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Trials for Reducing Aflatoxin B_1 Residues in Chicken Meat and Eggs using a Newly Developed Aflatoxin B_1 Vaccine

Heidy Abo El-Yazeed, Mai, H. Hanafy, R. Soliman and M. Refai

Department of Microbiology, Faculty of Veterinary medicine, Cairo University, Giza Egypt
Mohrefai@ yahoo.com

Abstract: *Fifty broiler chicks (seven days old) and 30 egg laying hens in the production phase (130 days old) were used in this study. Both of the broiler chicks and the egg layers were divided into test and control groups. Chickens and hens in the test groups were immunized S/C with aflatoxin B_1 (AFB1) vaccine adjuvanted with Isa 70 oil adjuvant. Vaccinated birds received 2 doses of the vaccine at two weeks interval. The control groups were kept non-immunized. One week following the booster dose, the birds of both vaccinated and control groups were challenged with aflatoxin contaminated diet (78µg/kg) for 17 days. Serum samples were collected at 0, 14, 28, 45 days of the experiment for measurement of aflatoxin B_1-specific antibodies production using ELISA. The titers of aflatoxin B1-specific antibodies in chicken sera of immunized birds ranged from 1/1600 - 1/3200 when measured 1 week after the booster dose. At the end of the experiment, samples of edible chicken products from both the immunized and non-immunized control groups were collected and tested for AFB_1 residues in an attempt to prove practically the ability of AFB_1- vaccine to reduce their level in these products. AFB1 residues were found in breast muscles of challenged non-immunized birds (34.3-49.0 pg/g) while no detectable levels were recorded in those collected from the immunized birds. Moreover, AFB_1 residues were found in liver of challenged non-immunized birds (16-25 pg/g), and were not detected in vaccinated birds. AFB1 residues were also detected in eggs of challenged non-immunized birds (0.2pg/g) and were not detected in eggs from AFB_1 vaccine immunized egg layers.*

Keywords: *Aflatoxin-B1 Vaccine, chicken eggs, chicken meat, Aflatoxin-B1 specific Antibodies, Aflatoxin B1residues*

1. INTRODUCTION

Aflatoxins are typically found as secondary metabolites of *Aspergillus parasiticus* and *Aspergillus flavus* **[1]**. It frequently contaminates cereal crops such as corn, beans, peanuts, and dried fruits **[2]**. Among all aflatoxins, aflatoxin-B_1 (AFB_1) has the highest toxicity. Epidemiological studies have shown that with prolonged exposure to AFB1 liver cancer may develop, especially in persons with hepatitis B antigens **[3-4].** Consequently, the World Health Organization (WHO) classifies AFB1 as a human carcinogen and proposes no safe dose **[5].** The induction of cancer by AFB1 has been extensively studied and the International Agency for Research on Cancer (IARC) has produced sufficient evidence of carcinogenicity of aflatoxin B1 in experimental animals.

No animal species is resistant to the acute toxic effects of aflatoxin B_1. A wide variation in LD50 values has been obtained in animal species tested with single doses of aflatoxins. For most species, the LD50 value ranges from 0.5 to 10-mg/kg body weight. Also the animal species respond differently in their susceptibility to the acute and chronic toxicity of aflatoxins. Environmental factors, exposure level, and duration of exposure beside age, health, and nutritional status of diet can influence the aflatoxins toxicity **[6].**

Poultry industry plays an important role in the economy of developing countries and although the demand of poultry meat is increasing day by day, still this industry is facing some of the major problems like bacterial, viral, metabolic disorders and mycotoxicosis. The latter is considered to be the second most alarming issue in the poultry industry after increased poultry feed prices.

The most prominent manifestations of experimental aflatoxicosis in layers include reduced egg production and egg weight, enlarged liver and increased liver fat are **[7-9].** High mortality and dramatic reduction of egg production were reported to occur during a natural outbreak **[10].** Egg size, egg weight and yolk as percent of total egg size also decreased **[11].** In Japanese quail, aflatoxicosis

was reported to induce decreased feed conversion, egg production, egg weight, hatchability and exterior and interior egg quality **[12].**

The potential hazard of aflatoxins to human health has led to worldwide monitoring programs for the toxin in various commodities as well as regulatory actions by nearly all countries.

