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Evaluation of the protective potentials of *Clostridium perfringens* necrotic enteritis NetB toxin-based vaccine in Broiler Chickens

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Clostridium perfringens induced necrotic enteritis in poultry and the related subclinical diseases have become economically significant problems for the broiler industry. Immunity to necrotic enteritis is not yet fully characterized. However, previous reports proved the immunoprotective potentials of *C.perfringens* vaccines. The present study was planned to evaluate in chickens the immunizing potentials of formalized *C.perfringens* type A alpha and netB toxins prepared from the culture supernatant of the used strains. Two forms of *C.perfringens* vaccine preparations were developed, tested and their immunizing efficacy was compared. The first vaccine formulation composed of formalized *C.perfringens* type A Net B^{-ve} culture supernatant. The 2nd vaccine formulation was prepared from formalized *C.perfringens* type A $NetB^{+ve}$ strain. The efficacy of two vaccine formulations in protection of broiler chickens against necrotic enteritis was investigated. Both vaccines were injected in two groups of chickens and determined as group 1 and 2 respectively. In addition, two groups (3and4) were considered as control positive and negative respectively. The birds were immunized with 1 ml of toxoids subcutaneously on days 3,7 and 14 post hatching followed by challeng with virulent NetB positive *C.perfringens* type A strain. The developed immune responses against the prepared vaccines in the immunized chickens were evaluated using ELISA, real time PCR (RT-PCR).The *C.perfringens* intestinal load and the antibody levels were measured to determine protection efficacy after challenge with virulent *C.perfringens* isolates. The obtained results revealed that, birds immunized with NetB toxin based vaccine were significantly protected against necrotic enteritis when challenged with a virulent strain of *C.perfringens*. Higher NetB-specific antibody titers were detected in birds immunized with NetB toxin positive vaccines (2nd group). Moreover, significant decrease in the intestinal *C.perfringens* microbial load in these birds was noted compared to group immunized by the NetB negative vaccine formulation (group No.1). In conclusion, NerB toxin plays a major role in causing necrotic enteritis in chickens and vaccine against this disease better to be produced from *C.perfringens* strain posses NerB toxin.

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INRODUCTION:

Necrotic enteritis (NE) is one of the most important disease in poultry and is very expensive for industry worldwide **(Bahram et al. 2012).**

Necrotic enteritis is primarily caused by *C.perfringens* type A and to lesser extent type C strains, producing both alpha and beta toxins (**Van Immerseel et al. 2009)**. It was first described in 1961 **(Parish 1961)** and has since been found in all poultry producing countries.

*C.perfringens*is a Gram-positive, anaerobic, fermentative spore-forming bacillus, which classified into five types (A, B, C, D and E) according to the production of the four major toxins (alpha α, Beta β, epsilon e and iotai). Alpha toxins are produced by all strains and involved in disease pathogenesis (**Cato et al. 1986).** Toxins B, NetB, were recently proposed as a new key virulence factor for the development of NE in broilers **(Abildgaard et al. 2010).**

Although it is clear that *C.perfringens* is etiologic agent of NE, a wide range of host and pathogen factors can influence the severity of the disease. These factors include the nature of the feedstuff, coinfection with various *Eimeria* species and the molecular make up of *C.perfringens* in gut **(Shojadoost et al. 2012).** The molecular basis of virulence of *C.perfringens* associated with NE is still being investigated.

However, almost all *C.perfringens* isolates from cases of NE possess the *net*B gene which encodes necrotic enteritis toxin B (NetB), a β-pore forming toxin **(Martin and Smyth 2009** and **Savva et al. 2013).**

Recently, a novel toxin has been described that is associated with necrotic enteritis in broilers**.** The *C.perfringens* necrotic enteritis B-like toxin (NetB) is a member of the b-barrel pore-forming toxin family. The toxin causes cell rounding and lysis in a chicken Leghorn male hepatoma cell line, and it was shown that NetB forms plasma membrane pores with an estimated pore diameter of 1.6 to 1.8 nm **(Keyburn et al. 2008).**

