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Influence of Bovine IGG-Rich Fraction on Growth Promotion, Gut Health and Clostridium Perfiringens Enumeration in Necrotic Enteritis Infected Broiler Chickens.

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Abbreviations: *NE=Necrotic Enteritis, C. perfringens= Clostridium perfringens, BW=Body weight, BWG= Body weight gain, FCR= Feed conversion ratio, FI=Feed intake, GIT=Gastrointestinal tract, AGP=Antibiotic growth promoters, PBS= Phosphate buffered saline, PC=Positive control, NC=Negative control, A group=Prophylactic group, B group=Therapeutic group, VH/CD ratio=Villi Hight/ Crypt Depth ratio.*

Abstract: *One day-old male Arbor Acres plus broiler chickens (n=240) were used to study the effects of bovine IgG-rich fraction preparation (intramuscularly inoculated) on mortality, production performance, gut integrity (Histomorphometric analysis), macroscopic and microscopic lesion scoring, C. perfringens enumeration from intestine and caecum under NE challenge. A marked prophylactic and therapeutic effect was noted against NE in broiler chickens by reducing mortality, macroscopic and microscopic lesion scores, improving productive performance variables BW, BWG, FCR, Production number and Histomorphometric analysis. Such protection was further reflected by reduced C. perfringens enumeration particularly in those birds treated prophylactically. Bovine IgG-rich fraction is recognized as safe status, it could be considered as drug-independent control strategy of NE and preventing contamination of poultry products with food borne pathogens.*

Keywords: *Bovine IgG-rich fraction, Broiler chickens, Performance, Gut integrity, NE, C. perfringens*

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I. Introduction

Ultimately; poultry industry is exposing to stressful conditions and problems related to diseases that often occur and result in serious economic losses **(Lutful Kabir, 2009)**. *C. perfringens* infection in broiler chickens result in NE which is a dreaded disease dramatically influence poultry industry worldwide causing serious economic losses, about of two dollar billions/year **(Kaldhusdal and Lovland; 2000, Van Immerseel et al, 2009, Cooper et al., 2009).** NE occurs in broiler chickens aging between 2-6 weeks **(Songer, 1996 and Cooper and Songer, 2010).** It threatens GIT health and livability of many poultry flocks (**Craven *et al.*, 2001**). Many recent studies of NE have focused on finding different ways to control this disease **(Shojadoost *et al.*, 2012).** The issue of controlling enteropathogens without the use of AGP became a big challenge. Accordingly; natural alternatives concepts based on natural ingredients for GIT integrity and antibacterial action became highly commendable and emerging the direction towards the use of natural control methods **(Awaad *et al.*, 2011, Awaad *et al.*, 2013)**. Feed additives such as enzymes, probiotics, prebiotics, symbiotics have utilized in order to find better circumstances to enhance poultry GIT health and prevent or limit production losses. The role of certain biological products such as immunoglobulins rich fractions prepared from pooled bovine blood has been previously used in prevention of colisepticaemia in calves and chickens **(Penhal *et al.*; 1971, Logan and Penhale, 1971, Awaad ,1975, Awaad *et al.*, 1992, Youseif *et al.*, 2005, Hassanain *et al.*, 2008**, **Samy *et al.*, 2012). Laurien *et al.* (2018)** reported that bovine immunoglobulins, in particular bovine IgG, have been studied since the 1970's for their potential effects on immunity and infection in humans. They added that it can be functionally active throughout the GIT and a large number of studies in infants and adults have shown that bovine IgG can prevent GIT and upper respiratory tract infections.

The objective of this study is to elucidate the effect of bovine IgG-rich fraction on growth performance, intestinal and caecal colonization of *C. perfringens*, and gut integrity of experimentally induced NE in broiler chickens.

II. Material And Methods

Bovine IgG-Rich Fraction*:*

Bovine IgG-rich fraction containing 80 mg/ml was intramuscularly inoculated on 2 consecutive days before *C. perfringens* infection (for Prophylactic study) and post *C. perfringens* (for Therapeutic study).

