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ARTICULO BREVE

MOULDY LUCERNE HAY SUSPECTED TO CAUSE BOVINE ABORTION

(Sospecha de aborto bovino por heno enmohecido)

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SUMMARY

In a farm at the province of Jujuy (Argentina), a cattle ate some mouldy bales of lucerne hay and some cows aborted immediatly after. Considering that there could be mycotoxicosis present, some samples of lucerne hay were collected and the following fungi were isolated: ***Myrothecium verrucaria*** *(29.2%),* ***Eurotium amstelodami*** *(28.8%),* ***Aspergillus versicolor*** *(19.5%),* ***Alternaria alternata*** *(10.1%),* ***Aspergillus ochraceus*** *(5.28%),* ***Penicillium aurantiogriseum*** *(1.64%),* ***Aspergillus flavus*** *(0.70%),* ***Aspergillus niger*** *(0.47%), and others (4.33%). Ochratoxin A and aflatoxin* B_1 *were detected in the fodder yet there was absence of macrocyclic trichothecenes. When the animals stopped eating the contaminated fodder, ochratoxin α was detected in the milk of one cow.*

RESUMEN

En una hacienda de la provincia de Jujuy (Argentina) el ganado vacuno consumió fardos enmohecidos de alfalfa henificada y algunas hembras abortaron. Como se consideró la posibilidad de micotoxicosis, se cultivaron muestras del heno, aislándose los siguioentes hongos: ***Myrothecium verrucaria*** (29,2%), ***Eurotium amstelodami*** (28,8%), ***Aspergillus versicolor*** (19,5%), ***Alternaria alternata*** (10,1%), ***Aspergillus ochraceus*** (5,28%), ***Penicillium aurantiogriseum*** (1,64%), ***Aspergillus flavus*** (0,70%), ***Aspergillus niger*** (0,47%), *y otros* (4,33%). *Se detectó ocratoxina A y aflatoxina* B_1 *en el forraje, pero no tricotecenos macrocíclicos. Después que los bovinos dejaron de consumir el forraje contaminado, sólo se detectó ocratoxina α en la leche de un vacuno.*

INTRODUCTION

During the growing season farm animals can graze on the living crop, but in semiarid regions they depend on winter, on grass or leguminous crops conserved as hay. After harvest, the development of the microbiota is controlled by storage conditions. ***Aspergillus*** and ***Penicillium*** spp. are characteristic on stored hay and conserved forage crops have been involved in mycotoxicoses (Lacey, 1991).

Aspergillus ochraceus in mouldy lucerne hay was involved in bovine abortion (Lacey, 1991). Ruminants are reported to be very resistant to the acutely toxic effects of ochratoxin A, because it was degraded to ochratoxin α and phenylalanine by ruminal microbiota (Raisbeck *et al.*, 1991). A cow given a single ochratoxin A dose had ochratoxins A and α in the milk the following day. After that, only ochratoxin α could be detected (Ribelin *et al.*, 1978).

During the dry season in Jujuy at northwest of Argentina, a herd of cows was being fed with mouldy *Medicago sativa* hay. The animals became ill and some of them aborted, but they did not suffer any infectious illness. The search of fungi and mycotoxins detection in the remnant fodder was carried out. Milk mycotoxins were analysed.

MATERIALS AND METHODS

Screening and enumeration of fungi

Samples of remaining fodder were collected from a cattle farm in the month of August. For the general screening of the moulds rose Bengal chloramphenicol agar was used (Jarvis, 1973). Each 25 g sub-sample was treated with a blender. Dilutions were made in aqueous 0.1% w/v

peptone and the inoculum for surface plating was 0.1 mL. After incubation at 25-27°C, the colony forming unity of each mould were counted. Streak and multiple points of inoculation were made on Czapek-yeast agar and malt extract agar (Pitt & Hocking, 1997), for purification of strains.

