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STUDY ON THE EFFECT OF AFLATOXICOSIS ON THE IMMUNE RESPONSE OF RABBIT TO *PASTEURELLA MULTOCIDA* VACCINE

(With 4 Tables and 9 Figures)

By

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دراسة تأثير الأفلاتوكسين على المستوى المناعي في الأرانب المحصنة بلقاح الباستيرلا ملتوسيدا

وفاء على غنيمي ، حسن عبد المقصود حسن ، سميرة عبد الكريم سليمان، ساهر مكين جرجس

أجريت هذه الدراسة لإختبار مدى تأثير التغذية بعلف ملوث بسموم الأفلاتوكسين من النـــوع ب١ على الإستجابة المناعية للأرانب المحصنة بلقاح الباستيرلا ملتوسيدا الميت وأيضاً إختبار مثل هذا التأثير على صورة بروتينات السيرم بأجسامها. وقد أظهرت النتائج أن تزامن مثـــل تلك التغذية مع التحصين المشار إليه أدى إلى حدوث تدهور مطرد في مســـتويات المناعـــة الخلوية والمصلية (عند إستخدام علف ملوث ب ٥٠ جزء في البليون من الأفلاتوكســين ب١) مع ضعف مقاومة تلك الأرانب لصد العدوى بالميكروب الضارى للباستيرلا ملتوسيدا. وقد تم قياس دلالات المناعة في الأرانب المغذاة على العلف الملوث بالأفلاتوكسين ب١ حيث كـــانت متوسطاتها تقل حوالي ٥ مرات (بالنسبة للمناعة الخلوية) وحوالي ٣ مرات (بالنسبة للمناعـــة المصلية) عن مثيلاتها المسجلة في الأرانب الضوابط (المحصنـــة والمغـــذاة علـــى العلـــف الطبيعي الخالي من الأفلاتوكسين ب١). أيضاً جاءت وفيات مجموعة الأرانـــب المحصنـــة والمغذاه بالأفلاتوكسين عالية وسريعة (٤٧%) مقارنة بوفيات الأرانـــب الضوابـــط (٢١%) وذلك عند التعرض للعدوى التجريبية بالميكروب الضارى. ويجدر الإشارة كذلـــك لـــهبوط مستويات بروتينات سيرم الأرانب المسممة بالأفلاتوكسين ب١ هبوطاً ملحوظاً عن الأرانـــب الضوابط . كما أن نماذج التحليل الكهربائي لتلك البروتينات كانت متميزة في صورتها العامة بدرجة قد يعتمد عليها في تشخيص الإصابة الكامنة في الأرانب بالتسمم الأفلاتوكسينى (سلبي الأعراض والمثبط لجهاز المناعة).

SUMMARY

This study was conducted to determine the effects of aflatoxin B_1 (AFB_1) contaminated feed on the immune response of rabbit to inactivated *Pasteurella multocida* vaccine as well as on serum protein profile. Concurrent exposure of rabbits to 50 part per billion (ppb) of AFB_1 in feed and vaccination against pasteurllosis resulted in progressive suppression of cellular and humoral immunity; and lack of adequate resistance to subsequent challenge with virulent *P.multocida* organism. Parameters of cellular and humoral immunity were about 5 times and 3 times lower respectively in AFB_1-exposed rabbits. Higher and accelerated mortalities were recorded in vaccinated, AFB_1-fed rabbits (47%) than in control fed-vaccinates (21%). Serum protein levels were markedly reduced in AFB_1-exposed rabbits than in controls. In this investigation, the serum protein electrophoretic pattern seen in AFB_1-exposed rabbits is possibly unique suggesting its valuable aid for diagnosis of subclinical, immunotoxic form of aflatoxicosis in rabbit.

