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ORIGINAL ARTICLE

Comparison of toxicogenic and immunosuppressive capacity of *Aspergillus fumigatus* strains isolated from clinical and corn silage samples

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Abstract

Aims: To compare clinical and environmental *Aspergillus fumigatus* strains on their toxicogenic and immunosuppressive capacity.
Methods and Results: A total of 51 strains of *A. fumigatus* isolated from clinical and corn silage samples were assayed. All *A. fumigatus* strains were assayed for gliotoxin production, therefore strains with different gliotoxin capacities and isolated from different sources were selected and assayed for their effects on bovine macrophages and lymphocytes. Spore diffusates (SDs) obtained from all *A. fumigatus* strains were able to inhibit macrophage phagocytosys, regardless of their gliotoxin production capacity. However, most but not all strains were able to inhibit bactericidal activity. SDs from all *A. fumigatus* strains reduced lymphocytes viability. The heat treatment was not always able to inhibit the negative effect on immune cells.
Conclusions: There was no difference between clinical and environmental isolates in their toxicogenic and immunosuppressive capacity. Gliotoxin would not be responsible for the immunosuppressive activity observed by the assayed *A. fumigatus* strains. However, gliotoxin could be present in the SD, together with some other substances.
Significance and Impact of the Study: The results obtained suggest that any environmental strain of *A. fumigatus* is a putative infectious strain. Prevention measures should be applied to control environmental *Aspergillus* conidia.

Introduction

Aspergilli species are saprophytes fungi that commonly grow on decaying plant material. *Aspergillus fumigatus* has been isolated from a wide range of substrates including plants, wood, air, seeds, compost, silage and especially ground. It is also considered an opportunistic pathogen for humans and animals (Kradin and Mark 2008). These fungi produce a large number of secondary metabolites such as fumagillin, fumitoxins, fumigaclavines A and C, fumitremorgins, gliotoxin, trypacidin, pseurotins, helvolic acid, pyripyropens, methyl-sulochrin, verruculogen and fumiquinazolines (Samson *et al.* 2007). Many of these compounds have been shown to be highly toxic (genotoxic, carcinogenic, immunosuppressive, apoptotic) for human and animal health (Khoufache *et al.* 2007). As opportunistic pathogen, *A. fumigatus* has become a prevalent species and may cause diseases such as allergic bronchopulmonary aspergillosis, aspergilloma or invasive aspergillosis depending on the base disease and immune condition of the host (Debeaupuis *et al.* 1997). It is estimated that human being inhale about 200 viable conidia of *A. fumigatus* every day, and people working in barns or silage may be highly exposed (Millner *et al.* 1994). In immunocompetent persons, conidia are removed by innate and acquired immunity mechanisms. However, in transplanted or under cancer therapy patients or persons exposed to an excessive amount of

spores, these mechanisms may not be efficient to remove them.

In ruminants *A. fumigatus* may cause mastitis, but the infection may diffuse from the mammary gland to supramammary lymph nodes and lungs causing respiratory processes, abortion and other clinical conditions (Aller *et al.* 2000). Concerning this pathogeny, *A. fumigatus* can escape from the local host defences by synthesizing toxic metabolites that may inhibit the immune response. Bertout *et al.* (2002) showed that the spores, especially a diffusible substance (SD) released by them, are responsible for phagocytosis inhibition of *A. fumigatus* conidia in alveolar macrophages. Also, they determined that this property is a virulence factor which has some strains. The SD activity may be or not related to the presence of gliotoxin.

Aspergillus fumigatus frequently pollutes silage, hay and cereals intended for animal causing economic losses due to the deterioration and loss their nutritional quality (Boudra and Morgavi 2005; El-Shanawany *et al.* 2005; González Pereyra *et al.* 2008, 2011). Several authors have demonstrated the presence of *A. fumigatus* strains and their mycotoxins in hay, silage, feed and various cereals intended for cattle (Dos Santos *et al.* 2002). Its prevalence was demonstrated in comparison with other toxigenic species present in silage and finished food for consumption by cattle under intensive rearing (González Pereyra *et al.* 2008). In Argentina, a high density of strains of *A. fumigatus* able to produce gliotoxin, fumitremorgins and fumigaclavin B in silage and finished feed consumed by dairy cows was observed (Pereyra *et al.* 2008; Alonso *et al.* 2013).