Bacterial Pore-forming toxins (PFTs) are important virulence factors and in general are produced as soluble precursors that bind to the host cell membrane and assemble as oligomers that subsequently form trans membrane pores **(Feil et al. 2010).**

The genetic studies, biochemical and biophysical analysis, strain surveys and vaccination studies have revealed the importance of NetB toxin in the pathogenesis of necrotic enteritis. NetB toxin fulfils molecular Koch's postulates **(Falkow, 1988)** and therefore has been demonstrated to be an essential virulence factor in the development of necrotic enteritis **(Keyburn et al. 2008).**

The immunization with either crude toxoids **(Saleh et al. 2011)** or culture supernatants **(Lanckrier et al. 2010)** can provide immune response but incomplete protection against experimental NE. Although these vaccines are simple to prepare, they suffer from the limitation that it is difficult to configure them for non - invasive dosing for example by oral delivery.

Belote et al. (2018) experimentally infected broilers with *Eimeria* and *C.perfringens* and exmined microscopically the intestinal lesions at early stages of necrotic enteritis. Strong inflammatory reactions were recorded. The lamina propria was hypremic and infiltrated with numerous inflammatory cells.

The objective of the present study was to determine the role of Net-B producing strains of *C.perfringens* in enhancing the susceptibility of avian species to necrotic enteritis and this will be achieved by:

Molecular characterization of Net-B gene in correlation to Necrotic enteritis.

Trial for immunization of chickens with the toxoid of Net-B producing *C.perfringens* strains and evaluation of the immune response formed.

MATERIALS AND METHODS:

Bacterial Strains

Two *C. perfringens* type A strains (previously isolated from cases of necrotic enteritis, **Abo El-yazeed et al. 2018**) were used. Both strains belonging to Alpha toxin gene were further analyzed for the presence of both virulence genes *net*B and *tpe*L by multiplex PCR. The identified strains were as follows: (Type A – NetB $^{-ve}$) and (Type A – NetB^{+ve}). A NetB positive strains of *C.perfringens* were used in challenge test. The stains were grown anaerobically at 37°C in brain heart infusion (BHI) broth (Oxoid, UK) and used for supernatant production.

Formalized toxoid Preparation: (Osman et al. 2010)

All bacterial strains of *C.perfringens* were first grown on sheep blood agar (SBA) then passed through broth culture. A single colony from SBA was inoculated into 20 mL of Trypticase-glucose yeast extract (TGY) medium and grown overnight at 37°C in an anaerobic condition. Ten ml of the resultant culture were inoculated into one liter of TPG medium and grown at 37°C to an OD 600 nm of 0.8 – 1.0. The culture was centrifuged at 6000 g for 10 min at 4°C, filtered through 0.45 µm membrane and concentrated by dialysis against a 20-kDa polyethyleneglycol (PEG, Sigma Aldrich, St. Louis MO, USA) solution, followed by further concentration and desalting. Protein concentrations from the supernatants were determined by the Bradford method with a commercially available Bradford reagent (Spin React, Spain). The concentrated supernatant samples were diluted in PBS to a final concentration of 140 µg/500 µl (Dose).

0.6 % Formalin was added to the supernatant. A vaccine-grade mineral oil, as adjuvant and Span 80 as an emulsifier were added.

Animal model development: Lee et al. 2011

A total of 100 chickens of one day old (Hubard) were used in the experimental study and housed with standard protocol for a period of one month.

The birds were distributed into 4 groups each of 25birds.Groups (1) and (2) were vaccinated with formalized preparation of (Type A – NetB $^{-ve}$and Type A –NetB^{+ve}) respectively.

On day 3, 7 and 14 post-hatching (Table1), subcutaneous injection in the neck with 1 ml of supernatant containing 140 µg total protein. At the same time, one control positive group (3) was taken unvaccinated and challenged. Another control negative group (4) was left unvaccinated and unchallenged. Nobilis Gumboro D78 vaccine (Schering-Plough Animal Health, Belgium) was given as eye drop water on day21in groups (1,2 and3) as immunosuppressive agent

Challenge test:

It was done using virulent *C.perfringens* type A Net B +ve strain isolated from diseased broiler and used for challenge of experimental birds on day 28 of the experiment as described by Fasina et al., 2016. Birds were challenged with an inoculums of 1 ml of 10^8 cfu/ml *C.perfringens* orally (**Keyburn et al. 2013**). Blood and intestinal samples were collected according to the table (1).