Key words: *Aflatoxicosis, Rabbit, Pasteurella multocida.*

INTRODUCTION

Aflatoxins (AF) are the most commonly occurring mycotoxins found in feed-stuffs. They are produced primarily by the mold Aspergillus flavus which may grow in feed during storage under conditions of high humidity and temperature (Bryden et al., 1980). Of the four major forms of aflatoxin (B_1, B_2, G_1 and G_2) AFB_1 is the most common, potent and biologically active component.

Description of the disease, aflatoxicosis, in different animal species and poultry indicated that the rabbit is extremely sensitive to aflatoxins (Edd, 1973 & Mehrota and Khanna, 1973). The acute LD_{50} for young rabbit is about 0.3 mg/kg of body weight, among the lowest of any species studied. In all animal species studied, hepatic damage is the principal injury induced by aflatoxins.

Data obtained primarily from poultry indicated that prolonged feeding of sublethal doses of AFB_1 causes subclinical immunotoxic form of aflatoxicosis. This form has been associated with decreased resistance to bacterial diseases (Boonchuvit and Hamilton 1975); immuno-suppression (Thaxton et al., 1974); and consequently vaccination failures

(Anjum, 1994; Batra et al., 1991 and Pier and Heddleston, 1970). No study appears to have been carried out on such form of aflatoxicosis in rabbits.Therefore, it was the aim of this investigation to study if the presence of low level of AFB_1 in feed of rabbits during vaccination with P.multocida vaccine, might impair the development of adequate immunity and resistance to subsequent challenge.Moreover, changes in the serum protein levels indicative of hepatic damage by AFB_1, were also determined.

MATERIAL and METHODS

Experimental rabbits:

A total of forty-eight Boscat rabbits (1.6 to 1.9 kg) were allotted into-four separate experimental groups and housed in stainless-steel cages. They were kept on specific feeding regimen and water was available ad libitum.

Assay of feed:

To ensure the absence of aflatoxins, the control feed was assayed fluorometrically using the Aflatest™ according to the manufacturer's procedures (Vicam, Services-4 Fluorometer, BBL-Source Scientific, CA, USA). Readout will be in part per billion (ppb) aflatoxin for the extracted feed sample.

Preparation and administration of Aflatoxin:

Pure crystalline AFB_1 (Aldrich Chemical Co., USA) was dissolved in chloroform under a hood. The dissolved AFB_1 was mixed with small batch of feed, which after solvent evaporation was mixed to reach 50 ppb AFB_1 in the final feed to be fed. Such dose did not caused clinical disease and/or mortality as reported in previous study (Clark et al., 1980).

Pasteurella multocida vaccine and challenge:

Formalin inactivated rabbit pasteurellosis vaccine was obtained from Serum and Vaccine Research Institute, Abbassia, Cairo. It contains four serotypes 5:A, 8:A, 9:A and 2:D mixed equally to form an aqueous polyvalent vaccine.Challenge was done by intranasal instillation (0.25 ml into each nostril) of broth culture of virulent *P.multocida* strain containing 2×10^{10} CFU/ml as described by *Borkowska et al. (1996).*

Blood samples:

Blood samples were collected from the jugular veins of individual rabbits in all groups at weekly intervals. A part of each sample was put in a vial with EDTA as anticoagulant and used for cell-mediated immunity assay. The other part of the blood sample was allowed to clot and sera were separated, inactivated at 56°C for 30 min and frozen at –20°C until tested serologically and biochemically.

Assay for cell-mediated immunity:

Lymphocyte blastogenesis assay was applied according to the method adopted by Lucy (1977). Results of the test were expressed as Delta Optical Density (DOD).

Assay for humoral immunity:

Indirect haemagglutination test (IHT) described by Carter and Rappey (1962) was used for measurement of serum antibody levels for *P.multocida*. Specific IgG antibodies to *P.multocida* were measured using ELISA test as described by Borkowska et al. (1997).

Assay for total protein:

Total serum protein was determined using the colorimeteric biuret method described by Cornall et al. (1949).