There remains a question concerning *A. fumigatus* pathogenicity strains as it varies and there are some discrepancies on this point. Some authors have suggested that *A. fumigatus* strains isolated from clinical cases are more virulent than those isolated from the environment (Lewis *et al.* 2005; Kupfahl *et al.* 2008). Testing this difference would be vital for rural workers continuously exposed to large amounts of concentrated *A. fumigatus* spores.

Therefore, the aim of this work was to compare the toxicogenic and immunosuppressive capacity of *A. fumigatus* strains isolated from corn silage with clinical strains ones isolated from human clinical cases.

Materials and methods

Aspergillus fumigatus strains

A total of 51 strains of *A. fumigatus* were assayed. Forty-six were isolated from corn silage intended for dairy cattle, and five of them were isolated from human pulmonary aspergillosis cases.

Aspergillus fumigatus identification

The morphological identification was made according to Samson *et al.* (2007). Identification for each species was performed with different culture media and growth temperature for the observation of microscopic and macroscopic characteristics. Colonies representative of *A. fumigatus* isolated were subcultured on to malt extract agar (MEA). Subsequently, suspensions of conidia of each strains were made in semisolid agar and used to inoculate three equidistant points in the plates with Czapek yeast extract agar CYA (25°C and 37°C), MEA (25°C), oatmeal agar OAT (25°C), yeast extract saccharose agar YES (25°C), creatine saccharose agar CREA (25°C) and Czapek agar CZ (25°C). The plates were incubated for 5–7 days at the indicated temperature.

The *A. fumigatus* strains were deposited in the National University of Río Cuarto, Córdoba, Argentina (RC) Collection Centre.

Gliotoxin production by *Aspergillus fumigatus* strains

All *A. fumigatus* strains were assayed for gliotoxin production. The strains were grown in YES broth (sucrose 40 g, yeast extract 20 g and distilled water to 1000 ml) at 37°C for 7 days. Grown broths were filtered with Whatman filter paper No. 1. The filtrate was extracted twice by stirring for 10 min with the addition of 50 ml of chloroform at 25° C. The chloroform fractions were collected and evaporated to dryness under N_2. The residue was resuspended 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 Waters e2695 with a loop of 20 μl equipped with Waters 2998 detector arrangements diodes and a C18 column Phenomenex Luna (150 × 4·6 mm, 5 mm 4 particle size; Phenomenex, Inc., CA) connected to a protection precolumn (Phenomenex, Inc., Buenos Aires, Argentina). The mobile phase was pumped at 1·5 ml min^{-1} and consisted of 75% (1% acetic acid in water) and 25% acetonitrile. The retention time was 8·5 min. Detection was carried out at 258 nm. The solution standards were prepared by dissolving pure gliotoxin (Sigma-Aldrich Co.) at 5 mg ml^{-1} in chloroform to obtain a working solution of 1 mg ml^{-1} and dryness with N_2. Standard solutions were made from the working solution diluted in mobile phase to obtain concentrations of 1, 5, 15, 25, 50 and 100 ng ml^{-1} and 5, 10, 15 and 20 μg ml^{-1}. Quantification of gliotoxin was performed by

measuring the areas followed by extrapolation to obtain a calibration curve using pure standard solutions of gliotoxin. The instrumental detection limits (LOD) and quantification limits (LOQ) were determined in fungal extracts and standard solutions of gliotoxin based on the signal/noise (S/N) relation of 3 : 1 for LOD and 7 : 1 for LOQ; being determined in 0·1 and 1 ng ml^{-1}, respectively.

Spore diffusate from *Aspergillus fumigatus*

Aspergillus fumigatus spores were obtained from cultures grown for 5 days at 37°C on MEA. Spore diffusates (SDs) were obtained according to Bertout *et al.* (2002). Briefly, spore suspensions were prepared in Hank's balanced salt solution (HBSS, Gibco) by gentle homogenization. Spore counts were performed using an improved Neubauer chamber. To obtain *A. fumigatus* diffusate, the spores were incubated at a concentration of 10^8 ml^{-1} in H BSS at 37°C for 3 h in an orbital incubator. There was no evidence of germination. The spores were removed by centrifugation (4000 ***g***, 10 min), and the diffusate was filtered through a 0·22-μm sterile filter (Millipore) and stored at 4°C. A part of this diffusate was heated (120°C, 15 min) for testing its stability.

