ORIGINAL ARTICLE MYCOLOGY

Improved clinical laboratory identification of human pathogenic yeasts by matrix-assisted laser desorption ionization time-of-flight mass spectrometry

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Abstract

The key to therapeutic success with yeast infections is an early onset of antifungal treatment with an appropriate drug regimen. To do this, yeast species identification is necessary, but conventional biochemical and morphological approaches are time-consuming. The recent arrival of biophysical methods, such as matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), in routine diagnostic laboratories holds the promise of significantly speeding up this process. In this study, two commercially available MALDI-TOF MS species identification systems were evaluated for application in clinical diagnostics, using a geographically diverse collection of 1192 clinical yeast and yeast-like isolates. The results were compared with those of the classical differentiation scheme based on microscopic and biochemical characteristics. For 95.1% of the isolates, all three procedures consistently gave the correct species identification, but the rate of misclassification was greatly reduced in both MALDI-TOF MS systems. Furthermore, several closely related species (e.g. *Candida orthopsilosis/metapsilosis/parapsilosis* or *Candida glabrata/bracarensis*) could be resolved by both MALDI-TOF MS systems, but not by the biochemical approach. A significant advantage of MALDI-TOF MS over biochemistry in the recognition of isolates novel to the system was observed. Although both MALDI-TOF MS systems employed different approaches in the database structure and showed different susceptibilities to errors in database entries, these were negligible in terms of clinical usefulness. The time-saving benefit of MALDI-TOF MS over biochemical identification will substantially improve fungal diagnostics and patient treatment.

Keywords: Biotyper 2.0, human pathogenic fungi, MALDI-TOF MS, Saramis, yeast identification
Original Submission: 1 July 2010; **Revised Submission:** 30 September 2010; **Accepted:** 30 September 2010
Editor: G. Greub
Article published online: 14 October 2010
Clin Microbiol Infect 2011; **17:** 1359–1365
10.1111/j.1469-0691.2010.03398.x

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Introduction

Yeast infections are a significant cause of morbidity and mortality in critically ill patients, e.g. those undergoing immunosuppressive therapy, those recovering from surgery, or in those infected by human imunodeficiency virus. Early therapeutic intervention is critical for successful treatment of yeast infections [1–3], and the optimal choice of antifungal drugs will ultimately be based on two key factors: (i) the fungal species and its intrinsic resistance; and (ii) the result of the *in vitro* resistance testing of the individual isolate [4].

The *Atlas of Clinical Fungi* lists approximately 400 fungal species with clinical relevance and human pathogenic potency [5], causing a wide range of clinical symptoms, ranging from local inflammation to life-threatening disseminated disease. Unfortunately, conventional laboratory differentiation of yeasts, involving, for example, microscopy and biochemical tests, not only requires up to several days, but is also cost-intensive and requires extensively trained laboratory personnel. At present, in those cases where classical methods give unclear results, the isolates need to be re-analysed by sequencing of species-specific regions. This is especially true for rare, potentially emerging yeast pathogens that are not identifiable by standard tests. Faster

TABLE 1. Species distribution within the test set

Species	No. of isolates	Biochemical	Biotyper	Saramis
Blastoschizomyces capitatus (*Geotrichum capitatum*)	3	Y	N	N
Candida albicans	512	Y	Y	Y
Candida bracarensis	1	N	N	N
Candida dubliniensis	8	Y	Y	Y
Candida glabrata	272	Y	Y	Y
Candida metapsilosis	1	N	Y	N
Candida orthopsilosis	8	N	Y	N
Candida parapsilosis	105	Y	Y	Y
Candida pararugosa	3	N	N	N
Candida rugosa	8	Y	Y	N
Candida tropicalis	88	Y	Y	Y
Candida viswanatii	1	N	N	N
Clavispora lusitaniae (*Candida lusitaniae*)	14	Y	Y	Y
Cryptococcus neoformans	7	Y	Y	Y
Galactomyces geotrichum (*Geotrichum candidum*)	1	N[a]	N	Y
Geotrichum clavatum	3	N[a]	N	N
Issatchenkia orientalis (*Candida krusei*)	53	Y	Y	Y
Kluyveromyces marxianus (*Candida kefyr*)	21	Y	Y	Y
Kodamaea ohmeri (*Candida guilliermondii* var. *membranaefaciens*)	2	Y	Y	N
Pichia anomala (*Candida pelliculosa*)	3	Y	Y	Y
Pichia cactophila (*Candida inconspicua*)	7	Y	Y	Y
Pichia fabianii (*Candida fabianii*)	7	N	N	N
Pichia farinosa (*Candida cacaoi*)	2	Y	Y	Y
Pichia fermentans (*Candida lambica*)	1	Y	Y	Y
Pichia guilliermondii (*C. guilliermondii* var. *guilliermondii*)	23	Y	Y	Y
Pichia jadinii (*Candida utilis*)	1	Y	Y	Y
Pichia membranifaciens (*Candida valida*)	1	Y	Y	Y
Pichia norvegica (*Candida norvegensis*)	5	Y	Y	Y
Rhodotorula mucilaginosa	2	Y	Y	Y
Saccharomyces cerevisiae	20	Y	Y	Y
Trichosporon asahii	4	Y	Y	Y
Yarrowia lipolytica (*Candida lipolytica*)	1	Y	Y	Y
Uncharacterized species	4	N	N	N

Y, represented in the database; N, not represented in the database.
A total of 1192 clinical yeast isolates across the fungal phylum representing 32 known and four uncharacterized species were represented in the test set. Teleomorph–anamorph relationships and conspecific species, as described by deHoog [5] and others [29,30], necessary to resolve ambiguous nomenclature between the databases used in this study are given in parentheses.
[a]Genus level only.

species identification, with rapid determination of the particular species-specific intrinsic resistance, would be an important step forwards in the successful management of life-threatening fungal diseases.