Electrophoresis:

Levels of serum protein fractions were determined according to the method described by Miller et al. (1984) using the cellulose acetate electrophoresis and computing densitometer (Gelman Deluxe electrophoresis system, USA). Scan were recorded graphically into albumin, and globulin peaks representative of the electrophoretic fractionation. The absolute value of each of the separate fraction can be obtained by multiplying its relative percentage value by the total serum protein concentration.

Experimental design:

Forty-eight Boscat rabbits were allotted into four groups and treated as follows:

Group A (Aflatoxin fed, unvaccinated).
Group B (Aflatoxin fed, vaccinated).
Group C (Control fed, unvaccinated).
Group D (Control fed, vaccinated).

Rabbits in each group were continued on their respective feed for the whole period of the experiment. At one and three weeks post receiving that feeds, rabbits of groups B and D received inactivated rabbit pasteurellosis vaccine. This vaccine was given subcutaneously as

1ml dose/rabbit. The rabbits in all groups were continued on that feeds until the 5th week post AFB_1 exposure, when the challenge inoculation was given. Challenged rabbits were observed for two weeks, where mortality, mean death time (MDT) and gross lesion suggestive of acute pasteurellosis were recorded (Ringler et al., 1985). Cultures for *P.multocida* were taken from lungs, trachea and liver of dead challenged rabbits.

RESULTS and DISCUSSION

The present study confirms the extreme susceptibility of rabbits to subclinical immunotoxic form of Aflatoxicosis induced by as low as 50 ppb of AFB_1. Such form of Aflatoxicosis has been observed in turkey fed higher level of AFB_1 (250-500 ppb) and also in chicks fed 300 ppb of AFB_1 (Pier and Heddleston, 1970 and Ghosh et al., 1990).

Data presented in Table (1) and illustrated in Figure (1) revealed marked and progressive suppression of cell-mediated immunity (CMI) in vaccinated, AFB_1-exposed rabbits compared to those of control-fed rabbits. The suppression of CMI (as measured by lymphocyte blastogenesis assay and expressed as DOD) was detected on the 1st week post vaccination (2nd week post AFB1 intoxication) and peaked on the 5th week post AFB_1-intoxication recording a mean of 0.21 compared with a mean of 0.305 in control-fed vaccinates. Lymphocytes from unvaccinated rabbits did not respond to pasteurella antigen in that assay indicating that the blast cell response only develops as a sequel to *P.multocida* immunization. This assay appears to be in vitro correlates of CMI and represent useful assay to quantitate the importance of CMI in bacterial immunity (Maheswaran et al., 1976). Our finding are in agreement with Pier (1981) who stated that the immunotoxic effects of AFB_1 are primarily on the cell-mediated immunity. Moreover, Ghosh et al. (1990) found a significant decrease in T lymphocyte count in AFB_1-fed chickens. In addition, Chang and Hamilton (1976) found an inhibition of both humoral and cellular factors involved in cellular immunity. It worth to state that aflatoxicosis has been found to increase susceptibility of chickens to *Candida albicans* infection (Hamilton and Harris, 1971), caecal coccidiosis and Marek's disease (Edds et al., 1973) as well as to Pasteurella infection (Refai et al., 1993). Immunity against these diseases is largely dependent on cellular immunity (Ghosh et al., 1990).

