RAPID DETECTION OF AFLATOXIGENIC PRODUCING STRAINS OF *ASPERGILLUS fLAVUS* FROM POULTRY FEEDS BY UV LIGHT AND AMMONIA VAPOR

Raed Najeeb Alkhersan, Mohammed H.Khudor, Basil A. Abbas

Microbiology Department, College of Veterinary Medicine, Uni. Of Basrah, Iraq.

(Received 14 January 2016 ; Accepted 9 March 2016)

Keywords; (PDA) , Poultry feed, *Aspergillus flavus*.

ABSTRACT

A total 180 samples of pellet poultry feed were collected from poultry feed stores and local markets .They were cultured on potato dextrose agar(PDA) and malt extract agar (MEA) for isolation and identification of *Aspergillus flavus*.They were Sub-cultured on sabouraud dextrose agar(SDA) .Then the isolates were cultured on coconut agar medium(CAM) . The rapid detection of 50 selected isolates of contaminated poultry feed samples with *A.flavus* were achieved by blue –green fluorescence under UV light and by ammonia vapor on CAM medium to determine the aflatoxigenic isolates of *A.flavus*. The detection by UV light revealed that 26 (52%) of isolates were aflatoxigenic (positive) by produce blue-green fluorescence under UV light at 356nm , and also 26 (52%) of isolates were aflatoxigenic (positive) by turned the colony reverse to pink color by exposure to ammonia vapor.

INTRODUCTION

Aspergillus flavus is widely distributed in nature and is largely found at cereal and grains. Before harvest or during storage, *A. flavus* grows on agricultural crops (1). Its growth is affected by the environmental condition such as temperature and relative humidity (2).

Aflatoxins are difuranocumarin derivatives.They are very slightly soluble in water (10–30 µg/mL), in non-polar solvents they are insoluble , and soluble in moderately polar organic solvents (e.g. chloroform and methanol) and extremely soluble in dimethyl sulfoxide (3) .Under the influence of ultraviolet light they are unstable and in the presence of oxygen, to extremes of pH (< 3, > 10) and to oxidizing agents (4).

Aflatoxins are produced only by a closely related group of Aspergilli: *A. flavus, A.parasiticus* and *A.nomius* strains (5).Other species such as *A.bombycis*, *A.pseudotamari* and *A.ochraceoroseus* are also aflatoxin-producing species, but they are found less frequently (6).Aflatoxins cause a problem concerning many commodities also aflatoxin B1(AFB1) act as carcinogenicity, mutagenicity and acute toxicology and determined it to be a human carcinogen . Aflatoxins are common occurrence in feedstuffs, feeds. Aflatoxicoses , the disease caused by exposure to aflatoxin have made severe economic losses in the poultry industry, affecting ducklings, broilers, layers, quail and turkeys to cause clinical signs include anorexia, decreased weight gain, decreased egg production, hemorrhage, embryotoxicity, and increased susceptibility to environmental and microbial stressors (7).

Blue fluorescence a method used for developing qualitative cultural methods for detecting aflatoxigenic *Aspergillus* species grown on suitable media. This techniques use either solid media, such as coconut agar medium (CAM) and potato dextrose agar (PDA) or liquid medium, like aflatoxin producing-ability medium(APA) , and a medium with steep liquor (8,9), and achieved by cut a small plugs from *Aspergillus* colonies on medium to culture on the other media . The aflatoxins producer *Aspergillus* were detected under long-wave UV light (365nm) . This rapid identification to determine aflatoxigenic isolates from non-aflatoxigenic by appear blue to blue – green fluorescent to aflatoxigenic , and nonaflatoxigenic is non-produce fluorescent (10). Ammonium hydroxide vapor-induced color change a rapid and sensitive method , also for detection of aflatoxigenic and nontoxigenic strains of *Aspergillus* (11). In this method a single colony was grown in the centre of Petri dish. The reverse of colony of aflatoxigenic *Aspergillus* strains turned to pink color when their medium were exposed to ammonia vapor by dropped of ammonia hydroxide on it but nonaflatoxigenic is no color produce (12).This study aims to detect the aflatoxigenic *Aspergillus flavus* by UV light test and by ammonia vapor test which were isolated from poultry feed.

