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In ovo Vaccination in Ducks against Aflatoxin-Ovalbumin Conjugate

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ABSTRACT

Background: Aflatoxicosis is a major disease affecting ducklings especially due to their high susceptibility during the post-natal stage. There is no vaccination schedule for this disease as aflatoxins are haptens and incapable of generating immune response by itself.

Methods: In this study to combat this malady, *in ovo* vaccination with an aflatoxin-chicken ovalbumin conjugate was done on 25th day of incubation to generate adequate immune response during the post-natal period of ducklings. An isolate of *Aspergillus flavus* was used for harvesting of the aflatoxin. The purified crude toxin was checked by TLC to observe the presence of all 4 isomers. Chicken ovalbumin was used as carrier protein due to its similarity with duck ovalbumin as well as to avoid autoimmunity. The conjugate was then encapsulated with chitosan to prevent clearance of the antigen as well as to provide a balanced Th1/Th2 response. A booster dose of the conjugate was given on 14th day post hatch along with sheep red blood cells. The antibody titre was checked by passive haemagglutination test.

Result: The results seen *i.e.*, Log_2 antibody titre of 1.33±0.21 proved that an immune response was generated. This could provide protection to the ducklings against aflatoxins. Also, this is the first time *in ovo* vaccination in ducks with aflatoxin-chicken ovalbumin conjugate has been carried out.

Key words: Aflatoxin-Ovalbumin conjugate, Ducks, *In ovo* vaccination.

INTRODUCTION

Aflatoxicosis is one of the major diseases affecting ducks as they are more susceptible to it compared to other species even in parts per billion concentrations, 200 times more compared to chickens (Han *et al*., 2008; Wogan, 1975). Aflatoxin exposure to ducks either through feed or environment, even at minute doses lead to carcinogenic effects in not only the liver but in other organs as well. They are genotoxic and teratogenic as well due to the formation of adducts. Aflatoxicosis predisposes to other infections mainly due to its immunosuppressive nature (Benkerroum, 2020). In tropical climates, aflatoxicosis is widely prevalent becoming a bane to duck farming industry. Being liposoluble, they are deposited in fatty tissues and thereby can indirectly affect humans as well (Bintvihok and Kositcharoenkul, 2006).

Aflatoxins being haptens, have low molecular weight (less than 1 kDa) and cannot generate an immune response. To elicit an immune response against aflatoxin, conjugation of the hapten to a carrier protein such as Bovine Serum Albumin or Ovalbumin have been seen to be successful (Langone and Van Vunakis, 1976). In this study, chicken ovalbumin was selected as the carrier protein to bind to purified crude toxin containing all 4 isomers *i.e.*, B_1, B_2, G_1 and G_2 so as to provide protection against all 4 isomers during the postnatal stage of growth of the ducklings. The selection of chicken ovalbumin was mainly due to the reason that it has subtle differences from duck ovalbumin (Miguel *et al*., 2005). Also, the use of duck ovalbumin may lead to autoimmunity in the vaccinated ducks. Prior to this study, there was no report of *in ovo* vaccination in ducks against aflatoxin using an aflatoxin-ovalbumin (AF-OVA) conjugate. Furthermore, *in ovo* vaccination on 25th day of incubation would confer protective immunity during the early post-natal period when ducklings are most susceptible.

The immune response to the conjugate was measured by Passive Haemagglutination test. Chitosan was used to encapsulate the conjugate to prevent clearing of the antigen without generating immune response in the vaccinated duck embryos. Chitosan is also known to generate a balanced Th1/Th2 response (Wen *et al*., 2011). A booster dose of the AF-OVA conjugate was given to the treatment group 14 days post hatch along with sheep red blood cells (RBCs). Haemagglutination test was done to measure the humoral response against sheep RBCs. The results revealed that an immune response was generated against the conjugate giving proof of concept. This would

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prove very useful in protection of ducks against this disease.

MATERIALS AND METHODS

Birds and housing

Khaki Campbell breed ducks were used for the experiment. Incubation of the duck embryos of the two experimental groups were done under identical conditions. The two groups were kept in battery brooder cages after hatch under identical conditions in the poultry house of Department of Veterinary Microbiology, College of Veterinary Sciences and Animal Husbandry, Central Agricultural University, Mizoram, India. Feed and water were given *ad libitum* during rearing till 6 weeks of age.

