REVALUATION OF AFLATOXIN PRODUCTION BY *Aspergillus candidus* AND *Eurotium chevalieri* ISOLATED FROM POULTRY FEED IN BRAZIL*

REAVALIAÇÃO DA PRODUÇÃO DE AFLATOXINA PRODUZIDA POR ISOLADOS DE Aspergillus candidus E Eurotium chevalieri DE RAÇÕES PARA AVES NO BRASIL

Marcelo Elias Fraga[1]; Glória Maria Direito[2]; Mario Jorge Gatti[3]; Áurea Maria Lage Moraes[4]; Lilia Renné Cavaglieri[5]; Ana Maria Dalcero[5] and Carlos Alberto da Rocha Rosa[6]

ABSTRACT. Fraga, M.E.; Direito, G.M.; Gatti, M.J.; Moraes, Á.M.L.; Cavaglieri, L.R.; Dalcero, A.M.; Rosa, C.A. DA R. **Revaluation of aflatoxin production by Brazilian *Aspergillus candidus* and *Eurotium chevalieri* isolated from poultry feed in Brazil.** [Reavaliação da produção de aflatoxina produzida por isolados de *Aspergillus candidus* e *Eurotium chevalieri* de rações para aves no Brasil.] *Revista Brasileira de Medicina Veterinária 30(2):86-90, 2008.* Departamento de Microbiologia e Imunologia Veterinária, Instituto de Veterinária, Universidade Federal Rural do Rio de Janeiro (UFRRJ), Km 07 da BR 465, Seropédica, RJ 23890-971, Brasil. E-mail: fraga@ufrrj.br

Commercial feedstuffs in Brazil are an important component in modern animal husbandry. *Eurotium chevalieri* and *Aspergillus candidus* have been reported as poultry feed contaminants in Brazil, however, their potential ability to produce aflatoxins on this substrate has not been evaluated. Therefore, the aim of this work was to evaluate the aflatoxin-producing ability by *A. candidus* and *E. chevalieri* strains isolated from poultry feed and raw materials in Brazil. Mycological analysis was done to isolate total mycobiota. *Aspergillus candidus* and *E. chevalieri* strains were sub-cultured in yeast extract sucrose for aflatoxin production. TLC and HPLC analyses were done to demonstrate the aflatoxins production. *Aspergillus candidus* was isolated at 7.56 and 8.19% from poultry feed and raw materials, respectively, while 26.05 and 26.33% of *E. chevalieri* strains were isolated. They were able to produce aflatoxin B_1, B_2, G_1 and G_2. *Eurotium chevalieri* produced higher amounts of aflatoxin B_1 and G_1 than *A. candidus*. AFG_1 and AFB_1 production were the higher produced aflatoxins. The potential production of aflatoxins by these fungi could be an unexpected hazard to animal health when poultry feed is consumed. The toxigenic ability knowledgement by these species is important to guarantee the quality of animal feeds.

KEY WORDS: *Aspergillus candidus*, *Eurotium chevalieri,* aflatoxin, HPLC, Brazil.

RESUMO. Ração comercial no Brasil é um componente importante na produção animal moderna. *Eurotium chevalieri* e *Aspergillus candidus* são isolados como contaminantes em ração de aves no Brasil, porém, o potencial desses fungos em produzir aflatoxinas neste substrato não é avaliado. Entretanto, a objetivo deste trabalho foi avaliar a capacidade toxígena de *A. candidus* e *E. chevalieri* isolado de

*Recebido em 17 de abril de 2008.

[1] Biólogo. D.Sc. Professor Adjunto II, Departamento de Microbiologia e Imunologia Veterinária (DMIV), Instituto de Veterinária (IV), Universidade Federal Rural do Rio de Janeiro (UFRRJ), Km 07 da BR 465, Seropédica, RJ 23890-971, Brasil. E-mail: fraga@ufrrj.br

[2] Médica-veterinária. D.Sc. Professor Adjunto IV, DMIV, IV, UFRRJ

[3] Biólogo. M.Sc. Pesquisador Titular, Laboratório de Avaliação e Promoção da Saúde Ambiental, Instituto Oswaldo Cruz (IOC), Fundação Instituto Oswaldo Cruz (Fiocruz), Manguinhos, Rio de Janeiro, Brasil.

