Inoculum Standardization for Antifungal Susceptibility Testing of Filamentous Fungi Pathogenic for Humans

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Two methods of inoculum preparation for filamentous fungi were compared: counting with a hematocytometer and spectrophotometric adjustment. One hundred eighty-two filamentous fungi pathogenic for humans were used. Colony counts were done for all inoculum preparations. The agreement between the hematocytometer counts and the colony counts (CFU per milliliter) was 97.2%. The reproducibility between the hematocytometer counts and the colony counts by means of an intraclass correlation coefficient was 0.70. Pearson's correlation index for hematocytometer counts versus colony counts was 0.56, whereas that for optical density versus colony counts was 0.008. Both methods can be used for inoculum size adjustment. However, the use of the spectrophotometric method requires that each species be standardized separately.

An increasing prevalence of infections caused by filamentous fungi has been detected in humans in recent years (1). This increasing prevalence has stimulated interest in a reliable and reproducible antifungal susceptibility testing method for molds. Recently, the National Committee for Clinical Laboratory Standards (NCCLS) proposed a reference method (the NCCLS M38-P method) for the susceptibility testing of conidium-forming filamentous fungi (7). The NCCLS method recommends inoculum preparation by a spectrophotometric procedure (2–4, 7). However, it is well known that larger objects such as spores or filaments do not obey the rule that established a proportional relationship between dry weight concentration and optical density (6). In addition, the inoculum size has a great influence on the MICs for filamentous fungi (3–5, 7). Other variables such as the color of the spores can also influence the optical density value.

In the work described here, two procedures for inoculum size adjustment were evaluated, and the results were compared with those obtained by colony counting.

MATERIALS AND METHODS

Isolates. A set of clinical isolates was tested. Each isolate was obtained from a different patient and was sent to the laboratory for identification or antifungal susceptibility testing.

The following species were included: 31 *Aspergillus fumigatus*, 28 *Aspergillus flavus*, 25 *Aspergillus terreus*, 16 *Aspergillus niger*, 18 *Scedosporium apiospermum*, 27 *Scedosporium prolificans*, 24 *Fusarium solani*, and 13 *Fusarium oxysporum* isolates. The isolates were maintained as a suspension in sterile distilled water at 4°C until testing was performed.

Inoculum preparation. The isolates were subcultured from the stock water suspensions on Sabouraud agar and on potato dextrose agar. Isolates of all species except members of the genus *Fusarium* were incubated at 35°C; members of the genus *Fusarium* were incubated at 30°C. Inoculum suspensions were prepared from fresh, mature (3- to 5-day-old) cultures grown on Sabouraud agar or potato dextrose agar slants. The colonies were covered with 5 ml of distilled sterile water. Tween 20 (5%) was added to facilitate the preparation of *Aspergillus* inocula. For *Aspergillus* spp., the inocula were achieved by carefully rubbing the colonies with a sterile loop; the isolates were then shaken vigorously for 15 s with a Vortex mixer and then transferred to a sterile tube. For slowly sporulating fungi, such as *Scedosporium* and *Fusarium*, the suspensions were obtained by exhaustive scraping of the surface with a sterile loop. Then, the inoculum was transferred to a sterile syringe attached to a sterile filter with a pore diameter of 11 μm (Millipore, Madrid, Spain). The suspension was filtered and collected in a sterile tube. This procedure removed the majority of the hyphae, producing a inoculum mainly composed of spores.

Inoculum adjustment. The inoculum size was adjusted to between 1.0×10^6 and 5.0×10^6 spores/ml by microscopic enumeration with a cell-counting hematocytometer (Neubauer chamber; Merck, S.A., Madrid, Spain). Then, the optical density at 530 nm (OD_{530}) of the suspensions was measured in a spectrophotometer (Bausch & Lomb, Pacisa, Madrid, Spain). All adjusted suspensions were quantified by plating on Sabouraud agar plates. The inocula were agitated for 15 s with a Vortex mixer and were diluted 1:1,000. Aliquots of 100, 50, and 25 μl were spread onto the Sabouraud agar plates with a glass hockey stick. The plates were incubated at 35°C (*Aspergillus* and *Scedosporium* spp.) or 30°C (*Fusarium* spp.) and were observed daily for the presence of growth. The colonies were counted as soon as possible after the observation of visible growth.

Statistical analysis. The proper inoculum size range, according to previous work (2), was established to be between 1.0×10^6 and 5.0×10^6 CFU/ml. The percent agreement between inoculum sizes determined by counting with a hematocytometer and colony counting was calculated by taking into account the colony counts in a range between 1.0×10^6 and 5.0×10^6 CFU/ml.

The reproducibility of the results obtained by counting with a hematocytometer and colony counting was evaluated by using the intraclass correlation coefficient (ICC). Reproducibility was calculated by means of a scales analysis in which reliability was the extent to which the counting with a hematocytometer yielded an equivalent colony count. The ICC assesses reliability as an internal consistency statistic by means of interitem correlations. A two-way mixed-effect model was used to calculate the ICC, which was expressed to a maximum value of 1 and with a confidence interval (CI) of 95%. When appropriate, the variables were transformed to $\log_{10}$ data. In addition, the correlation among the hematocytometer count; the colony count, and the OD_{530} was also determined with Pearson's coefficient (r), which was also expressed to a maximum value of 1. All statistical analyses were done with the Statistical Package for the Social Sciences (version 10.0; SPSS, S.L., Madrid, Spain).