The best strategy to avoid the build-up of aflatoxins in feedstuffs is to avoid contamination with aflatoxin producing fungi both in the field and during harvesting and storage [13], however, it is a difficult proposition particularly in developing countries where harvesting and storage conditions are far below the required standards. Several methods are reported that could be used to neutralize ill-effects of aflatoxins in poultry feeds. Addition of organic acids on inert support (e.g. vermiculite, zeolite, etc.) in feed results in fungistatic actions and thus reducing the chances of aflatoxin production [13-14].

In the present work it was planned to develop an immunologically based AFB1 clearance process through preparation of AFB1 vaccine. This vaccine was aimed to stimulate the synthesis of AFB1-specific antibodies that block initial absorption of the aflatoxin –B1 and neutralize the absorbed one.

2. MATERIAL AND METHODS

2.1. Standard Strain

As per gillus flavus ATCC 16875 obtained from Microbiological Resources Centre (Cairo MIRCEN), Faculty of Agriculture, ASU, Egypt.

2.2. Production of Aflatoxin B1 on different media [15]

2.2.1. Subculture of A. flavus

The of *A. flavus* standard strain was subcultured on slopes of potato dextrose agar (PDA) medium and was incubated at 25^0 C for 5 days.

2.2.2. Inoculation of the A. flavus strain

120 flasks, each containing 500 ml of sterile synthetic high salt medium (SH-medium) were inoculated with ***A. flavus*** strain and incubated for 8 days at 25°C as well as 60 flasks, each containing 500 ml YES semi-synthetic medium were inoculated and incubated for 15-20 days at 25°C.

2.2.3. Harvesting of the culture filtrate

The fungal mat was collected and discarded, and the filtrate of all flasks was poured into a collecting flask.

Determination of AFB1 concentration by Aflacheck test (semi-quantitative) and HPLC [22]

2.2.4. Concentration of culture filtrate containing AFB1 and toxin extraction [16]

The largevolume of aflatoxin culture filtrate was reduced by lyophilization in order to be able to perform toxin extraction.

The aflatoxins were extracted 3 times with 25 ml of chloroform, filtered through Whatman No. 1 filter paper, evaporated in a water bath at 60°C, and concentrated to a final volume of 5 ml according to the method of **Davis et al. [17]**. The extracts were dissolved in 50 ml methanol and transferred to a separation funnel. Fifty milliliters of 5% NaCl plus 50 ml of hexane were added. The mixture was shaken, the phases were separated, and the lower methanol phase was collected.

This process was repeated with the addition of 50 ml of hexane. The methanol portion was transferred to another separation funnel, and the toxin was extracted 3 times with 50 ml of chloroform. The lower (chloroform) layer was then collected, filtered through Whatman No#1 filter paper, and evaporated dry. After extracting the toxin, a sample was measured for AFB1 concentration.

2.3. Preparation of AFB1-Carboxymethyl Oxime

Since AFB1, has no reactive group for coupling reactions, AFB1 was first converted to AFB1-O-carboxymethyloxime (AFB1-Oxime) using the method described by **Polonelli *et al.*,[18]**.

2.4.Preparation of AFB1-protein conjugates

The AFB1 immunogen was prepared by conjugation of AFB1-Oxime with BSA in the presence of EDPC (1Ethyle,3,3 Dimethylaminopropyle,carbodiimide (water soluble Carbodiimide-Sigma) according to **Chu,F. S. and Ueno, I. [19].**

2.5. Confirmation of AFB1-BSA-conjugate formation

A. In order to confirm the success in conjugation process between AFB1 and bovine serum albumin and determination of the actual conjugation percentage, the prepared conjugate sample together with the AFB1-BSA conjugate standard (sigma), were analyzed using spectrophotometric method (U.V 1600 series).

B. The concentration of both preparations (standard and tested sample) was adjusted to 1mg/ml. Before testing, the samples were diluted with water (1:30) before being injected into the U.V apparatus. According to the available literature **[19]**, the target product (AFB1-BSA conjugate) was expected to give peaks at 357- 362 nm. The result of the standard and prepared conjugate are compared and the conjugation percentage is determined.

2.6. Vaccine preparation for injection

A water-in-oil emulsion was prepared using a sterilized Potter-Elvehjem type homogenizer and sterile Isa 70- montanid oil adjuvant (7:3 vol/ vol). The total amount of AFB1 was estimated as each bird received 0.5 ml of the vaccine emulsion containing 50 ug of AFB1 conjugate.