Data shown in Table (2) and Figure (3) reflected the adverse effect exerted by AFB_1 on the humoral immune responses. The pasteurella antibody titers were markedly higher in the vaccinated, control-fed rabbits than in those exposed to AFB_1. They were also considerably higher than the values of the negative controls. The AFB_1-suppressed antibody titers were considerably detected in IHA and ELISA tests starting from the 2nd week post vaccination (3rd week post AFB_1 intoxication) and thereafter. At challenge (5th week post AFB_1-intoxication) the mean antibody titers in AFB_1-exposed vaccinates were 288 and 717 as measured by IHA and ELISA test, respectively. Corresponding values in control fed-vaccinates were 80 and 290, respectively. These suppressed titers could be explained on the basis that AFB_1 directly inhibits protein synthesis and thereby could inhibit antibody production including the specific immunoglobulins IgG (Giamborne et al., 1978). An increase in lysosomal activity during aflatoxicosis resulted in immunoglobulins degradation, was also suggested by Tung et al. (1970). Aflatoxin is a potent nephrotoxin and the continuos exposure to it resulted in tubular renal damage (Newberne, 1973). In such cases it could be suggested that large amount of immunoglobulins get abolished through the impaired kidney, thus leading to hypoproteinaemia and decrease in the level of circulating immunoglobulins. It was also shown that several non specific humoral substances important to native defense and immunological mechanisms are affected during aflatoxicosis. These include production of significantly less haemolytic active complements (Richard et al., 1974); impairment of lymphokine production (Pier et al., 1979) and delay in the production of interferon (Pier et al., 1971).

Serum protein changes during aflatoxicosis are shown in Table (3) and Figures (4&5). The total albumin values continued to remain low at all intervals starting from the 3rd week post AFB_1-intoxication in vaccinated rabbits but considerably decreased at the 4th week post intoxication and thereafter compared with control-fed vaccinates. Similar observations were recorded by (Pier and Heddleston, 1970; and Huff et al., 1986) using a level of 250-500 ppb AFB_1 in turkey feed and as high as 6000 ppb level in chickens feed, versus 50 ppb level of AFB_1 used in the present study. Aflatoxin B_1 is a potent hepatotoxin that metabolized mainly in the liver, the major site of albumin and plasma protein synthesis (Kaneko, 1989). Therefore, it could be concluded that

the liver of rabbits is highly sensitive to the hepatic damage caused by AFB_1.The total globulin values decreased during aflatoxicosis at all the intervals but marked decrease was detected on the 3rd week post AFB_1 intoxication, being in agreements with Ghosh et al. (1990). Aflatoxin B_1 has been found to cause decrease in serum IgG and IgA (Giambrone et al., 1978). Therefore, the decrease in total globulins by AFB_1 could be the result of inhibition of synthesis of specific immunoglobulins as also suggested by Thaxton et al. (1974). A possible important sequel to the suppression of globulin synthesis apparent in aflatoxicosis is that the affected rabbits may be rendered more susceptible to many of the infectious diseases.

The electrophoretic pattern of serum protein revealed a striking difference between controls and AFB_1-intoxicated rabbits, which become more exaggerated as the intoxication progressed. This difference is readily appreciated by studying figures 6 to 9, which reveal electrophoretograms representative of the different groups of rabbits. On considering these figures it is apparent that the hypoproteinaemia recorded in AFB_1-exposed rabbits could be attributed to simultaneous reduction in all the serum protein fractions, in particular the albumin. That the latter should be the most severely reduced, in consistent with advanced liver damage. Our results coincided with those reported previously in chickens, ducklings (Brown et al., 1965 and Tung et al., 1975); and guinea pigs (Richard et al., 1974). It worth to state that the electrophoretic pattern of serum protein may prove valuable diagnostic aids in instances where aflatoxicosis is suspected as also suggested by Brown et al. (1965).

Regardless of the mechanism by which AFB_1 exerts its immunotoxic effects, challenge test is considered the most practical way for evaluating the actual immunological status and a resistance of specific host. As shown in Table (4) rabbit vaccinated against pasteurellosis while on feed containing 50 ppb of AFB_1 did not withstand experimental challenge of their acquired immunity as did rabbits vaccinated while on the AFB_1-free feed. Deaths in the former group (group B) started on the 3rd day of challenge and reached a total of 47% within mean death time (MDT) period of 4.1 days. On the other hand, deaths in non AFB_1-exposed vaccinates (group D) started on the 5th day of challenge and reached a total of 21% within MDT period of 6 days. Necropsy of dead challenged rabbits revealed gross lesions suggestive of acute rabbit pasteurellosis and in most of AFB_1-exposed

rabbits, body tissues were icteric and the liver in particular was yellowish, It worth to state that dead challenged rabbits of group B had a higher rate of *P.multocida* positive cultures than those of group D, as also observed by Dziuk et al. (1978).

