Egyptian Journal of Veterinary Sciences

https://ejvs.journals.ekb.eg/

Evaluation of Immunization with Native NetB in Addition to Reactive Proteins Against Necrotic Enteritis in Broiler Chickens

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Abstract

CLOSTRIDIUM PERFRINGENS (*CP*) induces necrotic enteritis (NE) and has re-emerged as one of the deadliest diseases in poultry industry after banning antibiotics in poultry feed. The study aimed to prepare and assess the protective potential of four native *CP* type A vaccines against necrotic enteritis (NE) in commercial broiler chickens; three are *NetB* +ve including; formalized toxoid (FTNB), sonicated supernatant (SSNB), boiled bacterin (BBNB) and the fourth is *NetB* -ve (formalized toxoid (FT). SSNB, intracytoplasmic proteins; perfringolysin O, glyceraldehyde-3-phosphate dehydrogenase (GPD), and fructose 1,6-biphosphate aldolase(FBA) while BBNB, cell wall proteins; hypothetical proteins (HPs), pyruvate ferredoxin oxidoreductase (PFOR), elongation factor-Tu (EF-TU), and collagen adhesion protein (CnaA). SSNB and BBNB proteins were electrophoretically divided. Western blotting was done to determine their capacity to elicit an immunological response against NE. 120 one-day-old broiler chicks had been categorized into six groups; control negative(C-ve), FTNB, SSNB, BBNB, FT, and control positive (C+ve). Vaccines were mixed with Montanide™ ISA 71 VG adjuvant. Chicks are vaccinated subcutaneously at 3 and 9 days. Orally challenged groups with *Eimeria maxima* on day 20 and *CP* at 24 to 26 days of age. Mortality was observed within 3 days post-challenge in the control positive group only (25%). Higher IgY antibody ELISA titres are found in SSNB and BBNB practically than FT group. The expression of a gene of interleukin-17 (IL-17) in spleen tissue was down-regulated in all groups except FT and C+ve groups. These show the efficiency of native *NetB* with other protective component antigens against NE in broilers.

Keywords: Clostridium perfringens, ELISA, Interlukien-17, Necrotic enteritis, Vaccine.

Introduction

Necrotic enteritis (NE), an economically significant intestinal disease of chicken caused by *Clostridium perfringens* (*CP*), has resurfaced as a major issue following the prohibition of antimicrobial feed additives [1].

CP is a gram-positive, anaerobic bacilli bacterium, spore-forming found in nature as a normal flora in animals and humans' gastrointestinal tracts [2]. Typing of *CP* from A to G based on the ability to produce four major toxins: Alpha, beta, epsilon, and iota [3]. More than 15 toxins are produced by *CP* including zinc metalloproteases, collagenase (κ-toxin), Phospholipase C, glycoside hydrolases, sialidases, bacteriocin, adhesins, proteolytic enzymes, and tpel [4]. The largest class of bacterial protein toxins is pore-forming toxins, which include perfringolysin O, *NetB* toxin, beta2 toxin, and enterotoxin. Pore-forming toxins are a common mechanism of cell death as they generate pores to access the enterocytes [5].

To control NE in broiler chickens, researchers have worked hard to comprehend the pathogenesis of *CP* and create vaccinations against it. Numerous vaccination platforms, such as toxoid, whole inactivated, live attenuated, immunogenic proteins, subunit, recombinant, and attenuated salmonella vector vaccines have been investigated [6].

Various NE vaccination programs, challenge models, and processes show that immunization with *NetB* (using recombinant *NetB*, toxoids and bacterin

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(Received 15 June 2024, accepted 24 August 2024)
DOI: 10.21608/EJVS.2024.297718.2175

vaccines containing *NetB*) makes an immunological reaction that, partially defends against NE [7].

Birds immunized with recombinant *NetB* (*rNetB*) were strongly protected against NE when treated with a moderate oral dose of virulent *CP*; however, when a more strong challenge was employed, there was no protection, these findings imply that *NetB* immunization by itself might not be adequate to shield birds against NE in the field, but when combined with other cellular or cell-free antigens, it can greatly reduce the risk of disease in chicks. Furthermore, without *rNetB* supplementation, bactererin, and cell-free toxoids did not shield birds against moderate and severe in-feed challenges. The only birds that demonstrated considerable protection against moderate and severe in-feed challenges were those that had received bacterin and cell-free toxoid vaccinations coupled with *rNetB* [8].

A good immunological response is thought to require multiple vaccination doses. As a result, twice (or three times) intramuscular or subcutaneous vaccination with toxoid, glyceraldehyde-3-phosphate dehydrogenase (GPD), hypothetical proteins (HPs), fructose 1,6-biphosphate aldolase(FBA), or pyruvate ferredoxin oxidoreductase (PFOR) resulted in a reduced intestine lesion score. In chickens, GPD and FBA provided very minor resistance when faced with a mild challenge, whereas HP and PFOR provided great resistance to a severe challenge [9].

A study compared four *CP* recombinant proteins as a vaccine using the Montanide™ ISA 71 VG adjuvant in an experimental NE. Broiler chickens were vaccinated subcutaneously with purified clostridial *rNetB* toxin, PFOR, α-toxin, and EF-Tu in conjunction with ISA 71 VG. Birds vaccinated with *NetB* toxin and PFO plus ISA 71 VG made significantly increased body weight gains compared with the ISA 71 VG alone group. Greater *NetB* toxin antibody titres were observed in the *NetB*/ISA 71 VG group, and greater PFO antibody titres were evident in the PFO/ISA 71 VG group as the best protective immunity was conferred when given recombinant proteins as a vaccine with Montanide^{TM} ISA 71 VG adjuvant [10].

Bacterial adhesions have specialized structures that make it easier for bacteria to link with host extracellular matrix molecules (ECM) and regulate environmental tropism with successful colonization. Collagen, a major component of the ECM, could be a key attachment target for gram-positive bacteria's virulence proteins [11]. Where the putative collagen-binding protein (CnaA) is found primarily in *NetB*-positive strains inside the pathogenicity locus VR-10B [12].

The only readily available vaccine (Netvax ®), which was delivered subcutaneously or intramuscularly to broiler breeder chicks, as well as it mainly relied on alpha toxoid, was no longer approved [1]; Later, the presence of the *NetB* gene in type A strains is essential for NE pathogenicity. In addition to *NetB*, other virulence factors have been studied as immunogenic proteins HPs, PFOR, GPD, EF-TU, FBA, Perfringolysin O, and predicted pilin structural subunit adhesion proteins (CnaA) [13].

So, in this paper, a native *NetB* with multi-component antigens (protective proteins) has been used with effective cost by preparing and evaluating the protective potential of four *CP* type A vaccines against NE in commercial broiler chickens

Material and Methods

Ethics Approval and Consent to Participate

All animal experiments were assessed and approved by Institutional Animal Care and Use Committee at the Faculty of Veterinary Medicine, Beni-Suef University (approval#022-374)

All methods were performed in accordance with the relevant guidelines and regulations.

CP Isolates and their Characterization

Two *CP* type A strains (alpha-positive, *NetB*-positive, and alpha-positive, *NetB*-negative strains) were previously isolated and characterized from 2-4 weeks of diseased broiler chicken farms in Egypt. The two strains were first refreshed in cooked meat media and then incubated for 24 hours in a gas pack anaerobic gar at 37°C with anaerobe kits (Oxoid). After that, the isolates were cultivated on blood agar base supplemented with 10% defibrinated sheep blood with neomycin sulphate at 200 µg/ml and incubated anaerobically at 37°C for 24 hours. Presumptive transparent colonies of the double zone of haemolysis [14].

Toxin Preparation

The two strains of *NetB*-positive and *NetB*-negative were inoculated into tryptose glucose yeast extract broth (TGY), a toxin production medium, under anaerobic conditions at 37°C. Briefly, trypticase and yeast extract were dissolved in 950 ml of water and autoclaved to make TGY. Glucose and L-cysteine were dissolved in 50 mL of water before being sterilized by filtering. After mixing the two solutions at 21°C, they were incubated under anaerobic conditions at 37°C for 15 minutes after centrifugation at 11,000×g for 15 minutes at -4° C [15, 16].

