Mycological survey and mycotoxins detection in equine feeds samples

Kelly M. Keller[1], Beatriz D. Queiroz[1], L. Antonio M. Keller[1], Jessika M. M. Ribeiro[1], Lilia R. Cavaglieri[2] and Carlos Alberto da Rocha Rosa[1]

Departamento de Microbiologia e Imunologia Veterinária. Universidade Federal Rural do Rio de Janeiro, Instituto de Veterinária, Rio de Janeiro[1] and Departamento de Microbiología e Inmunología, Universidad Nacional de Río Cuarto[2]

Introduction

Molds capable of producing mycotoxins present in raw materials will contaminate the final compound into which they are incorporated, such as mixed feeds. Because fungal and mycotoxin contamination of feed can lead to nutrient losses and can have detrimental effects on animal health and production, it is necessary to control their mycological quality. Aflatoxins are a group of secondary metabolites produced by the common molds Aspergillus flavus and A. parasiticus. Horse exposure to aflatoxins, particularly to aflatoxin B1 (AFB1) is characterized by decreased feed intake, loss of body weight, liver damage, centrilobular hepatic disease, brain, kidney and heart damage (*Hintz* 1990). The Fusarium species of greatest concern are those that produce mycotoxins in cereal grains used in animal feed, such as corn. Fumonisins are mycotoxins implicated in equine leukoencephalomalacia (ELEM) and porcine pulmonary edema and have been shown to promote cancer in rats (*Harrison* et al. 1990, *Marasas* et al. 1988). The mycobiota and the level of mycotoxin contamination in animal feed has been monitored worldwide (*Magnoli* et al. 1998, *Siame* et al. 1998, *Sydenham* et al. 1992). However, a detailed study of mycobiota and mycotoxins contamination in equine feed has not been done. The purposes of this study were: 1) to investigate the fungal biota in equine mixed feeds and 2) to determine the aflatoxin B1 (AFB1) and fumonisin B1 (FB1) natural contamination of this substrate.

Materials and methods

Pelletized, non-pelletized and oat samples from equine centers located in Rio de Janeiro, Brazil, were gathered at random every month from June 2003 to March 2004. Quantitative enumeration of fungal propagules was done according to *Magnoli* et al. (1998). Aliquots (0.1 ml) were inoculated in triplicates onto dichloran rose bengal chloranphenicol agar (DRBC), dichloran 18% glycerol agar (DG18) and dichloran chloranfenicol peptone agar (DCPA). The results were expressed as CFU per gram of sample. Aspergillus species frequency was defined as the percentage of samples in which each species was present. They were identified according to *Klich* and *Pitt* (1994), *Pitt* and *Hocking* (1997) and *Samson* et al. (2000). A commercially available enzyme-linked immunosorbent assay (ELISA) kit (Beacon Analytical Systems Inc., Portland, USA) was applied to separate and quantify AFB1 and FB1. Mycotoxins extraction and tests (ELISA) were performed according to manufacturer's instructions. A critical comparison between high-performance liquid chromatography (HPLC) and ELISA was carried out. The separation and quantification of AFB1 and FB1 by HPLC was done according to the methodology proposed by *Ribeiro* et al. (2005) and *Shephard* et al. (1990) and modified by *Doko* et al. (1995), respectively.

Results

Equine feeds fungal colony counts (CFU g-1) from equine centers are shown in Table 1.

Table 1 Equine feed total fungal counts (CFU g-1) obtained from different equine centers.

Equine centers samples		Culture media			
		DRBC (CFU g^{-1})		DG18 (CFU g^{-1})	
		Range	Mean value	Range	Mean value
1	Oats	$6 \times 10^3 - 4.8 \times 10^5$	1.2×10^5	$1.8 \times 10^4 - 3.8 \times 10^5$	1×10^5
	Feed	$1 \times 10^2 - 1 \times 10^4$	3.4×10^3	$1 \times 10^2 - 1 \times 10^5$	3.3×10^4
2		$< 1 \times 10^2 - 1.2 \times 10^4$	5×10^3	$3 \times 10^2 - 3.4 \times 10^4$	1.2×10^3
3		$< 1 \times 10^2 - 4 \times 10^4$	8×10^3	$< 1 \times 10^2 - 5 \times 10^4$	1×10^4
4		$5.1 \times 10^3 - 1.3 \times 10^4$	9×10^3	$< 1 \times 10^2 - 1.4 \times 10^4$	5×10^3
5 (non–pelletized)		$1.3 \times 10^4 - 2 \times 10^5$	1×10^5	$1.2 \times 10^4 - 8 \times 10^4$	4×10^4

