JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 2003, p. 5236–5237 Vol. 41, No. 11
0095-1137/03/$08.00+0 DOI: 10.1128/JCM.41.11.5236–5237.2003

Interlaboratory Evaluation of Hematocytometer Method of Inoculum Preparation for Testing Antifungal Susceptibilities of Filamentous Fungi

J. L. Rodriguez-Tudela,[1] Erja Chryssanthou,[2] Evangelia Petrikkou,[1] Juan Mosquera,[3] David W. Denning,[3] and Manuel Cuenca-Estrella[1]*

Unidad de Micología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Spain[1]; Karolinska Hospital, Stockholm, Sweden[2]; and University of Manchester, Manchester, United Kingdom[3]

Received 22 July 2003/Returned for modification 5 June 2003/Accepted 4 August 2003

A three-laboratory study was performed to evaluate conidium counting in a hematocytometer as a technique of inoculum preparation for susceptibility testing of *Aspergillus* spp. In addition, inocula were quantified by colony counting and optical density determination. The agreement and correlation coefficient between conidium and colony quantifications were 89.2% and 0.73 ($P < 0.01$). Correlations with optical density determination were not significant.

During recent years, filamentous fungi have gained importance as common pathogens in patients receiving immunosuppressive therapy or suffering from AIDS (2, 3). The most frequently identified fungi are *Aspergillus* species, although other species such as those of *Scedosporium* and *Fusarium* are becoming more relevant (5, 6). This increase in the prevalence of fungal infections has generated interest in reliable procedures for testing susceptibilities of filamentous fungi (8). One of the most significant aspects of antifungal susceptibility testing is inoculum size, which can influence susceptibility results (4, 5). Two previous works have demonstrated that inoculum preparation by counting conidia with a hematocytometer is an accurate and universal procedure, independent of the color and size of conidia (1, 7). However, the weakness of both studies was the lack of an interlaboratory comparison, a fundamental step in the process of standardizing an antifungal susceptibility testing procedure.

The aim of this study was to evaluate the interlaboratory reproducibility of *Aspergillus* sp. inoculum preparation by means of conidium counting in a hematocytometer.

Laboratories. Three different laboratories designated Lab 1, Lab 2, and Lab 3 participated in the study.

Isolates. A panel of 40 clinical isolates belonging to four different *Aspergillus* species was included (10 each of *A. fumigatus*, *A. flavus*, *A. terreus*, and *A. niger*). The isolates were distributed to the three participant laboratories and were maintained on potato dextrose agar slants until testing was performed.

Inoculum preparation. The isolates were subcultured, from the stock water suspensions or from petri plates, on potato dextrose agar slants and incubated at 35°C. Inoculum suspensions were prepared from fresh, mature (2- to 3-day-old) cultures. The colonies were covered with 5 ml of distilled sterile water containing 0.1% Tween 20. Then, the conidia were carefully rubbed with a sterile cotton swab and transferred to a sterile tube; the resulting suspensions were vigorously homogenized for 15 s with a vortex mixer. Appropriate dilutions were performed in order to get the right concentration for counting in a cell-counting hematocytometer. All inoculum preparations were checked for the presence of hyphae or clumps by a previous exam in the cell-counting hematocytometer chamber. If a significant number of hyphae were detected, 5 ml of the suspension was transferred to a sterile syringe attached to a sterile filter with a pore diameter of 11 μm and filtered and collected in a sterile tube. This step removes hyphae and yields a suspension composed of conidia. If many clumps were detected, the inoculum was shaken again in a vortex mixer for a further 15 s. This step was repeated as many times as necessary if clumps were noted again. These procedures were performed in a biohazard safety cabinet.

