is a rich nutrient substrate and favorable medium for *C. perfringens* proliferation and toxin production (Van Immerseel *et al*., 2004). Collier *et al*. (2008) suggested that coccidial infection induces mucogenesis as a result of a host mucogenic response, providing a growth advantage for *C. perfringens*.

Depression, anorexia, ataxia, brownish diarrhoea dehydration, cyanotic comb and ruffled feathers observed in the affected birds are in agreement with the observations of many authors (Al-Sheikhly and Truscott, 1977a; Al-Sheikhly and Al-Saieg, 1980). Sudden death

after showing clinical illness for shorter duration was observed in many flocks. This might be due to the highly potent alpha toxin produced by *C. perfringens*, which could cause cell membrane disorganisation (Naylor *et al.,* 1998; Rood, 1998; Titball *et al.,* 2000), and necrosis of epithelial cells as well as erythrocytes (Titball, 1993).

In the present study, gross lesions of friable, thickened mucosa, with firmly adhering yellowish brown diphtheritic membrane in the jejunum and ileum are well supported by the earlier observations of Long (1973); Malmurugan *et al*., (2012). These lesions in the small intestine might be due to the action of toxin produced by *C. perfringens* (Long and Truscott, 1976; Rood, 1998). Al-Sheikhly and Truscott, (1977a) and Kaldhusdal *et al*. (1995) also observed destruction of mucosal tissue frequently in the jejunum and ileum region of small intestine. The mean average lesion score in combined infection was 3.71 against 3.47 in NE alone affected birds indicate the concurrent diseases increases severity of NE lesions in commercial layers (Van Immerseel *et al*., 2004)

Microscopic examination of intestine revealed diffuse degenerationand excessive epithelial desquamation due to coagulative necrosis and the lumen contained masses of tissue fragments, necrotic cells, cell debris and numerous bacteria coincide well with the earlier report of Al-Sheikhly and Truscott (1977b). Characteristic NE morphological features of cytoplasmic and nuclear changes of enterocytes observed in this study has been supported by Olkowski *et al*. (2006), who reported that the differentiation of NE from other forms of enteritis could be made by adopting a set of strict histological criteria, which included cytoplasmic vacuolation and eosinophilia, intense nuclear basophilia, pyknosis, karyorrhexis and karyolysis.

Presence of dark golden brown color immunostaining within and around the necrosed and desquamated cells of the intestinal mucosa indicate that the proliferationof *C. perfringens* mainly occurred only in dead cells and absent in viable cells. Presence of positive reaction mainly in the jejunal region of the small intestine indicated the affinity of this organism to this region of intestine, which was also documented by Kaldhusdal *et al.,* (1995).

In conclusion, our results indicate that the *C. perfringens*can induce the necrotic enteritis in raised cage reared commercial layer chicken as alone or in combination with coccidiosis, worm infestation and Newcastle disease resulting in significant economic loss in terms of mortality, production loss and treatment cost. Hence continuous monitoring of *C. perfringens* and its associated pathological effect in poultry is prerequisite for decreasing the

prevalence of this disease in poultry and subsequent human illness resulting from consumption of products.

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