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ORIGINAL ARTICLE

Typing clinical and animal environment *Aspergillus fumigatus* gliotoxin producer strains isolated from Brazil by PCR-RFLP markers

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Significance and Impact of the Study: *Aspergillus fumigatus* is a well-known human and animal pathogen causing aspergillosis. In this study, clinical (human and animal) and animal environment strains were able to produce high gliotoxin levels and had band profiles according to *A. fumigatus* sensu stricto by PCR-RFLP markers. The results obtained here suggest that strains involved in human and animal aspergillosis could come from the animal environment in which *A. fumigatus* is frequently found. Its presence in animal environments could affect animal health and productivity; in addition, there are risks of contamination for rural workers during handling and storage of animal feedstuffs.

Keywords
animal environment, *Aspergillus fumigatus*, clinical (animal and human), restriction enzymes, taxonomic state.

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Abstract

Aspergillus fumigatus, a well-known human and animal pathogen causing aspergillosis, has been historically identified by morphological and microscopic features. However, recent studies have shown that species identification on the basis of morphology alone is problematic. The aim of this work was to confirm the taxonomic state at specie level of a set of clinical (human and animal) and animal environment *A. fumigatus* strains identified by morphological criteria applying a PCR-RFLP assay by an *in silico* and *in situ* analysis with three restriction enzymes. The *A. fumigatus* gliotoxin-producing ability was also determined. Previous to the *in situ* PCR-RFLP analysis, an *in silico* assay with *BccI*, *MspI* and *Sau3AI* restriction enzymes was carried out. After that, these enzymes were used for *in situ* assay. All *A. fumigatus* strains isolated from corn silage, human aspergillosis and bovine mastitis and high per cent of the strains isolated from cereals, animal feedstuff and sorghum silage were able to produce high gliotoxin levels. Also, all these strains identified by morphological criteria as *A. fumigatus*, regardless of its isolation source, had band patterns according to *A. fumigatus* sensu stricto by PCR-RFLP markers.

Introduction

Aspergillus fumigatus is a thermophilic fungus commonly found in contaminated animal environments and is the toxicogenic fungus that most frequently contaminates silages (dos Santos *et al.* 2003, Pereyra *et al.* 2008). The large number of spores from *A. fumigatus* is easily spread in the air indicating a high risk of exposure, both for animals and humans. In ruminants, this fungus causes pulmonary process, abortion, mastitis and other clinical cases (Gourreau *et al.* 1988; Smith 1989). Pepin (1988) isolated *A. fumigatus* from milk of cows without symptoms, some of which had subsequently aspergillar mastitis. This fungus is able to produce tremorgenic

mycotoxins and to induce neurological syndromes to farmworkers who manipulate mouldy feed containing it (Land *et al.* 1987, Gordon *et al.* 1993). One highly toxic metabolite that can be produced by *A. fumigatus* is gliotoxin. It 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 consuming contaminated hay (Gareis and Wernery 1994), and *in vivo* gliotoxin production by *A. fumigatus* was reported by Bauer *et al.* (1989). Recent studies have informed the gliotoxin presence in cattle feedstuffs (Pereyra *et al.* 2008).

Aspergillus fumigatus, a well-known human and animal pathogen causing aspergillosis, has been historically identified by morphological characteristics and microscopic features (Latgé 1999). However, recent studies have shown that species identification on the basis of morphology alone is problematic because some isolates are pigmentless or poorly sporulated, and growth conditions can influence the morphology making the identification difficult (Guarro *et al.* 1999). This fact has led to species misidentification and also to the discarding of organisms as contaminants (Balajee *et al.* 2004; Yaguchi *et al.* 2007). To resolve this issue, a number of different techniques have been developed and used to identify the species belonging to the section *Fumigati*. These include analysis of the profiles of secondary metabolites, isozyme electrophoretic pattern analysis and molecular data analysis (Varga *et al.* 2000; Yaguchi *et al.* 2007). Staab *et al.* (2009) devised and validated an identification scheme based on the β-tubulin gene (*benA*) to rapidly identify similar morphotypes in the section *Fumigati* (*A. fumigatus*, *A. lentulus* and *Neosartorya udagawae*) using PCR-restriction fragment length polymorphisms (RFLPs) with *BccI* restriction enzyme. Based on this study, the aim of this work was to confirm the taxonomic state at specie level of a set of clinical (human and animal) and animal environment *A. fumigatus* strains identified by morphological criteria applying a PCR-RFLP assay by an *in silico* and *in situ* analysis with three restriction enzymes. The *A. fumigatus* gliotoxin-producing ability was also determined.