Preparations of Vaccine

Formalized Toxoid Preparation (FTNB)

NetB-positive as well as *NetB*-negative cell culture supernatants were inactivated with formaldehyde solution (Sigma-Aldrich) at a final concentration of formaldehyde (0.4%) and incubated at 37°C for two days. The residual formaldehyde was then removed by overnight dialysis against Tris-

buffered saline (TBS) in a 10 KDa dialysis sac (Sigma Chemical Co.). The obtained toxoid was then concentrated with trichloro acetic acid (TCA), and a suspension with the final concentration of 200 mg/ml was obtained using the Bradford method with normal saline solution (NSS). The final FTNB preparation was filter-sterilized before use through a 0.45 µm syringe-driven filter (HI Media) [17].

Boiled Bacterin *NetB* (BBNB)

After centrifugation at 11,000×g for 15 minutes at -4°C, *CP* bacterial sediment was collected. Following that, the pellets were suspended in NSS at 1.2×10^9 CFU/ml using McFarland tube 4. The suspension was inactivated by boiling for 30 minutes then inoculated onto neomycin blood agar and incubated aerobically and anaerobically at 37°C for 16 hours to assure bacteria inactivation before being utilized as a vaccine. A 0.45 µm syringe-driven filter (HI Media) used to filter-sterilize the BBNB [3, 8].

Supernatant of Sonicated *NetB* (SSNB)

Bacterial cells were broken down using an optimized sonication process at 10 hertz ten times for one minute each. The sonicated bacteria were then centrifuged for 15 minutes at 11,000×g at - 4°C and the supernatant was subjected to TCA for precipitation of the proteins. Finally, a suspension with NSS was made to the final concentration of 200 mg/ml using Bradford method. Before being used as a vaccine, the SSNB was filter-sterilized using a 0.45 µm syringe-driven filter (Hi-Media) [3].

Electrophoretic Analysis and Western Blotting

Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS- PAGE)

Protein components of the SSNB and BBNB antigens were electro-phoretically separated using SDS-PAGE according to the method of [18]. After separation, the gel was stained with 0.1 % Coomassie blue R-250 at room temperature. Molecular weight standards (Vivantis chromatin prestained protein ladder 175-22 KDa code No. PR0602), electrophoresed on the same gel to calculate the relative molecular weights of the examined antigens after electrophoresis, the gel was removed from the tank carefully and stained overnight in staining solution (50%methanol, 7% glacial acetic acid and 0.2% coomassic brilliant blue dye) with gentle agitation. Distaining was done in a distaining solution containing 45% methanol and 10% glacial acid till the background of the gel became clear and the protein bands were obvious. The results were analysed by a gel analyser.

Blotting

The protein bands were electrophonically transferred from SDS PAGE to a nitrocellulose sheet using the modified method of [19]. A sheet of nitrocellulose membrane 0.45 µm pore size (Bio_Rad Laboratories) and the gel to be blotted were put on the nitrocellulose sheet. The assembly was put into an electrophoretic chamber containing a transfer buffer with a nitrocellulose sheet facing the anode. The nitrocellulose membrane was soaked in a blocking buffer for 2 hours. Infected serum with *CP* was added to the nitrocellulose membrane. After incubation labeled secondary antibody (anti-chicken IgY horse radish peroxidase (HRP) was added and after incubation, the substrate; Ortho phenylene diamine (OPD) was added to visualize the immunogenic bands in the four antigens.

Vaccination challenge study

Experimental Design

Animal and Housing Condition

One I00 hundred and twenty commercial day-old Ross broiler chicks were obtained from the Cairo poultry company, the parent flock had not received any *CP* vaccine, and the chickens were reared in a cage system in six groups (20 birds/cage) in the same room, the cages separated by solid borders to prevent contact between birds, the birds received ad libitum feed and drinking water, the first week, chicks were reared at a temperature of 32°c with a whole daylight program and subsequently, the room temperature decreased by 0.5°c each day to reach 25°c until the end of the experiment, and the light schedule adjusted to 16 h of light and 8 h of dark for the rest of the period with good ventillation. Bird experiments were carried out according to the recommendations and following approval of the Ethical Committee of the Faculty of Veterinary Medicine, Beni-Suef University, Egypt. The 6 groups included negative control group (C-ve), formalized toxoid *NetB*- positive group (FTNB), supernatant sonicated *NetB*- positive group (SSNB), boiled bacterin *NetB*- positive group (BBNB), formalized toxoid *NetB*-negative group (FT), Control positive group (C+ve). Based on the obtained protein concentrations determined by the Bradford method the dose of vaccine/bird was adjusted to be 40 µg\bird [20], mixed with an equal quantity of Montanide™ISA 71 VG adjuvant (1:1) added to each suspension [21]. The vaccination schedule was 2 doses at 3 and 9 days old subcutaneously. 5 random birds were euthanized humanely and examined excreta in addition to scraping of intestinal mucosa to ensure birds were free from coccidian infection.

Induce sub-lethal dose of *Eimeria maxim* which was orally taken with approximately ($1X10^4$) sporulated oocysts at 20 day-old-chicks [7]. Then the groups were challenged orally with *CP NetB*-positive strain by matching with Mackyferland tube 4(1.2×10^9 CFU/bird) for 3 successive days from 24 to 26 days [22]. Birds ' body weight individually and feed intake were recorded at 3,9,20, and 30-day-old chicks to calculate feed Conversion ratio. Furthermore, Blood samples were collected

aseptically from the wing vein and kept at room temperature for 2 hours followed by centrifugation for 10 min at 580xg, to determine IgY titer in serum by ELISA. Where, three birds from each group were randomly selected and euthanized humanely, and taken of small intestine samples were used for counting. Five birds were euthanized humanely for histopathology; intestinal severity index (ISI) for histological alterations was evaluated in the ileum and intestinal lesion score in the duodenum, jejunum, ileum, and cecum. In addition, spleen samples for interleukin 17 were taken at 20 and 30 days old chicks, and gross intestinal examination of NE lesions of the jejunum, ileum, and cecum was scored on a scale of 0(none) to 5 (high) as previously described. The experimental design, sampling and sample testing are shown in table (1).

CP Counting

At 3, 9, 20, and 30 days of broiler age were randomly selected chicks and humanely euthanized before necropsy. Samples from the jejunum to cecum content, one gram from pooled contents were diluted 1:9 (wt\vol) in peptone water and 10-fold serial dilution was prepared [23]. I ml from different dilutions were plated onto tryptose sulfite cycloserine (TSC) agar were prepared using Perfringens Agar Base (Sigma-Aldrich) supplemented with12 mg/L kanamycin sulphate and 400 mg/L D-cycloserine. Pour about 12 ml to 15 ml of TSC agar for 90 mm Petri dishes of TSC agar Petri dishes then carefully mix the inoculum with the agar by rotating the Petri dishes and allow the mixture to solidify by leaving the Petri dishes standing on a cool horizontal surface after complete solidification to pour about 5 ml of TSC agar to prevent the development of spreading colonies on the surface of the medium, allow to solidify, invert the plate and incubated anaerobically at 37c° for 20 hour. The numbers of typical colonies that show black colonies are counted in different serial dilutions of intestinal content samples [24].

Eimeria Maxima

Purchased *Eimeria maxima* strain from Animal Health Research Institute, Dokki, Giza. Where, putting *Eimeria maxima* with, potassium dichromate 2.5% which is used for sporulation and preservation of the parasite, in an incubator for 4 to 5 days at 28°c, washed 3 to 4 times by phosphate buffer saline and centrifuged 10 min for 3000rpm till clearance then thrown supernatant and used pellets (sporulated oocyst) for oral infection [25].

