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Use of a microbial toxicity test (Microtox®) to determine the toxigenicity of *Aspergillus fumigatus* strains isolated from different sources

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ABSTRACT

The toxic activity of *Aspergillus fumigatus* is attributable to substances secreted by its cells. Specific toxic compounds synthesized by the fungi such as gliotoxin, can be detected by sensitive chemical procedures like TLC or HPLC. Measuring the total toxigenicity of a strain extract, however, requires a bioassay. In the present study, we evaluated the possibility of using the Microtox® bioassay to determine the toxigenicity of *A. fumigatus*, using 32 strains from different sources. The Microtox® method is based on the ability of *Vibrio fischeri* to produce luminescence, and their sensitivity to toxins. *A. fumigatus* strains, grouped according to their original sources, showed differences in toxigenicity. Strains isolated from invasive aspergillosis patients proved to be more toxigenic than environmental strains, or strains from colonized patients. Since the strains that were more toxigenic were isolated from sick patients, it is not surprising they showed more virulence than the other strains, and as expected, virulence could be correlated with high toxigenicity. The Microtox bioassay could be a useful tool in the study of toxigenicity of the mycelial fungi and their possible pathogenic roles, and for rapid assessment of secreted toxic compounds.

1. Introduction

Aspergillus fumigatus is a ubiquitous mycelial fungus that can cause various infections in human and animals, among which invasive aspergillosis (IA) is the most important because of a high mortality rate in immunosuppressed patients (Latgé, 1999; Lumbreras and Gavaldà, 2003). Pathogenicity factors involved in IA development are not completely defined (Latgé, 1999; Tomee and Kauffman, 2000), although hypotheses have been proposed about the role of mycotoxin production in the establishment of illness (Sutton et al., 1996; Nieminen et al., 2002; Watanabe et al., 2004b; Kamei and Watanabe, 2005; Sugui et al., 2007).

Although gliotoxin could be the most important mycotoxin produced by *A. fumigatus* because of its immunosuppressing activity (Murayama et al., 1996; Watanabe et al., 2003), its effect is known to be reinforced by other secondary metabolites, most of which are not yet characterized (Watanabe et al., 2003). Therefore, fungal toxic activity must be viewed in the context of all the secreted substances that are present in the substrate where the fungus grows (Rementería et al., 2006). Sensitive chemical procedures, like TLC or HPLC, can detect specific toxic compounds. If every toxic compound is not known, however, bioassays are more informative.

Traditional acute toxicity bioassays using multi-cellular organisms are time-consuming, requiring lengthy exposure periods of up to 96 h. In contrast, microbial toxicity tests, such as the Microtox® Test, take no more than 45 min to conduct, and can measure temporal changes in bioavailability over relatively short time frames (Kaiser and

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Palabrica, 1991). The Microtox® system is based on the capacity of *Vibrio fischeri* to produce luminescence, and their sensitivity to different toxic products, such as mycotoxins.

In this study, we designed a method to measure fungal toxigenicity by monitoring the toxicity of culture medium in which fungi were growing. The culture filtrate toxicity was analyzed for possible virulence. To our knowledge, this is the first application of the Microtox® system to the study of fungal toxigenicity.

2. Materials and methods

2.1. Fungal strains

Thirty-two isolates of *A. fumigatus* from different sources were used. Twelve were from invasive aspergillosis (IA) patients, four from aspergilloma (A) patients, seven from environmental sources (EA) and nine from colonized (C) patients.

2.2. Culture filtrates

Each strain was cultured on Sabouraud-Dextrose agar (BioMerieux, Marcy l'Etoile, France) slants at 37 °C for seven days to ensure optimal sporulation. PBS was added to the tube, and mixed. The conidia suspension was recovered with a 10 ml sterile syringe and transferred to a sterile tube, where it was homogenized. Conidia were counted in a Thoma chamber. Erlenmeyer flasks with 100 ml Czapeck–Dox liquid medium were inoculated with 10^3 conidia and incubated at 37 °C with shaking in the dark. After four days of culture, medium was filtered through filter paper no. 240 (Labcenter, Madrid, Spain), separating the fungal mycelium from the liquid medium. The culture filtrate was then processed through a 0.22 μm filter (Labcenter).

