A fast, practical and reproducible procedure for the standardization of the cell density of an *Aspergillus* suspension

Ricardo Araujo,¹,² Acacio G. Rodrigues¹,² and Cidalia Pina-Vaz¹,²

¹Department of Microbiology, Faculty of Medicine, University of Porto, 4200-319 Porto, Portugal
²IPATIMUP, University of Porto, Portugal

The progressive increase of invasive disease and reports of resistance among *Aspergillus* species emphasizes the need for reproducible antifungal susceptibility testing. Inoculum standardization is a crucial step in such procedures. The objective of this study was to develop a fast and precise method of evaluating the cell density of an *Aspergillus* spore suspension, as an alternative to spectrometric readings or cell-counting with a haemocytometer. Densimat (bioMérieux) is a portable photometer that shows a good correlation with spectrometric readings and can advantageously replace the cumbersome, time-consuming method of cell-counting. Thus, Densimat brings significant improvement to the reproducibility and feasibility of standardization of a fungal inoculum.

**INTRODUCTION**

Recent outbreaks of infection caused by *Aspergillus*, particularly by *Aspergillus fumigatus* among neutropenic and critical care patients (Fridkin & Jarvis, 1996), led the scientific community to perform comprehensive research on this topic, and particularly on antifungal susceptibility testing (Espinel-Ingroff et al., 1995, 2001; Espinel-Ingroff, 2001a, b). In an attempt to enhance inter- and intra-laboratorial reproducibility, the National Committee for Clinical Laboratory Standards published the Document M38-A (NCCLS, 2002), which standardizes susceptibility testing for several fungal species, including members of the genus *Aspergillus*.

Inoculum concentration may significantly affect the determination of MIC and minimal fungicidal concentration values for filamentous fungi (Gehrt et al., 1995; Manavathu et al., 1999), as well as germination studies of *Aspergillus* sp. (Manavathu et al., 1999; Araujo & Rodrigues, 2004). Accordingly, both the NCCLS and the European Committee for Antifungal Susceptibility Testing (EUCAST-AFST) recommend that inoculum standardization should be based on determination of cell density by spectrometry (Espinel-Ingroff & Kerkering, 1991; NCCLS, 2002; Rodriguez-Tudela et al., 2001). Nevertheless, colour, shape and size of spores are morphological characteristics that can affect spectrometric readings of optical density (Petrikkou et al., 2001).

Taking into account the variance of spectrometric readings taken from different species of *Aspergillus*, Petrikkou et al. (2001) suggested that spectrometry might be used for inoculum standardization, provided that each species was standardized separately, resulting in a time-consuming methodology that makes cell-counting with a haemocytometer a better alternative.

The objective of the present work was to develop and standardize a fast and reliable procedure, based on photometric readings, for the standardization of an *Aspergillus* inoculum, as an alternative to the classical, haemocytometer-based counting method and devoid of the drawback of observer dependency.

**METHODS**

**Organisms and growth conditions.** Clinical isolates of *A. fumigatus* (39 strains), *Aspergillus flavus* (20 strains) and *Aspergillus niger* (15 strains), belonging to the collection of the Department of Microbiology of Porto Faculty of Medicine, were used. Organisms were cultivated in Sabouraud agar slants (Difco) at room temperature (20°C) for 5 or 11 days. Spores were harvested by flooding the agar surface with PBS (Sigma) and then filtered and suspended in PBS with 0·01 % Tween 80 (Difco), in serial concentrations. Spore suspensions were stored at 4°C for up to 5 days.

**Cell-counting.** Spore concentration of different spore suspensions was evaluated by using a Neubauer’s chamber (haemocytometer), according to the classical procedure.

**Optical readings.** Spore suspensions were submitted to spectrometric readings with a Shimadzu UV-160A spectrometer and to photometric readings with a Densimat photometer (bioMérieux). The suspensions were vortexed before reading. Spectrometric readings were taken at 550 and 620 nm; photometric readings were taken at 550 nm (as established by the manufacturer). All determinations were performed in triplicate and each value was entered individually for data analysis.

**Statistical analysis.** The program SPSS 11.5 was used for data
elaboration and analysis. The Wilcoxon signed-rank test (Bradford Hill, 1991) and Student’s t-test for paired samples were used for statistical analysis. Data were compared at a significance level of 0.05.