[4] Bióloga. D.Sc. Pesquisadora, Laboratório de Taxonomia, Bioquímica e Bioprospecção de Fungos, IOC, Fiocruz, Manguinhos, Rio de Janeiro, Brasil.

[5] Microbióloga. DSc, Professora Pesquisadora CONICET, Departamiento de Microbiologia e Inmunología, Universidad Nacional de Rio Cuarto (UNRC), Rio Cuarto, Córdoba, Argentina. E-mail:dalcero@exa.unrc.edu.ar

[6] Médico-veterinário. D.Sc. L.D, Professor Titular, DMIV, IV, UFRRJ. E-mail: shalako@ufrrj.br

alimento de aves e matérias primas no Brasil. Análise micológica foi realizada para isolar micobiota total. *Aspergillus candidus* e *E. chevalieri* foram inoculados em meio sacarose e extrato de levedura para produção de aflatoxinas. As análises foram realizadas em CCD e CLAE para demonstrar a produção das aflatoxinas. *Aspergillus candidus* foi isolado em 7,56 e 8,19% de ração para aves e matérias-primas, respectivamente. Enquanto 26,05 e 26,33% de *E. chevalieri* foi isolados de ração para aves e matérias-primas, respectivamente. Esses fungos produziram aflatoxinas B1, B2, G1 e G2. *Eurotium chevalieri* produziu quantidades mais altas de aflatoxinas B1 e G1 que *A. candidus*. As AFG1 e AFB1 foram as que apresentaram maior produção. Os potenciais de produção de aflatoxinas por estes fungos apresentam um perigo à saúde animal quando consumidas. O conhecimento da capacidade toxígena destas espécies é importante para garantir a qualidade da alimentação animal.

PALAVRAS-CHAVE: *Aspergillus candidus*, *Eurotium chevalieri,* aflatoxina, CLAE, Brasil.

INTRODUCTION

The worldwide contamination of foods and feeds with mycotoxins is a significant problem. Mycotoxins are secondary metabolites of moulds that have adverse effects on human beings, animals and crops, which result in illness and economic losses (Hussein & Brasel, 2001). Aflatoxins not only are the most studied group of mycotoxins but also are among the most carcinogenic natural compounds known, mainly produced by strains of *Aspergillus flavus*, *A. parasiticus* and *A. nomius* (Creppy, 2002). They produce hemorrhagic, hepatorenal syndrome and suppress immune function, besides it has been tested that aflatoxin B_1 (AFB_1) is carcinogenic. Several studies have demonstrated the aflatoxin-producing ability by different species in *Aspergillus* genera or related ascomycete (Aziz & Youssef, 1991; Goto et al., 1997; Abarca et al., 2000; Frisvad & Samson, 2000; Atalla et al., 2003). Poultry mixed feeds and raw materials (corn, soya, bean, peas) are commonly contaminated by aflatoxin-producing strains (Jan et al., 1995; Lozada, 1995; Dalcero et al., 1997; Magnoli et al., 1998).

These studies have demonstrated the aflatoxin producer's isolation; however, they did not consider the aflatoxin production ability by all *Aspergillus* or *Eurotium* potential toxigeneic species. Castro et al. (1995) and Magnoli et al. (1998) determined the *Eurotium* spp. and *A. candidus* occurrence in corn and poultry feed, respectively. The aflatoxin-producing ability of *E. chevalieri* and *A. candidus* were detected by Abarca et al. (2000) in a few strains and with only TLC method. Commercial feedstuffs in Brazil are an important component in modern animal husbandry.

Aflatoxins are frequently found in raw material used as primary feed components and poultry feed in this country. *Eurotium chevalieri* and *A. candidus* have been reported as poultry feed contaminants in Brazil (Castro et al., 1995; Rosa, 2002), however, their potential ability to produce aflatoxins on this substrate has not been evaluated. The toxigenic ability knowledgement by these species is important to guarantee the quality of animal feeds. Therefore, the aim of this work was to evaluate the aflatoxin-producing ability by *A. candidus* and *E. chevalieri* strains isolated from poultry feed and raw materials in Brazil.