In the present study, vaccination failure expressing immunological impairment by AFB_1 are clearly evident. This would explain the incidence of pasteurellosis as a field problem in rabbits which have been adequately vaccinated with an efficient *P.multocida* vaccine. Such vaccination failure and decreased resistance to *P.multocida* reinfection, has been observed by Pier and Heddleston (1970); Pier et al. (1971) and Hegazi et al. (1991). Moreover, susceptibility to other infectious diseases, were also increased in immunized birds as a consequence to AFB_1-exposure (Batra et al., 1991; and Gabal and Azzam, 1998).

The widespread distribution of *Aspergillus flavus*, the main fungal species which produces aflatoxins in feed, suggests that aflatoxin contamination must be seriously considered in rabbit industry. Extra prophylactic measures have been introduced to alleviate contamination problems. Such measures include regular quality controls of feed (Tabib et al., 1981); supplemental aluminosilicates in feed that reduce absorption of aflatoxin from the gut (Harvey et al., 1993); and preservation of feed with mold inhibitors (Dixon and Hamilton, 1981). The Food and Drug Administration (FDA) has established 20 ppb as the tentative maximal allowable tolerance of aflatoxin in feed stuffs.

From the present study, it can be concluded that AFB_1 even at as low as 50 ppb level is not safe because it caused immunosuppression without clinical effects. Consequently, the rabbits may experience mortality due to vaccination failures as well as an increasing susceptibility to various disease agents.

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Table (1) Cell mediated immune response with lymphocytes from rabbits vaccinated with inactivated pasteurellosis vaccine while consuming 50 ppb of aflatoxin B_1 (AFB_1) in their feed

Group	treatment	Weeks post receiving dietary AFB_1					5-weeks mean DOD
		1	2	3	4	5	
A	AFB_1 fed, non vaccinated	0.010 *	0.037	0.029	0.021	0.017	0.023
B	AFB_1 fed, vaccinated	0.023	0.041	0.023	0.064	0.021	0.034
C	Control fed, non vaccinated	0.019	0.063	0.051	0.034	0.018	0.037
D	Control fed, vaccinated	0.045	0.168	0.189	0.247	0.305	0.191

* Results of lymphocyte blastogenesis assay are expressed as Delta Optical Density (DOD).

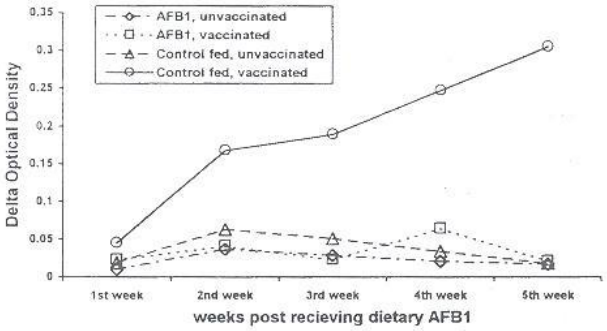


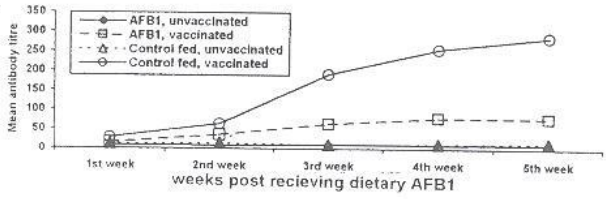
Figure (1) cell-mediated immune response in rabbits vaccinated with inactivated *P.multocida* vaccine while consuming 50 ppb of aflatoxin B_1 (AFB_1) in their feeds