Feed DRBC counts ranged between less than 1×10^2 to 4×10^4 CFU g^{-1} with mean values from 3.4×10^3 to 9×10^4 CFU g^{-1}. The highest values were obtained from non-pelletized feeds and oats, which ranged between 1.3×10^4 to 2×10^5 CFU g^{-1} and 6×10^3 to 4.8×10^5 CFU g^{-1} respectively. Counts of xerophilic fungi (DG18) ranged between less than 1×10^2 to 1×10^5 CFU g^{-1}. Non-pelletized feed counts were higher than pelletized feeds. Oat counts from DG18 ranged between 1.8×10^4 to 3.8×10^5 CFU g^{-1} with a mean value of 1×10^5 CFU g^{-1}. Seven fungal genera were identified. The most frequent isolated genus was Aspergillus spp. and its teleomorphs (40.54%) followed by Penicillium spp. (18.38%) and Fusarium spp. (16.22%). Alternaria spp. and Cladosporium spp., among other genera, were isolated in smaller frequency. The most prevalent Aspergillus spp. was A. flavus (36%). In decreasing order A. niger (16%), A. candidus (16%), A. ochraceus (8%), A. puniceus (8%), A. fumigatus (4%), A. caespitosus (4%), A. flavipes (4%) and A. tamarii (4%). Aflatoxin B1 and FB1 determined by ELISA method is shown in Table 2.

Table 2 Equine feed AFB1 (mg kg-1) and FB1 (mg g-1) natural contamination.

Equine centers samples	Aflatoxin B_1 (µg kg^{-1})		Fumonisin B_1 (µg g^{-1})	
	Range	Mean levels	Range	Mean levels
1	0.01 – 5.46	3.01	0.01 – 0.41	0.19
2	0.91 – 6.66	4.79	0.24 – 4.40	1.53
3	0.48 – 99.4	22.13	0.01 – 7.49	2.17
4	4.06 – 11.23	5.10	0.53 – 6.26	2.91
5	2.02 – 8.35	4.04	0.01 – 3.00	1.14

Values ranged between 0.01 to 99.4 mg kg^{-1} and AFB1 mean levels ranged from 3.01 to 22.13 mg kg^{-1}. Fumonisin B1 levels ranged between 0.01 to 7.49 mg kg-1. They varied between 0.19 to 2.91 mg kg^{-1}. HPLC and ELISA methods showed a positive correlation for AFB1 and FB1 determinations ($r=0.9851$ and $r= 0.9791$, respectively) (Fig. 1 and 2).

Discussion

Equine feeds used for equine nutrition were analyzed for fungal biota and natural contamination of AFB1 and FB1. In this

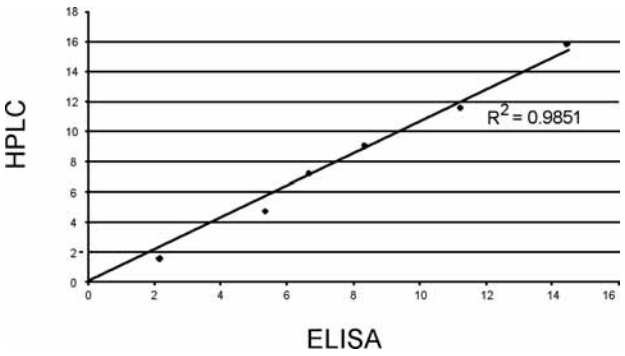

Fig 2 Correlation between ELISA and HPLC methods for fumonisin production.

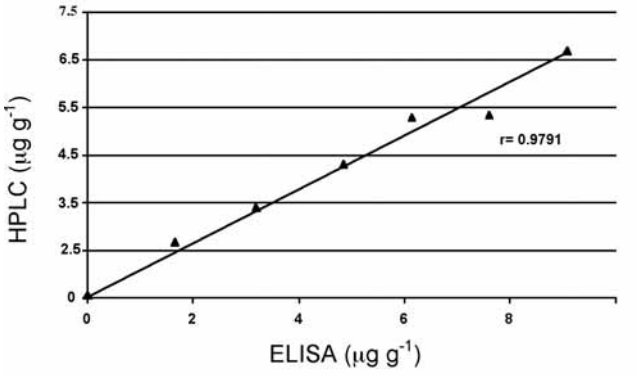

Fig 1 Correlation between HPLC and ELISA methods for aflatoxin production.

