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ORIGINAL ARTICLE

Aspergillus fumigatus toxicity and gliotoxin levels in feedstuff for domestic animals and pets in Argentina

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Abstract

Aims: To evaluate gliotoxin production by *Aspergillus fumigatus* strains isolated from feedstuff intended for domestic animals and pets, and to determine the amount of gliotoxin in these substrates.

Methods and Results: A total of 150 feedstuff samples were collected. They were composed of 30 samples each of five different feed types (pigs, poultry, cattle, horse and pets). *Aspergillus fumigatus* gliotoxin production ability and gliotoxin presence in feedstuff was determined by HPLC. *Aspergillus fumigatus* strains were isolated from all of the tested samples. Strains from cattle, horses and pet food were able to produce gliotoxin. Corn silage samples intended for cattle did not show gliotoxin contamination. All the other tested samples had gliotoxin levels ranging from 29 to 209 $\mu g\ g^{-1}$. Horse and poultry feed samples had the greatest contamination frequency.

Conclusions: Feed samples contaminated with gliotoxin are potentially toxic to animals.

Significance and Impact of the Study: The presence of gliotoxin could affect animal productivity and health. Moreover, there are risks of contamination to farm workers handling improperly stored animal feed. *Aspergillus fumigatus* strains isolated from different sources should be investigated to determine prevention and control strategies.

Introduction

The fungal invasion of raw materials and consequent mycotoxin production are thought to be a potential risk factor for feedstuff quality. In domestic animals, such as dairy cattle, pig and poultry, mycotoxin contamination reduces growth efficiency, lowers feed conversion and reproductive rates, impairs resistance to infectious disease, reduces vaccination efficacy and increases pathologic damage to the liver and other organs (CAST 2003).

A common thermophilic fungus found in contaminated animal feed is *Aspergillus fumigatus* (Scudamore and Livesey 1998; Schneweis *et al.* 2001; Cavaglieri *et al.* 2005; Pereyra *et al.* 2008). *Aspergillus fumigatus* spores are easily spread in the air and pose a high risk of exposure for both animals and humans (Land *et al.* 1987). This fungus is able to produce tremorgenic mycotoxins and to induce neurological syndromes in farm workers who have manipulated mouldy feed containing it (Gordon *et al.* 1993). In addition, this fungus is a well-known human

and animal pathogen causing aspergillosis (Sutton *et al.* 1996).

One highly toxic metabolite that can be produced by *A. fumigatus* is gliotoxin, a toxin that has potent immunosuppressive, genotoxic, cytotoxic and apoptotic effects (Nieminen *et al.* 2002; Upperman *et al.* 2003). Gliotoxin has been linked to intoxication and death in camels that had consumed contaminated hay (Gareis and Wernery 1994), and *in vivo* gliotoxin production by *A. fumigatus* was reported by Bauer *et al.* (1989). A recent study demonstrated the presence of gliotoxin in cattle feedstuff (Pereyra *et al.* 2008). However, information is scarce about the presence of *A. fumigatus* and its mycotoxins in other feedstuffs.

The aims of the present study were (i) to evaluate gliotoxin production by *A. fumigatus* strains isolated from feedstuff intended for domestic animals and pets and (ii) to determine the incidence of gliotoxin in these substrates.

Materials and methods

Sampling and feed composition

A total of 150 feedstuff samples (30 intended for pigs, 30 for horses, 30 for feedlot cattle, 30 for poultry and 30 for pets) were collected monthly over a 3-month-period from farms and factories located in Córdoba Province, Argentina. The sampling scheme is shown in Table 1. The storage period of poultry and pet samples in factories generally ranged from 8 to 15 days. The storage period was longer (between 3 and 8 months) for pig, horses and cattle feed samples in farms. Each of the feedstuff samples was homogenized, milled and quartered to get 1 kg of laboratory samples and immediately analyzed for fungal contamination. Then, the samples were stored at 4°C (for up to 7 days) until mycotoxin analysis.

Aspergillus fumigatus isolation and identification

Ten grams of each milled sample were blended with 90 ml of 0·1% peptone water solution and an aliquot inoculated in triplicate on to dichloran rose Bengal chloramphenicol agar (DRBC) (Abarca *et al.* 1994). The DRBC plates were incubated at 25°C for 7–10 days. Colonies representative of *A. fumigatus* were subcultured on to malt extract agar. Identification was made according to microscope observation criteria in accordance with Klich (2002). The frequency (percentage of samples in which *A. fumigatus* was present) was determined. Representative *A. fumigatus* strains were deposited in the culture collection of the National University of Río Cuarto, Córdoba, Argentina.

