

## *In vitro* testing of fungicidal activity of biocides against *Aspergillus fumigatus*

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The activity of biocides against *Aspergillus fumigatus* is unknown. In the European guidelines to evaluate the fungicidal activity of a biocide, the critical step concerning the preparation of conidial suspensions is cumbersome and time-consuming. The aims of this study were to evaluate a simplified procedure to prepare conidial suspensions to test a biocide in comparison with the recommended one and to investigate the *in vitro* activity of seven biocides by the suspension–neutralization method against *A. fumigatus* clinical isolates. The proposed simplified procedure proved reproducible, gave the same results and was quicker than that described in the European guidelines. Benzalkonium chloride (0.25%), glutaraldehyde (1.6%), polyvinylpyrrolidone iodine (1% available iodine) and polyester glycol iodine (0.18% available iodine) showed biocidal activity in ≤5 min contact time, and chlorine (0.14% available Cl) and chlorhexidine (0.06%) after 15 min and 30–60 min, respectively. In contrast, chloramine-T (0.01%) did not show biocidal activity. In addition, a simplified and reproducible procedure may be used for testing the fungicidal activity of new compounds or combined formulations. In conclusion, the biocides tested, which are commonly used in hospital settings, were shown to display biocidal activity against *A. fumigatus* and a simplified procedure may be adopted for testing the fungicidal activity of new compounds.

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## INTRODUCTION

*Aspergillus fumigatus* is a filamentous fungus that causes life-threatening invasive infections in immunocompromised patients. Soil is the natural ecological niche from which aerosols of conidia are released. Infection is usually acquired by inhalation of conidia. A rise in the concentration of airborne conidia, as occurs during renovation and construction of buildings, increases the risk of aspergillosis in susceptible individuals. Hospital and municipal water, inadequately sterilized nebulizers, ice-making machines, ornamental plants and flower arrangements may also be sources of aspergilli. In addition, contamination of food such as cereals, powdered milk, pepper, tea, chocolate and vegetables has been linked with infection in neutropenic patients (Bouakline *et al.*, 2000; Hajjeh & Warnock, 2001; Manuel & Kibbler, 1998; Nolard *et al.*, 1988; Pottecher, 2000).

Biocides have an essential role in the control and prevention of nosocomial infections. In addition, they are extensively used in the home environment of neutropenic patients. Biocides are reported to be less active against yeasts and considerably less active against filamentous fungi than

against non-sporulating bacteria (McDonnell & Russell, 1999; Russell & Furr, 1996; Russell *et al.*, 1997; Russell, 2003).

Several procedures have been proposed for evaluating the fungicidal activity of biocides (Association Française de Normalisation, 1987a, b). The most widely used is the suspension–neutralization method: a fixed volume of the biocide solution is mixed with the microbial suspension and, after a defined contact time, the product is neutralized and microbiocidal activity is evaluated. In 1997, the European recommendations concerning this method were published (European Standard, 1997). The critical step in these procedures is the preparation of conidial test suspensions, which is particularly cumbersome and time-consuming.

To establish the fungicidal activity of a product, *in vitro* testing of biocides against only a limited number of fungi (*Absidia corymbifera*, *Cladosporium cladosporioides*, *Penicillium verrucosum* and *Candida albicans* strains, or only *C. albicans* and *Aspergillus niger* strains) is required (Association Française de Normalisation, 1987a, b; European Standard, 1997). It is worth noting that knowledge of the activity of biocides against *A. fumigatus*, the most frequent pathogenic species, is quite poor.

The objectives of the present study, performed within the

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European Research Group on Biotypes and Genotypes of *Aspergillus* (EBGA) Network, were to: (i) evaluate a modification of the procedure reported in the European recommendations to test the activity of a biocide; and (ii) test the fungicidal activity of biocides against *A. fumigatus* clinical isolates.

## METHODS

**Isolates.** *A. fumigatus* isolates were obtained from the EBGA/IHEM culture collection. The isolates were collected during the EBGA project from patients from different European countries affected by invasive aspergillosis or bronchopulmonary colonization. Three isolates were used to evaluate the simplified procedure and 15 to test the fungicidal activity of biocides.

**Preparation of conidial test suspensions.** Conidial suspensions were prepared starting from a 5 day culture of *A. fumigatus* on malt agar, according to the European Standard (1997). Briefly, conidia were detached from the culture surface using a glass spatula and transferred into 10 ml sterile distilled water containing 0.05% (w/v) Tween 80 in a flask containing glass beads (3–4 mm diameter). After shaking for 1 min, the suspension was filtered through a filter (40–100 µm porosity). If mycelium was seen at microscopic examination, the filtered suspension was centrifuged at 2000 g for 20 min. The conidia were washed at least twice by resuspension and centrifugation and the number was adjusted to  $1.5-5 \times 10^7$  ml<sup>-1</sup>.

The simplified procedure to prepare the conidial suspension consisted of flooding the fungal colonies on the agar surface with 8–10 ml 0.05% Tween 80 in distilled water without touching the growth with the tip of the pipette. The wash was transferred into a tube and the number of conidia was adjusted to  $1.5-5 \times 10^7$  ml<sup>-1</sup>.

The numbers of c.f.u. obtained from plating conidial suspensions of three *A. fumigatus* clinical isolates prepared in triplicate on Sabouraud glucose following the two procedures were compared using the Student's *t* test. In addition, the activity of one biocide, chlorhexidine, against three *A. fumigatus* isolates tested with the two different preparations of conidial suspensions was compared.

In the following tests, the conidial suspensions obtained with the simplified procedure were used.