Table (2) Mean antibody titres to *P.multocida* in rabbits receiving 50 ppb of aflatoxin B_1 (AFB_1) during vaccination with inactivated *P.multocida* vaccine

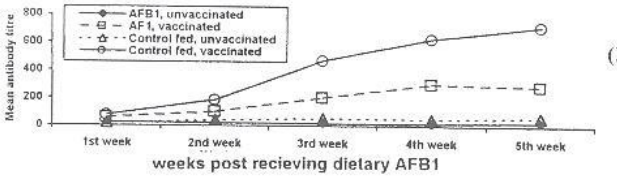
Group	treatment	Serological assay	Weeks post receiving dietary AFB_1 **					5 weeks mean titre
			1	2	3	4	5	
A	AFB_1 fed, non vaccinated	IHA*	10	8	12	10	12	10
		ELISA	22	24	21	19	28	22.8
B	AFB_1 fed, vaccinated	IHA	16	36	64	80	80	55
		ELISA	58	102	205	303	290	192
C	Control fed, non vaccinated	IHA	12	14	10	12	16	11
		ELISA	20	41	53	48	65	45
D	Control fed, vaccinated	IHA	28	64	192	256	288	166
		ELISA	77	188	473	627	717	416

* Mean antibody titres as mensured by indirect haemagglutination test (IHA).

** Rabbits were vaccinated at the end of the 1st and 3rd weeks post receiving dietary AFB_1.



(A)



(B)

Fig. (2) Changes of mean antibody titers of *P.multocida* in serum of rabbits exposed to concurrent aflatoxin B1 (AFB1) in feed (50 ppb) and vaccinated with inactivated *P.multocida* vaccine measured by indirect haemagglutination test (A) and ELISA test (B)

Table (3) Mean of total serum protein, albumin and total globulins from rabbits vaccinated with inactivated *P.multocida* vaccine while consuming aflatoxin B_1 (AEB_1) at a dietary level of 50 ppb

Group	Treatment	Serum protein (g%)	Weeks post receiving dietary AFB1 *					5 weeks mean A/G
			1	2	3	4	5	
A	AFB1 fed, non vaccinated	Total	5.78	5.74	4.20	4.54	4.39	2.39
		Alb.	3.96	4.10	3.07	3.20	3.12	
		T.G	1.82	1.64	1.23	1.35	1.27	
B	AFB1 fed, vaccinated	Total	5.49	5.58	4.65	4.99	4.70	1.93
		Alb.	3.72	3.76	3.10	3.14	3.08	
		T.G	1.77	1.80	1.55	1.85	1.71	
C	Control fed, non vaccinated	Total	5.73	5.60	5.65	6.13	6.51	1.88
		Alb.	3.78	3.93	3.79	4.03	3.89	
		T.G	1.93	1.78	1.86	2.10	2.61	
D	Control fed, vaccinated	Total	5.86	6.02	4.76	6.66	6.95	1.56
		Alb.	3.97	3.84	4.21	3.85	3.79	
		T.G	1.80	2.17	2.55	2.81	3.16	

A/G = Albumin globulin ratio. Alb. = Albumin T.G = Total globulin.

* Rabbits were vaccinated at the end of the 1st and 3rd weeks post receiving dietary AFB_1.