Inoculum adjustment and colony counts. The inoculum size was adjusted to a range of 1.0×10^6 to 5.0×10^6 conidia/ml by microscopic enumeration with a cell-counting hematocytometer. Five milliliters of this suspension was transferred to a crystal tube, and the optical density at 530 nm (OD_{530}) was measured in a spectrophotometer. All adjusted suspensions were quantified by plating on Sabouraud agar plates. The plates were incubated at 35°C 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 target inoculum size range was established as 1.0×10^6 to 5.0×10^6 CFU/ml. The percentage of agreement between inoculum sizes determined by counting with a hematocytometer and colony counting was calculated taking into account that both systems of measurement should produce colony counts in a range of 1.0×10^6 to 5.0×10^6 CFU/ml.

The correlation between the results obtained by counting with a hematocytometer and the colony counting data was evaluated by using the intraclass correlation coefficient (ICC), which was expressed to a maximum value of 1 and with a confidence interval of 95% (95% CI). The ICC is a reverse measurement of the variability of the counting values. Apart

* Corresponding author. Mailing address: Unidad de Micología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Ctra Majadahonda-Pozuelo Km 2, 28220 Majadahonda (Madrid), Spain. Phone: 34-91-5097961. Fax: 34-91-5097966. E-mail: mcuenca-estrella@isciii.es.

TABLE 1. Ranges and 95% CIs of colony counts, hematocytometer counts, and spectrophotometrical OD_{530}s

Species	Conidium diam (μm)	Colony count (10^6 CFU/ml)		Hematocytometer count (10^6 CFU/ml)		OD_{530}		ICC[a]	r[b]
		Range	95% CI	Range	95% CI	Range	95% CI		
Aspergillus furnigatus	2.5–3.0	1.17–6.13	2.40–3.31	1.70–5.0	2.93–3.58	0.04–1.06	0.06–0.20	0.36	−0.088
Aspergillus terreus	1.5–2.5	1.65–7.01	2.60–3.57	2.32–5.70	3.26–3.95	0.05–0.25	0.08–0.11	0.52	0.455
Aspergillus flavus	3.5	0.36–3.17	1.30–1.89	1.0–4.30	2.10–2.80	0.06–0.38	0.13–0.18	0.69	0.824
Aspergillus niger	3.5–5.0	0.93–5.77	1.75–2.55	1.50–4.80	2.25–2.97	0.09–1.15	0.26–0.46	0.61	0.437
Total	1.5–5.0	0.36–7.01	2.20–2.65	1.0–5.70	2.80–3.17	0.04–1.15	0.15–0.22	0.73	0.083

[a] Between colony and hematocytometer counts.
[b] Pearson coefficient between colony counts and optical density values.

from the agreement (concordance between counting values), the ICC also evaluates the correlation between values offering statistical significance since it takes into account the number of cases and the absolute value of the count. The ICC is a scale analysis and exhibits the highest statistical power for correlation studies. However, it cannot be used for correlating variables that are not expressed in the same units (i.e., optical density expressed in absorbance units and colony counts expressed in colonies per milliliter). Considering that limitation, the correlation between OD_{530} and counting was calculated by a simple correlation coefficient (Pearson's coefficient [*r*]). When appropriate, the variables were transformed to $\log_{10}$ data. All statistical analyses were done with the Statistical Package for the Social Sciences (version 11.0; SPSS, S.L., Madrid, Spain).

A total of 107 of 120 colony counts fell in the range of 1.0×10^6 to 5.0×10^6 CFU/ml, giving an overall agreement of 89.2%. There were disagreements within all participating laboratories. Thus, Lab 1 had two disagreements whereas Lab 2 and Lab 3 had four and seven disagreements, respectively. Furthermore, the 95% CI for colony counting was very narrow (2.20 to 2.65), indicating that hematocytometer counting has high reproducibility between laboratories.

Overall, the ICC obtained between colony counting and hematocytometer counting was 0.73 (95% CI, 0.62 to 0.81), this value being statistically significant ($P < 0.0001$). By laboratories, Lab 2 reached the best ICC value (0.84; 95% CI, 0.71 to 0.91). Although Lab 1 and Lab 3 had lower ICC values, both values were statistically significant ($P < 0.0001$).