Table 1: *Experimental animal groups, vaccination, challenge times and type of samples collected:*

Group no.	Vaccine	Vaccination AGE (Days)	Challenge Time (Days)	Sampling Time (Days post vaccination)	
				Serum	Intestine
1	Toxoid A	3 , 7 ,14	28	7 ,14, 37,44	7 ,14, 37,44
2	Toxoid NetB-(A)	3 , 7 ,14	28	7 ,14, 37,44	7 ,14, 37,44
3	+ve Control	Unvaccinated	28	37,44	37,44
4	-ve Control	Unvaccinated	No challenge	37,44	37,44

Keyburn *et al.,* 201

Table 2: Sequence of primers used for real-time quantitative RT-PCR[1]

Target gene	**Primer sequence (5′-3′)**		**PCR product size (base pairs)**	**Accession number**
	Forward primer	Reverse primer		
GAPDH	GGTGGTGCTAAGCGT GTTAT	ACCTCTGTCATCTCTCC ACA	264	K01458
IL-4	ACCCAGGGCATCCAG AAG	CAGTGCCGGCAAGAAG TT	258	AJ621735
IL-10	CGGGAGCTGAGGGTG AA	GTGAAGAAGCGGTGAC AGC	272	AJ621614

Hong *etal.,* 2006
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
IL: interlukein

C.perfringens **intestinal count (Fasina et al. 2016):**
Intestinal *C.perfringens* was enumerated in the intestinal content at 3, 7, 37and 44 day old onto TDC agar according to **Fasina et al. 2016**.

Dectetion of small intestine secretory immunoglobulin A (SIgA) ():
It was done using ELISA kit (NOVA, Beijing, China) kit according to the method described by **Gutzeit et al. 2014.**

Quantitative Real-Time RT-PCR:(Livak and Schmittgen, 2001)
The oligonucleotide primer sequences used for quantitative real-time PCR (qRT-PCR) were shown in Table (2). The various cytokines whose differential expression was evaluated in the intestine include interleukin IL4, IL10. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference gene. Amplification and detection were carried out using SuperScript® III Platinum® SYBR® Green One-Step qRT-PCR Kit, StepOnePlus™ Real-Time PCR System (Applied Biosystems). Each sample was analyzed in duplicate with the internal positive control on each plate (GAPDH). The reaction was performed according to the kit manufacturer instructions.

Statistical Analysis (Rajput et al. 2017):
Collected data of experiment were analyzed using SPSS (version 20) for mean ± slandered deviation and were statistically analyzed by

conducting analysisof variance (ANOVA) test by for least significance difference (LSD) for determination of the significance between means at $p < 0.05$.

RESULTS:

Effect of Toxoid on intestinal *C.perfringens* microbial load:

The intestinal *C.perfringens* concentrations are presented in Table(3). Before challenge (baseline) and post-challenge, *C.perfringens* concentration was affected in all vaccinated group compared with control positive. By day 7 and 14 post-challenge, the level of *C.perfringens* in the positive control treatment is higher than that of its corresponding vaccinated groups and logically than the unvaccinated group. Group2 vaccinated with alpha and NetB Toxoid showed significant decrease in the microbial load even after challenge (3.18, 3.00, 1.98, and 0.90) compared with group 1 with alpha only.

Table 3: Effect of Toxoid on intestinal C.perfringens microbial load (log_{10} cfu /g):

Vaccine	Baseline Pre challenge		Post challenge	
	3 Day old	10 Day old	37 Day old	44 Day old
Toxoid(α) (group1)	3.36	3.18	1.70	1.48
Toxoid (NetB+ α) (group 2)	3.18	3.00	1.98	0.90
+ve Control (group 3)	3.30	3.18	5.26	6.40
-ve Control (group 4)	3.28	3.26	3.32	3.38

The results are represented as Means

Effect of toxoid on immunoglobulin A in chicken sera and intestinal secretory immunoglobulin A in intestinal washings (SIgA):

