Published in final edited form as:

Diagn Microbiol Infect Dis. 2018 June ; 91(2): 130–135. doi:10.1016/j.diagmicrobio.2018.01.015.

Rapid One-Step Extraction Method for the Identification of Molds using MALDI-TOF MS

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Abstract

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) has revolutionized fungal identification. Previously, we developed a MALDI-TOF MS mold extraction procedure and comprehensive database. While MALDI-TOF MS has become routine in a few laboratories, it has not yet become widespread. A major obstacle is the lack of a simple, reproducible and uniform protein extraction procedure. In this study, we developed and validated a rapid one-step protein extraction protocol for filamentous fungi. Excised molds were placed into tubes containing zirconia-silica beads and extraction solution without washing or ethanol inactivation steps. Extraction solutions containing different ratios of acetonitrile and formic acid were evaluated. Samples were then processed using a PowerLyzer high power bead based homogenizer and supernatants spotted for MALDI-TOF MS. The rapid method was evaluated prospectively and in parallel to our current mold extraction protocol for 3 months. Analysis of 106 clinical mold isolates resulted in an improved performance and a decrease in extraction time by 30 minutes to a total of 5 minutes of hands-on time. Acceptable identification scores (≥ 2.00) were achieved for up to 63.0% of mold isolates by the rapid method compared with 52.8% of isolates by the current routine protocol. Score comparisons between duplicate spots showed higher reproducibility of the rapid method as compared to the routine method. The rapid extraction method allows efficient analysis of clinical mold isolates both in scheduled batch runs and on an in-demand basis while providing a simple starting platform for laboratories adopting MALDI-TOF MS for mold identification.

Keywords

MALDI-TOF MS; clinical mycology; mold identification; rapid diagnostic methods

1. Introduction

Classic identification of filamentous fungi is time-consuming and requires extensive training and expertise in the recognition of macro and microscopic features (1). However,

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⁶Conflicts of Interest
None.

morphologically similar molds are difficult to discriminate even by experienced microbiologists. Moreover, non-sporulating molds cannot be identified morphologically at all. Molecular methods, such as gene sequencing, are becoming valuable tools for mold identification but may be time consuming and relatively expensive. Matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been increasingly used for bacterial and yeast identification (2). Several laboratories, including our own at the National Institutes of Health (NIH) Clinical Center, have adopted MALDI-TOF MS for the identification of molds. In addition, we and others have evaluated commercial mold databases and also developed databases of spectra from clinical mold isolates (3–17). However, the use of MALDI-TOF MS for the identification of filamentous fungi has not yet gained similar traction. This may be due to a number of factors, including the lack of a comprehensive database and technical challenges in the extraction step, leading to the lack of a universal quick, simple and reproducible procedure for protein extraction (5, 6, 18).

Many of the studies that examined MALDI-TOF MS for the identification of molds used different extraction protocols, including commercially-developed and laboratory-developed procedures (4, 5, 7–9, 13, 18, 19). After growth, mold samples are inactivated via immersion in ethanol prior to analysis to prevent potential laboratory contamination. Following inactivation, a chemical solution is applied to extract the fungal proteins. To aid in the extraction process, manual and/or mechanical disruption of the fungal cell wall may be performed. Extraction protocols may involve multiple steps (i.e. wash, inactivation, sequential extraction with solvents) and require anywhere from minutes (9) to over an hour to perform (5, 6, 13) before the sample can be analyzed via MALDI-TOF MS. Due to the time burden of our published extraction procedure (requiring a minimum of 35 minutes per clinical isolate, with ~1 min added for each additional sample), mold identification by MALDI-TOF MS had to be batched and was performed only twice weekly at NIH.

To expedite and simplify the processing procedure, we developed a rapid, one-step extraction procedure for the identification of clinical mold isolates by MALDI-TOF MS. Analysis of clinical mold isolates found that the rapid extraction method reduced the 35 minutes per isolate processing time to only 5 minutes per isolate, with only a few seconds more for each additional sample. In addition, parallel analysis of 106 clinical isolates showed an increase in acceptable identification scores by the rapid method compared to the routine method. Reproducibility, which we defined as duplicate spots from the same extraction solution scoring within the same score threshold, was also higher with isolates extracted by the rapid method compared to isolates extracted by the routine method. Overall, the rapid method showed better performance and has replaced the routine method for extracting mold proteins for identification by MALDI-TOF MS at the NIH Microbiology Service.