2.7. Immunization of chickens

1. A total of 50-one day old Ross chicks were divided into 2 groups each contained 25 birds (Test and control). They were housed in an experimental shed, where installations and equipment were previously cleaned and disinfected.

2. A standard starter mash, grower mash, and water were supplied ad libitum. The chicks were allowed to acclimate for 4 days prior to initiation of the study.

3. During this adaptation period, the birds were submitted to conventional broiler chicken management in a heated battery and received a mycotoxin-free commercial ration that was tested by HPLC before feeding the birds, and received water ad libitum.

4. Thirty egg layers in the production phase (130 days old) were divided into 2 groups each 15 birds (Test and control groups). They were housed in an experimental shed where installations and equipment were previously cleaned and disinfected. The birds were distributed as 5 birds per cage in a total of 6 cages.

5. Commercial layers ration, pretested for its content of AFB1using HPLC was supplied as 112 g/bird/day divided into two meals, as well as water ad libitum was supplied during the whole period of the experiment.

6. Light was provided for 16 hours daily for egg production.

7. Birds were allowed to acclimatize for 4 days prior to administration of AFB1 first dose of the aflatoxin B1vaccine.

2.8. Vaccination (dose and route)

Each bird of the immunized groups was injected S/C with 0.5 ml AFB1 vaccine containing 50µg AFBI conjugate/dose.

2.9. Vaccination program

The primary immunization with the prepared AFB1 vaccine to the immunized group was administered at 4^{th} day after their arrival, followed by a booster dose 2 weeks later.

2.9.1. Aflatoxicosis induction in tested birds:

Preparation of aflatoxin contaminated diet was done according to **Alkonya, A. E.; Monda, E. O. and Ajana. S**., **[20]** as follows

A. Into a half liter conical glass flasks 200g of sterile corn kernels were added to each flask. The added crushed corn had been previously autoclaved at 121°C for one hour for two consecutive days to ensure its sterility and absence of ear rot fungi.

B. 200ml of sterile distilled water were added to the flasks, The aflatoxigenic *Aspergillus flavus* standard strain was cultured on PDA slants and incubated at 25°C for 10 days

C. Conidia were suspended in sterile water and 50 ml of spore suspension were inoculated on moistened corn, cultures were then incubated in the dark with shaking for 28 days at 25°C.

D. The cultures were then dried at 45°C for 72 hours. The dried samples were finally ground using blender and stored at 0°C until mixing.

E. The ration mixtures were prepared in a horizontal/ helicoidal mixer for 15 min at the concentrations proposed for the treatment (78ppb).

F. After preparation of the experimental rations, one kg sample was collected and analyzed for determination of AFB1 levels by HPLC for confirmation of the desired concentration (78ppb).

G. Feeding the birds: All birds of the tested and control groups were fed AFB1 contaminated diet (78ppb) starting from day 28 and for 17 days (45 day whole experiment period).

2.10. Evaluation of immunizing efficacy of AFB1 vaccine

2.10.1. Specimen

Serum samples

Blood samples were collected from birds of each group in dry sterile tubes without anticoagulant using wing vein puncture technique. The blood samples were collected before immunization and at day 21, 28, and 45 post- immunization from tested and control groups. Serum was separated and frozen until analysis. Adsorption of the albumin-specific antibodies from serum samples collected from the immunized birds was done using 1%BSA.

Breast muscle and liver samples: these samples were collected from the immunized and control birds according to[32].

2.10.2. Determination of the AFB1-specific antibody titers in the collected serum samples was done using ELISA[18] as follows

A. The wells of polystyrene microtiter plates (Costar) were coated overnight (4ºC) with 50 µl of AFB1- BSA conjugate (Sigma-Aldrich) 20 µg/ml, in 0.05 M sodium carbonate-bicarbonate buffer (pH 9.6).

B. The Plates were washed at this point and after each incubation step with of PBS containing 0.05% Tween 20 (200 µl/well) three times and then dried against tissue.

C. To each well, 50 µl of 2 fold serially diluted tested serum samples were added staring with 1/100 and incubated at 37ºC for 90 min.

D. 50 µl of goat anti-chicken IgG4 peroxidase conjugated antibody diluted 1:500 in PBS were added to each well.

E. After 2hrs incubation at 25ºC, 50 µl/well of ABTS substrate solution were added and kept in a dark place for 15 minutes.