MATERIALS AND METHODS

Collection of samples

A total 180 samples of pellet poultry feed were collected from poultry feed stores and local markets at Basrah governorate during one year from Sep. 2014 to Apr. 2015 . Samples were stored for 2-3 days in sterile plastic containers at room temperature (22-25ºC) in laboratory . After stored, they were prepared for fungi isolation and identification.

Isolation and identification of *A.flavus*

Suspension of 20 g of the poultry feed samples with 180 ml of saline solution (0.85% Sodium Chloride) with 0.05% Tween 80 on a horizontal shaker for 30 minutes. Then 0.1 ml of suspension was inoculated on PDA and MEA(13,14). The pure culture was incubated at 25 °C ± 2 and after 7 days (15). After incubation , the macroscopic and microscopic distinct colonies by using lactophenol cotton blue were done. The morphological characteristics of *A.flavus* isolates were described microscopically according to Domsch and Gams (16) and Klich (17) .Sub-culture on SDA was done , and also on CAM according to Davis *et al* (18). Then the cultures were incubated at 25◦C ± 2 for 7 days (19).

Coconut based medium test

It was done by fluorescence on coconut agar medium(CAM). A preliminary screen for aflatoxin producer *Aspergillus* was performed on the basis of emission of blue to blue – green fluorescence after UV light excitation at 365 nm after growing the isolates on coconut agar medium , because this agar is inductive of aflatoxin production (20). Producer isolates can be identified by fluorescence in the reverse side of the culture CAM in glass Petri dishes(18, 21).Five millimeter diameter sterile cork borer was used to make a hole in the center of CAM medium in petri dish .The isolate was inoculated of a mass of conidia by cork borer to the hole at the central point Petri dish of CAM , then they were incubated at 28 °C for 7 days. nonaflatoxigenic *Aspergillus niger* was used as control (20,22).

Ammonia vapor test

The fungal isolates were inoculated on CAM as single colonies by cork borer (5mm) diameter in the center of plate and incubated in the dark at 28 °C. for 7 days. The dish was inverted and 1 or 2 drops of concentrated ammonium hydroxide solution are placed on the inside of the lid of Petri dish. Then the Petri dish inverted over the lid containing the ammonium hydroxide. A control as was mentioned in previous test was prepared.