MATERIALS AND METHODS

Sampling. Thirty-six samples of poultry feed and 36 samples of raw materials 10 kg each were collected at random from factories located in Rio de Janeiro, Brazil, during April 2003-March 2004 period. The samples were taken during the production process. They were sent to the laboratory as soon as they were collected, and tested on arrival. These primary samples were homogenized and quartered to obtain a 1 kg laboratory sample. They were stored at 4ºC for fungal analysis.

Isolation and identification of *Aspergillus candidus* and *Eurotium chevalieri* from poultry feed and raw materials. Mycological analysis was done on solid media using the surface-spread method by blending 10 g portions of ground sample with 90 ml of 0.1% peptone water solution. Serial dilutions of 10^{-2}, 10^{3} and 10^{-4} concentration were made from each material and 0.1 mL aliquots were inoculated by triplicates on dichloran rose bengal chloranphenicol agar (DRBC) (Abarca et al., 1994), used for general fungal enumeration. Plates were incubated at 28ºC for 7 days.

Fungal colonies identified like *Aspergillus* were sub-cultured in malt extract agar (MEA) for posterior identification in species. They were identified according to taxonomic schemes proposed by Raper & Fennell (1965), Domsch et al. (1980) and Klich (2002). After the identification; the *A. candidus* (CMDB 0452/INCQS 40222, CMDB 0455/ INCQS

40224, CMDB 0464/INCQS 40226, CMDB 0465/ INCQS 40227) and *E. chevalieri* (CMDB 0453/ INCQS 40223, CMDB 0463/INCQS 40225), strains were preserved under lyophilized condition, and then deposited in the Mycological Culture Collection of the Department of Biology (CMDB) and National Institute for Quality Control of Health (INCQS) – Fundação Instituto Oswaldo Cruz, respectively.

Detection of aflatoxins. *Aspergillus candidus* and *E. chevalieri* strains were sub-cultured in yeast extract sucrose agar (YESA) and aflatoxin production was performed according to Samson et al. (2002).

TLC analysis. Agar plug (5 mm diam.) from YES Agar cultured during 7 days at 28ºC, was transferred to an eppendorf tube and 500 mL chloroform were added. The mixture was agitated for 20 min at 400 rev min^{-1}. The mycelia mass was extracted and the chloroform was evaporated at room temperature. This extract was used for qualitative thin layer chromatographic analyses (TLC). Five mL of each sample extracts were placed on TLC plates (Merck, Darmstadt, Germany) 2 cm from bottom. Plates developed in an unsaturated tank with toluene-chloroform-ethyl acetate-90% formic acid (70:50:50:20,v/v) as developing solvent at room temperature, according to Gimeno et al. (1980).

When the solvent front was 15 cm from the origin, the plates were removed and allowed to dry. Standard solutions of AFB_1, AFB_2, AFG_1, AFG_2 (Sigma, Chemical, St. Louis, MO, USA) were included in each plate. They were prepared according to Official Methods of Analyses (1990). AFB solutions were prepared in benzene-acetonitrile (98:2,v/v). Plates were then examined under long wave UV light for aflatoxins presence throughout visual comparison with standard solutions.

Detection of aflatoxins by HPLC analysis. The same extract samples used for TLC were filtered through MF-Millipore Membrane™ filters (0.22 mm), dried under nitrogen flow and quantitatively determined by HPLC, following the methodology of detection proposed by Seiber & Hsieh (1973). The dried extracts were re-dissolved in 500 µL. An aliquot (20 µL) was analyzed by using the HPLC system mentioned above.

Chromatographic separations were performed on a MICROSORB-MV C18 (15 x 4.6 mm id., 5-m particle size). Ethyl acetate:n-hexane (3:2.5) was used as mobile phase at a flow rate 1.5 mL min^{-1}. Fluorescence of aflatoxin derivatives was recorded at excitation and emission wavelengths of λ 360 nm and λ 460 nm, respectively (Direito, 1989). Standard curves were constructed with different levels of AFs. These toxins were quantified by correlating peak heights of sample extracts with those of standard curves. The detection limit of the analytical method was 0.05 $ng.g^{-1}$.