Effect of SD on the phagocytic and bactericidal capacity of bovine macrophages

A bovine macrophage cell line (BoMac) was used for this study. Cells were kindly donated by Dr. Silvia Mundo (Facultad de Ciencias Veterinarias, UBA, Argentina) and maintained with RPMI 1640 medium, (Sigma-Aldrich, St. Louis, MO) containing 10% heat-inactivated foetal calf serum (FCS) (Natocor, Córdoba, Argentina), 10 000 U penicillin, 20 μg ml^{-1} streptomycin and 2 mmol l^{-1} glutamine. The cell line was cultured at 37°C in a 5% CO_2 air atmosphere, and the culture media was changed daily.

Phagocytic activity

For the phagocytosis assay, BoMac cells were seeded (1×10^5 cell ml^{-1}; viability <98% by trypan blue exclusion) in immunofluorescence slides and led to adhered for 1 h. The culture medium was removed, and the cells were washed and cultured at 37°C for 1 h with RPMI 1640 medium without FCS or antibiotics plus SD (1 μg ml^{-1}) of each *A. fumigatus* strain. After that, slides were washed three times with sterile PBS, and macrophages were mixed with a yeast suspension at 1×10^6 CFU ml^{-1} in fresh RPMI 1640 medium without FCS or antibiotics. The slides were subsequently incubated for 2 h at 37°C in 5% CO_2 to allow the yeast to be taken by the macrophages. Afterwards, slides were washed three times with sterile PBS to remove the nonphagocytosed yeast. Cells were fixed with methanol for 5 min and then washed three times with sterile PBS, stained with Giemsa and observed under optical microscope. The percentage of phagocytosis was evaluated by counting 200 macrophage cells and differentiating cells with phagocytic activity and without phagocytic activity. The assay was carried out by triplicate. Slides without SDs or with autoclaved SDs were also assayed.

Bactericidal activity

For bactericidal activity, BoMac cells were seeded (1×10^5 cell ml^{-1}, viability <98% by trypan blue exclusion) in 96-well plates. The cells rapidly adhered and spread over the support. The plates were incubated overnight at 37°C in a CO2 (5%) humidified incubator. The culture medium was removed, and the cells were cultured at 37°C for 1 h with RPMI 1640 medium without FCS or antibiotics plus SD (1 μg ml^{-1}) of each *A. fumigatus* strain. After that, the medium was removed, and each well was washed three times with sterile PBS. Macrophages in each well were mixed with 200 μl of fresh RPMI 1640 medium without FCS or antibiotics containing 1×10^7 CFU ml^{-1} of *Salmonella* spp. obtained from calves with enteritis. The plate was subsequently incubated for 3 h at 37°C in 5% CO2 to allow the bacteria to be taken by the macrophages. The medium was removed, and fresh RPMI 1640 medium with penicillin/streptomycin was added to each well for 30 min, after that the wells were washed three times with sterile PBS, and the macrophages were lysed by adding RMPI 1640 medium with 0·5% of Tween 20. To recover the number of *Salmonella* spp. that survived inside the macrophages, 100 ml of each well were taken and decimal dilutions were carried out and spread onto MacConkey agar. The assay was carried out by triplicate. Wells without SDs or with autoclaved SDs were also assayed.

Cytotoxicity effect of SD on bovine lymphocytes

The methodology was based on the ability of viable cells to reduce methyl thiazole tetrazolium salt (MTT, Sigma-Aldrich) to a purple formazan dye that could be quantitated by spectrophotometric means and hence give a measure of the cytotoxicity of SDs. The methodology was carried out according to Mosmann (1983).

Blood (12 ml) was obtained from three healthy cows, belonging to the Camdocex, Facultad de Agronomía y Veterinaria, Universidad Nacional de Río Cuarto, into heparinized tube, overlaid on Histopaque 1077 (Sigma-Aldrich) and centrifuged at 800 ***g*** for 30 min. The interface layer consisting of mononuclear cells was removed using a sterile pipette. The isolated lymphocytes were washed three times with PBS. The washed pelleted cells

were then resuspended in RPMI 1640 medium containing SDs (1 μg ml^{-1}), cultured (1 × 10^6 cell ml^{-1}, viability <98% by trypan blue exclusion) in 96-well plates and incubated at 37 °C in a 5% CO2-buffered and humidified incubator for 12 h. The assay was carried out by triplicate. Wells without SDs or with autoclaved SDs were also assayed.