Gliotoxin production by *Aspergillus fumigatus* strains

All *A. fumigatus* strains were assayed for gliotoxin production. The strains were grown on YES (sucrose 40 g, yeast extract 20 g, agar 20 g and distilled water to 1000 ml) plates at 37°C for 7 days. Three agar plugs were removed from the central area of the colony, weighed and introduced into a small vial. Chloroform (1 ml) was added to each vial, and the sample–solvent mixture was centrifuged for 10 min at 1252 ***g***. The supernatant was filtered (Titan filtration system, 17 mm, 0·45 μm; Rockwood, TN, USA) and evaporated to dryness under N_2.

Table 1 Monthly sampling scheme of different kinds of feedstuffs

Feedstuff (30 samples each)	Kind of feed	Main composition	No. samples/ establishment	No. and type of establishment
Pig feed	Initial*	75% corn, 25% concentrate	2	Five farms
	Growing†	80% corn, 17% concentrate, 3% alfalfa	2	
	Final‡	82% corn, 15% concentrate, 3% alfalfa	2	
Horse feed	Standard	60% corn, 20% oat, 20% ensiled alfalfa	10	Three equestrian centres
Dairy cattle feed	Upper silo section Middle silo section Low silo section	100% ensiled corn	10	Three farms
Poultry feed	Broilers	Primarily: corn and soy Complementary: wheat, barley, oats, meat and bone meal	10	Three factories
Pet food	Standard	<21% protein	5	Two factories
	Premium	21–28% protein	5	
	Superpremium	>28% protein	5	

*Destined to 10–25-kg weight pigs.
†Destined to 25–60-kg weight pigs.
‡Destined to >60-kg weight pigs.

The residue was redissolved in the mobile phase and used for gliotoxin analysis by HPLC.

Detection and quantification of gliotoxin

Gliotoxin was determined following the methodology of Boudra and Morgavi (2005) with some modifications. The HPLC apparatus was a Hewlett Packard chromatograph with a loop of 20 μl, equipped with a UV detector and a C18 column (Supelcosil LC-ABZ, 150, 4·6, 5 mm particle size; Supelco, USA) connected to a precolumn (Supelguard LC-ABZ, 20, 4·6, 5 mm particle size; Supelco). The mobile phase was pumped at 2·0 ml min^{-1} and consisted of an isocratic system as follows: 75% (1% acetic acid in water) and 25% acetonitrile. The retention time was 5·5 min. Detection was carried out at 254 nm. The solution standards were prepared by dissolving pure gliotoxin (Sigma-Aldrich Co.) at 5 mg ml^{-1} in chloroform. The detection limit of the technique was 1·2 $\mu g\ g^{-1}$. Quantification of gliotoxin was performed by measuring the areas followed by extrapolation to obtain a calibration curve using standard solutions of gliotoxin.

Feedstuff incidence of gliotoxin

Gliotoxin was determined following the methodology of Boudra and Morgavi (2005). Samples were dried at 48°C for 72 h in a forced air oven. Distilled water (10 ml) was added to flasks containing 10 g of sample followed by addition of 40 ml dichloromethane. The sample–solvent mixture was soaked at room temperature for 2 h followed by 15 min of mechanical agitation and filtered through filter paper (Whatman, Inc., Clifton, NJ, USA). A volume of 5 ml of the filtrate extract was filtered (Titan filtration system, 17 mm, 0·45 μm) and evaporated to dryness under N_2. The residue was redissolved in 200 μl of the mobile phase and used for gliotoxin analysis by HPLC. Gliotoxin detection and quantification was performed by the methodology described earlier.

Results

Aspergillus fumigatus with gliotoxin production ability

Table 2 shows the gliotoxin levels produced by *A. fumigatus* strains isolated from feedstuff intended for cattle (corn silage), pigs, poultry, horses and pets.

Aspergillus fumigatus was isolated from all types of feedstuff. None of the strains isolated from poultry and pig feed were able to produce gliotoxin, while 28, 60 and 33% of the strains isolated from cattle, horse feed and pet food, respectively, were able to produce this mycotoxin. They were able to produce average levels, from nothing at all (ND, not detected) to 20 $\mu g\ g^{-1}$, and pet food strains were the highest producers.

Incidence of gliotoxin in feedstuff

The gliotoxin concentration and contamination frequency of feedstuffs intended for domestic animals and pets are shown in Table 2.