RESULTS AND DISCUSSION

No significant difference was noted when comparing spectrometric readings at 550 and 620 nm, for all tested strains of *A. fumigatus* (Fig. 1). Similar results were found with the other two *Aspergillus* species (data not shown). A correlation between spectrometric readings and cell-counting with a haemocytometer was established for each of the three tested species of *Aspergillus* (Fig. 2). Within each species, no significant differences were noticed.

A correlation between spectrometric readings (Shimadzu) and the MacFarland density scale, evaluated by the Densimat, is shown in Fig. 3.

A correlation between the MacFarland density scale and cell-counting was established for each of the different species of *Aspergillus* tested (Fig. 4). By using the correlation found with each of the three species tested, it was possible to define the upper and lower limits of evaluation of the concentration of a spore suspension with the Densimat, as shown in Table 1.

No significant difference was found between suspensions of spores that were 5 or 11 days old with either spectrometric or photometric readings (data not shown).

It is a general consensus that antifungal susceptibility testing of moulds represents an area of clinical interest. With resistance demonstrated in *A. fumigatus* (Denning et al., 1997; Mosquera & Denning, 2002), reproducible and standardized susceptibility methods are urgently needed in order to obtain meaningful data, particularly from a clinical perspective. Inoculum standardization represents one of the main pitfalls in antifungal susceptibility testing.

Previous reports emphasized that spectrometry could be used for evaluation of the cell density of an *Aspergillus* suspension in cases where each species had been standardized separately (Petrikkou et al., 2001), a fact that was confirmed by our results. Good correlation coefficients were found between Densimat and spectrometric readings, particularly in the cases of *A. fumigatus* and *A. flavus*.

Futhermore, by defining a correlation between cell-counting and the MacFarland scale by using the Densimat for all tested species of *Aspergillus*, a significant improvement was achieved from a technical perspective. Spectrometry involves the use of a very expensive and sturdy apparatus, which is unavailable in most clinical laboratories. Preparation of a large number of spore suspensions, e.g. for susceptibility testing, as will surely be needed in the near future (according to the most recent epidemiological findings of a steady increase of invasive fungal infections), makes cell-counting – also a much too time-consuming and observer-dependent procedure – an impractical method for clinical laboratories.

Densimat can replace, with considerable advantage, both spectrometric determination and cell-counting in the preparation of a spore suspension with a precise inoculum size,
to be used in antifungal susceptibility testing, as well as in other in vitro and in vivo studies. Nevertheless, uncommon species or atypical strains of Aspergillus, particularly with uncharacteristic pigmentation, may require specific standardization. It is important to emphasize that the limits of resolution of Densimat include the range of cell concentration values that are needed for microbial susceptibility testing, as well as for other laboratorial studies (Gehr et al., 1995; Bouchara et al., 1997; Wasylanka et al., 2001; Staudoher et al., 2002; Wasylanka & Moore, 2002). By reducing inter- and intra-laboratorial variation, this methodology could play an important role in inoculum standardization.

The portability, easiness-of-use and reproducibility of Densimat, together with its correlation with cell-counting, make it a valuable tool for use in clinical mycology laboratories.

**Fig. 3.** Correspondence between spectrometric readings at 550 nm and Densimat readings: (a) A. fumigatus (39 strains); (b) A. flavus (20 strains); (c) A. niger (15 strains).

**Fig. 4.** Correspondence between Densimat readings and spore-counting: (a) A. fumigatus (39 strains); (b) A. flavus (20 strains); (c) A. niger (15 strains).

<table>
<thead>
<tr>
<th>Species</th>
<th>Lower limit</th>
<th>Upper limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. fumigatus</td>
<td>$3 \times 10^6$</td>
<td>$6 \times 10^7$</td>
</tr>
<tr>
<td>A. flavus</td>
<td>$1 \times 10^6$</td>
<td>$3 \times 10^7$</td>
</tr>
<tr>
<td>A. niger</td>
<td>$1 \times 10^6$</td>
<td>$3 \times 10^7$</td>
</tr>
</tbody>
</table>

**REFERENCES**