Production of aflatoxin

An isolate of *Aspergillus flavus* from peanut was chosen for production of aflatoxin using sterile in-house Potato Dextrose Agar (50 g sliced potato, 5 g dextrose and 5 g agar in 250 ml distilled water). Extractions were done twice with chloroform after 10 days of incubation. The extract was dried, reconstituted and centrifuged using n-hexane to remove any impurities.

Thin layer chromatography to confirm presence of all isomers of aflatoxins

It was carried out using a Chloroform: Acetone (9:1) solvent system for 15 minutes runtime and visualised under Ultraviolet Transilluminator and Gel Documentation System (Alpha Imager).

Extraction of chicken ovalbumin

Chicken ovalbumin was extracted from chicken eggs by separation the egg white extract by drainage and the supernatant collected after centrifugation of the extract. Protein concentration was determined using UV spectrophotometry. Confirmation was done by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).

Conjugation of ovalbumin to aflatoxin

This was done as per Chu and Ueno (1977) with some modifications. First, the toxin was converted to its hemiacetal form as per Kononenko *et al.* (2002). Toxin was reconstituted in acetone to which 10 % H_2SO_4 was added. This was then incubated for 4 hours at 56°C in hot water bath and then evaporated to dryness. The hemiacetal was reconstituted in N, N-dimethylformamide. The hemiacetal solution was slowly added to the ovalbumin (OVA) solution in 0.05 M carbonate bicarbonate buffer. The solution was stirred with a magnetic stirrer for 4 hours. Then 0.2 ml sodium borohydride (1 mg/ml) was added and the solution was kept at 4°C for 8 hours with periodic stirring. This was then dialysed against Phosphate Buffer Saline (PBS). Then it was lyophilised to remove excess moisture and kept in -40°C. The hapten carrier coupling ratio was calculated using UV spectrophotometry at 355 nm using molar extinction coefficient 15180 (Langone and Van Vunakis, 1976).

Encapsulation of the conjugate by chitosan

Chitosan nanoparticles/microparticles were synthesised using ionotropic gelation of sodium tripolyphosphate and chitosan powder as per Mohammadpour Dounighi *et al.* (2012). Chitosan was dissolved in acetic acid at a concentration of 2 mg/ml. AF-OVA conjugate was dissolved in sodium tripolyphospate solution (1 mg/ml) at a concentration of 6 mg/ml which was then added to chitosan solution dropwise with magnetic stirring. This was then centrifuged at 6000G for 10 minutes and the supernatant collected and kept at -20°C.

Optimisation of the AF-OVA conjugate dose for *in ovo* vaccination

AF-OVA was given on 25th day of incubation at a dose of 3 mg and 6 mg per embryo to 2 different groups of 10 eggs each. The hatchability was used to assess the safe dose for *in ovo* vaccination.

In ovo vaccination of AF-OVA conjugate

In ovo vaccination was done on 25th day of incubation in 2 groups of 40 embryos each, one group acting as negative control and the other as AF-OVA treatment group. Sera was collected on 10th, 21st and 28th day post hatch.

Booster dose 14 days post hatch

A booster dose of 10% Sheep RBC was given by i/m route to both groups and 3mg AF-OVA conjugate to the treatment group only.

Passive haemagglutination tes

It was done as per Hay and Westwood (2002) with some modifications. Ox red blood cells (RBCs) were collected and mixed in equal volume of Alsever's solution and kept at a concentration of 10% v/v. For tanning, 5% Ox RBCs were taken and equal volume of 1/20000th dilution of tannic acid was added and incubated at 37°C for 15 minutes. Washing with Phosphate Buffered Saline were carried out thrice and the pellet was resuspended in Borate Succinate Buffer at 37°C for 30 minutes. The cells were then washed again and pelleted and kept at a final concentration of 2% v/v. The test was carried out on a 96 well V bottom plate with 25 µl of serially diluted sera and 25 µl of 1% sensitised AF-OVA conjugate per well. The reading was taken after 40 minutes of incubation at 20°C.