F. The reaction was stopped after 15 minutes with 25 ml of 0.5 M H_2SO_4. The optical density (OD) was read at 492 nm using a Multiskan Ascent (Labsystems, Helsinki, Finland). **The Cut off value** (COV) was calculated as = the Mean value of OD of negative control serum samples + standard deviation of the OD of these samples X 2). Any tested sample giving OD equal or higher than the COV is considered positive.

3. RESULTS AND DISCUSSION

3.1. Results

3.1.1. The total amount of aflatoxin B1

The total amount of prepared culture filtrate was reduced by lyophilization to 142 g powder and the total concentration of the AFB1 toxin was 2630.38 µg in the lyophilized materials.

3.1.2. Result of AFB1-BSA-conjugate formation

The results shown in **Figs. 1, 2 and 3** documented **AFB1-BSA** conjugation percentage of 64.41%

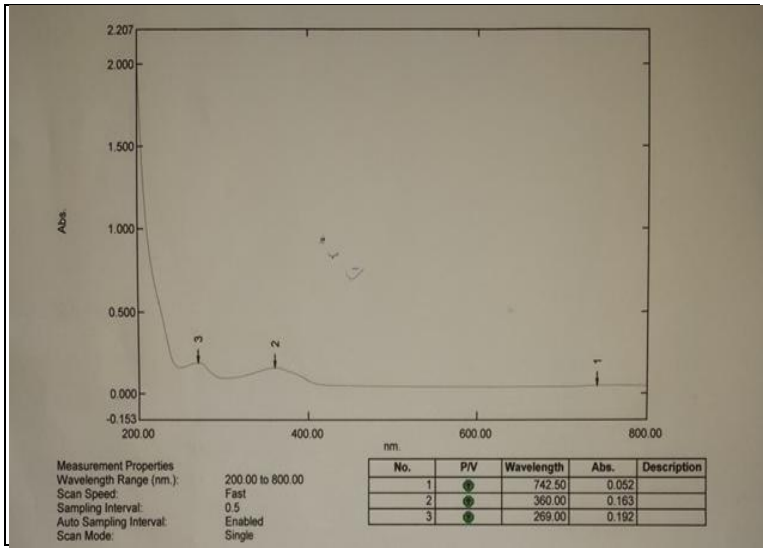

Fig.1: *The standard: the curve shows that at 360 nm the absorbance are 0.163, which represents the absorbance of AFB1-BSA standard conjugate (Sigma). This was considered as 100% value when compared with tested sample.*

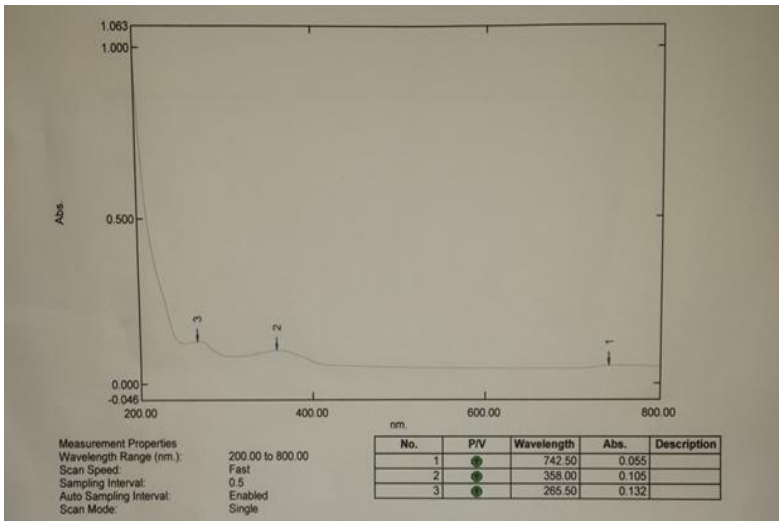

Fig2. *The examined sample of the prepared AFBI- BSA conjugate. The curve shows that at 358nm the absorbance is 0.105, which represents the absorbance of the prepared AFB1-BSA conjugate in the tested sample.*