Histopathology

Organ specimen obtained from the small intestine (0.5 to 2 cm long and 0.25 thick). All the specimens were immersed in 10% neutral formalin at room temperature for at least 48 hours before processing. The samples were trimmed and embedded in paraffin blocks for sectioning and staining. Section 5µm thick was routinely stained with haematoxylin and eosin [26]. Lesion scores were estimated according to [27] markedly thickened mucosa, haemorrhages, necrotic foci, congestion, haemorrhage, and ulceration.

(ISI)Total Histological Alterations Evaluated in Intestine

According to [28] the histopathology alteration is based on a numeric score evaluated through lamina propria thickness, epithelial thickness, enterocyte proliferation, epithelial plasma cell infiltration, lamina propria inflammatory infiltration, goblet cell proliferation, congestion, and presence of oocysts. In this methodology, an impact factor (IF) is expressed according to the degree of reduction of organ functional capacity, which ranges from 1 to 3, with 3 having the most impact on organ function. In addition, the extent of each lesion observed with score (S) ranges from 0 to 3: score. Where, 0 indicates the absence of a lesion, score 1, alteration up to 25% of the area, score 2, alteration ranges from 25 to 50%, and score 3, alteration extends to more than 50% of the area. To obtain the final value of the ISI index, the IF of each alteration is multiplied by the respective score number, and the results of all alterations are summed according to the formula ISI = Σ (IF*S).

Measurement of the Antibody Titers in Chicken Sera

Checkerboard Titrations

Use of 4 antigen concentrations of (40, 60, 80, and 120 µg/ml each concentration in 3 columns, for four clostridia antigens (FTNB, SSNB, BBNB, FT) on days 3,9,20 of the broiler chicken while adding the fifth antigen of control positive groups on day 30 of the broiler chick then 4 dilutions of serum was added to coat columns (1:25, 1:50, 1:100, and 1:200 µg/ml) then washing 3 times by 300 µL of washing buffer (0.8 M Tris-HCl, 0.15 M NaCl, 0.05% [v/v] Tween-20; pH 8) per well (stacked ELISA plate washer, BIOTEK Instruments, Winooski, VT, USA), 2 dilutions of anti-chicken IgY conjugated horse radish peroxidase (HRP (Biochek, Ascot, UK) were added, after incubation and washing the substrate; Ortho phenylene diamine (OPD) (Biochek, Ascot, UK) was added then the reactions were stopped by adding stop solution (BioChek) [29], and the optical densities (OD) were measured using a 450 nm spectrophotometer (BIO-Teck). The selection of OD values depends on the highest antigen dilution that allowed optimum binding capacity with a high dilution of primary antibody labeled secondary antibody which is 80 µg\ml and 1\100 µg\ml for antigen and serum respectively.

ELISA

Elisa was used to detect level of IgY antibody titre in all groups according to [30, 31]. Micro titer plates (PolySorp, Nunc, Roskilde, Denmark), on days 3,9,20 of broiler chicken, the antigens used 4

antigens (FTNB, SSNB, BBNB, and, FT) for coating while on day 30 of broiler chick (all vaccinated and the C+ve groups challenged with *CP*). These five clostridia antigens (FTNB, SSNB, BBNB, FT, and C+ve) were added at a concentration (80 µg/ml) diluted in coating buffer (Na2CO3 0.397 gm+ NaHCO 30.732 gm. in 250 ml distilled water), then blocking with blocking buffer (1% bovine serum albumin) (Sigma-Aldrich) followed by washing then addition of diluted serum (1/100 µg/ml) after incubation and washing added secondary antibody anti-chicken IgY conjugated horse radish peroxidase (HRP) (Biochek, Ascot, UK) was added then after incubation and washing the substrate; Ortho phenylene diamine (OPD) (Biochek, Ascot, UK) was added. The reactions were stopped by adding stop solution (BioChek) to each well, and the optical densities (OD) were measured using a 450 nm spectrophotometer (BIO-Teck).

Detection of Interleukin -17 Using Real-time PCR

RNA extraction from spleen tissue samples was applied using a QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH) when 30 mg of the tissue sample was added to 600 µl RLT buffer containing 10 µl β-mercaptoethanol per 1 ml. Tubes were inserted into adapter sets, which were then secured into the clamps of the Qiagen tissue lyser to homogenize the samples. A 2-minute high-speed (30 Hz) shaking step was used to cause disruption. One volume of 70% ethanol was added to the cleared lysate, and the processes were completed according to the QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH).

Oligonucleotide Primers, Primers used were supplied from Metabion (Germany) and are listed in table (2).

SYBR green rt-PCR, primers were utilized in a 25- µl reaction containing 12.5 µl of the 2x QuantiTect SYBR Green PCR Master Mix (Qiagen, Germany, GmbH), 0.25 µl of RevertAid Reverse Transcriptase (200 U/µL) (Thermo Fisher), 0.5 µl of each primer of 20 pmol concentration, 8.25 µl of water, and 3 µl of RNA template. The reaction was performed in a Stratagene MX3005P real-time PCR machine.

Analysis of the SYBR green rt-PCR results, amplification curves, and ct values were determined by the Stratagene MX3005P software. To estimate the variation of gene expression on the RNA of the different samples, the CT of each sample was compared with that of the positive control group according to the "ΔΔCt" method stated by[32] using the following ratio:(2-ΔΔct).

Statistical Analysis

All data were evaluated using a one-way nonparametric analysis of variance (ANOVA) test for mean ± SEM and statistically analyzed using an analysis of variance test for significance determination at a p-value ≤ 0.001.

Results

Bradford protein Titrations

The Bradford method was used to determine the protein concentration of each prepared vaccine[33]. Results revealed obtaining the following concentration of FTNB (228.36 µg\ml), SSNB (310.31 µg\ml), BBNB (385.7 µg\ml), and FT (436.01 µg\ml). The vaccines were mixed with an equal quantity of Montanide^{TM} ISA 71 VG adjuvant.

Detection of CnaA Using PCR

Four stains (3 of them were *NetB*-positive isolated from poultry farms suffering from NE and the other one was *NetB*-negative) were chosen for PCR for detection of CnaA (collagen adhesion gene) of *CP,* as strains tested showed thick bands for the CnaA gene in strains that where *NetB*-positive while no gene present in *NetB*- negative as shown in Fig.(1).

Electrophoretic Analysis and Western Blotting

Sonicated protein (SSNB) analysis revealed the intracytoplasmic proteins including perfringolysin O protein (52 KD), glyceraldehyde-3-phosphate dehydrogenase (GPD) protein (37 KD), and fructose1, 6-biphosphate aldolase (FBA) protein (33 KD). Bacterin protein (BBNB) analysis revealed the cell wall proteins including hypothetical proteins (HPs), pyruvate ferredoxin oxidoreductase (PFOR), collagen adhesion proteins, and elongation factor-Tu (EF-TU) sized 117, 55, 75, 34 KD, respectively. Western blotting revealed that the sonicated and bacterin proteins found in the same KD of electrophoresis as shown in Fig.(2).

Experimental challenge and vaccination approaches

Body Weight of Broiler Chickens

At 3 and 9 days of age, there were no statistically significant differences in mean body weight among treatment groups. However, at 20 days of age, all immunized groups showed a substantial increase in mean body weight, which further significantly rose by day 30 ($P<0.001$), particularly compared to the C+ve group as shown in table (3).

Feed Conversion Ratios (FCR)

In 20-day-old chicks (before the challenge) the FCR (average feed intake/ average chicken weight) was nearly the same in all groups while in 30-day-old chicks (after the challenge), the lowest FCR groups were SSNB and BBNB groups while the highest FCR was the C+ve group, there are significant differences in P value at ($P<0.001$) in all groups comparing C+ve group as presented in table (3).