On the other hand, Pearson's correlation index (*r*) between spectrophotometric adjustment and the colony counts was 0.083, a value without statistical significance ($P = 0.368$). The range was wide for all species although the 95% CI for *A. terreus* and *A. flavus* was narrow, having a better Pearson's correlation index (Table 1).

As Table 1 shows, the optical density of the inoculum containing 1.0×10^6 to 5.0×10^6 CFU/ml is species dependent. However, the adjustment of the inoculum by means of counting conidia in a hematocytometer can be used for any species, being a method independent of conidial color and size (1, 7). Therefore, this interlaboratory exercise adds a new step in the process of standardizing inoculum preparation, and we conclude that this methodology is universal and can be used for inoculum adjustment for antifungal susceptibility of filamentous fungi.

This work was supported by the EUROFUNG Network (EC-TMR-EUROFUNG network; ERBFMXR-CT970145). The EUROFUNG Network consists of the following participants: Emmanuel Roilides (coordinator) and Nicos Maglaveras, Aristotle University, Salonika, Greece; Tore Abrahamsen and Peter Gaustad, Rikshospitalet National Hospital, Oslo, Norway; David W. Denning, University of Manchester, Manchester, United Kingdom; Paul E. Verweij and Jacques F. G. M. Meis, University of Nijmegen, Nijmegen, The Netherlands; Juan L. Rodriguez-Tudela, Instituto de Salud Carlos III, Madrid, Spain; and George Petrikkos, Athens University, Athens, Greece.

REFERENCES

1. **Aberkane, A., M. Cuenca-Estrella, A. Gomez-Lopez, E. Petrikkou, E. Mellado, A. Monzon, and J. L. Rodriguez-Tudela.** 2002. Comparative evaluation of two different methods of inoculum preparation for antifungal susceptibility testing of filamentous fungi. J. Antimicrob. Chemother. **50:**719–722.
2. **Cuenca-Estrella, M., B. Ruiz-Diez, J. V. Martinez-Suarez, A. Monzon, and J. L. Rodriguez-Tudela.** 1999. Comparative in-vitro activity of voriconazole (UK-109,496) and six other antifungal agents against clinical isolates of *Scedosporium prolificans* and *Scedosporium apiospermum*. J. Antimicrob. Chemother. **43:**149–151.
3. **Denning, D. W.** 1998. Invasive aspergillosis. Clin. Infect. Dis. **26:**781–803.
4. **Espinel-Ingroff, A.** 2001. Germinated and nongerminated conidial suspensions for testing of susceptibilities of *Aspergillus* spp. to amphotericin B, itraconazole, posaconazole, ravuconazole, and voriconazole. Antimicrob. Agents Chemother. **45:**605–607.
5. **Espinel-Ingroff, A., and T. M. Kerkering.** 1991. Spectrophotometric method of inoculum preparation for the in vitro susceptibility testing of filamentous fungi. J. Clin. Microbiol. **29:**393–394.
6. **Moore, C. B., N. Sayers, J. Mosquera, J. Slaven, and D. W. Denning.** 2000. Antifungal drug resistance in *Aspergillus*. J. Infect. **41:**203–220.
7. **Petrikkou, E., J. L. Rodriguez-Tudela, M. Cuenca-Estrella, A. Gomez, A. Molleja, and E. Mellado.** 2001. Inoculum standardization for antifungal susceptibility testing of filamentous fungi pathogenic for humans. J. Clin. Microbiol. **39:**1345–1347.
8. **Rex, J. H., M. A. Pfaller, T. J. Walsh, V. Chaturvedi, A. Espinel-Ingroff, M. A. Ghannoum, L. L. Gosey, F. C. Odds, M. G. Rinaldi, D. J. Sheehan, and D. W. Warnock.** 2001. Antifungal susceptibility testing: practical aspects and current challenges. Clin. Microbiol. Rev. **14:**643–658.