Experimental Birds:

One day-old male Arbor Acres plus broiler chickens (n=240) were used in this study. Duration of the trial extended from one day of age up to slaughter (35 days). These birds allotted into 4 equal groups (1-4) consisting of 60 birds each and assigned into 3 equal replicates of 20 birds. All experimented chickens were floor reared in separate pens at a density of 10 birds/m^2 with fresh wood shavings as bedding with a thickness of approximately 10 cm on a concrete floor. The birds vaccinated against different diseases according to the vaccination programs usually adopted in Egyptian chicken broiler farms.

Experimental Design:

All groups ran contemporaneously. At d 14 of age; birds of groups 1-3 (A, B and PC groups) individually infected by crop gavage with 4×10^8 CFU/ml of *C. perfringens* in PBS for 4 successive days to induce NE **(Timbermont *et al.;* 2009).** The used strain of *C. perfringens* is type A B2 NET B, isolated from cases of chicken NE. Chickens of group 1 (A group) was intramuscularly treated with IgG-rich fraction at d 12 and 13 of age, while those of group 2 (B group) was similarly treated at d 18 and 19 of age. Chickens of groups 3 and 4 served as PC and NC controls respectively. The experimental design is illustrated in **Table 1**.

Diets:

Chickens feed *ad libitum* a crumbed started, grower and finisher diet. From d 1-16 of age, the birds received a starter diet (23% crude protein; 3000 kcal/Kg ME), from d 17-28 of age a grower diet (21% crude protein; 3100 kcal/Kg ME) and from d 29-35 of age a finisher diet (19% crude protein; 3150 kcal/ Kg ME). No antibiotics have been administrated in water or feed for the whole experimental period. Semduramycin was added at a rate of 25 ppm as a coccidiostat. Birds had free access to feed and water.

Measured parameters

1-Zootechnical performance assay:

For chicken performance response variables BW, BWG, FI (g/d/bird), FCR (g feed/g live body wt.) and production number were determined according to **Brady (1968), Sainsbury (1984), North (1984)** and **Timmerman *et al.* (2006).** All birds weighed individually at 1st day and weekly during the entire period of the trial. FI was measured on the same days of birds weighting and calculated to each pen.

2- Health status and Mortality assay:

During the evaluation; the health status and mortality of the birds were checked daily

3-Macroscopic lesion scoring of gut:

Necropsy has been adopted on 6 randomly selected birds (2 birds/replicate) at day 21 and 28 of age (one and 2 weeks PI) for gross pathological lesion scoring of the small intestine. The scoring system criteria was the six-point system of **Keyburn *et al.* (2006)** modified by **Shojadoost *et al.* (2012)** as follows:

0 =No gross lesions.
1 =Thin or friable walls, or diffuse superficial but removable fibrin.
2 = Focal necrosis or ulceration, or non-removable fibrin deposit 1 to 5 foci.
3 = Focal necrosis or ulceration, or non-removable fibrin deposit 6 to 15 foci.
4 = Focal necrosis or ulceration, or non-removable fibrin deposit 16 or more foci.
5 = Patches of necrosis 2 to 3 cm long variable.
6 = Diffuse necrosis typical of field cases variable, but extensive

4-Histopathological lesion scoring and Gut morphometry assays:

At day 35 of age specimens of major immune organs including thymus glands, spleen, BF and intestine were collected from sacrificed randomly selected 8 birds/group from all experimentally infected groups (2 bird/replicate). Fixed in 10% buffered formalin. Paraffin-embedded sections were routinely prepared and stained with Hematoxylin and Eosin **(Bancroft *et al.* 1996)**, and scored for histopathological lesions according to the method described by **Rosales *et al.* (1989).** For gut morphometry; one cm-thick samples were be taken from Duodenum and jejunum **(Samanya and Yamauchi, 2002).** Routine histological laboratory methods was adopted and villous histomorphometry for recording the histological indices measured using digital photography and light microscopy. The photos were taken and morphometric analyses was performed. The

villous height measured from the apical to the basal region and the crypts from the basis until the region of transition between the crypt and the villus. Five measurements per section has been made for each parameter and averaged into one value and the VH/CD ratio was determined.