2.3. Dry fungi mycelium weight

The mycelium was inactivated with 0.05% formalin and kept at 37 °C for 48 h. After this period, the mycelium was weighed and the dry mycelium weight (DMW) calculated for each strain.

2.4. Toxicity test

The Microtox® Basic Test was carried out using the following protocol from the manufacturer (Microbics Corporation, Carlsbad, USA). Briefly, a range of culture filtrate dilutions from 45% to 0.56% was made in solvent supplied by the manufacturer. For the reactions, freeze-dried *V. fischeri* were reconstituted with 0.01% sodium chloride and 10 μl was mixed with 500 μl of each culture filtrate dilution. A Microtox® Model 500 Analyser (AZUR Environmental, Carlsbad, CA, USA) was used to measure the luminosity from the reconstituted bacteria after 5 and 15 min of exposure to culture filtrate. The luminescence inhibition after a 15 min exposure was taken as the endpoint (Kaiser, 1998; Froehner et al., 2000). A 2% sodium chloride solution was used for bacterial regeneration, sample dilution and control. The osmotic control was made using OAS (Osmotic Adjusting Solution) of 22% sodium chloride.

In the Microtox® test, the inhibition of light emission was measured in relative units of luminescence. The data were used to calculate the EC_{50}, which is the mean sample concentration that causes a 50% reduction in bacteria bioluminescence (Chen and Que Hee, 1995; Guzzella et al., 1996). The behaviour of the bacteria was tested with reference toxins $ZnSO_4 \cdot 7H_2O$ and phenol, according to normative AFNOR T90-320 (AFNOR, 1991). Toxicity values are the average of four replicates of each filtrate sample, expressed as EC_{50}-15 min with 95% confidence limits. To simplify the comparison among the mean effective concentrations obtained for each of the selected *A. fumigatus* sets, values correspond to 100 mg of DMW. Although the filtrate samples contain a mixture of different toxins, to simplify the results, the values from the Microtox® test are expressed as ppb gliotoxin, obtained by comparison with the values obtained in a calibration curve for gliotoxin.

2.5. Calibration curve for gliotoxin

Stock solutions of Gliotoxin (Sigma–Aldrich Chemical Co., St. Louis, MO, USA) were prepared by dissolving pure mycotoxin in distilled water, to a final concentration of 5 mg/ml. Serial dilutions of 0.12-, 0.25-, 0.5-, 1-, 2-, 4-, 8-, 16-, and 32-μg/ml were made in the same solvent used for the mother solution. Each gliotoxin dilution was tested by the Microtox® Basic Test, according to the protocol above. Toxicity values were expressed as the percentage corresponding to each gliotoxin sample in the test medium (volume/volume %, v/v).

2.6. Statistical procedures

The inhibition effect was expressed as EC_{50} estimates with 95% confidence limits (CL 95%), determined as described by Litchfield and Wilcoxon (1949) using the Pharmacologic Calculation System (PCS version 4.0, NY, USA). The EC_{50} estimates were subjected to two-way analysis of variance (ANOVA) with replication within the subgroups followed by post-hoc contrast with the Bonferroni test. Differences were considered to be significant at a probability level of $P < 0.05$.

3. Results

3.1. Gliotoxin toxicity

Microtox® bioassays performed with a dilution series of pure gliotoxin are shown in Fig. 1. Previous results obtained at time exposures of 5 and 15 min showed that fluorescence reduction was greater at 15 min than at 5 min. Thus, the concentration–response experiments indicated that Microtox® test was sensitive to gliotoxin in both a concentration- and time-dependent manner. The EC_{50} value and corresponding confidence limit for pure gliotoxin were 0.36 (0.35−0.37) μg/ml.