Results and discussion

All *A. fumigatus* strains isolated from corn silage, human aspergillosis and bovine mastitis were able to produce gliotoxin, whereas 92, 84 and 71% of the strains isolated from cereals, animal feedstuff and sorghum silage, respectively, were able to produce it at levels between detectable (nonquantifiable concentration) to 29·6 μg g^{-1} gliotoxin (Table 1). High gliotoxin levels were produced by *A. fumigatus* strains of human aspergillosis and silage sources, whereas the highest amount of this mycotoxin was produced by a corn silage strain (57·8 μg g^{-1}). The analysis of variance of the effects of different isolation sources on gliotoxin production by *A. fumigatus* strains showed that there was a statistical difference ($P = 0{\cdot}0018$) among them. The Fischer's least significant difference (LSD) test showed that the isolation sources that had the greatest influence on gliotoxin production were corn silage and human aspergillosis (Table 1). Currently, there are few articles reporting *A. fumigatus* gliotoxin producer strains isolated from animal environments such as animal feedstuff and silages. Pena *et al.* (2010) studied the gliotoxin production ability of *A. fumigatus* strains isolated from feedstuffs intended to domestic animals and pets in Argentina. They found that several strains isolated from corn silage, equine feed and pet food were able to produce gliotoxin to 20·01 μg g^{-1} levels. Moreover, pet food strains were the highest producers. dos Santos *et al.* (2003) reported the gliotoxicogenic ability of 27 *A. fumigatus* strains isolated from corn silage, and they found that only three strains were able to produce gliotoxin. In this study, all strains isolated from corn silages produced gliotoxin at wide ranges; in addition, the highest gliotoxin

Table 1 *Aspergillus fumigatus* gliotoxin producer strains and PCR-RFLP patters

Source*	Positive strains†	Gliotoxin levels (μg g^{-1}) Range (min-max)‡	PCR-RFLP pattern
Animal feedstuff	11/13	1·2–29·6[a]	Similar to *A. fumigatus* sensu stricto
Cereals	12/13	0·8–21·7[a]	Similar to *A. fumigatus* sensu stricto
Corn silage	5/5	0·7–57·8[b]	Similar to *A. fumigatus* sensu stricto
Sorghum silage	5/7	0·4§–3·8[a]	Similar to *A. fumigatus* sensu stricto
Human aspergillosis	7/7	10·4–30·6[b]	Similar to *A. fumigatus* sensu stricto
Bovine mastitis	8/8	2·2–5·2[a]	Similar to *A. fumigatus* sensu stricto

*Source of strain isolation.

†Number of producer strains vs. total strains.

‡Range values with the same letter are not significantly different according to Fisher's protected LSD test ($P < 0{\cdot}05$).

§Detectable, under the limit of quantification (LOQ).

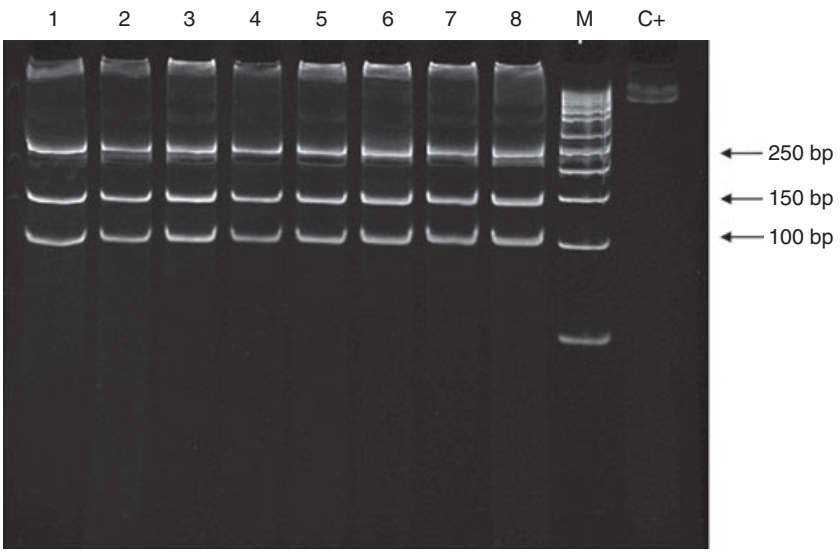

Figure 1 Polyacrylamide gel electrophoresis of the fragments generated from *benA* amplicons digestion with *BccI*. Lanes 1–4, strains isolated from sorghum silage, and lanes 5–8, strains isolated from corn silage. Lane M, 50-bp DNA ladder (Fermentas®). Line C+ *benA* PCR product not digested.