(Part of this work was presented at the 1st ASM Microbe meeting of the American Society for Microbiology, Boston, MA, 16 to 20 June 2016.)

2. Materials and Methods

2.1 Growth of clinical isolates

A total of 106 clinical mold isolates were tested in parallel with the routine and rapid method over a 3-month period. Specimens from which isolates were grown derived from our patient population including immunocompromised patients (e.g. severe aplastic anemia, prolonged neutropenia following hematopoietic stem cell transplantation, or hereditary immune defects such as chronic granulomatous disease) and those with impaired lung function (e.g. bronchiectasis and cystic fibrosis). The 106 isolates consisted of a total of 22 genera containing a total of 31 different species (Table 1). Of these, 70 (66.0%) were hyaline molds, 21 (19.8%) were dematiaceous molds, 3 (2.8%) were mucorales, 8 (7.5%) were basidiomycetes, 2 (1.8%) were dermatophytes, and 2 (1.8%) were dimorphic molds. Molds grew on average for 1–5 days, and were subcultured on Sabouraud dextrose agar at 27 °C in ambient air as per the standard laboratory procedures. To avoid interference due to primary specimen or bacterial colonies on the plate, most mold isolates were tested upon subculture to a new plate. However, due to clinical urgency in a few patients, four mold isolates were directly excised and extracted from the primary culture plates, which included two samples from inhibitory mold agar (IMA), and one each from chocolate agar and buffered charcoal yeast extract (BCYE) agar, instead of standard subculture and batched extraction and identification. Specimens on IMA were grown at 27 °C in ambient air and specimens on chocolate and BCYE agar were grown at 35 °C in ambient air or 8–10% CO₂.

2.2 Protein extraction

Routine extraction of proteins from clinical mold isolates was performed as previously described and as per laboratory standard procedure (4). Briefly, approximately 5 mm pieces of mold (including the hyphal bed) were removed from the agar and placed into 1.5 mL tubes containing 500 µL of 70% ethanol and 50 µL of silica-zirconia beads. The pieces were briefly ground into the beads and were then vortexed for 15 minutes, followed by centrifugation for 2 minutes at 13,000 rpm. The ethanol was then removed, 50 µL of 70% formic acid was added, and the tubes were vortexed for 5 minutes. Samples were spun briefly, 50 µL of acetonitrile was added, and the tubes were again vortexed for 5 minutes followed by a centrifugation at 13,000 rpm for 2 minutes.

Rapid extraction of proteins from clinical mold isolates was performed in parallel using the same cultures as the routine method. Two different rapid extraction solutions were tested. Approximately 5 mm pieces of mold (including the hyphal bed) were removed from the agar and placed into a 1.5 mL tube containing 50 µL of silica-zirconia beads and 100 µL of extraction solution A or B. Solution A consisted of acetonitrile (50%), 70% formic acid (35%) and water (15%). Solution B consisted of acetonitrile (50%) and 70% formic acid (50%). Mold pieces were briefly ground into the beads following excision. Tubes were then placed in a PowerLyzer24 (MoBio Laboratories, Inc.) bead-based homogenizer for protein extraction. Two cycles of 45 seconds at 4,000 rpm at room temperature were performed, with a 30 second pause between cycles. Samples were centrifuged for 1 minute at 13,000 rpm to pellet the solid material. Immersion of mold pieces in either extraction solution A or B for 5 minutes even without bead-beating rendered sample isolates non-viable as tested

upon subculture into both tryptic soy broth and on Sabouraud dextrose agar plates (data not shown). If not analyzed immediately after processing, protein lysates from both the rapid and routine methods were stored at -20°C .

2.3 MALDI-TOF MS and spectral analysis

Identification of clinical isolates was performed as published previously (4). One microliter of extracted supernatant was spotted in duplicate on MALDI-TOF BigAnchor target plates and was dried on a slide warmer at 42°C . In addition, $1\text{ }\mu\text{L}$ of calibration standard solution was spotted onto the plate. Dried sample and calibration solution spots were overlaid with $2\text{ }\mu\text{L}$ of matrix solution (50% acetonitrile and 2.5% trifluoroacetic acid solution saturated with α -cyano-4-hydroxycinnamic acid) and allowed to dry. Target plates were inserted into the MALDI-TOF Microflex LT mass spectrometer (Bruker Daltonics, Inc) and each sample spot was analyzed using 250 laser shots at 60 Hz. Shots were measured in groups of 50 (per sampling area). Spectra were acquired over a mass/charge (m/z) ratio that ranged from 2,000 to 20,000.