Corn silage samples intended for cattle did not show detectable gliotoxin contamination levels. All other types of feedstuffs had some degree of gliotoxin contamination. Average levels ranged from 29 $\mu g\ g^{-1}$ in pet food to 209 $\mu g\ g^{-1}$ in horse feed. Poultry feed showed the highest gliotoxin contamination frequency followed by horse feed, pig feed and pet food.

There was a positive relationship between the presence of *A. fumigatus* and levels of gliotoxin in feed intended for horses and pets. However, this relationship was negative for the other feedstuffs we studied.

Table 2 *Aspergillus fumigatus* strains gliotoxin producers and gliotoxin incidence in feedstuffs

	A. fumigatus gliotoxin producers		Gliotoxin incidence in feedstuffs	
Samples	Positive strains*	Gliotoxin levels† ($\mu g\ g^{-1}$) ± SD	Gliotoxin levels ($\mu g\ g^{-1}$) ± SD	Contamination frequency (%)
Cattle (corn silage)	4/14	14 ± 9	ND	0
Pig	0/1	ND	96 ± 57	30·0
Poultry	0/14	ND	70 ± 27	41·6
Horse	3/5	11 ± 2	209 ± 30	40·0
Pets	5/13	20 ± 13	29 ± 9	17·6

Detection limit of the technique: 1·2 $\mu g\ g^{-1}$.
Quantification limit of the technique: 2 $\mu g\ g^{-1}$.
ND, not detected.
*Number of producer strains *vs* total strains.
†Mean levels ± standard deviation (SD).

Discussion

The main objective of this study was to identify gliotoxin contamination in feedstuffs for domestic animals and pets. Moreover, the ability of *A. fumigatus* strains to produce gliotoxin was determined.

In this study, several strains isolated from corn silage, horse feed and pet food were able to produce gliotoxin. In a previous work, Pereyra *et al.* (2008) tested *A. fumigatus* strains isolated from corn silage intended for dairy cattle and found higher gliotoxin levels than we found. Boudra and Morgavi (2005) studied the ability of 14 strains of *A. fumigatus*, isolated from corn silage, to produce gliotoxin and found levels that were similar to those we have reported. Dos Santos *et al.* (2002) reported on the gliotoxin toxicogenic capacity of 27 strains of *A. fumigatus* isolated from silage and only three strains were capable of gliotoxin production.

In this study, except for corn silage intended for cattle, all the other four types of feedstuff were usually contaminated with gliotoxin. Poultry and horse feeds were the most often contaminated samples and horse feed had the highest gliotoxin levels. The incidence of gliotoxin in feedstuff for pigs, poultry, horses and pets does not appear to have been reported previously. Pereyra *et al.* (2008) demonstrated the presence of gliotoxin in 90 corn silage samples for dairy cattle, in contrast to our results where the toxin was not detected there.

There was a positive relationship between the gliotoxin-producing ability of *A. fumigatus* and the incidence of gliotoxin in feedstuffs for horses and pets, while this relationship was negative in corn silage, pig and poultry feed. This may be primarily because of the relatively low number of strains tested. The absence of gliotoxin in corn silage could be because of the acidic conditions present in correctly prepared silage. Boudra and Morgavi (2005) demonstrated, in *in vitro* studies, that acidic conditions and agitation exert a negative effect on gliotoxin production. However, the presence of the fungus does not imply the presence of mycotoxins just as the presence of mycotoxins does not imply the presence of the fungus. Environmental conditions that allow fungal growth are not always the same as those allowing mycotoxin production (CAST 2003).

Feeds are one of the main sources of gliotoxin contamination. There are no recommended levels for gliotoxin in feeds intended for domestic animals or pets. However, researchers have observed immunosuppressive and apoptotic effects in tissue cell studies at concentrations of $<0{\cdot}01$ μg ml^{-1} (Upperman *et al.* 2003; Watanabe *et al.* 2003). Levels greater than these were recorded in our study. These results were obtained on culture cells. Future studies are required to determine the gliotoxin levels able to produce immunosuppressive and apoptotic effects *in vivo*.

Gliotoxin was found in feedstuffs intended for different animal species in this work. The presence of the toxin could affect animal productivity and health. Moreover, there are risks of contamination to farm workers handling improperly stored animal feed.

Richard and DeBey (2005) studied gliotoxin production in an experimental infection of turkey poults by strains of *A. fumigatus* and concluded that gliotoxin was involved in avian aspergillosis. Remarkably, gliotoxin was recently found in conidia of environmental isolates of *A. fumigatus* (Fischer *et al.* 2006).

Future studies should focus on establishing any possible genetic and enzymatic profile similarities, as well as the toxigenicity, of *A. fumigatus* strains isolated from different sources to determine possible prevention and control strategies.

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