CP Counts

At 3, 9, and 20 days of age, there was no discernible difference in the counting of *CP* in the small intestine (jejunum to cecum) however, in 30 days -old- chicks, a variation in *CP* count was detected among groups, where the SSNB and BBNB groups have low in *CP* count, while, the FT and FTNB groups showed an increase in count, respectively. In contrast, the increase in *CP* count was dramatic (pathogenic) in the C+ve group with 25% mortality (3died \12 bird) within 3 days post-challenge from *CP* with ballowing, congestion, ulceration, blood clotting, and necrosis in the intestine. The p values on day 30 were more significant in all groups at P value $\leq$ 0.001 comparing C+ve, as shown in Fig.(3).

Intestinal lesion Score on Day 30

Intestinal gross lesion scores in broiler chickens according to[34, 35] with experimentally induced NE and vaccination prevention. The C+ve, showed necrosis patches with variable amounts indicating pathogenic lesions while the groups that take vaccines showed degrees of intestinal lesions from necrosis, as immunized groups from the *NetB*-positive had significantly fewer intestinal lesion scores than the *NetB*-negative.

The degree of intestinal lesion score is shown in table (4) and Fig.(4).

(0) No apparent injuries were found in the C-ve group.

(1) Thin or friable wall congested intestinal mucosa was found in the SSNB group.

(2) Focal necrosis or ulceration from 1 to 5 foci was found in the BBNB group.

(3) Focal necrosis or ulceration from 6 to 15 lesions was found in the FTNB group.

(4) Focal necrosis of 16 or more foci was found in the FT group.

(5) Necrotic patches 2-3 cm long with variable amounts were found in the C+ve group.

Histopathology of the Small Intestine at 30 Day Old Chicks

On 30-day-old chicks' small intestines, the best groups are SSNB and BBNB as mild degree of necrosis with a moderate degree of mononuclear cell infiltration in intestinal mucosa while stages of the parasite in enterocytes in intestinal villi are observed in FT and FTNB groups respectively but the worst group is C +ve where stages of the parasite are found either in the intestinal gland or in intestinal villi in addition to the presence of bacterial colonies as shown in Fig.5.

Histopathology of the Small Intestine

C-ve group showing: normal villi structure, normal intestinal glands, mild necrotic changes within intestinal gland cells. FTNB group showing: hypercellularity of the epithelial lining intestinal gland, mononuclear cell infiltration, and mild necrosis within the epithelial lining intestinal gland, mild stages of the parasite within intestinal gland cells, and mild necrotic changes within enterocytes. SSNB group showing: normal villi, and mild necrotic changes within the intestinal gland. Infiltration of mononuclear cells. and BBNB group show moderate hypercellularity of the intestinal villi and some necrotic changes within the epithelial lining intestinal gland. FT group showing: hypercellularity of the epithelial lining intestinal gland with severe necrotic changes, hypercellularity within villi, inflammatory cell infiltration, and stages of the parasite within the enterocyte. C +ve group showing: stages of the parasite in enterocytes that are found either in the intestinal gland or in intestinal villi, bacterial colonies, hypercellularity within epithelial lining intestinal gland within villi, and severe infiltration of mononuclear cells with villi atrophy, as represented in Fig.(5).

ISI Total Score in Ileum at 30 Day of Age

The average of 20 villi observed in each bird will reach the final value for this parameter and the sum of the average of all parameters presented in table (5) will give the intestinal severity index (ISI) in the ileum at 30 days of age, based on villi observations, significantly differed in *NetB*-positive groups compared to the C+ve and FT groups.

Measurement of the Antibody Titers in Chicken Sera Using ELISA

Checkerboard Titrations

According to checkerboard titration, the highest antigen dilution that allowed optimum binding capacity is 80 µg\ml and 1\100 µg\ml for antigen and serum respectively.

ELISA

The IgY titre in 6 groups at 3, 9, 20, and 30, days showed an increased level throughout the experiment in groups (SSNB, BBNB, and FTNB). However, little increase in the IgY titre was observed in FT and C+ve groups. The p values were most significant in vaccinated groups on 20 and 30-day-old chicks when compared to the C+ve group as shown in Fig.(6). The cut-off value was subtracted from each OD value and calculated according to [29].

Cell Mediated Immunity Interleukin-17

In 20 day-old-chicks, the expression of interleukin-17 in the vaccinated groups (SSNB, BBNB, FTNB, and FT groups) were upregulated while in 30 day-old-chicks after challenge with *CP* in

the presence of a stress factor (*Eimeria maxima*), this expression was downregulated in SSNB, BBNB and FTNB while in FT and C+ve groups, IL-17 was upregulated**.** The p values were significant in all vaccinated groups at 20 comparing the C+ve group, as shown in Fig.(7).

Discussion

Acute necrotic enteritis (NE) was found to be a cause of suddenly increased mortality in broilers during the last weeks of rearing (4th-5th) [36].

This study looked to prepare and asses four types of vaccines formulated with the ISA71VG adjuvant, focusing on positive native *NetB* including FTNB, SSNB, BBNB groups, and negative *NetB* including FT group. Protective immunogenic proteins that either intracytoplasmic (SSNB) include; perfringolysin O, GPD, and FBA or cell wall proteins (BBNB) include; HPs, PFOR, EF-TU, and CnaA that interacted with serum from immunized broilers by western blotting in the same Kilodalton separated by electrophoresis, indicating the importance of these proteins in immunity. These findings corroborated the findings of [37], those who demonstrated the importance of immune reactive proteins in immune system activation and protection against NE in broilers [38] .

The presence of collagen adhesion gene (CnaA) using PCR in *CP NetB*-positive strains and the absence of it in *CP NetB*-negative strains agreed with [12]. Who revealed that the CnaA as a pilus component of *CP* contributes to enteritis in chickens by promoting the adhesion to damaged intestinal tissue and inactivation of pilus genes results in the inhibition of pilus production, greatly reducing the capability of *CP* to bind collagen and initiate disease.

In the present study, the high body weight with low conversion rate is observed in groups that had the lowest bacterial count which was found in groups that were *NetB*-positive (SSNB, BBNB, FTNB), particularly in SSNB and BBNB (which have immune reactive proteins) compared with the group that was *NetB*-negative (FT). This result nearly agrees with that obtained by [36, 39], while a marked decrease in body weight was noticed in the C +ve group. These findings agree with [28, 40], who revealed that the accumulation of inflammatory processes led to anaemia, low weight, and low feeding efficiency especially when broilers were experimentally infected with *Eimeria* as a stress factor for *CP*.

highly immunogenic proteins are employed to protect birds against NE by SSNB and BBNB vaccination and the protection is most likely determined by an effective combination of diverse bacterial immunogens, these findings concurred [41], who assessed the effectiveness of *CP* type A and C vaccination regimens and discovered that immunization with *CP* type A with *NetB* as a formalized toxoid, resulted in a reduction in the number of *CP* and intestinal lesions in broilers. Particularly when combined with immunogenic and protective proteins, and the decrease was more significant in the SSNB and BBNB vaccinated groups. These findings were mirrored in intestinal scoring lesions and histological examination in the small intestine, whereas *NetB*-positive groups, particularly in SSNB and BBNB vaccinated groups, exhibited mild necrotic changes within the intestinal gland leading to improvement in the absorption of macro and micronutrients which reflected on body weight and FCR as highest BW and lower FCR detected in these vaccinated groups, but the FTNB vaccinated group had little increase in counting after challenge reflected on lesion score as focal necrosis or ulceration from 1 to 5 lesions have been found in the small intestine. Moreover, the *NetB* negative group showed a rise in counts (FT vaccinated group) which was reflected in intestinal lesion score as multifocal areas of necrosis from 6 to 15 lesions were found moreover its effect in histopathology as hypercellularity of epithelial lining intestinal glands with sever necrotic changes. While the highest counting of *CP* was in the C+ve group after the challenge which was reflected in lesion score as more ulceration and necrosis patches 2-3 cm long variable amount in the intestine, these findings agreed with [13].