producer was a strain from this substrate. Furthermore, strains of human aspergillosis and animal feedstuff were able to produce more than 20 μg g^{-1} of gliotoxin. Gliotoxin had been found in lung and sera of mice and humans infected with *A. fumigatus* (Lewis *et al.* 2005; Kupfahl *et al.* 2008). Reeves *et al.* (2004) studied the correlation between gliotoxin production and virulence of *A. fumigatus* on the *Galleria mellonella* larvae. They found that *A. fumigatus* strains that produced higher amounts of gliotoxin were more virulent (produced higher larvae mortality) than those who had low ability for this toxin production. In this study, the fact that clinical and animal environment strains produced similar amounts of gliotoxin is extremely important because this toxin has been considered as one of the most important virulence factors involved in invasive aspergillosis produced by *A. fumigatus* (Waring *et al.* 1991, Sutton *et al.* 1994). Therefore, strains that are prevalent in animal environments could be involved in the development of illness in both human and animals. The presence of this toxin in animal environments could affect animal health and productivity; in addition, there are risks of contamination for rural workers during handling and storage of animal feedstuffs.

The digestion of *benA* amplicons of the set of clinical and environmental *A. fumigatus* strains with *BccI*, *MspI* and *Sau3AI* restriction enzymes was found to be in 100% agreement with that predicted DNA fragment sizes of *in silico* analysis. Figure 1 shows band patterns obtained after cleavage of *benA* amplicons with *BccI* enzyme of the strains isolated from sorghum and corn silages. Three DNA fragments of approximately 99, 144 and 249 bp were originated from all assayed strains (band patterns from remaining isolation sources are not shown). Similarly, the digestion of *benA* amplicons with *MspI* and *Sau3AI* produced characteristic band patterns of *A. fumigatus* sensu stricto on *in silico* analysis (Fig. 2 and Fig. 3). Therefore, all strains by PCR-RFLP technique identified by morphological criteria as *A. fumigatus* result in banding patterns corresponding to that obtained for *A. fumigatus* sensu stricto by *in silico* analysis. The *benA* digestion pattern for each organism was stable, as judged with independent DNA samples prepared from biological replicates (at least two independent cultures; data not shown). Debeaupuis *et al.* (1997) suggested that any environmental strain of *A. fumigatus* is a putative infectious strain that can cause invasive aspergillosis when it encounters an immunosuppressed host. Staab *et al.* (2009) reported that *benA* PCR-RFLP is predicted to be a useful tool for distinguishing most known section *Fumigati* members, including *A. fumigatus*, *A. lentulus* and *N. udagawae*. The species discrimination is very important because these species have different antifungal susceptibility patterns. Hong *et al.* (2005) examined the variability within *A. fumigatus* Fresenius and found that DNA sequence analyses separated strains, considered as *A. fumigatus* before, into four groups including *A. fumigatus* sensu stricto, *A. lentulus*, *A. fumigatiaffinis* and *A. novofumigat*us. In this study, the studied strains were found to be gliotoxin producers and had PCR-RFLP patterns according to *A. fumigatus* sensu stricto profile on *in silico* PCR-RFLP analysis. Furthermore, DNA sequencing of *benA* regions of six strains (one from each isolation source) confirmed its identification as *A. fumigatus* sensu stricto because they had 99–100% maximum identities with published *A. fumigatus* sequences in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The obtained *benA* sequences have been deposited in GenBank under accession numbers KF410677–KF410682. RFLP-PCR technique has been previously applied only for typing human *A. fumigatus* strains (Verweij *et al.* 1996; Semighini *et al.*

