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Trial of *Aspergillus fumigatus* Vaccine in Broiler Chicks

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Abstract: There is a scarcity of information on the effect of vaccine against aspergillosis in Zaria and Kaduna areas. Thus, this study was conducted to determine the efficacy of an *Aspergillus fumigatus* germling vaccine. The methodologies used in this study included Aspergillus spore production, determination of LD_{50}, preparation of the germling vaccine and testing the efficacy on broiler chicks. Three groups of 4 days old broilers were exposed to 4.2-5.8×10^{8} cfu g^{-1} lung tissue of *A. fumigatus*. The calculated amount of *A. fumigatus* that killed 50% of the broilers was 5.8×$10^{7.4}$. *A. fumigatus* was recovered 4 weeks post exposure. A germling vaccine of *A. fumigatus* was administered to 4 days old broilers using the ocular route. The trial vaccine showed 40% protection when administered to chicks 2 weeks prior to challenge with *A. fumigatus* spores. It is recommended that levels of *A. fumigatus* spores should not rise to 5.8×$10^{7.4}$ cfu g^{-1} lung tissue in poultry houses. Adjuvants can be added to the vaccine to raise the level of protection using different routes of administration and vaccinating at an older age.

Key words: Aspergillosis, aflatoxin, aspergillus, poultry, vaccine

INTRODUCTION

Aspergillosis is an important mycotic disease that affects many species of birds including poultry and although the term aspergillosis usually refers to pulmonary aspergillosis (lung or air sacs), the disease is also manifested as systemic, ophthalmic or encephalitic syndromes (Akan *et al*., 2002; Abdu, 2007). All species of the genus *Aspergillus* (*A. fumigatus*, *A. flavus*, *A. niger*, *A. nidulans* and *A. terreus*) are known to cause aspergillosis with *A. fumigatus* being the most commonly encountered and most pathogenic *Aspergillus* species in poultry (Greenacre *et al*., 1992; Abdu *et al*., 1995; Joseph, 2000; Jones and Orosz, 2000; Deem, 2003). Aspergillus is a common and ubiquitous soil saprophyte and is worldwide in distribution (Khosravi *et al*., 2009).

The disease manifests either as an acute condition with high mortality rate in chicks or as a chronic condition in older birds. Predisposing factors to aspergillosis include stress, bacterial or viral diseases, poor housing and long time treatment with antibiotics or immunosuppressive drugs. Control of *Aspergillus* species and hence the control of aspergillosis is to remove all management practices that predispose birds to the disease (Jodas and Hafez, 2000). There are also antifungal agents available for the treatment of aspergillosis such as fluconazole and caspofungin (Deem, 2003; Orosz and Frazier, 1995; Rambach *et al*., 2005; Beernaert *et al*., 2009). The use of anti fungal agents have some limitations such as drug residue in the meat and eggs of the chickens that is consumed by man Kolaczkowska *et al*. (2010), long term use of the anti fungal agent can lead to colonization of the gastrointestinal tract of the chickens by opportunistic bacteria resulting in bacterial infections such as septicemia and possible emergence of resistant strains (Reece, 1988). Since, *Aspergillus* species are ubiquitous and the uses of anti fungal agents have limitations, the possibility of producing a vaccine that will protect birds from aspergillosis needs to be studied.

So far there are no known vaccines against fungal diseases possibly because of the complex structure of fungi making it difficult to identify and target antigenic sites and also difficulty of accessing infection sites by drugs and antibodies. Attempts at producing vaccines against fungal infections is only just began (Richard *et al*., 1982; Cenci *et al*., 2000; Ito *et al*., 2006).

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This trial of a crude germling vaccine of *A. fumigatus* is an attempt to that gap to determine LD_{50} of *Aspergillus fumigatus*, prepare *Aspergillus fumigatus* vaccine and determine its efficacy in broiler chicks.

MATERIALS AND METHODS

Ethical clearance for use of the chicks for the experiment was obtained from the faculty board and ethics committee for experiments in animals of the Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria.

Production of *Aspergillus fumigatus* spores: *Aspergillus fumigatus* isolated from the trachea of apparently healthy chickens in Zaria and identified as strain C000 by Dr. Libero Ajello of the CDC, Atlanta, Georgia, USA was used. The *A. fumigatus* was grown on 250 mL Erlenmeyer flask slants of Sabourauds' dextrose agar containing 0.05 mg mL^{-1} of chloramphenicol for the control of bacteria. The flasks were incubated at room temperature for 2 weeks to allow for maximum sporulation and maturation of spores. At the end of 2 weeks the spores were harvested by using a vacuum suction pump connected to a bottle. The harvested spores in the bottle were capped and stored in the fridge at 4°C for use. About 60 days old broiler chicks were purchased from a licensed dealer and housed in a room. They were placed in a cage (65×40 cm) and fed pfizer broiler starter *ad libitum* for 4 days to settle and acclimatize. On day 4 they were divided into 6 groups of ten birds each.