Haemagglutination test

It was done as per Bhanja *et al.* (2012). The test was carried out on a 96 well V bottom plate with 25 ìl of serially diluted sera and 25 µl of 1% Sheep RBCs per well. The reading was taken after 40 minutes of incubation at 20°C.

RESULTS AND DISCUSSION

After production and purification of the crude toxin, Thin Layer Chromatography confirmed the presence of all 4 isomers. The extraction of ovalbumin was also confirmed by SDS-PAGE where bands of 42.7 kDa were seen and no

Table 1: Mean±SE Log_2 anti AF-OVA conjugate antibody titre of serum by passive haemagglutination test.

Day	Group 1 (Negative control)	Group 2 (Treatment group)
10	0	1.33±0.21
21	0	1.33±0.21
28	0	1.33±0.21

Table 2: Mean±SE log_2 anti-sheep rbc antibody titre of serum by haemagglutination test.

Day	Group 1 (Negative control)	Group 2 (Treatment group)
21	4.76±0.21	1.50±0.22
28	5.5±0.22	4.17±0.16

bands of 32-37 kDa corresponding to globulin fraction were seen. The molar coupling ratio for the AF-OVA conjugate was found to be 2.79. Chicken ovalbumin was used as the carrier protein for the hapten aflatoxin as being from chicken species they would elicit a strong immune response in ducks. Another reason is that if antibodies get raised against the carrier, then chicken ovalbumin being structurally different to duck ovalbumin would be a safer choice to prevent autoimmunity (Miguel *et al*., 2005). The encapsulation by chitosan as per Mohammadpour Dounighi *et al.* (2012) resulted in a final concentration of 6 mg/ml.

For *in ovo* dose optimisation, the dose of 3 mg was found to be safe for the *in ovo* vaccination experiment wherein all the eggs hatched. *In ovo* vaccination in both the groups yielded similar hatch results *i.e*., 32 for the control group and 26 for the treatment group. An immune response was observed from the PHA test as seen in Table 1. Anti-Sheep RBC antibody titre is shown in Table 2, which shows the immunocompetence of the ducklings against the T-dependent antigen *i.e*., Sheep RBCs after chitosan encapsulation due to the stimulation of immune Th1/Th2 response (Wen *et al*., 2011).

Mucosal administration of Aflatoxin B1 conjugate was reported to produce a significant IgG immune response in chickens (Yang *et al*., 2021). *In ovo* vaccination stimulates mucosal immunity (Wilkinson *et al*., 2003). The current study further showed that *in ovo* vaccination with AF-OVA conjugate generated a humoral immune response. The immune system was also enhanced by chitosan encapsulation (Wen *et al*., 2011). This combination when administered by *in ovo* route is helpful in combating aflatoxin exposure during the post-natal period of ducklings. This would provide protection to the ducklings from aflatoxins in feed or environment during their early growth stage when their immune system is not fully developed. There are no reports of *in ovo* vaccination in ducks against aflatoxins using an aflatoxin-ovalbumin conjugate till date and thereby, this research opens up a plethora of opportunities for *in ovo* vaccination in ducks.

CONCLUSION

From the above results, this study can be seen as a novel approach to combat the conundrum of aflatoxicosis in young ducklings by use of an effective and uniform vaccination system such as *in ovo* vaccination which is less taxing, cheaper and easier compared to vaccinations given post hatch which is quite laborious and where the dose may not be uniform. *In ovo* vaccination is commercially used in hatcheries against Marek's disease in USA and some other countries (Sharma and Burmester, 1982). Commercial application of *in ovo* technology has also been applied against Infectious Bursal Disease and Fowl pox as well (Sharma *et al*., 2002). But there has been no report of ducks being vaccinated either by *in ovo* route or any route against a disease like aflatoxicosis using an aflatoxin-ovalbumin conjugate. And the use of chitosan encapsulation would further help to stimulate the Th1/Th2 response giving a balanced humoral and cell mediated immune response as well as prevent antigen clearance before generating a proper immune response. Commercial application of the *in ovo* technology would be a great gift to the duck farming industry.

Conflict of interest: None.

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