**Biocides.** The antifungal activity of seven biocides was studied using the following commercial preparations: benzalkonium chloride (Neomedil; Farmec), chloramine-T (Euclorina; SmithKline Beecham), chlorhexidine plus cetrime (Farvicet forte; Farmec), chlorine (Amuchina; Amuchina), glutaraldehyde (Cidex; Johnson & Johnson Medical), polyester glycol iodine (Eso-jod 25; Esoform) and polyvinylpyrrolidone iodine (Eso-jod 100; Esoform).

**Determination of antifungal activity of biocides.** The activity of the biocides was tested three times with the suspension–neutralization method against 15 *A. fumigatus* isolates. Briefly, 1 ml distilled water and 1 ml fungal test suspension were added to 8 ml biocide. The concentrations of the tested biocides, except for glutaraldehyde, were those recommended by the manufacturers: benzalkonium chloride, 0.25%; polyvinylpyrrolidone iodine, 1% available iodine; polyester glycol iodine, 0.18% available iodine; chlorine, 0.14% available chlorine; chlorhexidine, 0.06%; and chloramine-T, 0.01%. Glutaraldehyde, available at a concentration ready to be used (2%), was used in the test at a concentration of 1.6% as a result of the dilutions in the method.

Biocidal activity was determined for contact times of 5, 15, 30 and 60 min at room temperature. At the end of each contact time, 1 ml test mixture was added to 8 ml neutralizing compound and 1 ml distilled water.

A neutralizer containing a combination of 3% Tween 80, 0.3% lecithin, 0.1% histidine and 3% saponin was used for all of the tested biocides except for glutaraldehyde and polyvinylpyrrolidone iodine, for which 2% glycine and 0.5% sodium thiosulphate were employed, respectively. The neutralizers were demonstrated to be effective and non-toxic to the fungal cells in preliminary tests.

After a neutralization time of 5 min, 1 ml neutralized mixture, in duplicate, was transferred into 15 ml melted Sabouraud glucose agar and plated. Plates were incubated at 32 °C. After 48 h incubation, c.f.u. were counted and the number of c.f.u. ml<sup>-1</sup> in the test mixture ( $N_a$ ) was determined as the ratio of the mean of the c.f.u. on both plates and the dilution factor ( $10^{-1}$ ).

The fungal test suspension was diluted to final concentrations of  $1.5-5 \times 10^2$  and  $1.5-5 \times 10^1$ , and 1 ml suspension, in duplicate, was then transferred into 15 ml melted Sabouraud agar and plated. After 48 h incubation, c.f.u. were counted and the number of c.f.u. ml<sup>-1</sup> in the test suspension ( $N$ ) was determined using the formula  $N = c / (n_1 + 0.1n_2) \times d$ , where  $c$  was the sum of the c.f.u. on all four plates,  $n_1$  and  $n_2$  were the number of plates considered at the first and the second dilution, respectively, and  $d$  was the dilution factor corresponding to the first dilution considered ( $10^{-5}$ ).

For each contact time, the reduction in viability was calculated as the ratio of  $N \times 10^{-1}$  to  $N_a$ . If the reduction in viability was  $\geq 10^4$ , the product was deemed fungicidal after the selected contact time.

## RESULTS AND DISCUSSION

Conidial suspensions prepared according to both the European standard and the proposed simplified procedure did not show any difference in the number of c.f.u. (Table 1). Both procedures proved reproducible in three different tests. This meant that the conidia were well scattered. In addition, the proposed procedure was shown to give the same results as the standard one in testing the biocidal activity of chlorhex-

**Table 1.** c.f.u. obtained from conidial suspensions prepared according to European recommendations and to a simplified procedure

Values are the mean ( $\pm$  SD).

Isolate	European procedure	Simplified procedure	<i>P</i> value
IHEM 1363	35.00 $\pm$ 5.00	33.67 $\pm$ 3.21	0.717
IHEM 9417	58.33 $\pm$ 5.03	61.00 $\pm$ 4.58	0.535
IHEM 13936	33.00 $\pm$ 7.94	30.33 $\pm$ 8.02	0.96

**Table 2.** Antifungal activity of biocides against 15 *A. fumigatus* clinical isolates

Biocide	No. of strains killed after different contact times			
	5 min	15 min	30 min	60 min
Benzalkonium chloride	15			
Glutaraldehyde	15			
Polyvinylpyrrolidone iodine	15			
Polyester glycol iodine	15			
Chlorine	0	15		
Chlorhexidine	0	0	3	12
Chloramine-T	0	0	0	0

idine against three *A. fumigatus* isolates. Furthermore, the proposed procedure was quicker than that described in the European guidelines. A limitation of *in vitro* methods is that they test activity of biocides against conidia and not against hyphae, which might be present in some substrates. However, evaluation of the biocidal activity as a reduction in viability requires a well-scattered inoculum, which cannot be achieved using hyphae. In addition, the *in vitro* method does not discriminate the biocidal activity against metabolically active conidia from that against dormant conidia. The latter, being dehydrated, could be more resistant to chemical compounds.

The activity of the seven tested biocides is shown in Table 2. Benzalkonium chloride, glutaraldehyde, polyvinylpyrrolidone iodine and polyester glycol iodine caused a  $10^4$  or more reduction in viability of *A. fumigatus* strains in less than 5 min contact time. Chlorine caused a similar reduction in viability within 15 min, whereas chlorhexidine proved fungicidal in 30–60 min contact time. In contrast, chloramine-T did not demonstrate cidal activity after 60 min.

In conclusion, with the exception of chloramine-T, the tested biocides, widely used in hospital settings, were shown to display a high fungicidal activity against *A. fumigatus* isolates. These data offer a scientific basis for recommendations to outpatients at risk of aspergillosis to clean their homes and eliminate the main indoor sources of aspergilli. In addition, a simplified and reproducible procedure may be adopted for testing the fungicidal activity of new compounds or combined formulations.

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