Six groups of 10 broiler chicks each were aerosolized with spores of *aspergillus fumigatus* as follows:

- Group 1: aerosolized with phosphate buffered saline solution (PBS pH, 7.4)
- Group 2: aerosolized with 0.75 g of *A. fumigatus* spores
- Group 3: aerosolized with 1.2 g of *A. fumigatus* spores
- Group 4: aerosolized with 1.5 g of *A. fumigatus* spores
- Group 5: aerosolized with 2.0 g of *A. fumigatus* spores
- Group 6: aerosolized with 2.5 g of *A. fumigatus* spores

The infection of chicks was conducted in an inoculating chamber using a sprayer. The inoculating chamber was fitted with a UV fluorescent tube for sterilization. A beaker of boiled water set to the side to increase the humidity of the chamber and a small 1.5 amp fan was put in the chamber to increase air movement. This was to simulate the conditions of humidity necessary for maximum inhalation of the spores by the chicks. Each group of 10 chicks was placed in the inoculating chamber one at a time and infected with the different quantities of *Aspergillus fumigatus* spores. The infection was via aerosol delivery and the birds left for 15 min for maximum inhalation before being returned to their cages which had earlier been labeled. In between exposure the UV light was left on for 1 h to sterilize the chamber which was subsequently cleaned out and mopped down with Izal(R) soaked cotton wool. Each cage was labeled and the chicks were observed for a period of 10 days or until they died. Dead birds were examined for lesions and lungs samples harvested for laboratory isolation of *A. fumigatus*. The birds from the first cage (controls) were sprayed with 10 mL Phosphate Buffer Saline solution (PBS pH 7.4) and returned to their cages for observation.

Two birds were immediately euthanized and their lungs removed and ground in 5 mL of 1/10 dilution of PBS solution (pH 7.4). About 1 mL of the grounded lungs was plated on potato dextrose agar and incubated at room temperature until growth was noticed. The equation $LD_{50} = V\text{-}d\ (v_1\text{-}50/v_1\text{-}v_2)$ of Reed and Muench (1938) was used to calculate the LD_{50} of *A. fumigatus* where LD_{50}-lethal dose that kills 50% of test chicks, V-log of the highest last dilution that gave 100% response, d-$\log_{10}$ successive dilution, v_1% proportion of response that is >50%, v_2% proportion of response <50%.

Vaccine preparation: Germling (germinating spores) vaccine was prepared according to the method of Richard *et al.* (1982) by inoculating 100 mL asparagine broth as prepared by Paik and Suggs in 250 mL Erlenmeyer flasks to which was added 1 mL of a spore suspension of *A. fumigatus*. The inoculum was prepared by suspending 400 mg of *A. fumigatus* spores in 30 mL of a 1×10^{-4} concentration of polysorbate 80 (tween 80) in PBS (pH 7.4).

The flask was then fastened to a flask shaker with the bottom dipped into a water bath at 42°C. Samples from the flask were obtained every 30 min interval and examined microscopically for germinations. When at least 90-95% of the spores had swollen and germ tubes were evident without branching (usually 7-10 h) further growth was stopped using 3 mL of 10% formalin was then added giving a total volume of 104 mL of the asparagine broth in the flask. The germling was then harvested by filtering through a 0.45 μm APD millipore filter using vacuum

pressure pump. The germling was washed twice on the filter paper with 100 mL volumes of sterile distilled water. After washing they were rinsed into a sterile bottle and suspended in a 1 mL of water and stored in the refrigerator at 4°C for use.

