Effect of Aluminium Hydroxide Adjuvante Toxoid and Bacterin Combined Vaccine for Maternal Vaccination of Broilers Against Necrotic Enteritis


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Necrotic Enteritis (NE) caused by massive proliferation of *Clostridium perfringens* in the small intestine of the chicken has been a persistent problem in commercial poultry, especially in rapidly growing broiler chickens. It is an acute disease of 2 to 4 week old broiler chickens, causing high mortality and poor feed conversion ratio [Williams, *et al.*, 1999]. Generally, *C. perfringens* type A and to lesser extent type C causes NE. The type A strains produce the chromosomal encoded alpha toxin and is considered to be a major contributing factor towards the development of intestinal necrosis, the characteristic lesion of NE in poultry [Wages and Opengart, 2003].

The reported predominance of NE occurrence at two to five weeks of age [Ficken and Wages, 1997] might partly be explained by the disappearance of maternal antibodies, while active production of antibodies at this stage is still low [Tizzard, 2000; Lovland, *et al.*, 2004]. In lambs and piglets, the maternal vaccination against *C. perfringens* type C necrotizing or haemorrhagic enteritis is an effective and commonly used preventive measure [Taylor, 1999; Lewis, 2000]. The present was undertaken on vaccination of broiler breeder hens with aluminium hydroxide adjuvanted toxoid and bacterin combined vaccine against necrotic enteritis and assessment of their efficacy.

Materials and Methods

The isolates of alpha toxigenic strains of *C. perfringens* (confirmed by alpha toxin specific PCR) maintained at the Department of Veterinary Microbiology, Veterinary college and Research Institute, Namakkal, India were used in this study. The isolates were preserved in 15 per cent glycerol broth at -20°C. Alpha toxin was extracted and purified from toxigenic strains of *C. perfringens* as per the method described by Hofshagen and Stenwig [1992].

Each toxigenic strain of *C. perfringens*, preserved at -20°C, was suspended in fluid thioglycollate broth, inoculated onto a 5 per cent sheep blood agar plate and incubated anaerobically for 24 h at 37°C. Colonies from pure culture were inoculated on Brain heart infusion broth (10ml) and incubated an aerobically overnight at 37°C. The broth was then transferred to another 490 ml of brain heart infusion and incubated anaerobically for 48 h at 37°C. The broth was centrifuged at 400 × g for 30 min. The supernatant was collected and immediately subjected to purification by the ammonium sulphate precipitation method.

The toxin activity was checked by sub culturing on 5 per cent sheep blood agar for double haemolysis and onto Egg yolk agar for lecithinase activity. Cultures were incubated at 37°C and the purified toxin was kept at -20°C for later use.

For quantitative estimation of alpha toxin the total protein kit- Lowry’s Method (Genei, Bangalore) was used. Bovine albumin fraction V was used as standard. Optical density values were measured at 660 nm in a
Systronics double beam UV-VIS spectrometer :2202. The SDS-PAGE in a discontinuous buffer system with a 10 per cent (w/v) acrylamide resolving gel with a five per cent 0 (w/v) stacking gel according to the protocol described by Hale and Stiles (1999) was used to test the purity of alpha toxin. Lecithinase C Type IX (M/s Sigma laboratories) was used as control to check the purity of alpha toxin.

Formalin to a final concentration of 0.06 per cent v/v was added to the toxin and this was incubated at 37°C for 48 h in an orbital shaker incubator for inactivation of the toxin. After 48 h a loopful of inactivated toxin was spotted onto egg yolk agar plate and sheep blood agar plate and kept anaerobically for overnight at 37°C to check the inactivation.

The method described by Wilkie et al. [2006] was followed for preparation of broth Bacterin. Each toxigenic strain of C. perfringens was suspended in fluid thioglycollate broth and incubated anaerobically for 18 h at 37°C. For bacterin production, they were centrifuged at 3640 × g for 15 min, cells were harvested and pooled together. The cells were washed twice in phosphate buffered saline (PBS, pH 7.2), and resuspended in PBS containing 0.06 per cent formalin. The concentration was adjusted with a McFarland standard tube no. 4 (1.2 × 10⁹ cfu/ml). The cells were incubated at 37°C for 24 h in an orbital shaker incubator. After 24 h, a loopful of bacterin was streaked onto a sheep blood agar plate and incubated under anaerobic condition for 48 h to check the inactivation. Sterility was assessed by inoculating the inactivated bacterin onto Perfringens agar plates under aerobic and anaerobic incubation at 37°C for 48 h. The bacterin was stored at 4°C.

Clostridium perfringens type A toxoid vaccine was developed, combined with bacterin and aluminium hydroxide adjuvant and the final concentration of toxoid was adjusted to a 100 µg per dose and final concentration of bacterin was adjusted to 1.2 × 10⁹ cfu per dose. This combination was stored at 4°C until further use.

For preparation of the vaccine the toxoid and bacterin were mixed together first and then blended with aluminium hydroxide. The blending was done by slow stirring at 20°C for 4 h and subsequent storage at 4°C. The aluminium hydroxide was added at 0.5 mg per ml of vaccine. Purity, sterility and safety of the vaccine were checked as per the OIE manual of standards for diagnostic tests and vaccines (OIE, 2003). This vaccine was designated as ‘M’.