RESULTS

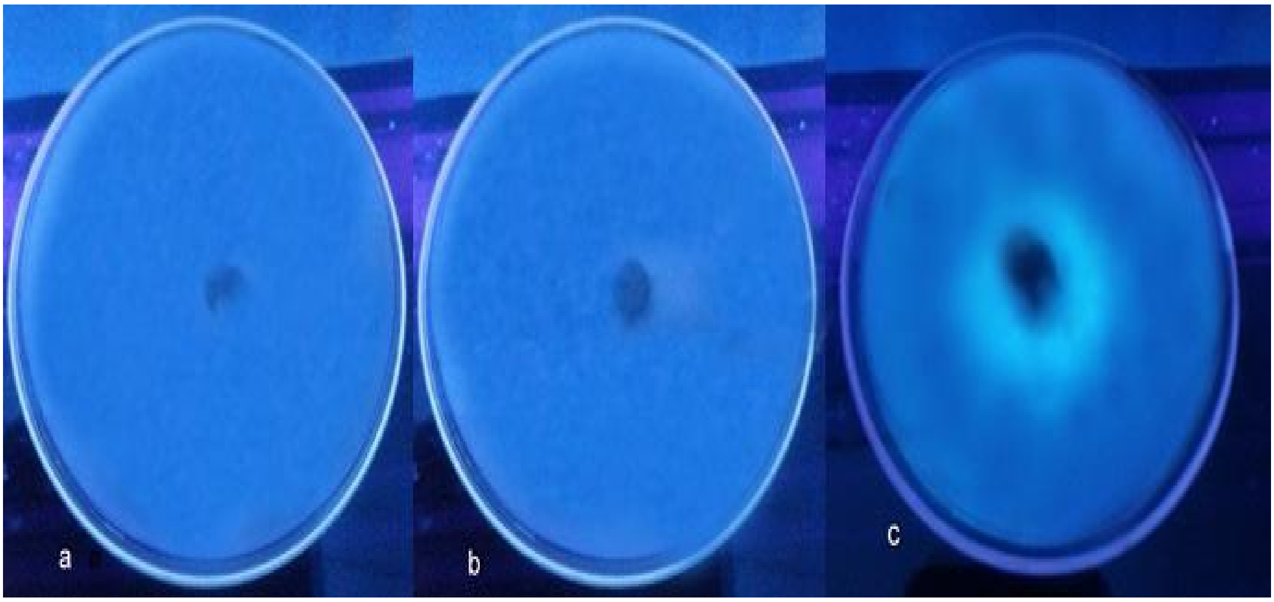
Fifty *A.flavus* isolates were considered out of 180 samples. The detection of aflatoxigenic and nonaflatoxigenic *Aspergillus flavus* by using UV light and ammonia vapor revealed that 26 (52%) of isolates were aflatoxigenic (positive) and 24(48%) of isolates were nonaflatoxigenic (negative) for both methods (table1,2) . The detection by UV light 365nm recognized aflatoxigenic by produce blue-green fluorescent colonies in the center of glass Petri dish of CAM in the reverse , from nonaflatoxigenic which were nonproducing fluorescent colonies , similar to the control isolates of nonaflatoxigenic *A.niger* ,(figure 1).

Table(1): Number of aflatoxigenic –producing isolates of *A.flavus* on the CAM

Detection method	Number of detected isolates	Positive isolates	Positive isolates(%)	Negative isolates
Coconut based medium detection	50	26	52	24
Ammonia vapor detection	50	26	52	24

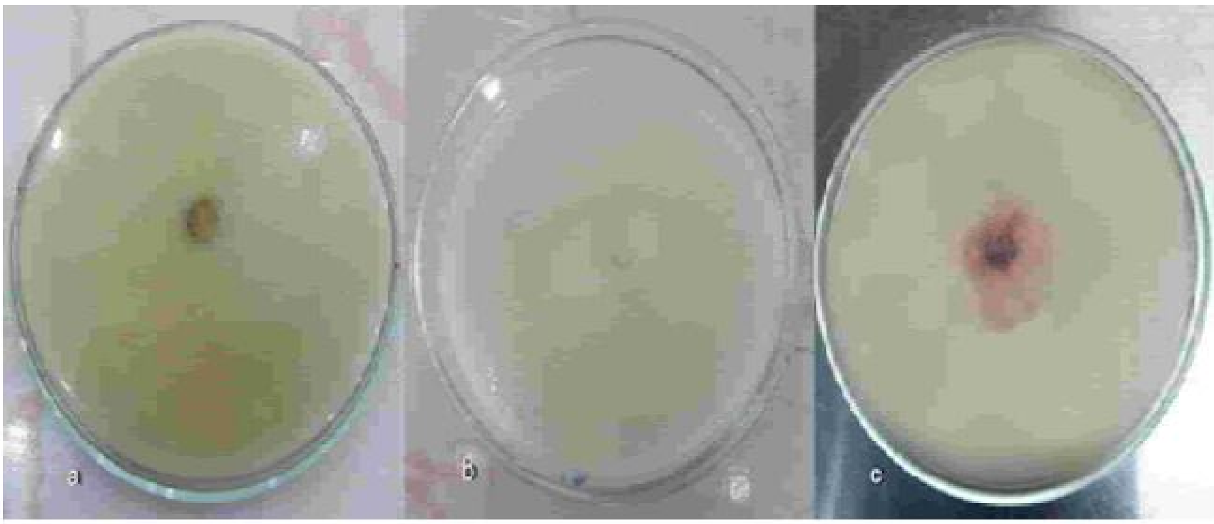
Table (2) : The results of aflatoxigenic *A.flavus* isolates recovered from poultry feed samples which was obtained by UV light detection , ammonia vapor detection on CAM.

