J. Clin. Microbiol. doi:10.1128/JCM.01481-17

Evaluation of Copan ESwab Transport System for Viability of Pathogenic Fungi Using Modification of Clinical and Laboratory Standards Institute M40 -A2 Document

Bharat Gandhi [1,4*], Richard Summerbell [2,3], Tony Mazzulli [1,4]

Department of Microbiology, Mt. Sinai Hospital and University Health Network, Toronto, Ontario, Canada[1]

Sporometrics, Toronto, Ontario, Canada[2]

Dalla Lana School of Public Health, University of Toronto, Toronto, Ontario, Canada[3]

Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada[4]

Viability of pathogenic fungi in the Copan ESwab system.

Bharat Gandhi
Department of Microbiology,
Mt. Sinai Hospital and University Health Network
600 University Avenue
Toronto, Ontario, Canada
Bharatxgandhi@gmail.com

(T) 647 234 0597

ABSTRACT:

The Copan ESwab system was evaluated for its ability to maintain viability of pathogenic fungi. Tests followed the Clinical and Laboratory Standards Institute (CLSI) M40 -A2 roll plate method at both room and refrigerator temperatures. A system was devised for standardizing homogeneous inoculum suspensions of variously sized conidia and sporangiospores of filamentous fungi. A total of 19 clinical and reference strains were standardized to 0.5 McFarland turbidity standard with a simple photometer. Corresponding optical densities were measured with a spectrophotometer. Colony counts equal to or greater than those seen at time zero were obtained for the entire test panel. Results indicate that the Copan ESwab system effectively maintains prevalent opportunistic fungal organisms for at least 48 hours.

INTRODUCTION:

The Clinical and Laboratory Standards Institute (CLSI) document M40 -A2 (1) describes methods for assessing the ability of transport medium devices to maintain potentially pathogenic microorganisms in a viable condition for up to 48 hours during transport at room temperature (RT) (20 to 25ºC), and at refrigerator temperature (FT) (2 to 8ºC). Typically, the standard panel of bacterial strains chosen to validate the transport media represents a diverse group of bacterial challenge organisms. However, the panel does not include any fungal pathogens. Also, there are no formally published data on the viability of filamentous fungi

during transport using swab-based systems. Preliminary data, however, were presented in 2010 in a poster at the American Society for Microbiology 110th General Meeting (2). This 2010 study was conducted only at room temperature. In this study 100 µL of standardized inoculum was delivered to each flocked swab directly and 10µL aliquots were dispensed onto plates of medium for control colony enumeration. Contrary to the work performed by Snyder et al (who also tested the Copan ESwab kit), CLSI-recommends allowing the flocked swabs to absorb material from the well of a microtiter plate containing 100 µL of inoculum (1). It is recommended that swabs be held at RT and FT. The present investigation was designed to adopt CLSI-recommended methods to allow a canonical evaluation of the Copan ESwab (Copan Diagnostics Inc., Murrieta, CA) transport system for fungal pathogens described below. Due to labor, resource intensity and incubator space restriction, these types of studies are seldom conducted.

MATERIALS AND METHODS:

A test panel was prepared consisting of frequently isolated organisms in the clinical laboratory, as well as a comprehensive selection of clinically important opportunistic pathogens. Viability studies followed CLSI M40-A2 methods with minimal modifications made necessary by the physical properties of fungal inoculum. Most importantly as described below, methods were developed for making uniform suspensions of hydrophobic fungi like *Aspergillus* spp. (3); in addition, the problem of counting rapidly expanding colonies accurately in growth

studies were addressed. Techniques for suspension standardization was modified and validated to ensure reliable test evaluation. Specifically, a fast and practical photometric procedure for cell density standardization was developed as described in the materials and methods section. This study is the first to employ the methods

STUDY ISOLATES:

A test panel of yeasts and filamentous fungi was assembled, including quality control reference strains, clinical isolates cultured from swab transport systems and superficial specimens in routine high volume clinical microbiology laboratories. This collection of 19 test isolates consisted of five yeast reference isolates, (*Candida albicans* ATCC 10231, *Candida krusei* ATCC 6258, *Candida guilliermondii* ATCC 6260, *Candida glabrata* ATCC 66032 and *Cryptococcus neoformans* ATCC 66031), three reference dermatophytes, (*Trichophyton mentagrophytes* ATCC 9533, *Trichophyton tonsurans* ATCC 28942 and *Trichophyton rubrum* ATCC 28188), five opportunistic hyaline moulds (*Aspergillus niger*, *Aspergillus fumigatus*, *Lecythophora* sp., *Fusarium solani* and *Trichosporon* sp.), three Zygomycetes (*Lichtheimia corymbifera*, *Mucor circinelloides* and *Rhizopus microsporus*) and three dematiaceous moulds (*Curvularia clavata*, *Phialophora americana* and *Alternaria alternata*). The identification at species level of the opportunistic moulds were confirmed by an independent reference laboratory.