Since these compounds have not been previously reported as a metabolite of *A. candidus* and *E. chevalieri* in Brazil, aflatoxin B_1, B_2, G_1 and G_2 were confirmed by HPLC coupled with a diode array detector.

RESULTS

Isolation and identification. A total of 119 *Aspergillus* strains were identified from poultry feeds. About 7.56% (9/119) belonged to *A. candidus* and 26.05% (29/119) belonged to *E. chevalieri*. One-hundred and ten *Aspergillus* strains were isolated from raw materials. *Aspergillus candidus* and *E. chevalieri* percents were 8.19 (9/110) and 26.33% (29/110), respectively. Table 1 shows the mycological counts of 36 samples of poultry feeds and 36 samples of raw materials. The count of *Aspergillus* genera was defined as the percentage of samples in which each fungus was present.

Table 1. Strains isolated and aflatoxin producers.

Strains	Source	Isolation		Aflatoxin production	
		S	F %	S	F´ %
Aspergillus candidus	Poultry feed	9	25	4/18	22.2
	Raw materials		9	25	
Eurotirum chevalieri	Poultry feed	31	80.5	2/60	3.3
	Raw materials		29	80	

S: number of strains
F: frequency percent of isolation
F´: frequency percent of aflatoxin-producing strains

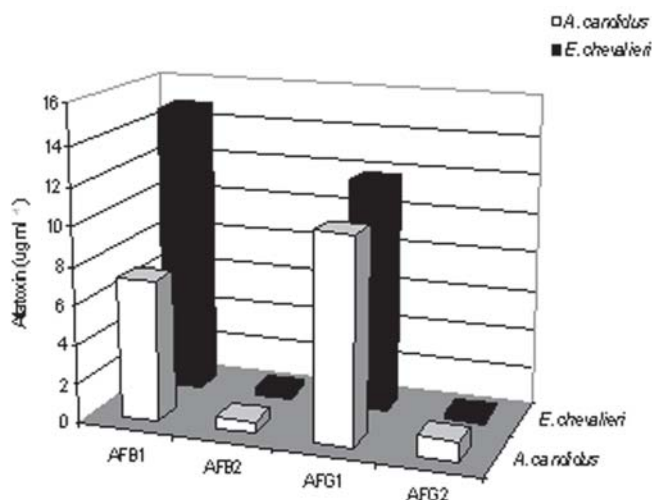

Figure 1. Aflatoxins production by *Aspergillus candidus* and *Erotium chevalieri*.

Aflatoxins determinations. Figure 1 shows the aflatoxin B_1, B_2, G_1 and G_2 by *A. candidus* and *E. chevalieri*. Although, *E. chevalieri* produced higher amounts of aflatoxin B_1 and G_1 than *A. candidus,* aflatoxin B_2 and G_2 was similar between the two microorganisms tested. AFG_1 and AFB_1 production were the higher aflatoxins produced by *A. candidus* and *E. chevalieri*, respectively. HPLC analysis using fluorescence detection showed AFs peaks at a retention time identical to that of standards (Figure 2). They were confirmed by HPLC coupled with a diode array detector.