5- C. perfringens load assessment:

Randomly sacrificed 6 birds of each group (1 bird/replicate) at d 21 and d 28 of age (d 7 and 14 PI) were collected, following their euthanasia, birds necropsied and 1g of intestinal and caecal contents from each bird weighed, pooled and serially diluted (10^{-1}-10^{-8}) in sterile peptone saline for colonization of *C. perfringens* colonization. 0.1 ml of each dilution of peptone saline was plated on 10 % neomycin sheep blood agar and tryptose sulfite-cycloserine (TSC) agar (supplemented by D-cycloserine) with egg yolk emulsion. These were overlaid with the same medium but without egg yolk. After anaerobic incubation at 37°C for 24 hours; typical *C. perfringens* colonies (black colonies) on TSC agar or large dome-shaped colonies with a double zone of haemolysis on blood agar plates were counted and reported as colony-forming units (CFU) per gram. The colonies picked and confirmed by criteria of **Harmon (1984)** and **Carrido *et al.* (2004), Garrido *et al*.(2006).** All quantitative data converted into log (CFU) values.

Statistical Analysis:

One-way analysis of variance using SAS software general liner models procedure **(SAS Institute 1999)** has been adopted. The main factor was the bovine IgG treatment. Mean values were assessed for significance using Duncan's multiple range test with significance set at $P<0.05$.

III. Results And Discussion

NE infected groups showed variable degrees of diarrhea, inappetence, different degrees of enteritis and mortalities. Significant increase in cumulative mortality rate of PC group (10.83 %) over other groups A, B and NC (5.00 %) has been recorded ($P<0.05$) (**Table 2)**. Significant reduction in final BW, BWG and production number with significant increase in FCR was also obtained in PC group vs. A, B and NC groups ($P<0.05$) **(Tables 2-3).** The macroscopic lesion scoring revealed significant reduction in groups A and B vs. PC group at d 7 and 14 PI ($P<0.05$) **(Table 4)**.

Histopathological examination of PC group revealed severe alterations involving the intestine and major immune organs. Concerning the intestine; severe necrosis of intestinal mucosal epithelium with fusion of intestinal villi associated with hemorrhages and marked congestion of blood vessels were detected in intestinal segments. The lesion was more severe in jejunum than duodenum **(Fig.1a).** Significant reduction in intestinal pathology was achieved in bovine IgG-rich fraction treated groups A and B **(Figs.1b and 1c).** The marked Amelioration in intestinal pathology was observed in group A than group B except in one case where massive hemorrhages was detected in duodenum and jujenum of group A. PC group revealed severe depletion of immune organs specially in cecal tonsils and spleen that showed severe depletion of lymphoid cells **(Fig.1d and 1g)**. On the other hand, there was marked immunostimulant effect on immune organs in the treated groups A and B where cecal tonsils of group A showed marked hyperplastic proliferation of lymphoid elements **(Fig.1e)** while there was less effect in group B **(Fig.1f)**. Splenic follicles of group A showed mild depletion of lymphocytes (**Fig.1h**) while the splenic follicular depletion was more evident in group B than in group A **(Fig.1i).** No significant change in either BF or thymus glands was noticed in groups A and B. The marked immunostimulant effect of bovine IgG-rich fraction on the immune organs in treated groups elucidated in the present work are on line with those described by **Ulfman *et al.* (2018)** who provided an overview of the current knowledge of the effects of bovine immunoglobulins on the human immune system.

The microscopic lesion scoring of different segments of intestine revealed significant reduction in sum of NE lesions in different organs of groups A and B as well as in spleen and cecal tonsils vs. PC group ($P<0.05$). Moreover; there was numerical reduction in lesion score in thymus glands and BF in A and B groups vs. PC group **(Table 2)**.

Histomorphological analysis of gut revealed significant increase in VH/CD ratio in duodenum and jejunum in both treated A and B groups vs. PC group **(Table 6)** ($P<0.05$).

C. perfringens load assessment revealed severe reduction in *C. perfringens* enumeration in intestine and caecum in both treated A and B groups vs, PC group at 7 and 14 days PI **(Table 7).**

Aforementioned results are on line with those reported by **Awaad *et al.* (2019)** in opposing of NE by phytonutrients and/or acidifiers in broiler chickens. **Henderson *et al.* (2015)** mentioned that mice receiving bovine serum IgG from non-immunized cows in a bacterial-induced colitis model had reduced weight loss and improved histological score compared to controls.