Table (4) Challenge results for rabbits vaccinated with *P.multocida* vaccine while consuming aflatoxin B_1 (AFB_1) at a level of 50 ppb in feed

Group	Treatment	Immunological status at challenge			No. of dead / No. of challenged	Mortality %	Mean death time (days)	Lesion score	*P.multocida* isolation (positive/total)
		ELISA	DOD	T.G					
A	AFB_1 fed, unvaccinated	28	0.017	1.27	10/10	100	2.6	+++	10/10
B	AFB_1 fed, vaccinated	290	0.021	1.71	6/14	43	4.1	++	4/6
C	Control fed, unvaccinated	65	0.018	2.61	10/10	100	3.0	+++	10/10
D	Control fed, vaccinated	717	0.305	3.16	3/14	21	6.0	+	0/3

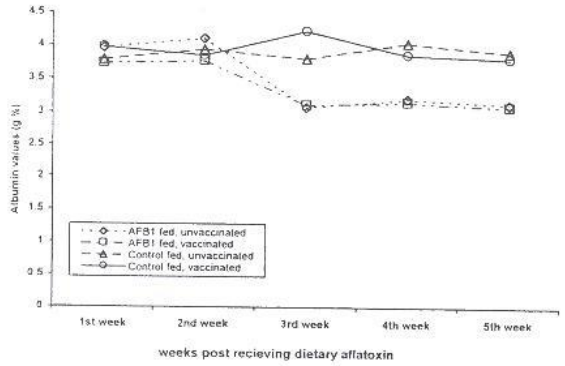


Figure (4) Changes of mean albumin values at different time intervals in rabbit receiving aflatoxin B_1 (AFB_1) during pasteurellosis vaccination

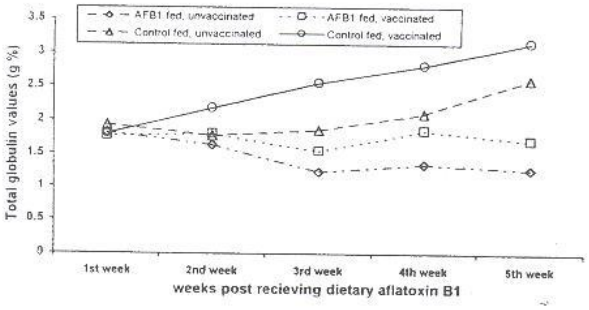


Figure (5) Changes of mean total globulin values at different intervals in rabbit receiving aflatoxin B_1 (AFB_1) during pasteurellosis vaccination

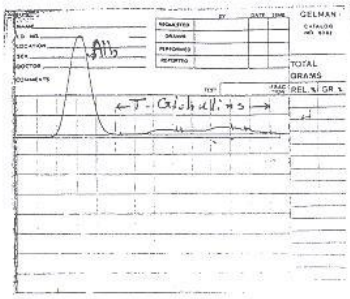


Fig.(6) AFB_1 fed, unvaccinated

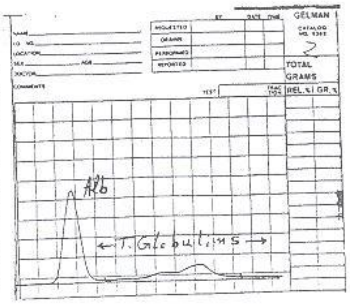


Fig. (7) AFB_1 fed, vaccinated

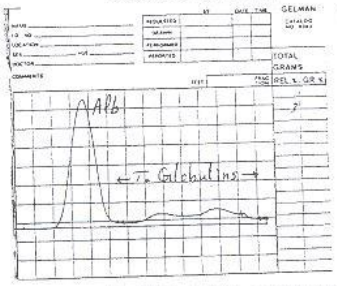


Fig.(8) Control fed, unvaccinated

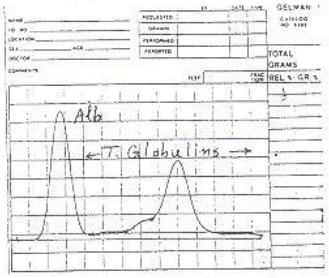


Fig. (9) Control fed, vaccinated

Figures (6-9) represent cellulose acetate electrophoretograms of serum protein of rabbits continuously exposed and non exposed to 50 ppb of dietary aflatoxin B_1 (AFB_1) during immunization period with inactivated P.multocida vaccine. These figures represent data obtained on the 5th week post AFB_1 intoxication