Spectra were analyzed by the BioTyper program (version 3.1; Bruker Daltonics, Inc) utilizing both the Bruker database and a previously created NIH database. A logarithmic score cut-off of ≥ 2.00 for acceptable clinical identification was applied (4). *Aspergillus ustus* (CBS 261.67T) was included as a positive quality control for all experiments, and required a log score ≥ 2.00 for results to be acceptable. Identification results were reported at genus, complex or species level following laboratory protocols.

2.4 Genomic sequencing of mold isolates

Isolates that scored below 2.00 on repeat testing with MALDI-TOF MS were identified by sequencing as per standard laboratory protocol. Briefly, primers complementary to the internal transcribed spacer (ITS) regions 1 and 4 were used to amplify DNA with a Big Dye Terminator X kit (Life Technologies). Additional targets were used as necessary. Sequencing was performed on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) and the resulting sequences were analyzed for quality and compared against the GenBank database and the MycoBank database through Nucleotide BLAST and MycoBank queries, respectively.

2.5 Statistical analysis

Scores generated by the routine method were compared against those generated either by the rapid method with solution A or with solution B via a paired t-test using GraphPad Prism software. This method was also used to compare scores generated by solution A versus those generated by solution B. Differences were considered statistically significant if the P value was less than 0.05.

3. Results

During the study period, 106 clinical mold isolates were analyzed by the routine method, the rapid method using solution A (50% acetonitrile, 35% (70%) formic acid, 15% water), and the rapid method using solution B (50% acetonitrile, 50% (70%) formic acid). Results for

each method were categorized by logarithmic score cut-offs assigned by the Bruker BioTyper program using a database composed from both NIH and Bruker spectra (4). Current practice by the NIH Microbiology Service dictates that a score of 2.00 is required for reporting MALDI-TOF MS results to the species or genus level, depending on the organism. For this study, isolates were sorted into three different score thresholds: scores 2.00, scores between 1.999 and 1.700, and scores 1.699. Using these criteria, 57 (53.7%) of the clinical isolates achieved scores at the reporting level (2.00) through use of the routine method. In contrast, using the rapid method produced reporting level scores for 61 (57.5%) isolates with solution A and 68 (64.1%) isolates with solution B. There were 26 (24.5%), 18 (16.9%) and 20 (18.9%) isolates by the routine method, rapid method with solution A, and rapid method with solution B, respectively, that achieved scores between 1.999 and 1.700. Scores less than 1.699 were produced by 23 (21.6%) isolates with the routine method, 27 (25.4%) isolates by the rapid method with solution A, and only 18 (16.9%) isolates by the rapid method with solution B. Duplicate spots from each isolate produced scores within the same score threshold for 81 (75.0%) isolates by the routine method, 91 (84.3%) isolates by the rapid method with solution A, and 86 (79.6%) isolates by the rapid method with solution B (data not shown).

Spectra generated from all three extraction methods were analyzed using only the Bruker supplied database to determine whether the rapid extractions allowed for identifications without a user-supplemented database. Following analysis, 3 (2.8%) isolates by the routine method and 1 (0.94%) isolate each by the rapid methods was identified with a score 2.00 (data not shown). Of these isolates, four consisted of *A. fumigatus* isolates (routine method and rapid method with solution A) and one isolate was an *Arthrographis* species (rapid method with solution B). Scores between 1.99 and 1.70 were obtained for 19 (17.9%), 13 (12.3%), and 15 (14.2%) isolates by the routine, rapid method with solution A, and rapid method with solution B, respectively (data not shown). Of these isolates, *A. fumigatus* represented all but two, which were *A. niger* and *Arthrographis* species scored in this range by use of the rapid method with solution B.