Human immunoglobulin therapy first occurred in the 1930s and a formulation for injection into a vein was approved for medical use in the United States in 1981**(Section on Breastfeeding, 2012).** It is on the World

Health Organization's List of Essential Medicines, the most effective and safe medicines needed in a health system **(van Neerven *et al.*, 2012).**

The precise mechanism by which immunoglobulin therapy suppresses harmful inflammation is likely multifactorial. Perhaps a more popular theory is that the immunosuppressive effects of immunoglobulin therapy are mediated through IgG's Fc glycosylation. By binding to receptors on antigen presenting cells, IVIG can increase the expression of the inhibitory Fc receptor, FcgRIIB and shorten the half-life of auto-reactive antibodies **(Köhler *et al.*, 2002; Yel Yel, 2010; Munblit *et al.*,2017). Bayry *et al.* (2003)** described this mechanisms including the possibility that donor antibodies may bind directly with the abnormal host antibodies, stimulating their removal; the possibility that bovine IgG stimulates the host's complement system, leading to enhanced removal of all antibodies, including the harmful ones; and the ability of immunoglobulin to block the antibody receptors on immune cells (macrophages), leading to decreased damage by these cells, or regulation of macrophage phagocytosis. They added it is becoming more clear that immunoglobulin can bind to a number of membrane receptors on T cells, B cells, and monocytes that are pertinent to autoreactivity and induction of tolerance to self. **Janki and Yong (2006)** gave a report stating that immunoglobulin application to activated T cells leads to their decreased ability to engage microglia. **Maverakis *et al.* (2015)** reported that the ability of immunoglobulin therapy to suppress pathogenic immune responses is dependent on the presence of a sialylated glycan at position CH2-84.4 of IgG. **Naaber *et al.* (1996)** and **Brooks *et al.* (2006)** stated that Immunoglobulins from non-immunized cows can prevent adhesion of some pathogens. **An *et al.* (2009)** reported that bovine immunoglobulins can also support intestinal barrier function and when this function is compromised, bacterial pathogens can passively cross the epithelial layer and cause inflammation and infection in the mucosa. **Detzel *et al.* (2015)** mentioned that bovine IgG can also have anti-inflammatory effects by preventing translocation of bacterial components across the epithelial layer. **Pérez-Bosque *et al.* (2015)** concluded that serum-derived bovine IgG reduces mucosal expression of pro-inflammatory cytokines and prevents reduction of barrier function. **den Hartog *et al*. (2014)** reported that bovine IgG is able to form immune complexes with bacterial and viral pathogens, mediates antigen presentation, as well as phagocytosis and killing in phagocytes.

Brynestad (2002) mentioned that *C. perfringens* is responsible for severe food borne enteritis in man and its presence in foods such as meat of poultry may be unavoidable **(Rhodehamel and Harmon, 1998, Ghadban *et al.*, 1998).** It is already known that food safety is probably the biggest issue facing poultry production systems today and preventing contamination of poultry products with food borne pathogens remains a considerable challenge for producers and integrations (**Awaad *et al.*, 2011).**

In conclusion; bovine IgG-rich fraction used in the present study can make a valuable contribution to flock health and safety of food as well. It could be considered a strong candidate for supporting broiler performance. Regarding our obtained results; it could be assumed that it could help in sustaining GIT integrity damaged by *C. perfringins* infection and could be a tool for impedance of NE in broiler chickens.

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Conflict of Interest

The authors declare that they have no conflict of interests.

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Table 1. Experimental design.

Group No.	No. of birds*	*Clostridium perfringens* challenge
1-A	60	+
2-B	60	+
3PC	60	+
4-NC	60	-

*Each group assigned into 6 replicates.

Table 2. Results of zootechnical performance.