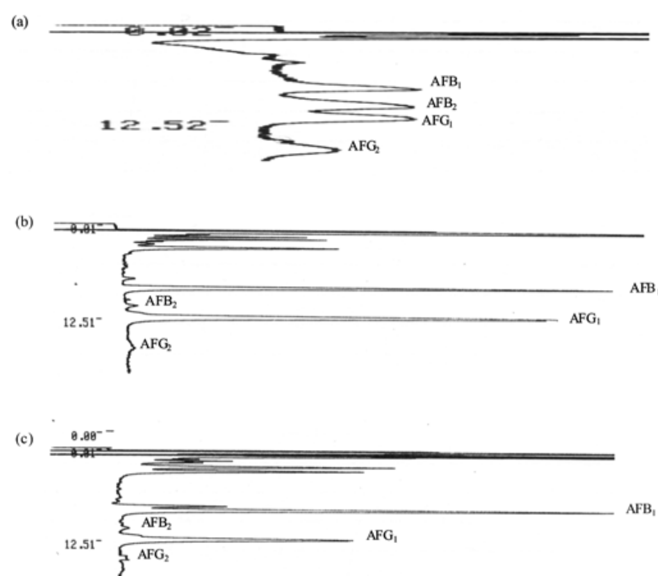

Figure 2. HPLC chromatograms for aflatoxins B1, B2, G1 and G2 standards (a), AFBs and AFGs produced by *Aspergillus candidus* (b) and AFBs and AFGs produced by *Eurotium chevalieri* (c) on YES medium.

DISCUSSION

This study reveals that *A. candidus* and *E. chevalieri* strains isolated from poultry feeds and raw materials are able to produce aflatoxins B_1, B_2, G_1 and G_2. *Aspergillus candidus* strains (22%) produced AFB_1, AFB_2, AFG_1 and AFG_2. Aflatoxin G_1 production was higher than AFB_1. Aflatoxins B_2 and G_2 were produced at lower concentrations. Bragulat et al. (1995) had reported the occurrence of *A. candidus* (24%) in mixed poultry feeds and mixed rabbit feeds. Bauduret (1990) found high incidence (60%) of *A. candidus* in raw materials and poultry feeds.

The previously cited researchers have not determined the aflatoxins incidence or aflatoxin production by *A. candidus* strains isolated. Jayaraman and Kalyanasundaram (1990; 1994) observed that a high incidence of AFB_1 corresponded to a dominance of *A. candidus* in rice bran, but they have not studied the AFs accumulation by this fungus. In this work, *E. chevalieri* strains (3%) were able to produce AFB_1, AFB_2, AFG_1 and AFG_2. Aflatoxin B_1 was the highest, followed by AFG_1. Low concentrations of AFB2 and AFG2 were produced again.

This is the first report of the aflatoxin-producing ability by strains of *E. chevalieri* and *A. candidus* isolated from poultry feed and raw materials in Brazil. Although our strains can be considered weak producers under the conditions assayed, the potential production of AFs by these fungi could be an unexpected hazard to animal health when poultry feed is consumed.

REFERENCES

Abarca, M.L.; Bragulat, M.R.; Castella, G. & Cabañes, J. Mycoflora and aflatoxin producing strains in animal mixed feeds. *J. Food Prot*., 57:256-258, 1994.

Abarca, M.L.; Bragulat, M.R.; Castella, G.; Accensi, F. & Cabañes, J. Hongos productores de micotoxinas emergentes. *Rev. Iberoam. Micol*., 17:63-68, 2000.

AOAC. *Official Methods of Analyses Ochratoxins in barley*, 1990, 15th Ed., AOAC, Airlington, sec. 973.37.

Atalla, M.M.; Hassanein, N.M.; El-Beih, A.A. & Youssef, Y.A. Mycotoxin production in wheat grains by different Aspergilli in relation to different relative humidities and storage periods. *Nahrung,*. 47:6-10, 2003.

Aziz, N.H. & Youssef, Y.A. Ocurrence of aflatoxins and aflatoxin-producing molds in fresh and processed meat in Egypt. *Food Addit. Contam*., 8:321-331, 1991.

Bauduret, P. A mycological and bacteriological survey on feed ingredients and mixed poultry feeds in Reunion Island. *Mycopathology,* 109:157-164, 1990.

Bragulat, M.R.; Abarca, M.L.; Castella, O. & Cabañes, J. Mycological survey on mixed poultry feeds and mixed rabbit feeds. *J. Sci. Food Agric.,* 67:215-220, 1995.

Castro, M.F.P.M.; Soares, L.M.V. & Furlan, R.P.Z. Mycoflora, aflatoxigenic species and mycotoxins in freshly harvested corn (*Zea mays* L.): a preliminary study. *Rev. Microbiol*., 26:289-95, 1995.

Creppy, E.E. Update of survey, regulation and toxic effects of mycotoxins in Europe. Toxicol. *Letters,* 127:18-28, 2002.