Immunoglobulin IgY has the advantage of removing potentially hazardous pathogens from the host [36, 42]. The current investigation found that the *NetB*+ve group exhibited substantial anti-*NetB* antibodies (IgY using ELISA) till the end of the experiment, with the greatest levels observed post-challenge compared to the *NetB*-ve and C+ve groups. These findings agreed with [36], who stated that broilers immunized with bacterin supplemented with *NetB* had significantly higher anti-*NetB* antibody titres than the *NetB*- negative group and disagreed with [43], who investigated that, *alpha* toxin had higher antibody titres.

The higher level of IgY titre in *NetB*-positive groups (SSNB, BBNB, and FTNB) protect against *CP* challenges, these findings agree with [44]. While, little increase in IgY titre was observed in the C+ve and FT groups respectively, providing low protection against NE, these findings agreed with [3, 9], who determined the role of immunoglobulin IgY and protective antigens in the prevention of NE.

The antibody titre increased after the first week of immunization and continued to increase after the second immunization, reaching the highest protective level in the third and fourth weeks, which agreed with [45]. It was concluded that antibodies act against enteric pathogens by binding to them, immobilizing them, and thus reducing or inhibiting their growth, replication, or colony-forming abilities,

which is reflected in the counting of bacteria and body weight.

Various cytokines are secreted as a result of these signaling events, including proinflammatory interleukin (IL)-1 by naive T cells, interferon- (IFN-) by Th1 cells, IL-13 by Th2 cells, IL-17 by Th17 cells, and TNF- and IL-10 by Treg cells, *CP* infection causes an inflammatory response in the intestines of broiler chickens, and the mechanisms of inflammation are most likely mediated by Th2 and Th17 cells [46, 47].

Interleukin-17 as a marker of cell-mediated immunity was estimated by real-time PCR. At 20 day-old- chicks, before the challenge in the vaccinated SSNB, BBNB, and FTNB, the expression of the interleukin-17 gene was upregulated compared with the C-ve, however, following the *CP* experimental challenge proceeded by coccidian infection, this expression had decreased in *NetB*-positive vaccinated groups. and did not cause significant changes in the serum IL-17 level, This research was confirmed with [48], who found that IFN-alpha, IFN-beta, IL-1b, IL-2, IL-12, IL-13, IL-17, and TGF-b4 were repressed after challenge with *CP,* while this expression of interleukin-17 in the C+ve and *NetB*- negative groups respectively were upregulated with a mortality rate of 25% in C+ve group only after the challenge. These results agreed with [2], which indicated that a *CP* challenge with *Eimeria* as a stress factor induced a high inflammatory process by stimulating CD4, Th1, and Th17 lymphocytes, as inflammatory cytokines promote the recruitment of heterophiles, monocytes, and lymphocytes, as well as their translocation and migration between endothelial cells, and in conjunction with tight junction reorganization resulted in a considerable increase in vascular leakage as a result of an inflammatory mediator reorganization. Activated mast cells release histamine, 5-hydroxytryptamine, proteases, heparin, cytokines, and other inflammatory mediators from their granules, increasing vascular permeability, inducing vasodilation, and altering intestinal motility, resulting in increased transit time and intestinal expulsion content, all of which are reflected in their histopathology. In the C+ve group and FT group, respectively massive immune cell infiltration into the lamina propria in damaged intestinal tissue leads to the flattening of villi and hyperplasia of the crypts, with the degradation of the intestinal mucosa and alteration of enterocytes. All these changes occurred in the intestine reflected in body weight and FCR due to a decrease in the site of absorption of macro and micronutrients that impact the health status of the bird.

Conclusion and Recommendations

Presence of multicomponent antigens, such as immunogenic proteins (hypothetical proteins (HPs), pyruvate ferredoxin oxidoreductase (PFOR), EF-TU, and glyceraldehyde-3-phosphate dehydrogenase (GPD), fructose 1,6-biphosphate aldolase(FBA), Perfringolysin O*,* and collagen adhesion proteins*)* as well as native *NetB* and *alpha*-toxin, are needed in vaccination for protection against NE. It was recommended to use the cell component of native *NetB*-positive *CP* for immunization of poultry instead of formalized toxoid.

Declarations

*Consent for publication***:** Not applicable.

Funding statements: Not applicable

Competing interests: The authors declare that they have no competing interests.

Acknowledgment : The study was performed in the Anaerobic Microbiology Department, Animal Health Research Institute in Dokki, and Department of Poultry Diseases in the Faculty of Veterinary Medicine at Beni-Suef University, gypt, so all authors thank them for the validation and equipment laboratory where this research was performed and completed.

TABLE 1. Grouping and Experimental Design

120 one-day-old chicks (20 birds/group)								**Parameters**
Group*	**Dose**	**Vaccination**		**Sublethal**	***NetB*-positive *CP.***			
		3 days	**9**	**20 days**	**24**	**25**	**26**	
C-ve		No	No	No	No	No	No	IgY ELISA in 3, 9, 20, and 30 day-old Body weight and feed conversion ratio, bacterial count and histopathology Interleukin-17 in spleen
FTNB		Yes	Yes	Yes	Yes	Yes	Yes	
SSNB	40	Yes	Yes	Yes	Yes	Yes	Yes	
BBNB	µg\bird	Yes	Yes	Yes	Yes	Yes	Yes	
FT		Yes	Yes	Yes	Yes	Yes	Yes	
C+ve		No	No	Yes	Yes	Yes	Yes	

Groups: C-ve; control negative, FTNB; formalized toxoid *NetB*- positive group, SSNB; supernatant sonicated *NetB*- positive group, BBNB; boiled bacterin *NetB*- positive group, FT; formalized toxoid *NetB*-negative group, and C+ve; control positive group.

TABLE 2. Primers sequences, Target genes, Amplicon sizes and Cycling conditions for SYBR green rt-PCR

Target gene	Primers sequences	Amplification (40 cycles)					Dissociation curve (1 cycle)			Reference
		Reverse transcription	Primary denaturation	Secondary denaturation	Annealing (Optics on)	Extension	Secondary denaturation	Annealing	Final denaturation	
IL-17	CAGATGCTGGAT GCCTAACC CTTTAAGCCTGGT GCTGGAT	50˚C 30 min.	94˚C 15 min.	94˚C 15 sec.	51˚C 30 sec.	72˚C 30 sec.	94˚C 1 min.	51˚C 1 min.	94˚C 1 min.	[49]
B. actin	CCACCGCAAATG CTTCTAAAC AAGACTGCTGCT GACACCTTC				60˚C 30 sec.			60˚C 1 min.		[50]

TABLE 3. Body Weight (g) and Feed Conversion Ratios (FCR) of Broiler Chickens before and after the *CP* experimental infection and Vaccination approaches

Item	Groups	Age (days)			
		3	9	20	30
Body weight (g)	C-ve	88.3±5.47	199.3±22.11	875±52.22	1852±45.81***
	FTNB	88±3.28	190.9±18.83	860±38.89	1837±49.39***
	SSNB	87±5.45	225.6±23.79	943±27.244***	1896±18.66***
	BBNB	87±4.25	199.6±16.34	911±54.92**	1882±23.84***
	FT	86±4.28	189±20.23	823±30.22	1785±23.32***
	C+ve	86±4.27	191±15.72	864±43.84	766±28.86
Feed conversion ratio	C-ve	NA		1.23	1.51***
	FTNB			1.25	1.52***
	SSNB			1.21	1.47***
	BBNB			1.22	1.48***
	FT			1.27	1.59***
	C+ve			1.25	2.34

Abbreviations: formalized toxoid *NetB*-positive (FTNB), supernatant of sonicated *NetB*-positive (SSNB), boiled bacterin *NetB*-positive (BBNB), and formalized toxoid *NetB*-negative (FT)), and non-vaccinated non-infected (C-ve), non-vaccinated infected (C+ve) Challenge with *CP* type A *NetB*-positive culture once daily on 3 consecutive days (days 24, 25, and 26). Mean ± SD.