After that, 20 μl of MTT solution (5 mg ml^{-1} in RPMI 1640) was added to each well, and the microplates were further incubated at 37°C for 4 h. To solubilize the released purple formazan dye, 50 μl of DMSO was added to each well. This was measured at 570 nm on an Elisa plate reader. Data were expressed as mean absorbance value (OD) of triplicate samples + standard error of the mean.

Results

Aspergillus fumigatus identification and gliotoxin production

The gliotoxin production capacity of *A. fumigatus* strains is shown in Table 1. An overview of the toxin levels and production rate is shown in Table 2. Gliotoxin levels produced by strains isolated from corn silages varied from 0·986 to 36222·91 ng kg^{-1}, whereas those obtained from clinical cases ranged from 6·68 to 4430·85 ng kg^{-1}, respectively. For further studies, strains with different gliotoxin capacities isolated from different sources were selected. Therefore, strains RC009; RC010; RC017; RC020; RC391; RC 537; RC 548; RC 621 and RC 676 were assayed for their effects on bovine macrophages.

Table 1 Sources and gliotoxin production of *Aspergillus fumigatus* strains

Source	*A. fumigatus*	Gliotoxin production (ng g^{-1})
Corn silage	RC001	78·58
	RC002	1·50
	RC003	2·03
	RC004	16·73
	RC005	1·47
	RC006	16767·60
	RC007	31876·16
	RC008	19678·10
	RC009	31907·54
	RC010	0·98
	RC011	13446·75
	RC012	34987·67
	RC013	8623·14
	RC014	36222·91
	RC015	24366·96
	RC016	3·04
	RC017	397·35
	RC018	1506·29
	RC019	82·32
	RC020	109·59
	RC021	24·29
	RC022	17·42
	RC023	27·23
	RC025	25·89
	RC026	1212·16
	RC027	68·53
	RC028	279·65
	RC029	290·50
	RC030	100·74
	RC031	8636·55
	RC032	4·56
	RC034	7·89
	RC035	5·68
	RC036	31·32
	RC037	4987·86
	RC038	2·36
	RC039	1·46
	RC040	6540·48
	RC041	1·02
	RC042	1·30
	RC043	522·17
	RC044	1·02
	RC045	1·08
	RC046	1·50
	RC048	225·87
	RC049	1621·71
Clinical cases	RC391	6·68
	RC537	17·87
	RC548	4430·85
	RC621	714·39
	RC676	35·85

Effect of spore diffusate on phagocytic activity of bovine macrophages

The results showed that SDs released from all the tested strains were capable of inhibiting BoMac cells phagocytic activity (Fig. 1). Some of the SDs (SDs from RC009, RC017 and from RC548 strains) lost or diminished significantly their immunosuppressive capacity after heat treatment (autoclaved SDs), increasing their phagocytosis percentage. However, this percentage was always less than that obtained by the control group (macrophages without SDs).

Effect of spore diffusate on bactericidal activity of bovine macrophages

The effects of SDs on the bactericidal activity of macrophages are shown in Fig. 2. The antimicrobial activity was determined by calculating the number of bacteria (*Salmonella* spp.) able to survive intracellular killing. According to that, the count of CFU per ml of *Salmonella* spp. intracellular was performed after macrophages lysis, which corresponds to the remaining viable bacteria into cells that were not destroyed by the BoMac cells.

Table 2 Gliotoxin levels according to the source of the isolates

Source	Gliotoxin production (ng kg^{-1})	
	Range	Mean
Corn silage	0·986–36222·91	14270·86
Clinical cases	6·68–1506·29	1041·13

Most of the SDs (SD from RC017, RC020, RC391, RC537, RC548, RC621 and from RC676 strains) were able to reduce the bactericidal activity of macrophages, compared with the control group. The heat treatment (SDs autoclaved) caused the inhibition of this reduced macrophage activity on some of the SDs tested, showing an increase in the intracellular *Salmonella* sp. counts that survived the bactericidal activity of macrophages.