Clinical mold isolates were next grouped by type (hyaline molds, dematiaceous molds, dermatophytes, mucorales, basidiomycetes, or dimorphic (Table 1)) and scores were evaluated for each group (Table 2). The majority of the 106 clinical isolates were hyaline molds [70 (66.0%)], with dematiaceous [21 (19.8%)] the only other mold group with greater than ten isolates, dermatophytes 2 (1.8%), dimorphic, 2 (1.8%); mucorales, 3 (2.8%); basidiomycetes, 8 (7.5%). Overall, the rapid method with solution B identified isolates from each mold type to the clinically reportable level (score 2.00) equal to or better than the routine method (Table 2). One exception was one *Trichophyton rubrum* isolate that resulted in scores less than 2.00 by the rapid method with solution B, but greater than 2.00 by the routine method. Compared to the rapid method with solution A, the rapid method with solution B was superior for hyaline, mucorales, and basidiomycetes molds, while equal for dematiaceous and dermatophyte molds (Table 2).

The most common molds to be isolated during the study period were in the genus *Aspergillus* with 53 (50.0 %) of 106 isolates (Table 1). The range and mean scores for each species or genus that had at least two clinical isolates is shown in Figure 1. Overall, the rapid

method with solution B achieved higher maximum scores for the *Aspergillus* species with the exceptions of *A. sydowii* (Figure 1A). In addition, the mean score was higher by the rapid method with either solution for *A. flavus*, *A. fumigatus*, *A. nidulans*, and *A. ustus*, approximately equal for *A. niger*, and lower than the routine method for *A. sydowii*. Statistically higher mean scores were obtained by the rapid method with solution B for *A. flavus* and *A. fumigatus* as compared to the routine method. The rapid method with solution A showed inferior performance for *A. fumigatus* and *A. sydowii* as compared to solution B and/or the routine method.

The range of scores produced for the molds other than *Aspergillus* was similarly analyzed (Figure 1B). As observed for the *Aspergillus* species, the rapid method generally achieved higher maximum scores and higher mean scores with these isolates. Statistically higher mean scores were obtained by the rapid method with solution B as compared to the routine method for *Coccidioides immitis-posadasii*, *Verruconis gallopava*, *Fusarium solani*, *Irpex* species, and *Rhizopus* species. The rapid method with solution A produced mean scores comparable or lower than those produced by solution B.

A few isolates resulted in no identification (scores <2.00) by any of the MALDI-TOF extraction methods. These included 6 of the 7 *Penicillium* species, one dematiaceous *Scopulariopsis* species that could only be identified as “most closely related to *Microascus cinereus* and *Scopulariopsis gracilis*”, the basidiomycete *Volvariella volvacea*, 1 of the 2 *Arthrographis* species isolates, all 3 of the *Cladosporium* species isolates, 4 of the 7 *Fusarium solani* isolates, 1 of the 2 *Rhizopus* species isolates, and some of the *Aspergillus* isolates (2 *A. flavus*, 1 *A. nidulans*, 1 *A. niger*, 1 *A. sydowii*) (data not shown). Several of these species are absent or underrepresented in our composite MALDI-TOF database and were identified by morphology and/or ITS sequencing. Lowering the acceptable cut off score from >2.00 to >1.8 for the abovementioned organisms would have yielded correct identification of an additional *F. solani* and all *A. flavus*, *A. nidulans*, and *A. sydowii*.

4. Discussion

MALDI-TOF MS identification is slowly gaining popularity as a fast and inexpensive method for the routine identification of clinical mold isolates. Unlike bacteria and yeast, which can easily be prepared for analysis in one extraction step (20, 21), the current cell lysis extraction procedures for filamentous fungi require a multitude of washing, inactivation, and chemical extraction steps (5). As observed in our laboratory, a multi-step cell lysis extraction procedure limits the MALDI-TOF MS workflow for molds to a twice-weekly batched schedule. In order to improve our workflow and turn-around-time, we developed a one-step rapid extraction procedure that utilizes a high-speed homogenizer. Prior to this protocol, each isolate of mold required a minimum of 35 minutes of dedicated hands-on time before MALDI-TOF MS analysis. The implementation of the rapid procedure, however, has drastically reduced the hands-on time to 5 minutes. This easy, one-step extraction protocol will allow us to move to on-demand mold identification as they begin to grow in culture rather than waiting for a batched schedule.