No. of isolate	Coconut based medium test	Ammonia vapor test	No. of isolate	Coconut based medium test	Ammonia vapor test
1	+	+	26	+	+
2	+	+	27	-	-
3	+	+	28	+	+
4	+	+	29	+	+
5	-	-	30	+	+
6	-	-	31	-	-
7	-	-	32	+	+
8	-	-	33	+	+
9	+	+	34	+	+
10	+	+	35	+	+
11	+	+	36	+	+
12	-	-	37	-	-
13	-	-	38	-	-
14	+	+	39	+	+
15	+	+	40	-	-
16	+	+	41	-	-
17	+	+	42	+	+
18	+	+	43	-	-
19	-	-	44	-	-
20	-	-	45	-	-
21	+	+	46	-	-
22	-	-	47	-	-
23	+	+	48	-	-
24	-	-	49	-	-
25	+	+	50	-	-



Figure(1): Showed the detection of aflatoxigenic *A.flavus* by CAM under UV light at 365nm .(a) control of nonaflatoxigenic isolate of *A.niger* , (b) nonaflatoxigenic *A.flavus* (negative) isolate, and (c) aflatoxigenic *A.flavus* (positive) isolate , showing a blue-green fluorescent ring around the colony .

Aflatoxin detection by ammonia vapor to characterize as aflatoxigenic isolates *A.flavus* which produced pink to red color colonies in inverted petri dish by applying 1or 2 drops of concentrated ammonia hydroxide solution on the inside of the lid , but no color change occurred in nonaflatoxigenic isolates, (figure 2).



Figure(2): Showed the detection of aflatoxigenic *A.flavus* by ammonia vapour.(a) control of nonaflatoxigenic isolate of *A.niger* , (b) nonaflatoxigenic *A.flavus* (negative) isolate, and (c) aflatoxigenic *A.flavus* (positive) isolate , showing a pink-red ring around the colony .

DISCUSSION

By using UV light technique in this study , 26 isolates (52%) from a total 50 isolates of *A.flavus* were aflatoxigenic can colored with blue –green fluorescence (positive) on reverse of glass Petri dish of CAM with compared to nonaflatoxigenic showed no color (negative) results and considered as negative. The same results above by ammonia vapor detection were reported in which the colony of aflatoxigenic *A.flavus* turned to pink color , while no change in color with non aflatoxigenic isolates. This mean that the number of aflatoxigenic isolates of *A.flavus* were equal by UV light at 365 nm and ammonia vapor on CAM. This result is similar with those obtained by Yazdani *et al* (11) , Saito *et al* (12) , Zarari *et al* (24), Nair *et al*(25) and Sudini *et al* (26). While there was difference with Riba *et al* (23) whose confirmed that the cultures of aflatoxigenic *Aspergillus* were tested for 365 nm UV light fluorescence and for bright orange-yellow colony reverse coloring, and also this study disagrees with study of Fani *et al* (27) , which reported only (25.6 %) positive isolates of aflatoxigenic *A.flavus* by fluorescence detection on CAM , while less isolates(12 %) were identified as aflatoxigenic using ammonium vapor detection.

CONCLUSIONS

Aflatoxin is a major problem in developing countries where contaminated food commodities may readily reach food stores and homes . It is important to know that the effect of aflatoxin on animals extend beyond the symptoms. There are high percentage of aflatoxigenic *A.flavus* in poultry feed product.