* P value ≤ 0.05 ** P value ≤ 0.01 *** P value ≤ 0.001

TABLE 4. NE Lesion Scores (Mean ± SD) in Chicks challenged with *CP* strains with predisposing factor (*Eimeria maxima*)

Groups	Chicks (No)	Lesion Score (No of Chicks)						Mean ± SD	Median ± SIR
		0	1	2	3	4	5		
C -ve	5	4	1					$0.2^{a} \pm 0.45$	$0^{a} \pm 0$
FTNB	5			1	3	1		$3^{b} \pm 0.71$	$3^{b} \pm 1.5$
SSNB	5	1	3	1				$1^{c} \pm 0.71$	$1^{c} \pm 0.5$
BBNB	5		3	2				$1.4^{c} \pm 0.55$	$1^{c} \pm 1.5$
FT	5				1	3	1	$4^{d} \pm 0.71$	$4^{d} \pm 2$
C +ve	5					1	4	$4.8^{e} \pm 0.45$	$5^{e} \pm 2.5$

SD: standard deviation SIR: semi-interquartile range

Abbreviations: FTNB (formalized toxoid *NetB*-positive), SSNB (supernatant of sonicated *NetB*-positive), BBNB (boiled bacterin *NetB*-positive), FT (formalized toxoid *NetB*-negative), non-vaccinated non-infected (C-ve), and non-vaccinated infected (C+ve). Challenge with *CP* type A *NetB*-positive culture once daily on 3 consecutive days (days 24, 25, and 26). There are significant differences (P<0.001) between means (medians) that have different letters

TABLE 5. ISI Total Score in Ileum at 30 Day of Age

Groups	Mean ± SD
C-VE	7.0 [a] ± 2.55
FTNB	16.0 [b] ± 3.32
SSNB	12.0 [ab] ± 2.65
BBNB	13.0 [ab] ± 2.74
FT	28.0 [c] ± 7.11
C+VE	40.0 [d] ± 6.67

Abbreviations

FTNB (formalized toxoid *NetB*-positive), SSNB (supernatant of sonicated *NetB*-positive), BBNB (boiled bacterin *NetB*-positive), FT (formalized toxoid *NetB*-negative), non-vaccinated non-infected (C-ve), and non-vaccinated infected (C+ve). The challenge with *CP* type A *NetB*- positive culture once daily on 3 consecutive days (days 24, 25, and 26). 5 birds from each group were sacrificed for ISI total score in ileum at 30 days of age. There are significant differences (P>0.001) between means having different letters.

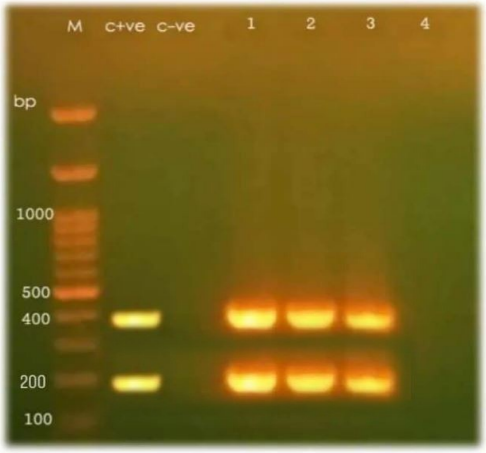

Fig.1. Detection of CnaA Using PCR
The presence of the *NetB* gene at 383 bp and the CnaA gene at 200 bP in *CP NetB*-positive and the absence of it in *CP NetB*-negative.

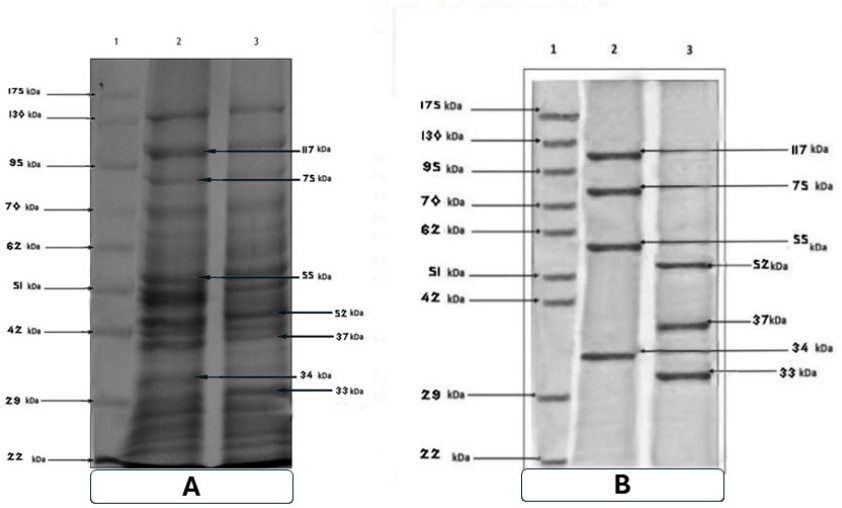

Fig. 2. Analysis of Bacterin and Sonicated Proteins in *CP* strain

(A) electrophoresis and (B) Western blotting using serum from immunized birds. (A) electrophoresis, lane 1; the marker ladder (22-175 Kilodalton), lane 2; *CP* bacterin showing hypothetical proteins (HPs) 117 KD, pyruvate ferredoxin oxidoreductase (PFOR) 55 KD, collagen adhesion proteins at 75 KD and elongation factor-Tu (EF-TU) protein at 34 KD) and, Lane 3; sonicated *CP* showing perfringolysin O protein 52 KD, glyceraldehyde-3-phosphate dehydrogenase (GPD) protein 37 KD and fructose1,6-biphosphate aldolase (FBA) protein at 33 KD). (B) Western blotting, the sonicated and bacterin proteins are found in the same KD of electrophoresis.

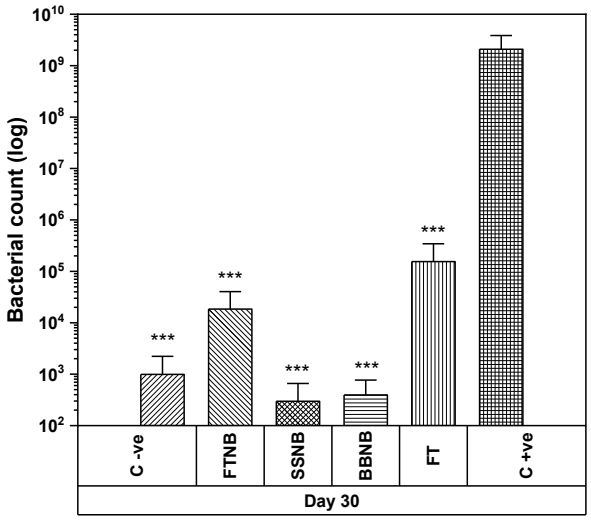

Fig. 3. Counting of *CP* in experimental Broilers on Day 30 of Broiler Chickens

Abbreviations: formalized toxoid *NetB*-positive (FTNB), supernatant of sonicated *NetB*-positive (SSNB), boiled bacterin *NetB*-positive (BBNB), and formalized toxoid *NetB*-negative (FT)), and non-vaccinated non-infected (C-ve), non-vaccinated infected (C+ve) Challenge with *CP* type A *NetB*-positive culture once daily on 3 consecutive days (days 24, 25, and 26). 3 birds from each group were sacrificed with the counting of *CP* in the jejunum, ileum, and cecum. Mean ± SD, the error bars represent the SEM