Antibody titers in intestinal mucosa:

As shown in Table (4), the concentration of secretory immunoglobulin A was evaluated in all experimented groups at 28 days, 37 day and 44 day of age by ELISA and fixed optical density (450nm) was applied. The level of SIgA was high in group (2) vaccinated with toxoid of alpha and NetB compared with group (1) as well as the control groups. Immunoglobulin level in group (2) was gradually increase at 28, 37 and 44-day old to reach (9.43 ±0.22, 12.73 ±0.33, 18.27±0.02 ng/ml)respectively , moreover, group (1) vaccinated with toxoid A, the level were (8.13 ± 0.13, 9.53 ± 0.33, 10.5 ± 0.23 ng/ml) respectively.

Table 4: Effect of toxoids administration on secretory immunoglobulin (SIgA) concentration expressed as ng/ml:

Age	α-Toxoid	α and NetB Toxoid	+ve Control	-ve Control
28 days	8.13 ± 0.13	9.43 ±0.22	5.32 ± 0.33	1.45 ±0.25
37 days	9.53 ± 0.33	12.73 ±0.33	4.49 ± 0.43	2.12 ±0.26
44 days	10.5 ± 0.23	18.27±0.02	5.6 ± 0.10	1.05 ±0.18

The results are represented as Means± SD (n = 5). P value ≤ 0.05: Significant; Value more than P-value: Non – significant

Detection of specific immunoglobulin against *C.perfringens* in serum:

All the supernatant proteins were used to immunize birds in the vaccination trial described, produce significant antigen-specific serum antibody titers (Table 5) in comparison to the preimmunization titers. Birds immunized triplicate with three supernatant filtrates.

Sera collected from the blood samples of birds in all groups at 7,14,28,37 and 44day old, were stored at -08ºC until the end of experiments, and tested for specific immunoglobulins using a single in house ELISA kit. Table (5) shows the results of serum antibody titres expressed as optical density values in different tested groups. It was noted the increasing of serum antibody titre at 37 and 44 days old in immunized groups comparing with the control –ve group. The highest antibody titer was recorded in NetB + ve based toxoid immunized group 2 in comparison to Alpha toxin Net B – ve toxoid (group 1). The increasing levels of serum IgA in group 2 based on NetB toxin reached to (2.097at day 37) and (2.103at day 44) however, group 1 showed lower titers at the same ages (1.838, 1.924).

Table 5: Detection of specific immunoglobulin against *C.perfringens* in serum of chicks vaccinated and challenged with *C.perfringens*:

Age	Toxoid A	Toxoid NetB-(A)	+ve Control	-ve Control
7 days	0.454	0.234	0.867	0.0181
14 days	0.716	0.851	0.543	0.234
28 days	1.759	1.967	0.835	0.303
37 days	1.838	2.097	1.321	0.301
44 days	1.924	2.103	0.871	0.463

The results expressed in ELISA OD values

Effect of application of toxoid on different cytokines:

The current study measured the effect of immunization with toxoids on cytokines secretion of IL-4, IL-10 by StepOnePlus™ Real-Time PCR System (Applied Biosystems).

Measurment of interleukin-4 secretion in broiler intestine:

Relative expression of IL-4 in different groups was showed in Figure (1). The results revealed that birds toxoid immunized had extremely up regulation of IL-4 gene expression at all ages while, the highest up regulation was recorded at 37 day of age as compared with

control, While at 44 day of age, birds immunized with NetB toxoid showed high up regulation which is 2.8 and compared with control (0.4) and alpha toxoid group(1.8). Also, toxoid Net B group had a steady up regulated level of IL-4 as compared with other immunized groups. The results are represented as Means of 2ΔΔCt (n = 5) using Step OnePlus™ Real-Time PCR System (Applied Bio-systems) and the comparative CT method was used to determine changes in gene expression calculated as 2ΔΔCt.