الكشف السريع عن سلالات الفطر *Aspergillus flavus* المنتجة للافلاتوكسين المعزولة من أعلاف الدواجن بواسطة ضوء الأشعة فوق البنفسجية وبخار الأمونيا

رائد نجيب الخرسان، محمد حسن خضر ،باسل عبد الزهرة عباس

فرع الاحياء المجهرية، كلية الطب البيطري ، جامعة البصرة

الخلاصة

تم جمع 180 عينة من اعلاف الدواجن المركزة من مخازن أعلاف الدواجن و الأسواق المحلية . تم زرعها على الوسطين (PDA) و (MEA) لعزل وتحديد سلالات الفطر *Aspergillus flavus* . وقد تم اعادة زرعها على وسطين ثانويين (SDA) و (CAM). تم الكشف السريع لـ 50 سلالة مختارة من عينات علف الدواجن المتلوثة بـ *A.flavus* بواسطة ضوء الأشعة فوق البنفسجية بطول موجي 356نانومتر وبخار الأمونيا على الوسط CAM لتحديد سلالات الفطر *A.flavus* المنتجة للافلاتوكسين وغير المنتجة .اثبت الكشف بواسطة الأشعة فوق البنفسجية أن 26 (52٪) من العزلات كانت aflatoxigenic (إيجابية) من خلال انتاج الضوء الفلورسنتي الاخضر المزرق تحت ضوء الأشعة فوق البنفسجية في 356 نانومتر، وأيضا لوحظ

ان 26 (52٪) من العزلات كانت aflatoxigenic (ايجابية) من خلال تحول لون ظهر المستعمرة الى اللون الوردي عند التعرض لبخار الأمونيا.

REFERENCES

1- Saini, S. S. and Kaur, A. (2012). Aflatoxin B1: Toxicity, characteristics and analysis. Global Advanced Research Journal of Chemistry and Material Science, 1(4): 063-070.

2- Giorni, P.; Leggrieri, M.C.; Magan, N. and Battilani, P. (2012). Comparison of temperature and moisture requirements for sporulation of *Aspergillus flavus* sclerotia on natural and artificial substrates. Fungal Biology ,116(6):637-642.

3- Bertuzzi ,T.; Rastelli, S.; Mulazzi ,A. and Pietri ,A. (2012). Evaluation and improvement of extraction methods for the analysis of aflatoxins B1, B2, G1 and G2 from naturally contaminated maize. Food AnalyticalMethods,5(3):512-519.

4- Mostafa ,M. K.; Ahmed, H.; Gomaa ,M. and Ahmed Salem Sebaei(2013). Reliable HPLC determination of aflatoxin M1 in eggs. J. Anal. Methods Chem.,817091.

5- Moss M.O.(2002). Risk assessment for aflatoxins in foodstuffs. International Biodeterioration and Biodegradation ,50(3-4) 137-142.

6- Ito,Y.; Peterson S.W.; Wicklow, D.T. and Goto, T. (2001).*Aspergillus pseudotamarii*, a new aflatoxin producing species in *Aspergillus* section Flavi. Mycological Research ,105(2): 233-239.

7- CAST. (2003). Mycotoxins: risks in plant, animal, and human systems. Council for Agric. Sci. Technol. Task Force Report , 139. Ames, IA.

8- Abbas, H.K.; Zablotowics, R. M.; Weaver ,M.A.;Horn ,B.W.; Xie ,W.and Shier, W.T. (2004). Comparison of cultural and analytical methods for determination of aflatoxin production by Mississippi Delta *Aspergillus* isolates. Can. J. Microbiol., 50(3):193-199.

9- Atanda ,S. A.; Pessu ,P. O.; Agoda ,S.; Isong, I. U.; Adekalu, O. A.; Echendu, M. A. and Falade ,T. C.(2011). Fungi and mycotoxins in stored foods. Afr. J. Microbiol. Res. ,5(250): 4373-4382.

10- Rodrigues, P.; Soares ,C.; Kozakiewicz, Z.; Paterson, R.R.M.; Lima, N. and Venâncio, A. (2007). Identification and characterization of *Aspergillus flavus* and aflatoxins.

Communicating Current Research and Educational Topics and Trends in Applied Microbiology, 527-534.

11- Yazdani, D.; Zainal Abidin, M. A.; Tan, Y. H. and Kamaruzaman, S.1(2010). Evaluation of the detection techniques of toxigenic *Aspergillus* isolates. Afr. J. Biotechnol., 9 (45): 7654-7659.