INOCULUM PREPARATION.

Strains previously stored at -80°C in cryopreservative fluid were subcultured twice before testing. First, they were cultured on Sabouraud dextrose agar (SDA) (Biomedia Unlimited Ltd), to ensure viability and purity followed by a second subculture on potato dextrose agar (PDA) (Biomedia Unlimited Ltd) to optimize sporulation. All plates were incubated aerobically at 30 °C until confluent growth was observed with incubation time ranging from approximately two to fourteen days. The inoculum was prepared by zeroing 5.0 mL (0.85%) of sterile saline (Oxoid Inc.) containing one drop of tween 20 (Biomedia Unlimited Ltd) as per CLSI M38 -A2 (3), in a spectrophotometer (WP-100DPlus, Walter Products Inc.) at a mark scribed on the tube. The addition of tween surfactant is not required to produce uniform suspensions of bacteria (1) but is used to prepare uniform suspensions of hydrophobic fungi (3). This slightly modified sterile saline diluent was poured onto a mature culture plate. A mature culture is when point inoculation in the centre of a plate produces only one colony which spreads outwards. Once coloured powdery conidia are observed visually after ~ 3 days, the mould is sufficiently mature to carry out laboratory investigations. Initially organisms were identified to the genus level by way of scotch tape and tease mount preparations. After initial evaluation of these techniques confirming their reliability and reproducibility, it was felt that it was not necessary to continue to perform these. Propagules were harvested by gently rubbing the surface of the plate with a swab. The coarse suspension was pipetted back into the original empty tube, vortexed and allowed to sit for 30minutes. The top portion of the

suspension was separated from the heavier hyphal sediment by means of a sterile transfer pipette. This supernatant was then manually adjusted to 0.5 McFarland turbidity standard using a Wickerham card to produce approximately 1×10^6 to 5×10^6 colony-forming units (CFU)/ml (4). Fine adjustments were performed on the DensiChek Plus photometric device to 0.5 McFarland (between 0.45 McF and 0.55 McF). Corresponding absorbance and % transmission values were obtained using round glass tubes containing saline as they came with screw caps to contain any aerosols and fitted well into the spectrophotometer. As per the manufacturer's directions, a line was scribed near the top of the tube so the absorbance/ transmission readings could be determined at the same spot as where the zeroing was determined at 530nm using the spectrophotometer. The readings were recorded for comparison with published values (4).

CLSI M40-A2 ROLL PLATE METHOD.

For the modified roll plate method, inocula were prepared as follows. For each prepared inoculum tube adjusted to the 0.5McFarland turbidity standard (~1×10^6 to 5×10^6CFU/mL), three 10-fold dilutions were performed at a concentration of 10^5,10^4 and 10^3 CFU/mL. Using an Eppendorf repeater pipette, 100 µL of the dilutions were dispensed into a round-bottomed 96-well microtiter plate in triplicate for each organism suspension and dilution. Swabs were immersed into the dispensed suspension, absorbed for 10 s and transferred into the ESwab device containing one ml of liquid Amies transport medium. One set of inoculated swabs was held at RT (20 to 25ºC), and a duplicate set at FT (2 to 8ºC). After 0, 24 and 48 hours, the swabs were removed. Initially, three swabs were tested at time zero for each organism and each dilution by removing the selected swabs from the

transport device after approximately 5-15 minutes. The ESwab transport devices, including the swab, were vortexed to express residual fluid from the swabs and discarded. The transport devices were vortexed again for 5 s. For 10^5 concentrations, to minimize potentially confluent growth of rapidly extending fungal colonies, only 50 µL aliquots (instead of 100µL) were pipetted and then evenly distributed with a sterile 10µL loop on three plates for each duplicate set. The resulting colony counts from each set of three plates were multiplied by two to give equivalent counts for 100 µL inoculum. For inocula at a concentration of 10^4, 100 µL was pipetted and evenly distributed on two plates for each duplicate set; colony counts were then totalled. For inocula at10^3 concentration, 100 µL was pipetted on a single plate; since two parallel inocula were tested, this test was effectively done in duplicate. The plates were then sealed with parafilm and incubated at 30ºC aerobically and observed for growth each day until distinct colonies are formed on the entire plate. Once growth is observed, they were counted manually (by eye) by marking on the reverse side of the plate with a black marker (Sharpie) and using a mechanical counter. Once growth is observed, fungal colonies can be counted (countable colonies) similar to bacterial colonies. Zygomycetes however grow rapidly and confluently within 2 days. They also do not produce a darkening at the point where the colony originates on the reverse side of the plate. This makes it difficult to distinguish individual colonies and therefore, to count. CFU were averaged and tabulated. Similar steps were followed for growth controls by transferring the working inoculum onto plates.