study, the fungal colony counts gave moderate values which did not achieve the maximum limit proposed (10^5 CFU g^{-1}) to ensure food hygienic quality. The present results might suggest a relation between some of mycotoxin producer genus frequencies and the occurrence of their related mycotoxin. Aspergillus spp., Penicillium spp. and Fusarium spp. were the most prevalent genera isolated. A. flavus was the most frequent species isolated. It includes potential aflatoxin producers. The frequency of aflatoxicogenic strains shows the potential risk of aflatoxigenic production in equine feeds. Fumonisin B1 in feedstuff suggests that potentially fumonisin producers among Fusarium spp. could be present. The recommended maximum concentration of total fumonisins in corn and corn by-products is 5 ppm for equids (FDA advisory guidelines 2001). In general, all feedstuffs have an action level of 20 ppb. In this work, some samples from equine center 3 had higher AFB1 levels than the established limit (20 ppb). In contrast, FB1 levels did not exceed the limit levels. This study showed good agreement between the methods tested (ELISA and HPLC). The amounts of toxins detected in our country on feedstuff, except one equine center, were lower than the regulation limit established. It is also necessary to establish maximum limits of fungal counts of potential AFs and FBs species since the presence of toxin-producing fungi is a possible risk. These data from Brazil would be of interest worldwide.

References

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

Dalcero A., Magnoli C., Chiacchiera S., Palacios G. and *Reynoso M.* (1997): Mycoflora and incidence of aflatoxin B1, zearalenone and deoxinyvalenol in poultry feeds in Argentina. Mycopathology 137, 179-184

Federal Register. Guidance for industry: Fumonisin levels in human foods and animal feeds. (2001): Federal Register 66 (218), 56688-56689

Harrison L. R., Colvin B. M., Greene I. T., Newman L. E. and *Cole J. R.* (1990): Pulmonary edema and hydrothoraxin swine produced by fumonisin B1, a toxic metabolite of Fusarium moniliform. J. Vet. Diagn. Invest. 2, 217-221

Hintz H. F. (1990): Molds, mycotoxins, and mycotoxicosis. Vet Clinic N America: Equine Practice 6, 419

Klich M. A. and *Pitt J. I.* (Eds.). (1994): A laboratory guide to common Aspergillus species and their teleomorphs. Australia: Commonwealth Scientific and Industrial Research

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

Marasas W. F. O., Kellerman T. S., Gelderblom W. C. A., Coetzer J. A. W., Thiel P. G. and *Van der Lugt J. J.* (1988): Leukoencephalomalacia in a horse induced by fumonisin B1 isolated from Fusarium moniliforme. Ondertepoort J. Vet. Res. 55, 197-203

Meireles M. C. A., Correa B., Fischman O., Gambale W., Paula C. R., Chacon-Reche N. O. and *Pozzi C. R.* (1994): Mycoflora of the toxic associated with equine leokoencephalomalacia (ELEM) outbreaks in Brazil. Mycopathology 127, 183-188

Pitt J. I. and *Hocking A. D.* (Eds.). (1997): Fungi and Food Spoilage. Blackie Academic Press, London. 2nd edition.

Ribeiro J. M. M., Cavaglieri L. R., Fraga M. E., Direito G. M.; Dalcero A. M. and *Rosa C. A. R.* (2005): Influence of water activity, temperature and time on mycotoxins production on barley rootlets. Letters Appl. Microbiol. (in press).

Samson R. A, Hockstra E. S., Frisvad J. C. and *Filtenborg O.* (2000): Introduction to food and airborne fungi. The Netherlands: Centaalbureau Voorschimmelculturs-Utrecht Ponson & Looyen, Wageningen Press

Siame B. A., Mpuchane S. F., Gashe B., Allotey J. and *Teffera G.* (1998): Occurrence of aflatoxin, fumonisin B1 and zearalenone in foods and feeds in Botswana. J. Food Prot. 61, 1670-1673

Sydenham E. W., Marasas W. F. O., Shephard G. S., Thiel P. G. an Hirooka E. Y. (1992): Fumonisin concentrations in Brazilian feeds associated with field outbreaks of confirmed and suspected animal mycotoxicoses. J. Agric. Food Chem. 40, 994-997

C. A. da Rocha Rosa
Departamento de Microbiologia e Imunologia Veterinária
Universidade Federal Rural do Rio de Janeiro
Instituto de Veterinária
23890-000 Rio de Janeiro
Brazil
shalako@ufrrj.br