Cytotoxicity effect of spore diffusate on bovine lymphocytes

The MTT assay is a cell viability assay often used to determine the cytotoxicity following exposure to toxic substances. Cytotoxicity effects of SDs of *A. fumigatus* strains are shown in Fig. 3. The results demonstrated that the SDs from all strains assayed were able to affect the viability of bovine lymphocytes. The heat treatment (autoclaved SDs) inhibited this cytotoxic effect.

Discussion

The present work was carried out to compare the gliotoxin production and immunosuppressive capacity of *A. fumigatus* strains isolated from corn silage and human clinical cases. *Aspergillus fumigatus* produces a number of toxins being gliotoxin, a hydrophobic metabolite that belongs to the class of epipolythiodioxopiperazine compounds, the most abundant (Waring and Beaver 1996). Gliotoxin exhibits diverse biological activities on the immune system. In murine models of invasive aspergillosis, gliotoxin was shown to inhibit macrophage and polymorphonuclear cell function, including phagocytosis and bactericidal activity (Watanabe *et al.* 2003; Coméra *et al.* 2007). Other important functions of the host immune defence are also impaired by gliotoxin, including induction of cytotoxic and alloreactive T cells (Yamada *et al.* 2000). It was previously reported that *A. fumigatus* spores release a diffusible

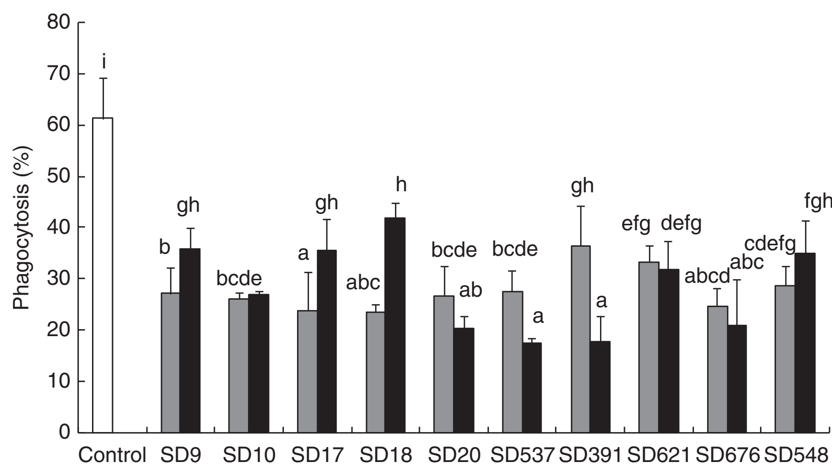

Figure 1 Phagocytic activity of BoMac cells. Values represent the percentage of phagocytosis of BoMac untreated cells (control) or treated with SDs or autoclaved SDs. Values are means of $n = 3 \pm$ SD (standard deviation). Different letters mean significant differences ($P < 0{\cdot}05$). (▤) SDs and (■) Autoclaved SDs.

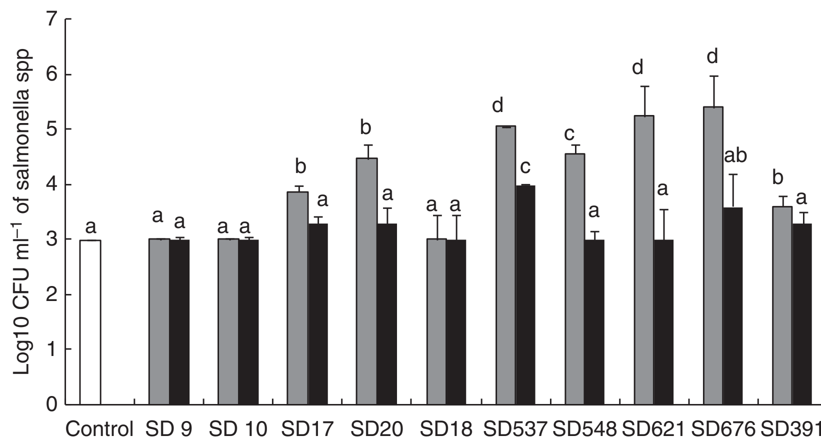

Figure 2 Microbicidal activity of BoMac cells.Results are expressed as $\log_{10}$ CFU per ml of intracellular *Salmonella* spp. Values are means of $n = 3 \pm$ SD (standard deviation). Different letters mean significant differences ($P < 0{\cdot}05$). (▤) SDs and (■) Autoclaved SDs.