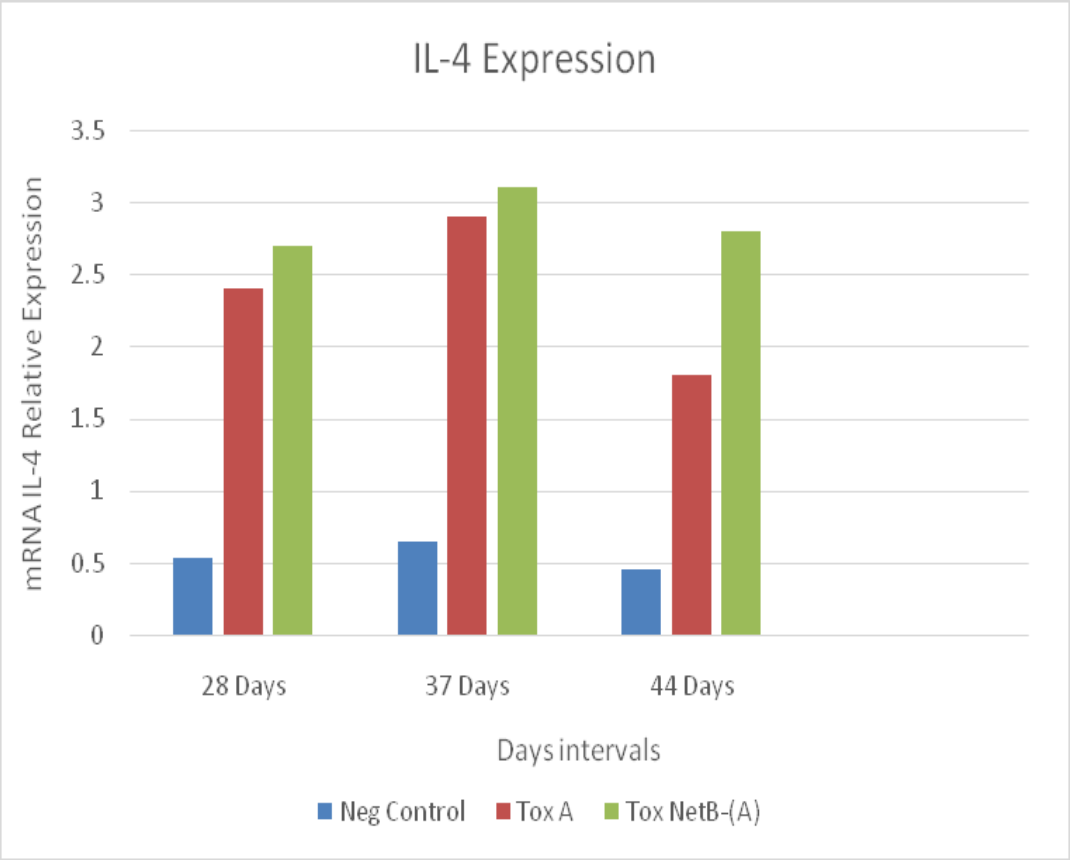

Fig 1. The mRNA expression levels of IL-4 , observed in intestine of negative control (C), groups and other toxoid immunized groups at different time interva

Effect of toxoid immunization on interleukin-10 secretion in broiler intestine:

Relative expression of IL-10 in different groups is showed in Figure (2). The results of current study revealed that birds toxoid immunized had an up regulation in IL-10 gene expression at 28 and 37 day of age while, the highest up regulation was at 44 day of age as compared with control. The results are represented as Means of 2ΔΔCt (n = 3) using Step OnePlus™ Real-Time PCR System (Applied Bio-systems) and the comparative CT method was used to determine changes in gene expression calculated as 2ΔΔCt.