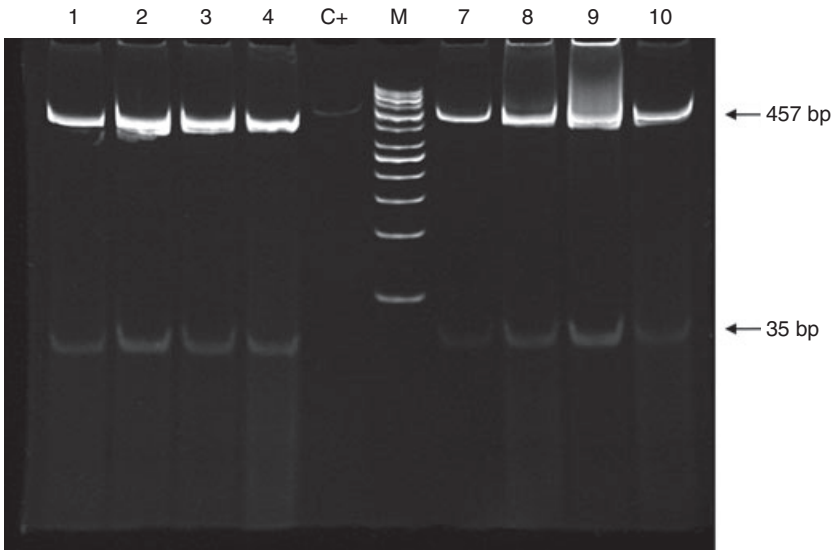

Figure 2 Polyacrylamide gel electrophoresis of the fragments generated from *benA* amplicons digestion with *MspI*. Lanes 1–4, strains isolated from animal feedstuff, lane C+ *benA*, PCR product not digested, lane M, 50-bp DNA ladder (Thermo Fischer Scientific Inc., Waltham, MA, USA), and lanes 7–10, strains isolated from sorghum silage.

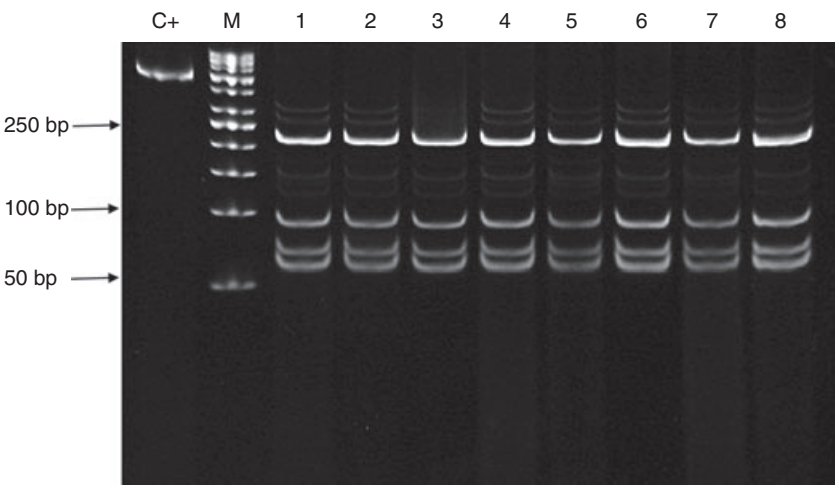

Figure 3 Polyacrylamide gel electrophoresis of the fragments generated from *benA* amplicons digestion with *Sau3AI*. Lane C+ *benA*, PCR product not digested, and lane M, 50-bp DNA ladder (Thermo Fischer Scientific Inc.). Lanes 1–8, strains isolated from sorghum silage.

2001), despite this technique was not previously used for comparing neither animal nor animal environments *A. fumigatus* strains, as was done in this work.

In conclusion, in this study, strains isolated from clinical (human and animal) and animal environment were able to produce high gliotoxin levels and had band patterns according to *A. fumigatus* sensu stricto by PCR-RFLP markers. These results suggest that strains involved in human and animal aspergillosis could come from the animal environment in which *A. fumigatus* is frequently found.

Materials and methods

Aspergillus fumigatus strains

A set of 53 clinical (human – aspergillosis and animal – bovine mastitis) and animal environment (feedstuffs, cereals and sorghum and corn silages) *A. fumigatus* strains originally from the Núcleo de Pesquisas Micológicas e Mitoxicológicas Collection Centre was used in this study (Table 2). These strains were morphologically identified as *A. fumigatus* by the classical taxonomy according to Klich and Pitt (2002) and maintained on malt extract agar (MEA) slants at 4°C until gliotoxin and PCR-RFLP analysis.

Aspergillus fumigatus gliotoxin production ability

Gliotoxin production and extraction

All clinical and environmental *A. fumigatus* strains were assayed for gliotoxin production. The strains were grown on YES (sucrose 15%, yeast extract 2%, agar 2%) plates at 37°C for 7 d. After that, 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

Table 2 *Aspergillus fumigatus* strains used in this study

Isolation source	No. of isolates	Location	Year of isolation
Animal feedstuff*	13	Sao Pablo, SP, Brazil	2011
Cereals†	13	Mato Grosso, RJ, Brazil	2011
Corn silage	5	Sao Pablo, SP, Brazil	2011
Sorghum silage	7	Sao Pablo, SP, Brazil	2011
Human aspergillosis	7	Rio do Janeiro, RJ, Brazil	2010
Bovine mastitis	8	Seropedica, RJ, Brazil	2011

*Animal feeds intended to poultry, pigs and cows.
†Barley, malt and rice.

each vial, and the sample solvent mixture was centrifuged for 10 min at 7567·46 ***g***. The supernatant was filtered through nylon filters (Titan filtration system, 17 mm, 0·45 μm, Rockwood, TN, USA) and evaporated to dryness under N_2 flow. The residue was re-dissolved in 300-μl mobile phase and used for gliotoxin analysis by high-performance liquid chromatography (HPLC).