BW (g)						
Groups	**Age**					
	1 day	**7 days**	**14 days**	**21 days**	**28 days**	**35 days**
1-A	42.55±0.38	178.13±2.32	463.97±8.27	898.68±16.76[a]	1533.24±25.01[a]	2274.42±37.61[a]
2-B	42.85±0.39	180.00±1.17	463.13±9.80	915.26±18.11[a]	1506.57±32.72[a]	2280.63±35.70[a]
3PC	41.75±0.42	176.76±2.38	453.35±3.09	833.76±11.08[*b]	1360.33±11.01[b]	1991.48±20.78[b]
4-NC	41.99±0.40	178.66±1.34	459.90±5.13	877.69±9.17[a]	1487.20±15.75[a]	2217.70±25.26[a]
Probability	0.4174	0.7845	0.5245	0.0001	0.0001	0.0001
BWG (g)						
	1 – 7 days	**7 – 14 days**	**14 – 21 days**	**21 – 28 days**	**28 – 35 days**	1-35 days
1-A	135.58±2.25	286.28±9.20	433.16±17.79[a]	623.97±28.55[a]	725.38±46.35[a]	2231.35±37.63[a]
2-B	137.15±1.22	283.13±10.05	450.00±17.28[a]	591.43±38.43[ab]	702.81±42.61[a]	2237.79±35.64[a]
3PC	135.00±2.40	276.40±4.04	380.58±11.61[*b]	527.12±15.96[b]	624.13±23.58[b]	1949.64±20.58[b]
4-NC	136.68±1.38	281.13±5.16	417.45±10.62[ab]	609.54±18.68[a]	731.00±28.83[a]	2175.65±25.29[a]
Probability	0.8907	0.7352	0.0037	0.0044	0.0243	0.0001
FI (g/bird/day)						
	1 – 7 days	**7 – 14 days**	**14 – 21 days**	**21 – 28 days**	**28 – 35 days**	1-35 days
1-A	28.08±1.23	61.83±1.33	94.08±1.64	135.31±3.20	178.44±7.42	3484.18±55.34
2-B	28.55±0.27	61.42±2.26	91.771.86	137.50±1.97	181.89±5.82	3507.91±41.78
3PC	28.17±0.33	62.31±0.45	90.45±2.52	140.52±2.47	187.27±4.73	3561.03±41.57
4-NC	28.94±0.91	62.77±0.78	91.18±2.38	134.90±2.02	183.35±1.84	3507.96±35.31
Probability	0.9448	0.0942	0.7110	0.1459	0.6154	0.3058
FCR (g feed/g live body weight)						
	1 – 7 days	**1 – 14 days**	**1 – 21 days**	**1 – 28 days**	**1 – 35 days**	
1-A	1.120±0.041	1.356±0.019	1.433±0.037[ab]	1.458±0.032[b]	1.532±0033[b]	
2-B	1.140±0.028	1.360±0.039	1.390±0.021[b]	1.483±0.021[b]	1.538±0.025[b]	
3PC	1.121±0.027	1.397±0.014	1.521±0.030[a*]	1.654±0.018[a]	1.790±0.032[a]	
4-NC	1.135±0.040	1.400±0.041	1.459±0.022[ab]	1.497±0.024[b]	1.583±0.032[b]	
Probability	0.8411	0.0942	0.0321	0.0001	0.0001	

Mortality Rate (%)						
	1 – 7 days	**7 – 14 days**	**14 – 21 days**	**21 – 28 days**	**28 – 35 days**	**1 – 35 days**
1-A	1.25±1.25	0.00±0.00	1.25±1.25	1.25±1.25^{b}	1.25±1.25	5.00±1.34^{b}
2-B	1.25±1.25	0.00±0.00	1.25±1.25	1.25±1.25^{b}	1.25±1.25	5.00±1.35^{b}
3PC	0.83±0.83	0.83±0.83	0.00±0.00	5.00±0.00^{a*}	4.17±1.54	10.83±2.01^{a}
4-NC	0.83±0.83	0.83±0.83	0.83±0.83	0.83±0.83^{b}	1.67±1.05	5.00±1.29^{b}
Probability	0.9662	0.9881	0.7332	0.0369	0.2193	0.0301

* Means with different, superscripts, within age, are significantly different ($P \leq 0.05$).

Table 3. Production Number.