RESULTS AND DISCUSSION

Table 1 shows the range, 95% CI, ICC, and Pearson's correlation index (r) of the colony counts versus the hematocytometer counts and OD_{530}s. The percent agreement between the hematocytometer counts and the colony counts was 97.2%.

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TABLE 1. Range, 95% CI, correlation index, and ICC among colony counts, hematocytometer counts, and OD_{530}s

Species	No. of isolates tested	Colony count (CFU/ml [10^6])		Hematocytometer count (CFU/ml [10^6])		r^a	ICC (95% CI)^b	OD_{530}		r^c
		Range	95% CI	Range	95% CI			Range	95% CI	
A. fumigatus	31	1.11–5.55	1.97–2.67	1.40–4.40	2.75–3.42	0.26	0.44 (−0.15–0.73)	0.03–0.13	0.06–0.08	0.38
A. flavus	28	1.02–3.69	1.70–2.26	1.85–3.92	2.48–2.94	0.24	0.35 (−0.40–0.69)	0.07–0.27	0.16–0.19	0.49
A. terreus	25	1.21–4.32	2.39–2.94	1.81–4.48	2.78–3.41	0.58	0.73 (0.40–0.88)	0.01–0.15	0.05–0.08	0.18
A. niger	16	0.45–4.36	1.72–2.86	1.31–4.46	2.19–3.27	0.71	0.80 (0.44–0.93)	0.20–0.50	0.29–0.39	0.42
S. apiospermum	18	1.01–2.73	1.43–1.94	1.04–4.10	1.97–2.81	0.76	0.85 (0.60–0.94)	0.13–0.50	0.25–0.36	0.44
S. prolificans	27	0.61–4.70	1.93–2.67	1.07–4.90	2.38–3.14	0.61	0.75 (0.47–0.89)	0.15–0.53	0.26–0.34	0.42
F. solani	24	0.55–3.84	1.60–2.42	1.22–4.79	2.50–3.32	0.57	0.70 (0.31–0.87)	0.13–0.61	0.23–0.36	0.01
F. oxysporum	13	1.45–4.71	2.24–3.42	1.65–3.98	2.43–3.40	0.90	0.94 (0.81–0.98)	0.09–0.57	0.13–0.28	0.42
Total	182	0.45–5.55	2.11–2.37	1.04–4.90	2.72–2.97	0.56	0.70 (0.60–0.78)	0.01–0.61	0.18–0.22	0.008

^{a} Pearson's correlation index for colony counts versus hematocytometer counts.
^{b} ICC and 95% CI for colony counts versus hematocytometer counts.
^{c} Correlation index for colony counts versus OD_{530}.

As expected, some values for the colony counts were out of the established range (1.0×10^6 to 5.0×10^6 CFU/ml). The colony count for one *A. fumigatus* isolate was above 5.0×10^6 CFU/ml. The colony counts for one *A. niger* isolate, one *S. prolificans* isolate, and two *F. solani* isolates were below 1.0×10^6 CFU/ml. Nevertheless, the 95% CIs of the colony counts were between 1.0×10^6 and 5.0×10^6 CFU/ml for all isolates tested (Table 1). Pearson's correlation indices between hematocytometer counts and colony counts were low for *A. flavus* ($r = 0.24$) and *A. fumigatus* ($r = 0.26$). For the remainder of the filamentous fungi, Pearson's correlation indices were above 0.57 (Table 1). This fact can be explained by the hydrophobic nature of *Aspergillus* species, which causes more dilution mistakes with *aspergillus* species than with hydrophilic filamentous fungi. The exceptions were *A. niger* ($r = 0.71$) and *A. terreus* ($r = 0.58$). The reproducibility, analyzed by determination of ICC, between the hematocytometer counts and the colony counts was adequate for all species tested. Thus, an ICC of 0.70