For identification the cultures were grown for 7 days on potato dextrose agar, Czapek yeast extract agar, malt extract agar and 25% glycerol nitrate agar at 25°C, and on malt extract agar at 5 and 37°C. Petri dishes were inoculated at three points (Pitt & Hocking, 1997). Wet mounts were prepared for microscopic examination.

Extraction of mycotoxins.

Fifty g of fodder were extracted by 250 mL acetone-H_2O (85+15). One hundred of filtrate was treated with 20 mL zinc acetate - aluminum chloride solution for aflatoxin analysis. This solution was prepared dissolving 200 g $Zn(CH_3COO)_2$ and 5 g $AlCl_3$ in H_2O to 1L. After flocculation, filtrate was shaken with 50 mL hexane into a separator. Aqueous layer was reextracted three times with 25 mL $CHCl_3$. Lower layer was drained through anhydrous Na_2SO_4. Solvent was evaporated on steam bath (Scott, 1996).

For ochratoxin analysis, fodder was extracted with chloroform - aqueous phosphoric acid. The chloroform layer was extracted with aqueous sodium bicarbonate. The aqueous phase was acidified and reextracted with chloroform. Fodder was extracted with aqueous acetonitrile and the extract was applied to charcoal - alumina - diatomaceous earth column for trichothecene analysis. Milk was extracted with salt solution - chloroform. The lipid material was removed by washing with hexane (Scott, 1996).

Detection of mycotoxins.

Dry extracts were dissolved in 0.2 ml $ClCH_3$. Thin layer chromatography on silicagel G60 plates (Merck) were developed with the solvent systems: acetone - chloroform, benzene - methanol - acetic acid or toluene - ethyl acetate - formic acid (Betina, 1985; Scott, 1996).

After development, plates were exposed to longwave UV light for aflatoxins and ochratoxins (Scott 1996) and to short-wave UV light for macrocyclic trichothecene detection (Habermehl *et al.*, 1985). Plates were sprayed with aluminum chloride solution or chromotropic acid solution before exposition to longwave UV and visible light for noncyclic trichothecenes detection (Baxter *et al.*, 1983).

Ochratoxin α standard was prepared by acid hydrolysis of ochratoxin A according to Xiao *et al.* (1995).

Myrothecium verrucaria isolates were also analyzed for their ability to produce mycotoxins on yeast extract sucrose agar. The agar plug TLC method was used (Filtenborg *et al.*, 1983).

RESULTS AND DISCUSSION

Outbreak

The dairy farm was placed in San Antonio, province of Jujuy, Argentina. Holstein breed animals grazed on natural pasture and supplemental balanced feeding was supplied during the milking time. In dry season, cows were fed with bales of *Medicago sativa* hay. As cattle aborted before the 5th month of gestation the veterinarian was consulted. The laboratory analysis for brucellosis, leptospirosis and neosporosis was negative.

Fungi and toxins in fodder

Three samples of the remaining fodder pool were serially diluted and plated in triplicate. The mean enumeration was 2.96 ± 0.49 x 105 cfu g^{-1}. Table 1 lists the fungal species isolated from fodder and mycotoxins detected.

Table 1. Fungal species found on fodder

MICROORGANISMS	% CFU	MYCOTOXINS DETECTED
Myrothecium verrucaria (Alb& Schw.) Ditm.:Steudel	29.2	*in vitro* verrucarins, roridins
Eurotium amstelodami Mangin	28.7	
Aspergillus versicolor (Vuill.) Tiraboschi	19.5	
Alternaria alternata complex (Fr.) Keissler	10.1	
Aspergillus ochraceus Wilhelm	5.28	in fodder ochratoxin A
Aureobasidium pullulans (de Bary) Arnaud	3.28	
Penicillium aurantiogriseum Dierckx	1.64	
Aspergillus flavus Link ex Gray	0.70	in fodder aflatoxin B_1
Aspergillus niger van Tieghem	0.47	
Epicoccum nigrum Link	0.35	
Chaetomium sp.	0.35	
Drechslera sp.	0.23	
Curvularia sp.	0.12	

Aflatoxin B_1 and ochratoxin A were detected in fodder samples but macrocyclic trichothecenes were not found. The TLC agar plug method revealed macrocyclic

trichothecene production by ***M. verrucaria*** isolates. One strain was analysed at INAME (Bs.As.) and it produced verrucarins A and J; roridins A,D and E. Other fungi were not analyse because they were not suspected to produce significant mycotoxins.