12- Saito, M. and Machida ,S. (1999). A rapid identification method for aflatoxin producing strains of *Aspergillus flavus* and *A. parasiticus* by ammonia vapor, Mycoscience ,40 (2):205-208.

13- Pitt, J. I. and Hocking, A. D. (2009). Fungi and Food Spoilage, 3rd ed, Springer, New York, USA.pp:23.

14- Greco, M.V.; Franchi ,M.L.; Rico Golba ,S.L.; Pardo ,A.G.and Pose, G.N.(2014). Mycotoxins and mycotoxigenic fungi in poultry feed for food-producing animals. Scientific World Journal.

15- Beuchat, L.R.and Cousin, M.A.(2001). Yeasts and molds. In: Downes, F.P., Ito, K. (Eds.), Compendium of methods for the microbiological examination of foods, 4th ed. American Public Health Association, Washington, D. C., 209-215.

16- Domsch, K.H.; Gams ,W. and Anderson , T.H.(1980) .Compendium of soil fungi .Academic press. London, New York, Toronto, Sydney, San Francisco,1:859.

17- Klich,M.A.(2002).Identification of common *Aspergillus* species. Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.pp:46.

18- Davis, N.D.; Iyer, S.K.and Diener, U.L.(1987). Improved method of screening for aflatoxin with a coconut agar medium. Appl. Environ. Microbiol. 53(7):1593–1595.

19- Tokhadze, E.V.; Kvachadze, L. L. and Kvesitadze, G.I. (1975). Effect of the composition on nutrient medium on the synthesis of acid fast alpha amylase by different strains of *Aspergillus*. Journal of Microbiology, 11(4): 515-518.

20- Dyer ,S.K. and Mccammon, S. (1994). Detection of toxigenic isolates of *Aspergillus flavus* and related species on coconut cream agar. Journal of Applied Bacteriology 76: 75-78.

21- Hara, S.; Fennell ,D.I. and Hesseltine, C.W.(1974). Aflatoxin-producing strains of *Aspergillus flavus* detected by fluorescence of agar medium under ultraviolet light. Appl .Microbiol. ,27(6):1118-1123.

22- Lin ,M.T. and Dianese, J.C.(1976). A coconut-agar medium for rapid detection of aflatoxin production by *Aspergillus* spp. Phytopathology 66, 1466-1469 .

23- Riba, A.; Matmoura, A.; Mokrane, S.; Mathieu ,F. and Sabaou, N. (2013). Investigations on aflatoxigenic fungi and aflatoxins contamination in some nuts sampled in Algeria. African Journal of Microbiology Research, 7(420): 4974-4980.

24- Zrari ,T.J.O. (2013). Detection of aflatoxin from some *Aspergillus* ssp. isolated from wheat seeds. Journal of Life Sciences, 7 (10): 1041-1047.

25- Nair ,S. C.; Bhagobaty, R.K. ;Nmpoothiri,K.; Kalaighandhi ,V. and Menon, K.R.K. (2014). Detection of aflatoxin production by fungi in spice samples using HPLC and direct visual cultural methods. Innovative Romanian Food Biotechnology, 14, 1-12.

26- Sudini ,H.; Srilakshmi ,P. ; Kumar, K. V. K.; Njoroge, S. M. C.;Osiru ,M.; Seetha, A. and Waliyar, F. (2015).Detection of aflatoxigenic *Aspergillus* strains by cultutral and molecular methods: A critical review. Afr. J. Microbiol. Res.,9(8): 484-491.

27- Fani ,S.R.; Moradi ,M.; Probst ,C.; Zamanizadeh, H. R.; Mirabolfathy, M. and Haidukowski ,M.(2014). A critical evaluation of cultural methods for the identification of atoxigenic *Aspergillus flavus* isolates for aflatoxin mitigation in pistachio orchards of Iran. Eur. J. Plant Pathol.,140(4):631-642.