Trait **Treatment**	**Production Number**
1-A	402.97±24.32^{a}
2-B	402.49±22.09^{a}
3PC	284.50±12.70^{b*}
4-NC	382.11±16.99^{a}
Probability	0.0001

* Means with different, superscripts, within age, are significantly different ($P \leq 0.05$).

Table 4. Macroscopic lesion score organs of infected treated chicken groups..

Trait **Treatment**	**Age**	
	21 days (7 days PI)	**28 days (14 days PI)**
1-A	1.92±0.36^{bc}	1.17±0.39^{b}
2-B	1.00±0.33^{cd}	0.92±0.26^{b}
3PC	3.25±0.49^{a}	3.00±0.37^{a}
Probability	0.0001	0.0002

* Means with different, superscripts, within age and trait, are significantly different ($P \leq 0.05$).

Table 5: Microscopic lesion score in different organs of infected treated chicken groups.

Trait **Lesion**	**Infected groups**		
	A	**B**	**PC**
Villous fusion	1.78^{a}±0.24	2.20^{a}±0.35	3.66^{b}±0.23
Dilated blood vessels	1.79^{a}±0.27	1.80^{a}±0.32	2.1544^{a}±0.41
Capillary hemorrhages	1.36^{a}±0.34	1.10^{a}±0.45	1.22^{a}±0.14
Mucosal necrosis	2.42^{a}±0.25	3.10^{a}±0.23	3.66^{b}±0.23
Sum. Of NE lesions	**7.33^{a}±5.29**	**7.80^{a}±5.81**	**11.00^{b}±0.76**
Cecal tonsils depletion	0.42^{a}±0.20	1.33^{b}±0.33	2.50^{c}±0.28
Thymic depletion	0.83^{a}±0.30	1.00^{a}±4.33	1.50^{a}±0.00
Bursal depletion	1.85^{a}±0.40	1.87^{a}±0.29	2.60^{a}±0.284
Splenic depletion	1.42^{a}±0.70	2.00^{a} ±0.70	3.00^{b}±0.28

* Means with different, superscripts, within age, are significantly different ($P \leq 0.05$).

Table 6: Histomorphometric analysis of different intestinal segments in infected treated chicken groups ($p \leq 0.05$).

Chicken Groups	**Intestinal segments**					
	Duodenum			**Jejunum**		
	Villi hight (µm)	**Crypt depth (µm)**	**VH/CD ratio**	**Villi hight (µm)**	**Crypt depth (µm)**	**VH/CD ratio**
1-A	635.87^{c}±11.53	56.36^{b}±2.75	11.99^{b}±0.79	429.55^{b}±9.43	51.35^{a}±3.07	8.89^{b}±7.89
2-B	534.59^{b}± 1.11	46.39^{a}±2.17	12.05^{b}±0.70	396.17^{a}±13.54	48.74^{a}±2.09	8.37^{b}±7.49
3PC	470.18^{a}±11.95	57.55^{b}±2.84	8.733^{a}±0.46	388.4^{a}±12.50	59.74^{b}±2.41	6.60^{a}±0.46

* Means with different, superscripts, within age, are significantly different ($P \leq 0.05$).

Table 7. Enumeration of *C. perfringens* after experimental induction of NE [Log (CFU) values].

Trait **Treatment**	**Age**			
	21 days (7 days PI)		**28 days (14 days PI)**	
	Intestine	**Caecum**	**Intestine**	**Intestine**
1-A	64	57	110	167
2-B	4	74	110	116
3PC	133	451	304	252