Gliotoxin detection and quantification

Gliotoxin was determined following the methodology proposed by Pena *et al.* (2010). The HPLC apparatus used for gliotoxin determination was a Perkin-Elmer 200 Series HPLC System equipped with autosampler and UV detection. Briefly, gliotoxin separation was performed at room temperature on a Phenomenex Luna RP C18(2) column (150 × 4·6 mm, 5 μm, Phenomenex Inc., Torrance, CA, USA) fitted with a C18 guard column using an isocratic mode: 75% (1% acetic acid in water) and 25% acetonitrile. A column washing of 5 min at 95% of acetonitrile followed by 5 min of stabilization at the running conditions was performed between chromatographic runs. The retention time was properly checked by co-injection technique. Detection was made at 254 nm. The standard solutions of gliotoxin in mobile phase were prepared from a 5 mg ml^{-1} solution of pure gliotoxin (Sigma-Aldrich Co. St Louis, MO, USA) in methanol (stock solution), after solvent evaporation. The stock solution was prepared dissolving pure gliotoxin in the ampoule of the manufacturer with 1 ml of methanol HPLC grade, following the manufacturer's instructions. A calibration curve was obtained injecting 20 μl of gliotoxin standards of 3–50 μg ml^{-1} in mobile phase (75 : 25) in triplicate. Good linearity with a correlation coefficient higher than 0·997 was obtained for the calibration range. The detection limit determined as a rate s/n = 3 was 0·2 μg g^{-1}, and the limit of quantification, as a rate s/n = 7, was 0·6 μg g^{-1}, where s means signal (intensity of the toxin peak) and n means signal noise.

PCR-RFLPs

Previous to the *in situ* PCR-RFLP, an *in silico* assay with *BccI*, *MspI* and *Sau3AI* restriction enzymes was carried out through a NEBcutter V2.0 (http://tools.neb.com/NEBcutter2/) online tool. Before generating restriction maps, *benA* sequences (GenBank) from *A. fumigatus*, *Neosartorya udagawae* and *A. lentulus* were aligned using the ClustalW algorithm within BioEdit v7.0.9.0 software (Thompson *et al.* 1994) (http://www.mbio.ncsu.edu/RNasaP/info/programs/BIOEDIT/bioedit.html). The expected fragment sizes obtained by *in silico* restriction with *BccI*, *MspI* and *Sau3AI* of the 492-, 493- and 494-bp *benA* region of *A. fumigatus* FC025, *N. udagawae* GM02/58 and *A. lentulus* GM02/57, respectively, are shown in Table 3. Those restriction enzymes were chosen because they produce different band patterns that allow distinguish these closely related species into *Aspergillus* section *Fumigati*.

In situ PCR-RFLP

Fungal biomass production. From each *A. fumigatus* strain grown on MEA, spores were harvested and used to inoculate Erlenmeyer flasks of 250 ml containing 50 ml of sterile Wickerham medium (Mulé *et al.* 2006). These flasks were incubated on an orbital shaker (150 rev min^{-1}) at 25 ± 1°C for 2–3 days. Mycelia were harvested by filtration through nongauze milk filters (Ken AG, Ashland, OH, USA) under negative pressure created by vacuum and washed with sterile distilled water. The excess water was removed by blotting mycelia between clean paper towels, and finally, dried mycelia were frozen at −20°C until ground. The time between mycelium storage and fungal DNA extraction did not exceed 7 days.

Table 3 Expected sizes of the DNA fragments generated with *BccI*, *MspI* and *Sau3 AI* restriction enzymes over *benA* gene.