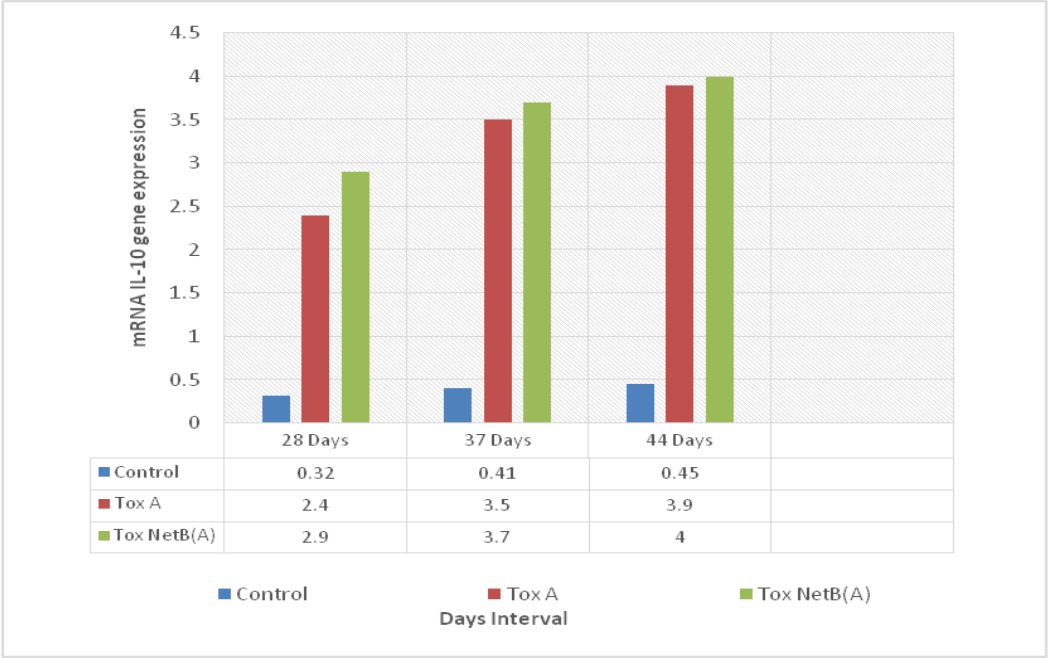

Fig 2. The mRNA expression levels of IL-10 , observed in intestine of negative control (C), groups and other toxoid immunized groups at different time interval.

DISCUSSION

In broilers, acute necrotic enteritis is often evidenced as a sudden increase in flock mortality during the last weeks of the rearing period (5^{th}-6^{th} week) and at necropsy, large necrotic foci and extensive necrosis are found at the mucosal surface of the gut (**Fernandes da costa et al. 2013).** This study has evaluated whether *net*B, a major virulence factor in NE, is an effective protective antigen when used as NetB positive *C.perfringens* type A and C crude toxoid vaccine.

The study was carried out using 100 chicks distributed in four groups (n=25/group). Groups 1and 2 were received subcutaneous injection of 1 ml of type A and NetB positive type A as a 1^{st} dose at 3 days old then two pooster doses were given at 7^{th} and 14^{th} day. Groups 3 and 4 are positive and negative groups respectively.

Challenge with direct oral gavage with 1 ml of 1 x10^{8} cfu/ml of *C.perfringens* culture was carried out at day 28^{th} of age. Serum and intestinal samples were taken after each treatment for immunological examination.

Count of intestinal load of *C.perfringens* in each group was done to investigate the effect of toxoid injection on the population of this microbe in ration to the control –ve group. Table (3) revealed that group 2 taken toxoid of *C.perfringens* type A having alpha and NetB toxins harboured the least number of *C.perfringens* (3.18, 3.00, 1.98, and 0.90 cfu/gm) in relation to group 1 receiving Alpha toxoid alone . This result is nearly agreed with that obtained by **Saleh and Mosaad (2011)** who evaluated the efficacy and safety of three vaccination regimes of *C.perfringens*; type A, C and combined A and C toxoids based on their clinical signs and immunological effects. Their results revealed that immunization of broilers with *C.perfringens* type A, having netB toxoids resulted in a significance decrease in numbers of chickens with intestinal lesions.