Fusarium spp. and ***A. ochraceus*** were isolated in Argentina by Saubois *et al.*(1988), from *Medicago sativa* and *Sorghum halepense* fodder during a mycotoxic outbreak, but ***Myrothecium*** species were not found. Gaggiotti *et al.* (2001), reported deoxynivalenol producing ***Fusarium*** in fodders during 1997-1998 season, and toxicogenic ***A. flavus*** in 1998-1999 season.

Myrothecium sp. did not produce trichothecenes in a mixed culture with ***Aspergillus*** spp. and ***Penicillium*** spp. (Reddy & Reddy, 1992; Reddy *et al.,* 1998). Some outbreaks of myrotheciotoxicosis in cattle were reported in Asia but the fodder was infected with Mucorales besides ***Myrothecium*** sp. (Prudhvi-Reddy *et al.* 1996).

Lucerne hay is a variable substrate for the growth and development of ***A. flavus*** and its production of aflatoxin (Lacey, 1991). Only a minority of ***A. ochraceus*** isolates are toxigenic and other species closely related also produce ochratoxin A (Pitt & Hocking, 1997). ***A. flavus*** inhibits the toxins production by ***A. versicolor*** in mixed cultures (Devi & Polasa, 1987) and ***A. alternata*** toxins are completely inhibited in cultures co-inoculated with ***Aspergillus parasiticus*** (Etcheverry *et al.,*1998).

Production of significant mycotoxins by ***A. pullulans*** and ***Epicoccum*** has not been reported. It is generally assumed that ***E. amstelodami*** and ***A. niger*** are benign fungi (Pitt & Hocking, 1997) but some ***Chaetomium***, ***Curvularia***, and ***Drechslera*** are suspected to produce toxins (Udagawa, 1983).

Toxins in milk

Ten milk samples were taken fifteen days after the end of contaminated fodder feeding. Aflatoxin M_1 was not found and one sample had traces of ochratoxin α.

Two weeks after oral administration, ochratoxin was detected in milk (Ribelin *et al.,* 1978). Aflatoxin M_1 is detected in cow milk within 72 h of ingestion of naturally contaminated feed (Raisbek *et al.,* 1991). These facts explain the absence of aflatoxin M_1 and the finding of ochratoxin α in the analysed milk. Ochratoxin α is 25-fold less toxic than the parent toxin ochratoxin A (Størmer, 1992).

CONCLUSION

We concluded that mycotoxicosis occurred as a result of a prolonged ingestion of mouldy fodder, because the outbreak finished when the mouldy fodder was exhausted. It might result from a combination of several mycotoxins, mainly aflatoxin B_1 and ochratoxin A. The milk can contain the less toxic ochratoxin (?) and this is a hazard for humans.

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REFERENCES

Baxter, J.A.; Terhune, S.J.; Qureshi, S.A. (1983) Use of chromotropic acid for improved thin layer chromatographic visualisation of trichothecene mycotoxins. J. Chromat. 261:130-133

Betina, V. (1985) Thin-layer chromatography of mycotoxins. J. Chromat. 334: 211-276

Devi, G.R.; Polasa, H. (1988) Interference in toxin production among toxigenic *Aspergillus* species. J. Stored Products Res. 23:1149-150

Ellis, M.B. (1971) Dematiaceous hyphomycetes. Kew: C.M.I.