RESULTS:

The isolates were tested once using one ESwab lot number. Even at the highest dilution, it was possible to produce colony counts similar to 0-hour counts at 24 and 48 hours (Table 1). All isolates yielding 0-hour counts of ≥ 5CFU at a given inoculum dilution level also yielded 24- and 48-hour counts ≥ 5CFU at RT and FT. The majority of the test isolates produced CFU in the 30 to 300 range at the 10^4 dilutions. As detailed above, this enumeration was facilitated in the more rapidly growing fungi by using several plates and reducing the volume of inoculum. Two of the three Zygomycetes – *Mucor circinelloides* and *Lichtheimia corymbifera* – and one dematiaceous mould, *Curvularia clavata,* produced countable colonies only from the 10^5 dilutions; these were species with notably broadly spreading colonies. *Rhizopus microsporus* produced colonial growth that was not countable even under those conditions, though it remained vigorously viable. All nineteen organisms tested from 0.5 McFarland-adjusted suspensions produced transmission values of approximately 71 to 80% transmission on the spectrophotometer using the saline -tween 20 diluents, similar to values cited in the literature of approximately 68 to 82 % transmission (4).

DISCUSSION:

Typically transport media systems are utilized for the collection of fungi from primary specimen which includes ear, throat, vagina, urethra, penis and wounds other than dermatophytic specimen such as skin, nail and hair. However, scalp

Journal of Clinical Microbiology

specimen is frequently received on swab transport media for KOH examination and culture in the laboratory. There are many studies of this method versus the traditional method of scrapings received in black paper with comparable results. From a scientific standpoint, it was important to validate a broad spectrum of pathogenic fungi to assess the viability by this mode of transport to allow for successful work up. Fungi are eukaryotic organisms with cells that are mostly relatively large and robust compared to bacterial cells. Nonetheless, it cannot be taken for granted that these organisms, when transported from bedside to laboratory via standard specimen collection methods, will retain viability. In addition, there is a question as to whether their relatively large sizes or their physical properties, such as hydrophobic exteriors or mucoid coatings, might interfere with detachment from swab-based transport systems. In the present study, potentially clinically important fungi with a wide range of physical propagule characters (including hydrophobic and hydrophilic cell walls, unicellular and multicellular propagules, smooth and echinulate integuments), cell colors (hyaline, melanised), and colony growth rates and forms (discretely colonial, diffuse) were tested. Inoculum standardization and colony counting methods were modified to accommodate these differences. More importantly these accommodations were designed to produce an equivalent to the bacterial CLSI standard for clinically important fungi. The criteria set by the newly revised second edition CLSI M40-A2 standard for the roll plate method states that for compliance of viability, any specimen held at 4°C and RT should yield ≥ 5CFU

Journal of Clinical Microbiology

after a specified holding period. Results suggest that the Copan ESwab transport system is able to maintain and recover a broad range of fungi after 48 hours at 4°C and RT. However, the one limitation of this study was that it was not performed on actual clinical specimens that might be harboring yeast or filamentous fungi pathogens. Therefore, the organism counts in a clinical specimen might be different than what was contrived in this study. Recovery at 24 and 48 hours might vary depending on organism load in a specimen. This supports the CLSI M40- A2 document standardized for validation using the modified procedure. The CLSI-recommended spectrophotometric methods of standardization for conidial and sporangiospore suspensions are difficult to perform with fungi because variability in spore colour and size causes different fungi to have differing optical densities (OD) at the same levels of colony-forming units. (4). Species-dependent standardization processes are not routinely used in microbiology laboratories and are not familiar to lab personnel. Several studies have compared widely used methods such as cell counting by hemocytometer – a procedure stipulated in EUCAST E.DEF9.1 standard, (6,7,8) but few studies have tested photometers as an alternative for hemocytometric and spectrophotometric measurements, (5,8). In our study, density standardization was found to be streamlined by using a simple photometric device, DensiCHECK Plus, for inoculum adjustments; it proved to be a very good alternative to more expensive and less user-friendly spectrophotometers. It may be imperative to address these issues in a further study; however, we are confident that the ESWAB is suitable for fungal viability and

Journal of Clinical Microbiology

transport in accordance with the CLSI standard for clinically important fungi.

ACKNOWLEDGEMENT:

The results of this work were partially presented at the 117th General Meeting of the American Society for Microbiology, New Orleans, Louisiana 3 to 6 June 2017.

This study was supported by Copan Diagnostics Inc. (Murrieta, CA).