		Fragment sizes (bp)		
		Restriction enzyme		
GenBank accession number	Species	*BccI*	*MspI*	*Sau3 AI*
HQ588166.1	*Aspergillus fumigatus*	99, 144, 249	35, 457	57, 70, 86, 222
HQ127276.1	*Neosartorya udagawae*	39, 46, 60, 346	24, 35,432	57, 70, 85, 279
HQ127275.1	*Aspergillus lentulus*	39, 105, 348	24, 35,433	70, 144, 278

Genomic DNA extraction. For DNA extraction of each *A. fumigatus* strain, 200–300 mg of mycelium was transferred to 2-ml microtubes containing 200 mg of glass beads (425–600 mm diameter, Sigma-Aldrich®, St Louis, MO, USA), 600 μl of extraction buffer (Tris–HCl 100 mmol l^{-1} pH 8, EDTA 20 mmol l^{-1} pH 8, NaCl 1·4 mol l^{-1}), 50 μg ml^{-1} proteinase K (Sigma-Aldrich®) and 7 μl of β-mercaptoethanol. After that, the microtubes were vortexed 3 min and incubated in a water bath at 50°C for 30 min. Cetyltrimethylammonium bromide (CTAB) was added at 2% of final concentration, followed by incubation at 65°C for 30 min. After this period, the microtubes were centrifuged at 12396 ***g*** for 10 min, and the supernatant was transferred to a new microtube. For deproteinization, the same volume of phenol/chloroform (1 : 1) was added, followed by 2 min of homogenization and 10 min of centrifugation at 12396 ***g***. The upper phase was transferred into a new microtube, and the same volume of chloroform/isoamylalcohol (24 : 1) was added, and then, the same steps of homogenization and centrifugation described above were applied. Finally, for DNA precipitation, the upper phase was transferred to a new microtube and ammonium acetate was added to final concentration of 2·5 mol l^{-1} and equal volume of cold (−20°C) isopropanol, followed by incubation at 20°C for 2 h. Subsequently, the microtubes were centrifuged at 12396 ***g*** for 30 min, the supernatant was discarded and the pellet was washed with 70% ethanol. After drying at room temperature in a laminar flow hood, the pellets were resuspended in 50 μl of ultrapure water and stored at −20°C.

To quantify the total DNA extracted, the samples were subjected to electrophoresis on 0·8% agarose gel stained with ethidium bromide (0·5 μg ml^{-1}). After the run, the gel was visualized under UV transilluminator and images were scanned in a photo documentation system (Gel Doc EQ™ BIO-RAD Laboratories®, Hercules, CA, USA). The estimation of the DNA concentration was made by comparison with bands intensity of the DNA marker lambda (λ) (Promega®, Madison, WI, USA) and quality determined by the absence of trace along the gel.

PCR-RFLP assay. Amplification of *benA* was performed using primers βtub1 (5′-AATTGGTGCCGCTTTCTGG-3′) and βtub2 (5′-AGTTGTCGGGACGGAATAG-3′) (Staab *et al.* 2009). PCRs were made in a final volume of 25 μl, containing DreamTaq buffer 1× with $MgCl_2$ (at final concentration of 2 mmol l^{-1}), 0·2 mmol l^{-1} of each dNTP, 0·4 mmol l^{-1} (each) primer, 1 U of DreamTaq DNA polymerase (Thermo Fischer Scientific Inc.) and 10 ng of total DNA. The amplifications were conducted according to the following cyclic conditions: initial denaturation at 94°C for 2 min, followed by 30 cycles consisting of 94°C for 30 s, 56°C for 30 s and 72°C for 45 s and a final extension step of 72°C for 5 min and then held at 4°C indefinitely. The amplification of *benA* was verified by agarose (1·5%) gel electrophoresis of a portion (5 μl) of each reaction mixture with 1X Tris–acetic acid EDTA buffer (pH 8). Digestion of the *benA* amplicons with *BccI*, *MspI* and *Sau3AI* (Promega®) was performed in a final volume of 15 μl containing 10 μl of the PCR product, 1× buffer specific for each enzyme, 0·1 mg μl^{-1} bovine serum albumin (BSA) and 0·5 U of restriction enzyme. The reactions were incubated at 37°C for 2 h in a thermomixer (Eppendorf®, Hamburg, Germany). DNA fragments were visualized after electrophoretic run on agarose and polyacrylamide gels of 3 and 12%, respectively, and stained in a solution of 0·5 μg ml^{-1} ethidium bromide. The fragment sizes were measured by comparison with GeneRuler 50-bp DNA marker (Fermentas®).