Intact cell extraction methods, in which spores and mycelial material are deposited directly on the target plate, offer reduced extraction times (5, 11, 18, 22, 23). Although good results with these extractions have been documented in the literature, cell lysis extractions have become the method of choice for many laboratories that have implemented MALDI-TOF MS for filamentous fungi due to possible difficulties resulting from the more complex fungal cell wall (18). Furthermore, cell lysis extractions are recommended for use with the two currently FDA approved mass spectrometry instruments (Bruker Biotyper (used in this study and laboratory) and Vitek MS), which may result in additional preference for performing this method. Our routine method has some differences when compared to the two-manufacturer's recommended methods. The Bruker method recommends the use of liquid cultures for growth of isolates and does not include beads. It is possible that the discrepancies observed in this study with the Bruker database alone are due to both the construction of this database with molds grown in liquid culture and differences in extraction protocols.

Evaluation of the rapid method not only revealed a decrease in processing time, but also led to more samples achieving clinically acceptable identification scores (≥ 2.00) compared to the routine method. Our data suggests that the extraction solution composition was important, as the rapid method with solution B (rather than solution A) yielded a greater number of isolates with scores ≥ 2.00. The rapid method with solution B also produced similar or higher mean scores when compared to the rapid method with solution A. Furthermore, analysis of scores of duplicate spots from the same isolate protein extract showed that the rapid method with either solution is generally more reproducible than the routine method. Due to the decreased hands-on time, increased isolate identification scores, and increased reproducibility, we believe that the rapid method using extraction solution B is superior to the current routine method. Following completion of this study, we have evaluated a modification of protocol B by increasing the volume of solution B to 200 µL (instead of 100). This larger solvent volume allows for an easier submersion of the excised mold into the solution and minimizes solvent loss while having no significant impact on scores.

Although increased identification numbers were observed when the rapid method with either solution was used, the percent of isolates with scores ≥ 2.00 as generated by all methods was lower than what was previously reported when the routine method was originally developed (4). There are several possibilities for why the lower amount of identifications occurred. First, it is plausible that person-to-person variability can occur in each step of the extraction process. To mitigate variability, the routine procedure was performed by trained clinical laboratory technologists as part of standard patient care; the rapid procedures were performed on the same mold isolates by the first author of this study. Second, since many of the same mold taxa included in this study were also included in the database generation and challenge study, it is possible that specific strain types included in this study had some differences in the spectra produced (compared to conspecific entries in the database).

This study provided a snapshot of how the rapid extraction method performs prospectively in real time at our institution. Over half of the data in this study is composed of *Aspergillus* species, which, along with *Fusarium* species and *Scedosporium apiospermum*, constitute the

most common molds isolated from our largely immunocompromised and/or impaired lung function patient population. In contrast, this same patient population resulted in the testing of only two dermatophytes, which are very commonly isolated in other clinical microbiology laboratories. Additional testing of molds with limited representation in this study, such as the dermatophytes, may be warranted to further evaluate the rapid method with each mold group.

Some of the isolates included in the study would be considered very uncommon taxa in most clinical microbiology laboratories (i.e. the basidiomycete *Volvariella volvacea* or a dematiaceous *Scopulariopsis* species that could only be identified as “closely related to *Microascus cinereus* and *Scopulariopsis gracilis*”) and thus would not be likely to be included in either commercial- or laboratory-developed databases. However, they do represent the diversity of fungi in our institution. As such, genomic sequencing remains an important tool to speciate isolates that could not be identified by MALDI-TOF MS. Continuous updates to MALDI-TOF MS databases are necessary to reduce the number of organisms that require sequencing while increasing the identification percentage of common clinical isolates.

In summary, we developed a rapid, one-step method for extracting proteins from clinical mold isolates that not only decreases processing time but also improves identification scores and reproducibility over the previously used method. We believe the implementation of this procedure will improve laboratory workflow and allow for fast, on-demand identification of fungal isolates when clinically indicated without the need for batching. Furthermore, the ease and simplicity of this method presents an attractive starting point for those laboratories adopting or considering fungal identification by MALDI-TOF MS.

5. Acknowledgements

We would like to thank Christina Henderson and the mycology technologists of the Microbiology Service at the NIH Clinical Center for their help in culturing the clinical isolates used in this study. We would also like to thank Frida Stock and Leslie Calhoun for performing the routine mold protein extraction method. This research was supported by the Intramural Research Program of the NIH Clinical Center, Department of Laboratory Medicine.

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A

B

Figure 1.