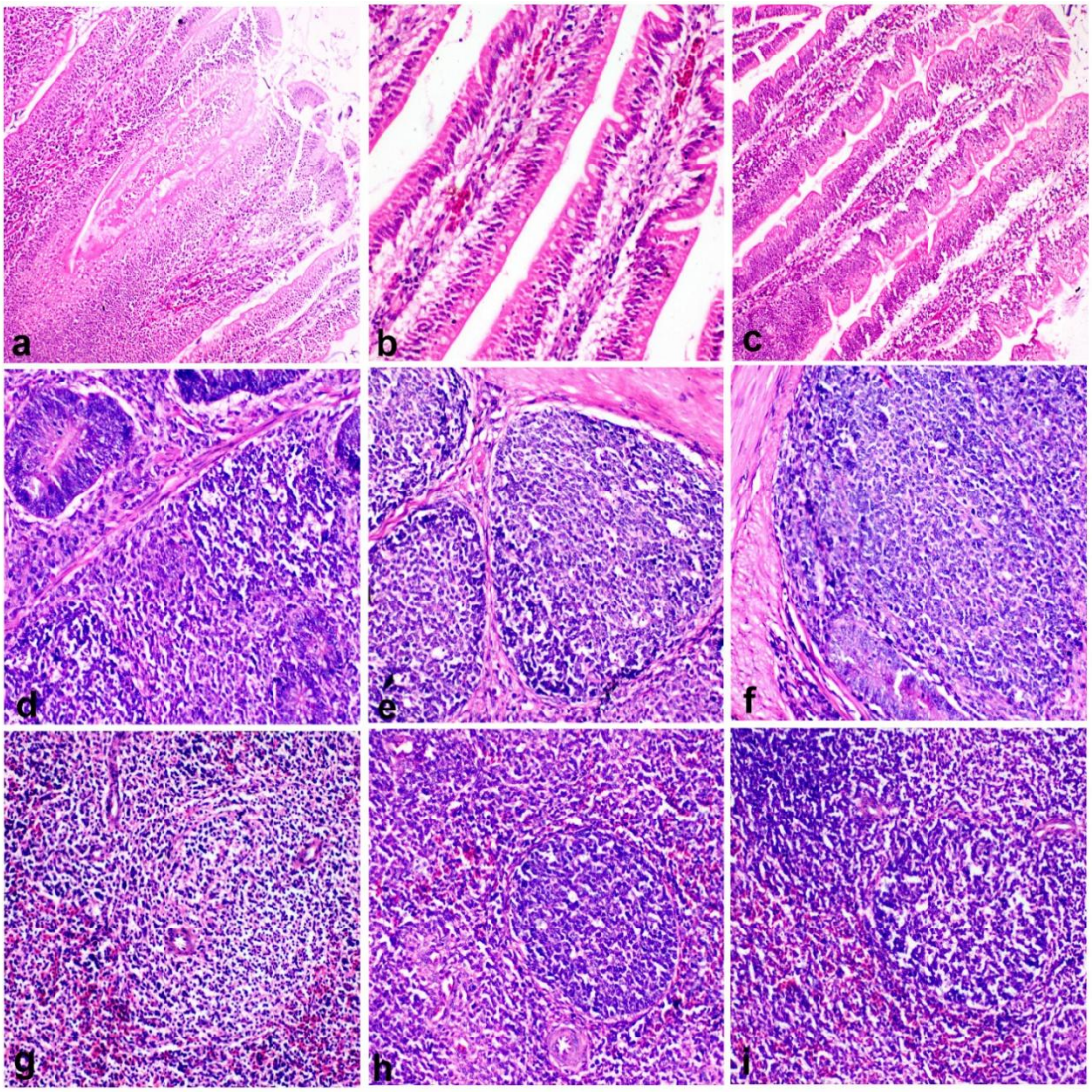

Fig.1: Histopathological sections of experimentally induced NE in broiler chickens

a-c)Duodenum:
a)Duodenum of control positive group showing massive villous fusion and necrosis of mucosal epithelium, capillary hemorrhages and marked inflammatory cells infiltration b)Duodenum from group A showing mild congestion of capillaries associated with mucosal edema and apparently intact mucosal epithelium.
c)Duodenum from group B showing infiltration of inflammatory cells in lamina propria with mild blunting of villi tips.
d-f)Cecal tonsils:
d)Control positive group showing atrophy and depletion of cecal tonsils.
e)Group A showing hyperplastic proliferation of cecal tonsils and individual depletion of lymphocytes.
f)Group B showing moderate increase in tonsilar size with mild depletion.

g-i) spleen:
g)Splenic follicle from control positive group showing severe depletion of lymphocytes
h)Spleen of group A showing individual mild lymphocytic depletion.
i)Spleen of group B showing moderate lymphocytic depletion.

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