Sequencing of *benA* regions of representative strains evaluated by PCR-RFLPs was carried out to definitively confirm its taxonomic state at specie level. Amplifications were performed according to Samson *et al.* (2004). The PCR containing Bt2a and Bt2b primers (Glass and Donaldson, 1995) was set up in a 50-μl reaction mixture containing 2·5 μl of genomic DNA (5 ng μl^{-1}), 10 μl of colourless reaction buffer 5× with $MgCl_2$ (1·5 mmol l^{-1}) (provided with GoTaq®, Promega Corporation, Fitchburg, WI, USA), 28·25 μl of ultrapure sterile water, 5 μl dNTPs (0·2 mmol l^{-1}), 2 μl of each primer (10 pmol μl^{-1}) and 0·25 μl GoTaq DNA polymerase (5 U μl^{-1}, Promega Corporation). Amplification was performed in a MJ Research PTC-200 thermocycler (GMI Inc. Minnesota, MN, USA), programmed for 5 min at 94°C followed by 35 cycles of 1-min denaturation at 94°C followed by primer annealing 1 min at 58°C and primer extension 1 min at 72°C and a final 7-min elongation step at 72°C. PCR products (5 μl) were detected after electrophoretic run on 1·5% agarose gels stained with ethidium bromide (0·5 μg ml^{-1}). The DNA 100-bp ladder (New England Biolabs, Inc., Ipswich, MA, USA) was used as molecular size marker. For DNA sequencing, template DNA (45 μl) was directly prepared from PCR products by purifying it with a commercial kit (DNA Wizard DNA Clean-Up kit, Promega) according to the manufacturer's instructions and sequenced by Applied Biosystems Sanger Sequencing Chemistry with the ABI/Hitachi Genetic Analyzer 3130 sequencer.

Statistics

Data analysis of gliotoxin production by clinical and environmental *A. fumigatus* strains was performed by analysis of variance (ANOVA). Means were compared by Fisher's LSD test to determine the influence of the source of isolation on gliotoxin levels produced by these studied *A. fumigatus* strains. The analyses were conducted using PROC GLM in SAS (SAS Institute, Cary, NC, USA).

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Conflicts of interest

No conflict of interest declared

References

Balajee, S.A., Weaver, M., Imhof, A., Gribskov, J. and Marr, K.A. (2004) *Aspergillus fumigatus* variant with decreased susceptibility to multiple antifungals. *Antimicrob Agents Chemother* **48**, 1197–1203.

Bauer, J., Gareis, M., Bott, A. and Gedek, B. (1989) Isolation of a mycotoxin (gliotoxin) from a bovine udder infected with *Aspergillus fumigatus*. *J Med Vet Mycol* **27**, 45–50.

Debeaupuis, J.P., Sarfati, J., Chazalet, V. and Latgé, J.P. (1997) Genetic diversity among clinical and environmental isolates of *Aspergillus fumigatus*. *Infect Immun* **65**, 3080–3085.

dos Santos, V.M., Dorner, J.W. and Carreira, F. (2003) Isolation and toxigenicity of *Aspergillus fumigatus* from moldy silage. *Mycopathologia* **156**, 133–138.

Gareis, M. and Wernery, U. (1994) Determination of gliotoxin in samples associated with cases of intoxication in camels. *Mycotoxin Res* **10**, 2–8.

Glass, N.L. and Donaldson, G. (1995) Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Appl Environ Microbiol* **61**, 1323–1330.

Gordon, K., Masotti, R. and Wadell, W. (1993) Tremorgenic encephalopathy: a role of mycotoxins in the production of CNS disease in humans. *Can J Neurol Sci* **20**, 237–239.

Gourreau, J.M., Laval, A. and Badinand, F. (1988) Les aspergilloses des bovins. *Point Vét* **20**, 589–608.

Guarro, J., Gené, J. and Stchigel, A.M. (1999) Developments in fungal taxonomy. *Clin Microbiol Rev* **12**, 454–500.

Hong, S.B., Go, S.J., Shin, H.D., Frisvad, J.C. and Samson, R.A. (2005) Polyphasic taxonomy of *Aspergillus fumigatus* and related species. *Mycologia* **97**, 1316–1329.

Klich, M.A. and Pitt, J.Y. (2002) *A Laboratory Guide to Common Aspergillus* Species and their Teleomorphs. Australia: CSIRO, Division of food Research Sydney, Academic Press.

Kupfahl, C., Michalka, A., Lass-Flörl, C., Fischer, G., Haase, G., Ruppert, T., Geginat, G. and Hof, H. (2008) Gliotoxin production by clinical and environmental *Aspergillus fumigatus* strains. *Int J Med Microbiol* **298**, 319–327.

Land, C.J., Hult, K., Fuchs, R., Hagelberg, S. and Lundstrom, H. (1987) Tremorgenic mycotoxins from *Aspergillus fumigatus* as a possible occupational health problem in sawmills. *Appl Environ Microbiol* **53**, 787–790.

Latgé, J.P. (1999) *Aspergillus fumigatus* and aspergillosis. *Clin Microbiol Rev* **12**, 310–350.