Etcheverry,M.; Cavaglieri,L.; Chulze,S. (1998) Microbial interaction of *Aspergillus parasiticus* and *Bacillus subtilis* with *Alternaria alternata*. Production of alternariol, alternariol monomethylether and tenuazonic acid on sunflower seeds. Mycotoxin Res. 14: 2-8

Filtenborg, O.; Frisvad, J.C.; Svendsen, J.A. (1983) Simple screening method for molds producing intracellular mycotoxins in pure culture. Appl. Environ. Microbiol. 45: 581-585

Gaggiotti M, Romero L, Basílico JC. (2001) ¿Conoce las micotoxinas? Infortambo 145: 60

Habermehl, G.C.; Busam, L.; Heydel, P.; Mebs, D.; Tokarnia, C.H.; Döbereiner, J.; Spraul, M. (1985) Macrocyclic trichothecenes: cause of livestock poisoning by the brazilian plant *Baccharis coridifolia*. Toxicon 23: 731-745

Jarvis, B. (1973) Comparison of an improved rose Bengal-chlortetracycline agar with other media for the selective isolation and enumeration of moulds and yeasts in foods. J. Appl. Bacteriol. 36: 723-727

Klich, M.A.; Pitt, J.I. (1992) A Laboratory Guide to Common *Aspergillus* species and their Teleomorphs. North Ryde: CSIRO.

Lacey, J. (1991) Natural occurrence of mycotoxins in growing and conserved forage crops. In Mycotoxins and Animal Foods (J.E. Smith and R.S. Henderson, eds) pp. 363-397. Boca Ratón: CRC Press.

Pitt, J.I. (1991) A Laboratory Guide to Common *Penicillium* species. North Ryde: CSIRO.

Pitt, J.I.; Hocking, AD. (1997) Fungal and Food Spoilage. London: Blackie Academic & Professional

Prudhvi Reddy K.; Radhakrishnaiah K.; Hafeez Md.; Ramakrishna A. (1996) Myrotheciotoxicosis in cattle. Indian Vet. J. 73: 1231-1234

Raisbeck, M.F.; Rottinghaus, G.E.; Kendall, J.D. (1991) Effects of naturally occurring mycotoxins on ruminants. In Mycotoxins and Animal Foods. (J.E. Smith & R.S. Henderson, eds.) pp. 647-677. Boca Ratón: CRC Press

Reddy, OL.; Reddy, S.M. (1992) Production of roridin by *Myrothecium roridum* in mixed cultures. Indian J. Microbiol. 31: 281-284

Reddy, V.K.; Kumari, D.R.; Reddy, S.M. 1998. Effect of carbon and nitrogen sources on the interaction of mycotoxigenic fungi and mycotoxin production. J. Food Science Technol. 35: 268-270

Ribelin, W.E.; Fukushima K.; Still, P.E. (1978) The toxicity of ochratoxin to ruminants. Can. J. comp. Med. 42: 172-176

Saubois, A.; Umansky, G.N.; Basilico, J.C. (1988) Estudio micotoxicológico de fardos implicados en una enfermedad del ganado vacuno. Revista Arg. Micología 11 (2): 21-24

Scott, P.. (1996) Natural toxins. In AOAC Official Methods of Analysis. Chapter 49. Washington: AOAC International

Størmer, F.C. (1992) Ochratoxin A - a mycotoxin of concern. In Mycotoxins in Ecological Systems (D. Bhatnagar, E.B. Lillehoj, D.K. Arora, eds.) pp. 403-432. New York: Marcel Dekker

Udagawa S. (1983) Taxonomy of mycotoxin-producing *Chaetomium*. In Toxigenic Fungi. Their Toxins and Health Hazard (H. Kurata & Y. Ueno, eds.) pp. 139-147. Amsterdam: Elsevier

Xiao, H.; Marquardt, R.R.; Frohlich, A.A.; Ling, Y.Z. (1995) Synthesis and structural elucidation of analogs of ochratoxin A. J. Agric. Food Chem. 43: 524-530