* P value ≤ 0.05 ** P value ≤ 0.01 *** P value ≤ 0.001

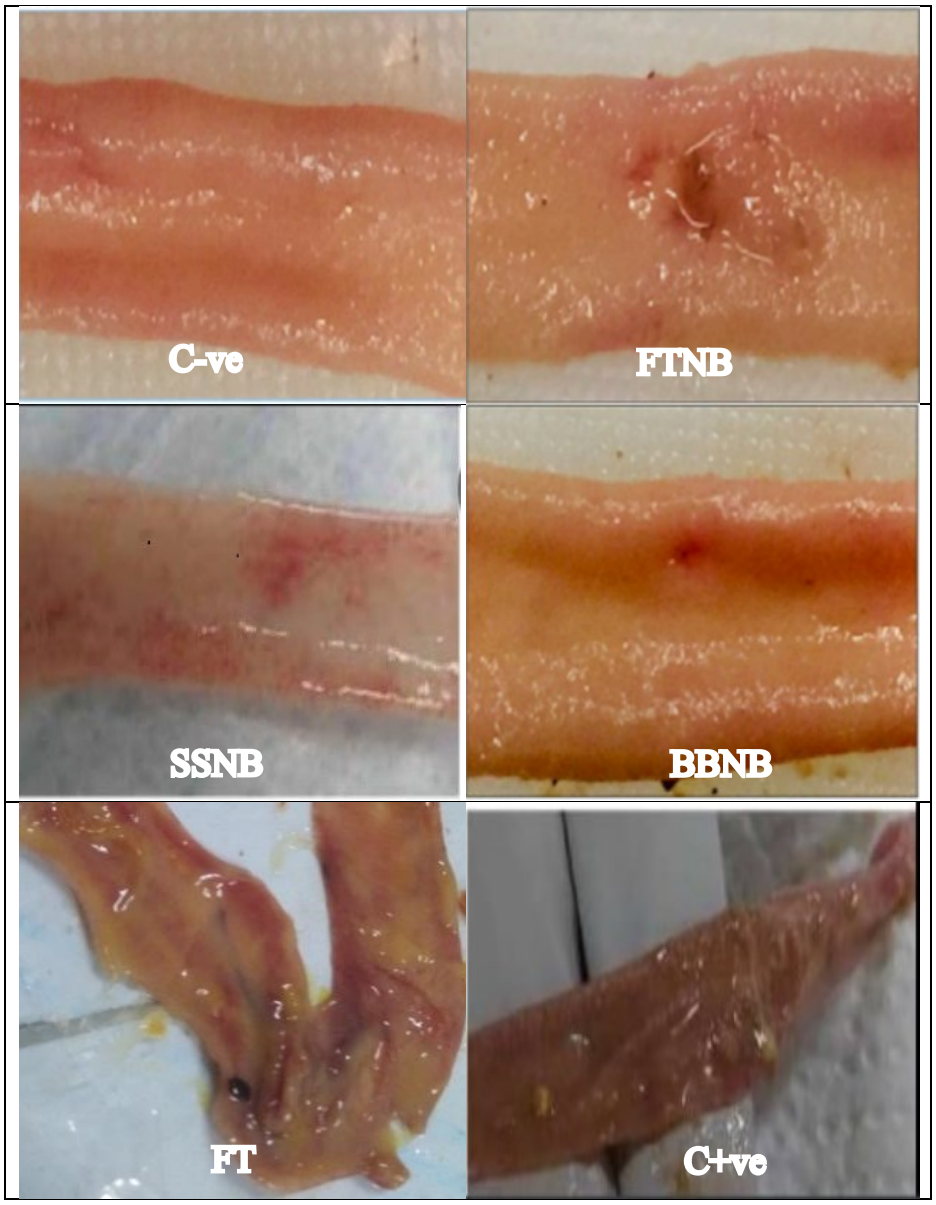

Fig. 4. Intestinal Gross Lesion Scores on Day 30.

Abbreviations: FTNB (formalized toxoid *NetB*-positive), SSNB (supernatant of sonicated *NetB*-positive), BBNB (boiled bacterin *NetB*-positive), FT (formalized toxoid *NetB*-negative), non-vaccinated non-infected (C-ve), and non-vaccinated infected (C+ve). 5 birds from each group were sacrificed with the examination of grossly intestinal lesion score of *CP* in the jejunum, ileum, and cecum.

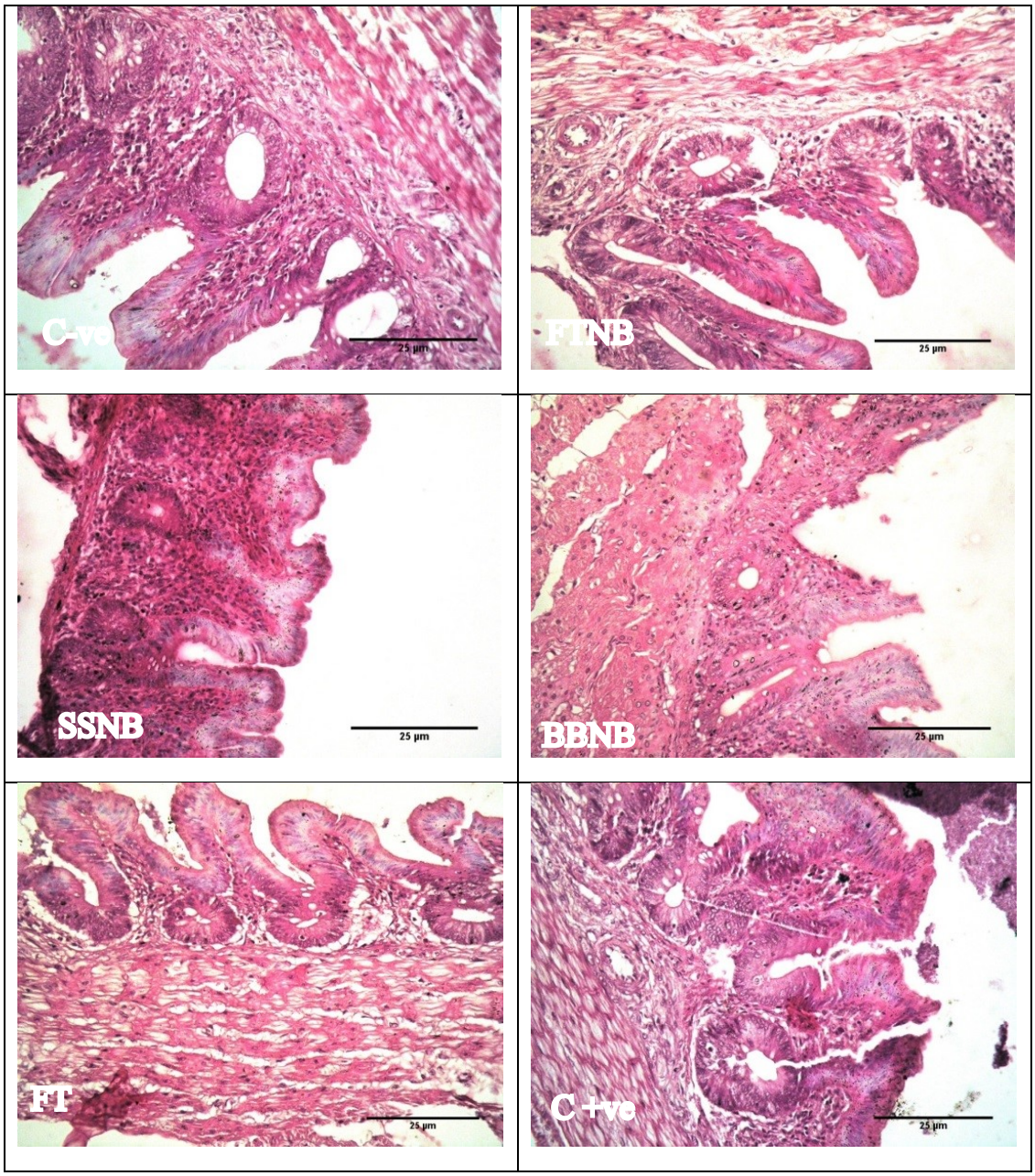

Fig. 5. Histopathology of Small Intestine Samples on Day 30 of Broiler Bhickens
Abbreviations:
FTNB (formalized toxoid *NetB*-positive), SSNB (supernatant of sonicated *NetB*-positive), BBNB (boiled bacterin *NetB*-positive), FT (formalized toxoid *NetB*-negative), non-vaccinated non-infected (C-ve), and non-vaccinated infected (C+ve). The challenge with *CP* type A *NetB*- positive culture once daily on 3 consecutive days (days 24, 25, and 26), 5 birds from each group were sacrificed with the counting of *CP* in small intestine, the magnification was x25, stained by Hematoxylin and Eosin (H&E) stain**.**