Vaccination of broiler chicks: The germling vaccine produced was given to the chicks via the ocular route. About 44 days old broiler chicks were placed in 4 groups and vaccinated as follows:

- Group 1: each eye inoculated with 25 μL of germling vaccine
- Group 2: each eye inoculated with 50 μL of germling vaccine
- Group 3: each eye inoculated with 75 μL of germling vaccine
- Group 4: each eye inoculated with 75 μL of PBS (This group was challenged but not vaccinated)

Challenge to test the efficacy of vaccine: About 14 days post vaccination the chicks in all the groups were aerosolized with approximately 5.8×10^{8} cfu g^{-1} of lung tissue. The chicks were observed for 2 weeks for signs of aspergillosis and mortality. All dead chicks were examined at postmortem and the lungs harvested for laboratory culture of *A. fumigatus*. Also, the percentage protection was calculated using the equation:

$$\frac{\text{Percentage dead in non vaccinated} - \text{Percentage dead in vaccinated}}{\text{Percentage dead in non} - \text{vaccinated}} \times 100$$

RESULTS AND DISCUSSION

The result showed that when 4 days old broiler chicks were aerosolized with the various concentrations of *A. fumigatus*, there were varying degree of mortality and the LD_{50} calculated from the results was 5.8×10^{74} (Table 1). This calculation was based on the number of dead chicks in groups 4 and 5 showing that 50% mortality was between the two concentrations of 4.9×10^{8} and 5.8×10^{8} cfu g^{-1} of lung tissue. The result also showed that surviving chicks had *A. fumigatus* in their lungs weeks after inoculation (Table 2). The ocular vaccination of broiler chicks using the crude germling vaccine preparation offered a maximum of 40% protection to the chicks (Table 2). The gross lesions observed at postmortem of the dead chicks were nodules on the air sacs, miliary nodules on the peritoneum and congestion of blood vessels.

The number of *A. fumigatus* spores that killed 50% of experimental broiler chicks was 5.8×10^{74} cfu g^{-1} of lung tissue in this study. This is probably the first time of trial and documentation of such vaccine in poultry in Nigeria known to the researcher. It represents an effort to develop a preventative approach to aspergillosis in place of treatment with drugs with their many side effects such as toxicity to host tissue, residue and cost of production (Richard *et al*., 1991; Kunkle *et al*., 1999). The LD_{50} in this study was found to be higher than that which was found by Richard *et al*. (1982) using 2 weeks old turkey poults. This showed that turkey poults may be more susceptible to infection by *A. fumigatus* than broiler chicks. The percentage protection in this study was <50% and this could be due to a number of factors such as the germling vaccine used in this study was not purified due to lack of facility so impurities could have reduced efficacy of the vaccine, the particulate nature and volume of the vaccine used in each group possibly was probably not enough for the chicks to marshal a high antibody response.

Recently there has been an effort to target specific Aspergillus antigens and to produce in commercial quantities, vaccine against aspergillosis through the proposed production and use of recombinant variants of *A. fumigatus* allergen Aspf3. The recombinant variant Aspf3 was found to protect mice against invasive aspergillosis (Ito *et al*., 2006). The result of this study showing a 40% protection is a step in the right direction and more research is needed to increase the percentage protection such as the incorporation of adjuvants like glycerin, *Pastuerellra multocida* lipopolysaccharide and avridine (Richard *et al*., 1991).

Table 1: Determination of LD_{50} of *Aspergillus fumigatus* in broiler chicks

Groups	Concentration of spores used for challenge (g)	Viable *Aspergillus fumigatus* in lungs cfu/g lung tissue	No. death/ challenged	No. with *A. fumigatus*/ No. examined
1	0.00	None	0/10	0/10
2	0.75	4.2×10^{8}	1/10	8/9
3	1.20	4.7×10^{8}	2/10	7/8
4	1.50	4.9×10^{8}	3/10	6/7
5	2.00	5.8×10^{8}	6/10	1/4
6	2.50	Not done	10/10	0/0

Table 2: Efficacy of crude *A. fumigatus* germling vaccine

Groups	Volume of vaccine	No. dead/challenged with 5.8×10^{8} cfu g^{-1} lung tissue	Protection (%)
1	75 μL (PBS)	5/5	0
2	25 μL	5/5	0
3	50 μL	4/5	20
4	75 μL	3/5	40

CONCLUSION

Aspergillus germling vaccine prepared by using *A. fumigatus* C000 offered inadequate protection to broilers when challenged with the same *Aspergillus* sp. post vaccination. Researchers recommend that poultry farmers be educated on the dangers of allowing fungi and especially *A. fumigatus* spores to rise above $5.8\times10^{7.4}$ cfu g^{-1} lung tissue in their poultry houses and the need to avoid all forms of stress which are predisposing factor for Aspergillus colonization on the birds.

RECOMMENDATIONS

Researchers also recommend that more research be conducted to develop an anti aspergillus vaccine for the control of aspergillosis in poultry.

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