and 95% CIs between 0.60 and 0.78 were obtained (Table 1 and Fig. 1). On the other hand, Pearson's correlation index for hematocytometer counts versus colony counts was 0.56 (Fig. 1 and Table 1). Pearson's correlation indices between the OD_{530}s and the colony counts were lower than those obtained by enumeration with a hematocytometer. The exceptions were *A. flavus* ($r = 0.49$) and *A. fumigatus* ($r = 0.38$). A larger range of OD_{530}s was obtained for each different species. As expected, the range of OD_{530}s was species dependent. Thus, among *Aspergillus* species, *A. fumigatus* had an OD_{530} range between 0.03 and 0.13 absorbance units. On the other hand, the range for *A. niger* was between 0.20 and 0.50 absorbance units. The size of the spores (*A. fumigatus*, 2.5 to 3.0 μm; *A. niger*, 3.5 to 5.0 μm) and the color of the species (green versus black, respectively) can explain the differences in the OD_{530}. In addition, each species of filamentous fungi does not have a uniform color. These differences in color tonalities can influence the OD_{530} and can explain the large range of values obtained
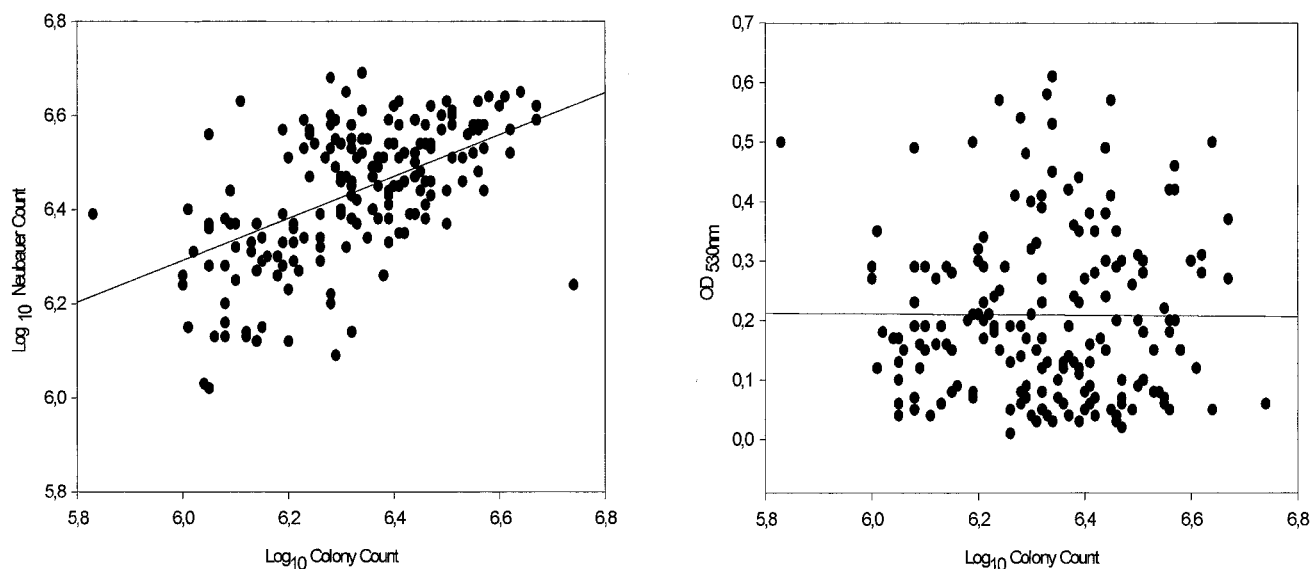
FIG. 1. Scatter plots of $\log_{10}$ colony counts versus $\log_{10}$ hemacytometer (Neubauer) counts (left) ($r = 0.56$; ICC = 0.70) and $\log_{10}$ colony counts versus OD_{530} ($r = 0.008$) (right) for 182 strains of filamentous fungi pathogenic for humans.

for each species (Table 1). However, the 95% CI showed a narrow range, sustaining the possibility that the inoculum can be prepared by this procedure. Nevertheless, a correlation index of 0.008 was obtained when all strains were analyzed together, suggesting that each species must be standardized independently (Table 1 and Fig. 1). NCCLS document M38-P (7) recommends the spectrophotometric adjustment of the inoculum. The original article (2) showed that at certain OD_{530}s the size of inoculum was between 1.0×10^6 and 5.0×10^6 CFU/ml (2). Unfortunately, the number of strains used was small, precluding an adequate statistical analysis (2). Further work showed that only 90% of the inocula prepared by the spectrophotometric procedure had between 4.0×10^5 and 3.3×10^6 CFU/ml (4). Observations described in a subsequent article (3) sustained the previous observation. Thus, only 87% of the inoculum preparations for *S. apiospermum* had between 4.0×10^5 and 3.2×10^6 CFU/ml. For *Aspergillus*, *Fusarium*, and *Rhizopus* species, more than 92% of the the inoculum suspensions had between 4.0×10^5 and 5.0×10^6 CFU/ml (3).

By the spectrophotometric method for inoculum preparation, the best scenario is retrieval of an inoculum that varies between 4×10^5 and 5×10^6 spores per ml. This wide range of sizes of inoculum suspensions could have a significant influence on MICs. Nevertheless, the level of agreement between laboratories that use NCCLS document M38-P (7) is adequate (3).

In any case, the range of OD_{530} obtained in the present work included the OD_{530} recommended by NCCLS document M38-P (7). However, proper identification of the filamentous fungi should be done by using the spectrophotometric method for inoculum preparation. Although many laboratories can identify usual pathogens like *A. fumigatus*, very experienced staff is needed for the identification of less common species.

In summary, two methodologies for inoculum adjustment have been compared by using 182 strains of fungi pathogenic for humans. The results indicate that both methodologies can be used. However, counting with a hematocytometer is a universal procedure that is independent of the color of the strain and the size of the spores. Use of this procedure could save a lot of time in the process of standardization of antifungal susceptibility testing of filamentous fungi.

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