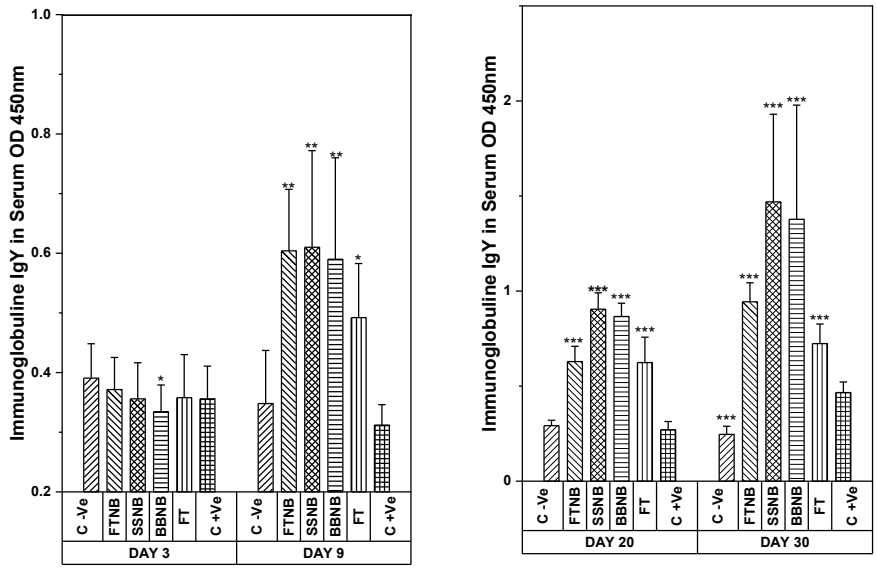

Fig. 6. Detection of IgY Using ELISA

Serum antibody (IgY) response to *CP* on days 3, 9, 20 and 30 of broiler chickens.
Abbreviations FTNB (formalized toxoid *NetB*-positive), SSNB (supernatant of sonicated *NetB*-positive), BBNB (boiled bacterin *NetB*-positive), FT (formalized toxoid *NetB*-negative), non-vaccinated non-infected (C-ve), and non-vaccinated infected (C+ve)**.** Challenge with *CP* type A *NetB*- positive culture once daily on 3 consecutive days (days 24, 25, and 26). The error bars represent SEM.
* P value ≤ 0.05 ** P value ≤ 0.01 *** P value ≤ 0.001

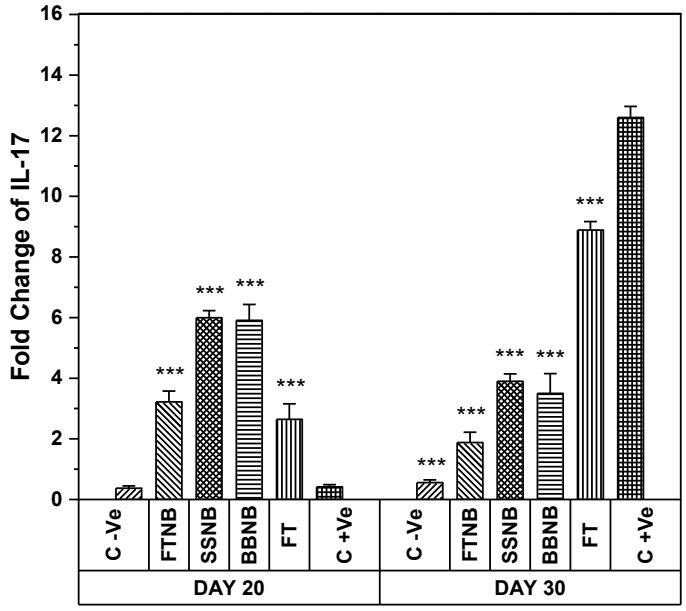

Fig. 7. Expression of IL-17 gene of *CP* on Days 20 (before challenge) and 30 (post-challenge) from Spleen tissue
Formalized toxoid *NetB* positive (FTNB), supernatant of sonicated *NetB*-positive (SSNB), boiled bacterin *NetB*-positive (BBNB), and formalized toxoid *NetB*-negative (FT)), and non-vaccinated non-infected (C-ve), non-vaccinated infected (C+ve). Challenge with *CP* type A *NetB*- positive culture once daily on 3 consecutive days (days 24, 25, and 26). All vaccinated groups had significant p values at 20 comparing the C-ve group, and 30 days in the vaccinated and C-ve groups comparing the C+ve group. The error bars represented SEM**.**
* P value ≤ 0.05 ** P value ≤ 0.01 *** P value ≤ 0.001

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تقييم التحصين باستخدام NetB الأصلي بالإضافة إلى البروتينات التفاعلية ضد التهاب الأمعاء النخري في الدجاج اللاحم

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الملخص

تسبب المطثية الحاطمة (*CP*) التهاب الأمعاء النخري (NE) وقد عاودت الظهور كواحدة من أكثر الأمراض فتكًا في صناعة الدواجن بعد حظر المضادات الحيوية في علف الدواجن. هدفت الدراسة إلى إعداد وتقييم القدرة الوقائية لأربعة لقاحات محلية من النوع *CP* من النوع A ضد التهاب الأمعاء النخري (NE) في الدجاج اللاحم التجاري؛ ثلاثة هي *NetB* +ve بما في ذلك؛ ذوفان رسمي (FTNB)، طاف صوتي (SSNB)، بكتيريا مسلوقة (BBNB) والرابع هو *NetB* -ve (ذوفان رسمي SSNB. (FT، بروتينات داخل الهيولى، بيرفرينجوليزين O، جليسيرالديهايد 3-فوسفات ديهيدروجينيز (GPD)، و الفركتوز 1،6 ثنائي الفوسفات ألدولاز (FBA) بينما BBNB، بروتينات جدار الخلية؛ بروتينات افتراضية (HPs)، بيروفات أوكسيدوريدوكتيز (PFOR)، عامل الاستطالة--Tu (EF TU)، وبروتين لاصق الكولاجين (SSNB و تم تقسيم بروتينات BBNB بالرحلان الكهربي لتحديد قدرتها على إثارة الاستجابة المناعية ضد NE. وقد تم تصنيف 120 من كتكوت الدجاج اللاحم عمرها يوم واحد إلى ست مجموعات؛ تم خلط لقاحات المكافحة الإيجابية (C+ve) مع مادة مساعدة MontanideTM ISA 71 VG للكتاكيت تحت الجلد عند 3 و9 أيام - المجموعات التي تم تحديها عن طريق الفم باستخدام Eimeriamaxima في اليوم 20 و*CP* عند عمر 24 إلى 26 يومًا. ولوحظت الوفيات خلال 3 أيام بعد التحدي في المجموعة الضابطة الإيجابية فقط (25%). تم العثور على عيارات ELISA أعلى من الأجسام المضادة IgY في SSNB وBBNB عمليًا مقارنةً بمجموعة FT. تم تنظيم التعبير عن جين إنترلوكين -17 (IL-17) في أنسجة الطحال في جميع المجموعات باستثناء مجموعتي FT وC+ve. تظهر هذه كفاءة *NetB* الأصلي مع مستضدات المكونات الوقائية الأخرى ضد NE في دجاج التسمين.

الكلمات الدالة: المطثية الحاطمة، ELISA، Interlukien-17، التهاب الأمعاء الناخر، لقاح.