Average MALDI-TOF MS score by species or genus-level and by method performed. The average MALDI-TOF MS score for each species or genus-level identification from this study as achieved by the routine (empty boxes), rapid with solution A (dotted boxes), and rapid with solution B (crossed boxes) methods are indicated. Horizontal bars represent the mean score achieved by each method for each identification. Boxes represent the range of scores achieved by all duplicate spots from isolates of the designated species or genus, with the bottom of the box representing the minimum score and the top of the box representing the maximum score. Species that were isolated at least twice from individual clinical samples representing the *Aspergillus* species (A) or all other molds from this study (B) are

shown. Statistically significant differences of mean scores between the routine method and either rapid method (bracket and *) are indicated. Statistically significant differences between the two rapid methods themselves (**) are also indicated. Statistical analysis was performed by a paired t-test and statistical significance was defined by p values less than 0.05.

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Table 1.

Number and Identification of Clinical Mold Isolates

Group	Isolate Identification (n)
Hyaline	
	<i>Arthrographis</i> species (2)
	<i>Aspergillus flavus</i> (6)
	<i>Aspergillus fumigatus</i> (27)
	<i>Aspergillus nidulans</i> (2)
	<i>Aspergillus niger</i> (6)
	<i>Aspergillus ochraceus</i> (1)
	<i>Aspergillus sydowii</i> (3)
	<i>Aspergillus terreus</i> (1)
	<i>Aspergillus unguis</i> (1)
	<i>Aspergillus ustus</i> (4)
	<i>Aspergillus versicolor</i> (1)
	<i>Aspergillus westerdjijkiae</i> (1)
	<i>Fusarium incarnatum-equiseti</i> species complex (1)
	<i>Fusarium solani</i> (7)
	<i>Penicillium</i> species (7)
Dematiaceous	
	<i>Alternaria</i> species (1)
	<i>Cladosporium</i> species (3)
	<i>Curvularia</i> species (2)
	<i>Verruconis gallopava</i> (2)
	Dematiaceous <i>Scopulariopsis</i> species (1)
	<i>Scedosporium apiospermum</i> (12)
Dermatophytes	
	<i>Microsporum</i> species (1)
	<i>Trichophyton rubrum</i> (1)
Mucorales	
	<i>Mucor</i> species (1)
	<i>Rhizopus</i> species (2)
Basidiomycetes	
	<i>Basidiomycete</i> , no further identification ^a (2)
	<i>Eutypella</i> species (1)
	<i>Irpex</i> species (4)
	<i>Volvariella volvacea</i> (1)
Dimorphic	
	<i>Coccidioides immitis-posadasii</i> (2)
	Total (106)

^aNo further identification as per NIH mycology lab standard operating procedures for those specimens.

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Table 2.

Performance of Routine and Rapid MALDI-TOF MS Extraction Methods for Identification of Molds by Group

Method	No. of Isolates (%) by Indicated Method and Score			
	Mold Group (n)	2.00	1.99–1.70	1.69
Routine	Hyaline (70)	39 (55.7)	20 (28.5)	11 (15.7)
	Dematiaceous (21)	14 (66.7)	2 (9.52)	5 (23.8)
	Dermatophytes (2)	2 (100.0)	0 (0.0)	0 (0.0)
	Mucorales (3)	0 (0.0)	2 (66.7)	1 (33.3)
	Basidiomycetes (8)	1 (12.5)	1 (12.5)	6 (75.0)
	Dimorphic (2)	1 (50.0)	1 (50.0)	0 (0.0)
Rapid A	Hyaline (70)	38 (54.3)	12 (17.1)	20 (28.5)
	Dematiaceous (21)	12 (57.1)	4 (19.0)	5 (23.8)
	Dermatophytes (2)	1 (50.0)	1 (50.0)	0 (0.0)
	Mucorales (3)	1 (33.3)	1 (33.3)	1 (33.3)
	Basidiomycetes (8)	7 (87.5)	0 (0.0)	1 (12.5)
	Dimorphic (2)	2 (100.0)	0 (0.0)	0 (0.0)
Rapid B	Hyaline (70)	43 (61.4)	15 (21.4)	12 (17.5)
	Dematiaceous (21)	14 (66.7)	2 (9.52)	5 (23.8)
	Dermatophytes (2)	1 (50.0)	1 (50.0)	0 (0.0)
	Mucorales (3)	2 (66.7)	1 (33.3)	0 (0.0)
	Basidiomycetes (8)	6 (75.0)	1 (12.5)	1 (12.5)
	Dimorphic (2)	2 (100.0)	0 (0.0)	0 (0.0)