Ten broiler breeder hens at 26th wk of age were obtained from a registered commercial broiler breeder farm in Namakkal and reared as per the standard management procedure. The birds were fed with standard broiler breeder feed. Ten broiler breeder hens were primarily vaccinated with 0.5 ml of ‘M’ (Toxoid + Bacterin + Aluminium hydroxide) vaccine i/m at the 28th week of age and a respective booster dose was given 2 weeks later. Artificial insemination was done every three days from the 28th week onwards. Eggs were collected between the 31st and 36th week and they were incubated for hatching as per standard hatching practices. During hatches, sixty recently hatched chicks were collected and reared up to 4 weeks at the experimental animal house of Department of Veterinary Microbiology, Veterinary College and Research Institute, Namakkal as per the standard management conditions.

Sera were collected at 30 and 31 weeks of age of breeder hens and every week up to fourth week from sixty chicks for assessment of maternal antibody levels against C. perfringens alpha toxin. The sera were heat inactivated and stored at -20°C until further analysis.

Alpha toxin specific indirect enzyme linked immunosorbent assay (ELISA) was performed essentially as described by Heier et al. (2001) Lecithinase C Type IX purchased from M/S. Sigma was used as the Antigen.
Hyper immune serum for toxoid raised in six, six weeks old cockerels was used as positive control. Serum samples collected from Serum Antibody Negative (SAN) birds were used as known negative control. Optimum concentrations of coating antigen, serum samples and conjugate were arrived at by a preliminary checker board titration.

Each well of the ELISA plate was coated with 100 μl of antigen (5 μg protein per ml) coating buffer (pH 9.6), and kept overnight at 4°C. The plate was then washed with PBST (Phosphate buffered saline in Tween 20) for three min. This process was repeated three times and blocked with 1 percent BSA (Bovine serum albumin) for 60 min at 37°C. The wells were washed as described above. The sera were diluted 1:100 in PBST and 100 μl was added to duplicate wells for optimum results and the plate was incubated for 60 min at 37°C. After another washing with PBST, 100 μl anti chicken peroxidase conjugate at 1:15000 dilution in blocking buffer was added and incubated for 60 min at 37°C. The wells were washed with PBST and 100 μl of OPD substrate was added and incubated at room temperature in the dark for 20 min for the development of a color reaction. 100 μl 1N HCl was added to all the wells to stop the reaction and the optical density values were measured at 496 nm in an automatic ELISA reader (Multiskan Ex, Labsystems). Statistical analysis was performed by Randomized Block Design.

Results and Discussion

In the present study, BHI broth was used for extraction of alpha toxin from pooled toxigenic strains of *C. perfringens* type A and incubated for 48 h at 37°C. Alpha toxin was separated and purified from culture supernatant by 30 per cent and 70 per cent saturation of ammonium sulphate precipitation and the protein concentration of alpha toxin obtained was 4 to 5 mg per ml. SDS – PAGE analysis indicated that the alpha toxin extracted in this study had protein bands of 43000 Da.

Lecithinase activity of alpha toxin was determined by opalescence production on egg yolk agar and haemolytic activity by haemolysis on sheep blood agar. 0.06 per cent v/v formalin was used to inactivate the ammonium sulphate precipitated; dialysed, alpha toxin and absence of lecithinase and haemolytic activity on egg yolk agar and sheep blood agar respectively confirmed the inactivation of toxin.

Clostridial toxoids are soluble proteins of relatively low antigenicity and traditionally, poor stability. Thus clostridial vaccines require adjuvants to increase antigenic potency and to enhance stability [David, 2000]. The final concentration of toxoid in vaccine used in the study was 100 μg per dose. Schoepf et al. [2001] used 50 μg of genetically constructed alpha toxin for immunizing twelve BALB/C mice and reported that the protective immunity was produced against wild type alpha toxin in mice. The final concentration of the bacterin, used in this study, was $1.2 \times 10^9$ cfu/dose. Absence of haemolysis on sheep blood agar and no growth on *perfringens* agar proved the bacterin inactivation and sterility respectively, Wilkie et al. [loc. cit] used $1.0 \times 10^9$ cfu of killed *C. perfringens* for hyper immunization of layers against *C. perfringens*.

The mean OD values of the sera collected from breeder vaccinated with ‘M’ vaccine were 0.372 and 0.492 at 30th and 31st week respectively. In progeny chicks, at first week of age, the highest mean OD value of 0.434 was observed and in the second week a mean OD value of 0.332 was observed. The lowest mean OD value of 0.094 and 0.088 was observed in the third and fourth weeks sera and it may be due to a short half life period of maternal (IgG) immunoglobulin in broiler chicks.

The incorporation of bacterin along with toxoid is helpful for suppression of colonization of *C. perfringens*, toxin production and neutralization of toxin for clostridial diseases in other animals.
Based on the results it is suggested that though the maternal vaccination against NE in a field situation resulted in only moderate protection against the disease, vaccination might be economically beneficial.

Summary

Broiler breeder hens were vaccinated with aluminium hydroxide adjuvanted C. perfringens type A toxoid and C. perfringens bacterin combined vaccine at 28th and 30th wk of age and in progeny chicks up to 4 wk. The mean OD values of the sera collected from breeders vaccinated with vaccine were 0.372 and 0.492 at 30th and 31st wk respectively. In progeny chicks, at first week of age, highest mean OD value of 0.434 was observed and in second week the mean OD value of 0.332 was observed. The lowest mean OD value of 0.094 and 0.088 was observed in third and fourth week sera and it may be due to a short half life period of maternal (IgG) immunoglobulin in broiler chicks. The results obtained in the present study showed that maternal immunity was highly associated with protection against the development of subclinical necrotic enteritis (NE) in progeny chicks. The maternal vaccination might be economically beneficial.

References


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