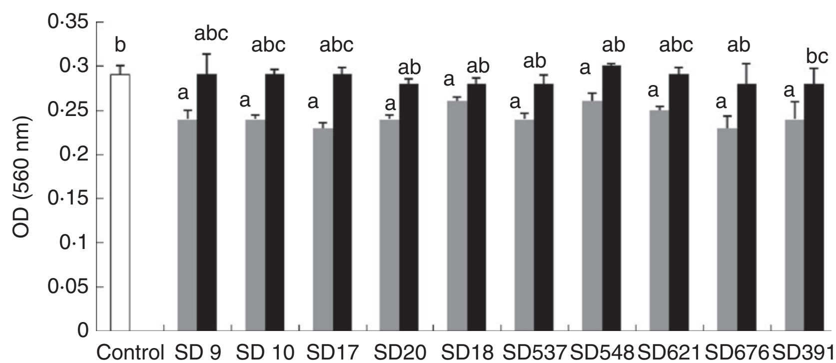

Figure 3 Cytotoxicity activity of SDs on bovine lymphocytes.Results are expressed as OD (560 nm). Values are means of $n = 5 \pm$ SD (standard deviation). Different letters mean significant differences ($P < 0{\cdot}05$). (▒) SDs and (■) Autoclaved SDs.

substance that inhibits certain functions of immune phagocytic cells (Hobson 2000; Bertout *et al.* 2002).

In this work, the effect of SD released from *A. fumigatus* spores (from two different origins) on bovine macrophages was investigated. To compare the negative effects on immune cells with the gliotoxin production capacity, strains with different gliotoxin levels production were selected and evaluated as inhibitors of phagocytic and bactericidal macrophage activity. All SDs from *A. fumigatus* strains were able to inhibit macrophage phagocytosys, regardless of their gliotoxin production capacity. However, when bactericidal activity was tested, the results showed that most, but not all strains, were able to inhibit this macrophage activity, suggesting that there may be more than one inhibitor or that the inhibitor is pleiotropic, affecting more than one macrophage activity. The heat treatment also supports the idea that this immunosuppressive activity is may be due to more than one substance, as some but not all of the autoclaved SDs lost their immunosuppressive capacity. When cytotoxicity activity was evaluated, it was shown that SDs from all *A. fumigatus* strains reduced bovine lymphocytes viability. However, autoclaved SDs did not show this effect. Some authors have suggested that SD with immunosuppressive ability is distinct from gliotoxin and other hyphal toxins (Slight *et al.* 1996; Mitchell *et al.* 1997). In addition, Bertout *et al.* (2002) demonstrated that not all diffusible substances obtained from the strains of *A. fumigatus* were able to inhibit phagocytosis, In the present work, the negative effects on immune cells of SDs from *A. fumigatus* strains, were not related to the gliotoxin production, as highly gliotoxin producer strains (SD from RC009) exhibited similar immunosuppressive activity to low or moderate gliotoxin producer ones (SDs from RC010 and from RC676). On the contrary, Kupfahl *et al.* (2008) showed culture supernatants of *A. fumigatus* strains lacking gliotoxin production with a significantly lower cytotoxicity on macrophage-like cells and T cells.

In the present work, no difference was observed in the gliotoxin production among clinical and environmental isolates. Although the gliotoxin production was assayed for a low number of clinical strains, other researchers found similar gliotoxin production to ours among clinical and environmental strains (Soleiro *et al.*, 2013). In contrast, Kosalec and Pepeljnjak (2005) found that gliotoxin production was detected only among clinical isolates of *A. fumigatus.* This fact could be related to genetic variability between the strains from different origins (Duarte-Escalante *et al.* 2009).

In conclusion, gliotoxin would not be responsible for the negative effect on the immune cells observed by the assayed *A. fumigatus* strains. This fact does not rule out the possibility that gliotoxin is present in the SD, together with some other substances. The immune inhibitor present in the SD could be more than one substance; some of them thermosensitive. Further studies are required to characterize SDs in all their constituents.

The significance of this study lies on the observation that there was no difference between clinical and environmental isolates in their toxicogenic and immunosuppressive capacity, suggesting that any environmental strain of *A. fumigatus* is a putative infectious strain. Prevention measures should be applied to any environmental *Aspergillus* conidia.

Acknowledgements

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Conflict of Interest

No conflict of interest has been declared.

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