Dalcero, A.; Magnoli, C.; Chiacchiera, S.; Palacios, G. & Reynoso, M. Mycoflora and incidendence of aflatoxin B_1, zearalenone and deoxinyvalenol in poultry feeds in Argentina. *Mycopathology,* 137:179-184, 1997.

Dalcero, A.; Magnoli, C.; Luna, M.; Ancasi, G.; Reynoso, M.; Chiacchiera, S.; Miazzo, R. & Palacio, G. Mycoflora and naturally occurring mycotoxins in poultry feeds in Argentina. *Mycopathology,* 141:37-43, 1998.

Direito, G.M. *Desenvolvimento de fase móvel para separação de aflatoxinas por cromatografia líquida de alta eficiência utilizando fase normal em sistema de compressão radial*. 1989. 50 f. Dissertation (MSc) – Universidade Federal do Rio de Janeiro, Itaguaí, 1989.

Domsch, K.H.; Gams, W. & Anderson, T. Compendium of soil fungi. Vol. I, Academic Press, New York, 1993. 860 p.

Frisvad, J.C. & Samson, R.A. Neopetromyces gen. nov. and an overview of teleomorphs of *Aspergillus* subgenus *Circumdati*. *Studies Micol.*, 45:68-89, 2000.

Gimeno, A. Improved method for thin layer chromathographic analyses of mycotoxins. *J. Assoc. Off. Anal. Chem.*, 63:182-186, 1980.

Goto, T.; Ito, Y.; Peterson, S.W. & Wicklow, D.T. Mycotoxin producing ability of *Aspergillus tamarii* strain. *Mycotoxins*, 44:17-20, 1997.

Hussein, S.H. & Brasel, J.M. Toxicity, metabolism and impact of mycotoxins on humans and animals. *Toxicology*, 167:101-134, 2001.

Jan, S.K.; Singh, P.P. & Amarjt, S. Observations of occurrence of poultry diseases associated with mycotoxins in feeds. *Indian J. Animal Sci.*, 65:1063-1067, 1995.

Jayaraman, P. & Kalyanasundaram, I. Natural occurrence of toxigenic fungi and mycotoxins in rice bran. *Mycopathology,* 110:81-85, 1990.

Jayaraman. P. & Kalyanasundaram, I. Changes in moisture content, mycoflora and aflatoxin content of rice bran during storage. *Mycopathology,* 126:115-120, 1994.

Klich, M.A. *Identification of common Aspergillus species*. Utrecht: CBS, 2002. 116 p.

Lozada, A.F. Isolation and identification of mycotoxicogenic fungi in selected foods and feeds. *Food Add. Contam.*, 3:509-514, 1995.

Magnoli, C.; Dalcero, A.; Chiacchiera, S.M.; Miazzo, R. & Sáenz, M. Enumeration and identification of *Aspergillus* group and *Penicillium* species in poultry feeds in Argentina. *Mycopathology,* 142:27-32, 1998.

Márcia, B.A. & Lázzari, F.A. Monitoramento de fungos em milho em grão, grits e fubá. *Ci. Tecnol. Alim.*, 18:363-367, 1998.

Raper, K.B. & Fennell, D.I. *The genus Aspergillus*. William and Wilkins, Baltimore, 1965. 686 p.

Rosa, C.A.da R. *Micobiota toxígena e ocratoxinas en raçãoes destinadas à alimentação de aves, bovinos, suínos e importância em saúde animal*. 2002. 184 f. Tese (PhD.) – Universidade Federal do Rio de Janeiro, Seropédica, 2002.

Samson, R.A.; Hockstra, E.S.; Frisvad, J.C. & Filtenborg, O. *Introduction to food and airborne fungi*. Utrecht: Centaalbureau Voorschimmelculturs- Ponson and Looyen, Wageningen Press, 2002. 389 p.

Seiber, J.M. & Hsieh, D.P.H. Application of highly speed liquid chromatography to the analysis of aflatoxins. *J. Assoc. Off. Anal. Chem.*, 56:827-830, 1973.