REFERENCES:

1. Clinical and Laboratory Standards Institute. 2010. Quality control of microbiological transport systems; approved Standard -second edition. CLSI document M40-A2. CLSI, Wayne, PA.
2. Snyder J.W., Munier G.K., Schiavi C.M., and Johnson C.L. 2010. Abstr. 110th Gen. Meet. Am. Soc. Microbiol., abstr. F-2141. Evaluation of the Copan liquid Amies elution swab (ESwab) for maintaining the viability of selected fungi and *Mycobacterium* spp.
3. Clinical and Laboratory Standards Institute. 2008. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi; approved Standard -second edition. CLSI document M38-A2. CLSI, Wayne, PA.
4. Espinel-Ingroff A, Kerkering T.M. 1991. Spectrophotometric method of inoculum preparation for the in vitro susceptibility testing of filamentous fungi. J Clin Microbiol 29:393-394.

5. Ricardo A, Rodrigues A.G. Pina-Vaz C. 2004. A fast practical and reproducible procedure for the standardization of the cell density of an *Aspergillus* suspension. Journal of Medical Microbiology 53: 783-786.
6. Aberkane M., Cuenca-Esterella A., Petrikkou E., Mellado E., Monzon A., Rodriguez- Tudela J.L., and the Eurofung Network. 2002. Comparative evaluation of two different methods of inoculum preparation for antifungal susceptibility testing of filamentous fungi. Journal of Antimicrobial Chemotherapy 50: 719-722.
7. Caligore-Gei P.F. and Valdez J.G. 2015. Adjustment of a rapid method for quantification of *Fusarium* Spp. Spore suspensions in plant pathology. Rev Argent Microbiol. 47 (2): 152-154.
8. Petrikkou E., Rodriguez-Tudela J.L., Cuenca-Estrella M., Gomez A., Molleja A. and Mellado E. 2001. Inoculum standardization for antifungal susceptibility testing of filamentous fungi pathogenic for humans. J Clin Microbiol 39 (4): 1345-1347.

Table 1. Recovery of pathogenic fungi after 24 and 48hours in CFU /100 µL. Average of triplicate tests.

	Concentration: 10^5 CFU/ml					Concentration: 10^4 CFU/ml					Concentration: 10^3 CFU/ml				
	0 Hour	24 Hour		48 Hour		0 Hour	24 Hour		48 Hour		0 Hour	24 Hour		48 Hour	
Organism	RT	4`C	RT	4`C	RT	RT	4`C	RT	4`C	RT	RT	4`C	RT	4`C	RT
Yeasts															
Candida albicans	723	842	822	>500	>500	171	133	283	152	>500	23	20	31	17	>500
Candida krusei	>500	>500	>500	>500	>500	297	208	>500	265	>500	34	34	295	22	>500
Candida guilliermondii	>500	>500	>500	>500	>500	456	387	>500	307	>500	98	67	294	75	>500
Candida glabrata	>500	>500	>500	466	>500	320	152	314	236	>500	38	25	79	31	321
Cryptococcus neoformans	>500	>500	>500	450	>500	207	241	309	306	>500	33	44	72	37	315
Dermatophytes															
Trichophyton mentagrophytes	332	375	378	405	401	61	28	26	20	33	8	7	6	9	11
Trichophyton tonsurans	441	>500	>500	399	375	39	37	32	41	34	4	3	4	2	2
Trichophyton rubrum	>500	355	333	346	431	229	43	35	20	49	14	8	5	4	19
Hyaline/Saprobes															
Aspergillus niger	388	>500	>500	>500	>500	80	81	112	92	80	13	11	12	13	12
Lecythophora sp	400	>500	>500	360	480	100	100	46	62	81	8	9	5	10	9
Fusarium solani	>500	>500	>500	>500	>500	269	260	249	269	274	32	25	34	29	32
Trichosporon sp	134	232	100	138	143	31	27	28	31	33	11	5	4	5	6
Aspergillus fumigatus	>500	>500	>500	>500	>500	260	197	239	264	213	15	13	19	14	13
Zygomycetes															
Lichtheimia corymbifera	62	61	78	63	75	12	7	7	12	7	1	2	1	1	0
Mucor circinelloides	112	120	100	46	41	14	25	15	20	6	3	2	3	3	0
Rhizopus microsporus	160	NC	NC	NC	NC	25	NC	NC	36	NC	2	3	2	2	2
Dermatiaceous															
Curvularia clavata	74	67	80	81	45	18	14	15	13	10	2	1	2	1	1
Phialophora americana	>500	>500	450	470	321	106	98	77	102	54	16	14	6	7	6
Alternaria alternata	>500	>500	>500	>500	>500	230	305	300	187	199	16	24	28	25	18

NC= not countable, RT= ambient temperature, 4°C = refrigerator temperature.