Moreover, **(Keyburn et al. 2013**) estimated that vaccination against NE disease is proposed to provide an alternative treatment for NE in poultry especially when more convincing evidences were revealed that the toxin NetB is responsible for the disease. Vaccination strategies have been put forward for the control of NE mainly in broiler chickens. Prior to the discovery of NetB, the earlier vaccination focused on toxins that may not be associated to NE

largely, for example, α-toxin. Thus, the vaccines developed only had limited success in controlling NE **(Cooper et al., 2010; Hoang et al. 2008; Zekarias et al. 2008).** Partial protective effect of α-toxin based vaccine may be due to the association of α-toxin protein with cell membrane that can have immune interaction to perform such protection **(Keyburn et al. 2013).**

The most important step forward to developing vaccines to immunize the birds against NE occurred following the discovery of NetB toxin **(Keyburn *et al.*, 2008).** A recombinant NetB *C.perfringens* (rNetB) was constructed and attenuated as a vaccine by **(Keyburn et al. 2013).** The birds immunized with rNetB were significantly protected against NE challenged with a mild dose of virulent bacteria, while the effectiveness of the vaccination was not so clear when a more robust challenge was performed. Alternatively, when the birds were immunized with a combination of rNetB, bacterin and cell free toxoid, significant protection against moderate and severe challenge was observed. It was suggested that in vitro levels of NetB produced by virulent *C.perfringens* isolates were too low to produce strong immune response in the birds and thus the combined vaccination of birds with rNetB and other cellular or cell-free antigens may be necessary **(Jang et al. 2012).**

The importance of immune modulation at the gastrointestinal level can be understood easily, considering that approximately 70% of the entire immune system is found in this site housed in structures buried in the delicate hair-like villi, which cover the intestinal wall. These structures, called Peyer's Patches, contain a variety of immune cells including B cells, T cells, macrophages and dendritic cells, and are involved in both innate and adaptive immune function. **(Weiner et al. 2000).**

Mucosal immunity is an important part of humeral immunity. As previously mentioned, GIT included about approximately 70% of the entire immune system and in the lamina propria there are about 80% of all plasma cells responsible for IgA antibody production **(Weiner**, **2000**; **Faria and Weiner**, **2005).**IgA released in intestinal lumen and mixed with the normal flora to inhibit pathogen colonization and allows the establishment of the normal flora **(Bos et al. 2001).** It well known that Intestinal commensal bacteria are major participant in the synthesis of mucosal IgA as IgA is absent in the intestine of germ free animals **(Stoel et al. 2005).** Therefore, it is important to modulate the intestinal bacterial ecosystem.

Secretory IgA is the principle weapon protecting animals from pathogens and toxins that might otherwise penetrate mucosal surfaces. These antibodies are key components of the mucosal mucus and other body secretions such as saliva and tears. The majority of the body's entire pool of activated B-cells is located near the mucosa and exocrine glands. Both in the lamina propria of the gut and the exocrine glands, about 80 % of all B-cells and plasma cells present produce polymeric IgA. This polymeric IgA is converted to secretory IgA as it is introduced into the mucus and body secretions via the poly- Ig receptor (pIgR) **Mak and Saunders (2006).**

This study also discussed the effect of different types of C*.perfringens* toxoid administration on intestinal secretory immunoglobulin A (SIgA) at 28^{th}, 37^{th} and 44^{th} day using sandwich enzyme-linked immunosorbent assay (ELISA). The results showed in table (5) revealed ($p \leq 0.001$) significance increase in SIgA in thevaccinated groups especially group 2 vaccinated with alpha and NetB toxoid compared with control negative group. This may explain the low level of intestinal damage and average lesion score compared with control positive group.

The present trail proved that *Clostridium perfringens* could affect secretion of SIgA and also delay decline of maternal immunoglobulin and keep constant level of immunoglobulin to protect the bird from invading by pathogen. This implies that toxoid could stimulate the humoral immune system to produce more localized antibodies than systematic humeral immunity. Increased antibodies cover the surface of intestinal mucosa and can protect villi from excessive immune mediated damage. As the

intestine is one of the organs subject to contact with exotic pathogens and toxins. Secretory IgA can function in eliminating antigens from tissues via immune complex formation **(Robinson *et al.,* 2001**) and intraepithelial neutralization of virus replication **(Fujioka et al. 1998).**

Furthermore, ELISA detected specific immunoglobulins against *C.perfringens* toxoid in serum. As well as, this study was conducted to test Net B positive *C.perfringens* type A toxoid for its potential to induce an immune response able to protect chicken against the subclinical form of necrotic enteritis comparing with those obtained by alpha toxoid only. As shown in table (5). Serum of four groups was tested for antibodies against *C.perfringens* using ELISA test, two groups were immunized by alphaand alpha+NetB toxoid respectively. A control +ve group was injected with *C.perfringens* type A, control –ve group was kept without injection. The antibody titres showed an increasing value in immunized groups at 7, 14, 28, 37 and 44 day olds. In addition, antibody levels to group 2 were significantly higher on day 37 and 44-day old (2.097, 2.103) respectively comparing with group (1) (1.838, 1.924) at the same period respectively.