Lewis, R.E., Wiederhold, N.P., Chi, J., Han, X.Y., Komanduri, K.V., Kontoyiannis, D.P. and Prince, R.A. (2005) Detection of gliotoxin in experimental and human aspergillosis. *Infect Immun* **73**, 635–637.

Mulé, G., Susca, A., Logrieco, A., Stea, G. and Visconti, A. (2006) Development of a quantitative real-time PCR assay for the detection of *Aspergillus carbonarius* in grapes. *Int J Food Microbiol* **111**, 28–34.

Nieminen, S.M., Maki-Paakkanen, J., Hirvonen, M.R., Roponen, M. and von Wright, A. (2002) Genotoxicity of gliotoxin, a secondary metabolite of *Aspergillus fumigatus*, in a battery of short-term test systems. *Mutat Res* **520**, 161–170.

Pena, G.A., Pereyra, C.M., Armando, M.R., Chiacchiera, S.M., Magnoli, C.E., Orlando, J.L., Dalcero, A.M., Rosa, C.A.R. *et al.* (2010) *Aspergillus fumigatus* toxicity and gliotoxin levels in feed stuff for domestic animals and pets in Argentina. *Lett Appl Microbiol* **50**, 77–81.

Pepin, G.A. (1988) Udder infections in cattle and goats caused by filamentous fungi. In *Proceedings of the X Congress of the International Society for Human and Animal Mycology*. ed. Torres-Rodriguez, J.M. pp. 313–317. Barcelona: JR Prous Science.

Pereyra, C.M., Alonso, V.A., Rosa, C.A.R., Chiacchiera, S.M., Dalcero, A.M. and Cavaglieri, L.R. (2008) Gliotoxin natural incidence of *Aspergillus fumigatus* isolated from corn silage and ready dairy cattle feed. *World Mycotoxin J* **1**, 457–462.

Reeves, E.P., Messina, C.G.M., Doyle, S. and Kavanagh, K. (2004) Correlation between gliotoxin production and virulence of *Aspergillus fumigatus* in *Galleria mellonella*. *Mycopathologia* **158**, 73–79.

Samson, R.A., Houbraken, J.A.M.P., Kuijpers, A.F.A., Frank, J.M. and Frisvad, J.C. (2004) New ochratoxin A or sclerotium producing species in *Aspergillus* section *Nigri*. *Stud Mycol* **50**, 45–61.

Semighini, P.C., Delmas, G., Park, S., Amstrong, D., Perlin, D. and Goldman, G.H. (2001) New restriction fragment length polymorphism (RFLP) markers for *Aspergillus fumigatus*. *FEMS Immunol Med Microbiol* **31**, 15–19.

Smith, J.M. (1989) *Opportunistic Mycoses of Man and Other Animals*. Wallingford, Oxon: CAB International Mycological Institute.

Staab, J.F., Balajee, S.A. and Marr, K.A. (2009) *Aspergillus* section *Fumigati* typing by PCR-restriction fragment polymorphism. *J Clin Microbiol* **47**, 2079–2083.

Sutton, P., Newcombe, N.R., Waring, P. and Mullbacher, A. (1994) In vivo immunosuppressive activity of gliotoxin, a metabolite produced by human pathogenic fungi. *Infect Immun* **62**, 1192–1198.

Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

Upperman, J.S., Potoka, D.A., Zhang, X.R., Wong, K., Zamora, R. and Ford, H.R. (2003) Mechanism of intestinal derived fungal sepsis by gliotoxin, a fungal metabolite. *J Pediatr Surg* **38**, 966–970.

Varga, J., Vida, Z., Toth, B., Debets, F. and Horie, Y. (2000) Phylogenetic analysis of newly described *Neosartorya* species. *Antonie Van Leeuwenhoek* **77**, 235–239.

Verweij, P.E., Meis, J.F., Sarfati, J., Hoogkamp-Korstanje, J.A., Latgé, J.-P. and Melchers, W.J. (1996) Genotypic characterization of sequential *Aspergillus fumigatus* isolates from patients with cystic fibrosis. *J Clin Microbiol* **34**, 2595–2597.

Waring, P., Kos, F.J. and Müllbacher, A. (1991) Apoptosis or programmed cell death. *Med Res Rev* **11**, 219–236.

Yaguchi, T., Horie, Y., Tanaka, R., Matsuzawa, T., Ito, J. and Nishimura, K. (2007) Molecular phylogenetics of multiple genes on *Aspergillus* section *Fumigati* isolated from clinical specimens in Japan. *Nihon Ishinkin Gakkai Zasshi* **48**, 37–46.