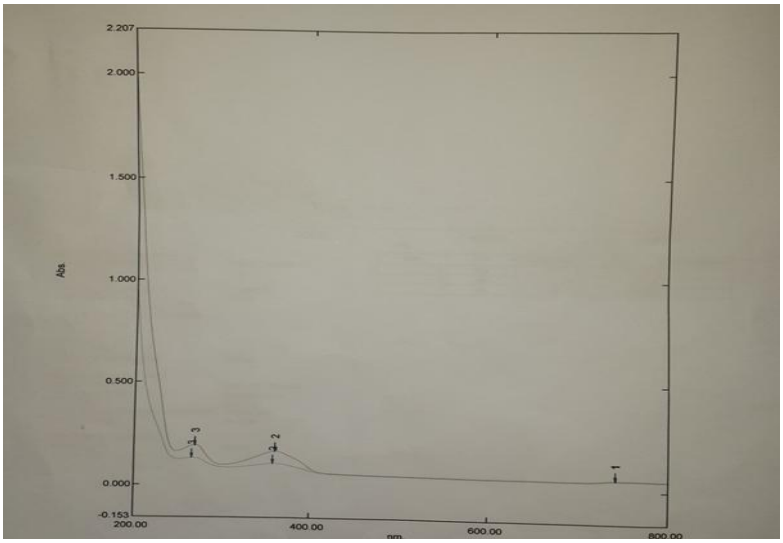

Fig3. *The overlay of Fig.1 and 2 with the peak (2) represents AFB1-BSA conjugate in both the standard and the examined sample. The percentage of conjugation in the examined sample in relation to the standard (100%) was equal to 64.41%.*

3.1.3. ELISA AFB1-specific antibody titers in immunized chickens

Serum samples from non-immunized birds were examined for AFB1-specific antibodies using ELISA and the determined Cut off value was equal to OD of 0.0855. Any OD equal or higher than the COV was considered positive. As shown in Table 1, AFB1-specific antibodies titers recorded in serum samples from immunized chicks and egg laying hens reached to 400 and 1600 Elisa units, respectively, when measured 2weeks post immunization. When measured 1 week after the booster dose it reached to 1600 and 3200, respectively. AFB1 specific antibodies measured 17 days post challenge reached to 3200 in immunized chicks and egg layers, respectively.

Table1. *ELISA titers of AFB1- specific antibodies measured in serum samples collected from immunized chickens at different phases of the experiment.*

Time of collection of serum samples	Immunized birds	ELISA Titers of AFB1-specific antibodies
2 weeks after the 1st dose of AFB1 vaccine (primary immunization).	Broilers	1/400
	Egg layers	1/3200
1 week after 2nd dose of vaccine (Booster dose)	Broilers	1/1600
	Egg layers	1/3200
17 days after AFB1 challenge period.	Broilers	1/3200
	Egg layers	1/3200

3.1.4. Level of AFB1 residues measured in chicken edibles including breast muscles, liver and eggs:

Table 2 shows the AFB1 concentration measured in samples collected from non- immunized birds after17 days post challenge with AFB1 ration. It reached to 34.3 -49.0 pg/g in breast muscle samples and to 16-25pg/g in the liver samples. While in samples collected from AFB1vaccinated birds no AFB1 residues could be determined. No AFB1 was detected in egg samples from immunized layers as compared to an amount of 0.2pg/g in egg from non immunized egg layer.

Table2. *HPLC measurements of AFB_1 residues in samples collected from breast muscles and liver of tested and control birds.*

Source of Samples	Concentration of AFB_1 pg/g	
	Muscles	liver
Non-Immunized birds (Challenged)	34.3 - 49	16.0 - 25.0
Immunized birds	ND*	ND*

**ND = not detectable*

Table3. *HPLC measurements of different aflatoxins in eggs samples collected from AFB1 vaccine- immunized and non immunized Egg laying hens at different phases of the experiment.*

Egg laying hens	Phase of sample collection	Aflatoxins conc. (pg/g) in egg samples		
		AFB1	AFG1	AFG2
Non-Immunized birds	At the start of the experiment	ND	6.4	3
	Post challenge	0.2	4.4	7.2
Immunized birds	2 weeks after 1st vaccine dose	ND	4.7	9.6
	1 week after booster dose (prechallenge)	ND	6.2	2.5
	Post challenge (end of experiment)	ND	0.9	ND

**ND = not detectable.*

3.2. Discussion

The presents study was conducted in an attempt to practically prove the ability of the prepared AFB_1vaccine to reduce AFB1 residues in chicken edible products (breast meat, liver, and table eggs) through production of neutralizing AFB1-specific antibodies in sera of chicken (immunological clearance mechanism). In our study the s/c route was used as route of injection and the titers of specific antibodies were as high as 3200 Elisa units. This route beside its convenience and easy technique, it is also proven to be effective route in eliciting the strong specific immune response.