The interleukin 4 (IL-4) is a cytokine that induced differentiation of naïve helper T cells (Tho cells) to Th2 cells. Upon activation by IL-4, Th2 cells subsequently produce additional IL-4. It also has many biological roles, including the stimulation of activated B-cell and T-cell proliferation, and the differentiation of B-cells into plasma cells. It is a key regulator in humeral and adaptive immunity. **Hershey et al. (1997) and Legard and predersen (2019)** stated that interleukin-4 (IL-4) is complex glycoprotein produced mostly by mast cell, basophils, and a subset of activated T-cell, eosinophils and neutrophils. Moreover, **Keegan, (1998)** showed that IL-4 elicits a diverse array of biological responses. These functions range from the regulation of helper T- cell phenotype and the production of immunoglobulin E (IgE) by B lymphocytes to the regulation of the adhesive properties of endothelial cells and the regulation of ion secretion by intestinal epithelial cells. In addition, **(Fasina and Lillehoj, 2019)** studied the expression levels of selected cytokines genes in the intestine and cecal tonsils of *C.perfringens* challenged broiler chickens. Using real- time PCR analysis, they determined the the expression levels of interleukin 1β (IL-1β) γ IFN, IL-2, IL-13, IL-17 and IL-10. They concluded that *C.perfringens* infection induced inflammatory response, in the intestine of broiler chickens and the mechanisms of inflammation are probably mediated via Th2 and Th17 cells. The results revealed that birds toxoid immunized had extremely up regulation of IL-4 gene expression at all ages while , the highest up regulation was recorded at 37 day of age as compared with control. While, at 44 day of age, birds immunized with alpha and net B toxoid show high up regulation which is 2.8 compared with control and other groups (Fig.1).

Interleukin 10 (IL-10) is a cytokine with potent anti-inflammatory properties that plays a central role in limiting host immune response to pathogens, thereby preventing damage to the host and maintaining normal tissue homeostasis. Immune response to pathogens involves the rapid activation of pro-inflammatory cytokines that serve to initiate host defence against microbial invasion. However, excess inflammation can give rise to systemic metabolic and hemodynamic disturbances harmful to the host. **(Sellon et al. 1998; O´ Garra et al. 2008).** Furthermore, relative expression of IL-10 in different groups was investigated and the results revealed that birds toxoid immunized had an up regulation in IL-10 gene expression at day 28 and 37 of age, while the highest up regulation was at day 44 of age (4.0) compared with control (Fig. 2). **Park et al. (2008)** discussed the intestinal expression of a panel of cytokine and chemokine genes following *Eimeria maxima* and *Clostridium perfringens* coinfection. They found that IFN-γ, IFN-α, IL-2, IL-12, IL-13, IL-17 and TGF-β4 were repressed, whereas, IL-8, IL-10, IL-15 LITAF were increased during coinfection compared with challenge by EM or *C.perfringens* alone. They concluded that EM and *C.perfringens* coinfection acts synergistically to create a more sever disease phenotype leading to an altered cytokine/ chemokine response than that

produced by infection with the individual pathogens.

CONCLUSSION

The pore forming toxin NetB is a key virulence factor in *C.perfringens* strains that cause necrotic enteritis disease in chickens.

NetB toxin is produced by most strains isolated from necrotic lesions, but is less commonly found in *C.perfringens* isolates from healthy birds.

The obtained results have shown that NetB based toxoid immunization can significantly protect birds against necrotic enteritis disease.

Necrotic enteritis vaccines better to be made from *C.perfringens* type A strains posses NetB toxin to induce good immunity for chickens against the disease.

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