Aflatoxins tend to infiltrate most of the soft tissues and fat depots of the chicken **[9].** One day after the administration of a single oral dose of 14C-labelled AFB1 to laying hens, the highest concentration of

14C activity was detected in the liver, followed by muscle, pancreas, skin, adipose tissue, lungs and spleen [**12, 24**]. In another study using 14C-labelled aflatoxin, **Harland and Cardeihac** [**25**] determined that the liver, kidney and bone marrow of chickens concentrated aflatoxins more readily than did brain, muscles or body fat. In the present study, however, AFB1 residues recorded in breast muscles were higher (34.3-49pg/g) than those of the liver samples (16-25 pg/g) in the AFB1-challenged non-immunized birds. These results might be attributed to the collection and examination of samples after short intoxication period, which was only 17 days.

In chickens, it is proven that free and conjugated AFB1 is the principal tissue residues **[26].** Although no evidence of other aflatoxins excretion in hen's eggs has been reported, other aflatoxin metabolites can be excreted with egg [**9**]. The aflatoxin residues in eggs have been AFB1 rather than any of its known metabolites **[30].** The results of our study, however, prove the presence of aflatoxins other than AFB1 in eggs and even in higher concentrations (Table-1), where the AFG1 concentrations were 0.9 - 6.6 pg/g, AFG2 concentrations were 2 - 7.2 pg/g. AFB2 was absent in all egg samples and AFB1 was found in a concentration of 0.2 pg/g. This might be due to that the transmission of AFB1 residues into eggs requires a considerably higher level of AFB1 in feed **[28]. Lotzsch and Leistner [29]** found that delectable residues in eggs occurred only when laying hens are exposed to feed containing more than 1000 ppb AFB1. While, **Jacobson and Wisman** [**31**] recorded that the carryover of AFB1 from layer feed to eggs was also demonstrated in hens where AFB1 dietary levels of 100-400 ppb resulted in AFB1 levels of 0.2 to 3.3 ppb in eggs. Despite the low levels of B1 in eggs the high carcinogenic potency of B1 makes its concentration in eggs a problem of concern **[30].**

Because the AFB1 has a low molecular weight, it has low immunogenicity acting as a hapten. This actually necessitates its transformation to immunogenic molecules through its conjugation with bovine serum albumin. According to the obtained results shown in **Figs. 1, 2 and 3**, the conjugation percentage calculated reached to 64.41%. Similar results were recorded by **Chu and Ueno** [**19**]. Also Isa-70 montanid adjuvant was used to potentiate the immune response instigated against the AFB1 component of the prepared vaccine. According to **Dungu *et al.*, [32]** ISA70 montanid proved to induce long duration of immunity and high protective antibody titer.

Before Elisa testing all serum samples, collected at different time intervals during the experiment, were adsorbed with 1% BSA in order to remove any BSA-specific antibodies produced during the immunization process. This process facilitates the measurement of AFB1-specific antibodies produced against the injected vaccine. Using Elisa the AFB1-specific antibodies produced in immunized chicks and egg layers reached to 400 and 1600 Elisa units, respectively at 2 week after the primary dose, and to 1600 and 3200, respectively, after 1 week from the secondary vaccine dose. Similar results concerning production of AFB1 specific antibodies were recorded [**19, 22**].

The produced AFB1 specific antibodies might stand behind the result of measurement of AFB1 in the chicken edible materials where no AFB1 residues were recorded in breast muscle samples and liver samples collected from immunized broilers and eggs from egg layers as compared to samples examined from control non-immunized birds. Other trials for successful immunization against AFB1 have been reported [**19**].

The present work proved the practical capability of freeing chicken edible products from the AFB1 residues; however, more studies are required to intensify the immune response and to induce mucosal production of AFB1 specific secretory antibodies.

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AUTHORS' BIOGRAPHY

Heidy abo El-yazeed, professor of microbiology, faculty of veterinary medicine, Cairo University

Mai Hamed Hanafy, assistant lecturer of microbiology, faculty of veterinary medicine, Cairo University.

Rafik Soliman, professor of microbiology, faculty of veterinary medicine, Cairo University.

Mohammed Refai, professor of microbiology, faculty of veterinary medicine, Cairo university.