Recently, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been successfully introduced for rapid species identification of microorganisms in the clinical laboratory. With this method, crude cell extracts can be used to identify the species of a given isolate by comparison of the mass patterns within approximately 2–20 kDa with a database containing the patterns of reference strains. These patterns are highly species-specific [6–8], and may even allow subspecies identification [9]. As this process takes only minutes, the introduction of MALDI-TOF MS into diagnostic laboratories holds the promise of significantly speeding up diagnostic processes while simultaneously leading to more accurate identification of pathogens [10,11]. Experimental data also indicate that MALDI-TOF MS can increase the resolution at which fungal species, such as different moulds [12–15] and yeasts [9,16], can be differentiated from each other. Therefore, in the clinical laboratory, MALDI-TOF MS-based differentiation could substantially improve the quality of and reduce the time needed for the identification of yeasts.

To evaluate the clinical use of the two currently commercially available MALDI-TOF MS systems (MALDI BioTyper2 (Bruker Daltonics, Bremen, Germany) and Saramis (AnagnosTec, Potsdam, Germany)) in yeast identification, a collection of 1192 clinically relevant yeast and yeast-like isolates was established (Table 1), reflecting a species distribution as it is encountered during clinical routine. Both mass spectrometry systems were compared with each other and with the classical approach for fungal species identification in diagnostic laboratories.

Materials and Methods

Cultivation of fungi

Yeasts were kept either as cryobank stocks (Mast Diagnostica, Reinfeld, Germany) or as snap-frozen liquid YPD-glycerol stocks (1% yeast extract, 2% peptone, 1% glucose, 25%

glycerol), stored at −70°C. Once thawed, strains were kept on agar slants (0.5% peptone (casein), 0.5% peptone (meat), 2% glucose, 2% agar) and re-streaked every 2 weeks. Prior to any of the experiments, strains were cultivated on Sabouraud agar plates (Oxoid, Wesel, Germany) overnight at 35°C or 30°C, as appropriate. All chemicals were from Roth (Karlsruhe, Germany), and media components were from BD (Heidelberg, Germany).

Strains used in this study

First, we collected all yeast and yeast-like isolates identified as human pathogens during routine in-house diagnosis from October 2008 to March 2009. In addition to these 324 isolates, predominantly obtained from primary sterile material, 310 strains were added from a survey in Sarh, Chad (Taverne-Ghadwal *et al.*, unpublished data). To evaluate the performance of the three methods in the correct identification of less frequent yeasts, we included 275 non-*Candida albicans* and non-*Candida glabrata* strains isolated from our MykoLabNet-D strain collection [17]. Also, 22 rare yeast strains kindly provided by F. Odds (Aberdeen, UK) were added. To further reduce potential bias in the strain set, we included 261 previously multilocus sequence-typed *C. albicans* and *C. glabrata* isolates obtained from different European laboratories (Bader, unpublished data). The collection is summarized in Table 1.

Identification procedure

The entire set was tested blindly and independently with all three procedures, as described below. Species identification was performed by starting with colonies from Sabouraud agar plates, as recommended by the manufacturers. All identifications with results below the defined thresholds were repeated, and if the species was still undetermined, it was classified as 'unknown'. In cases where the species identification did not match in all three test systems, sequencing of the ITS2 region [18] was used. To ensure the correctness of the concordant identifications, where available, two random isolates of each species were subjected to sequencing. No false identifications were found in this set (data not shown).

Biochemical fungal differentiation scheme

Fungi were differentiated by the combined use of microscopy and carbon assimilation testing, using the API 20 C AUX and ID 32 C systems (bioMérieux, Nürtingen, Germany). In detail, *C. albicans* and *Candida dubliniensis* were identified by formation of chlamydospores on rice agar (Oxoid) and on Staib agar (5% pulverized *Guizotia abyssinicia* seed, 0.1% glucose, 0.1% KH_2PO_4, 0.1% creatinine, 1.5% agar) [19,20]. All isolates other than *C. albicans* were further analysed with the ID 32 C (V3.0), and in some cases (e.g. to resolve *Pichia norvegica* and *Pichia cactophila*) additionally with the API 20 C AUX (V4.0).

MALDI-TOF MS yeast identification with the Bruker MALDI Biotyper 2.0 system

For yeast identification with the MALDI BioTyper 2.0 system (Bruker Daltonics), cells of approximately five colonies from Sabouraud agar plates were suspended in 300 μL of water and inactivated by addition of 900 μL of 96% ethanol [9]. The cells were spun down, and the pellet was air-dried at room temperature, resuspended in 50 μL of 70% formic acid, and extracted by addition of an equal volume of acetonitrile and thorough mixing. Cellular debris was removed by centrifugation (17 000 × ***g*** for 2 min), and 1 μL of the clear supernatant was spotted onto a polished steel carrier, allowed to dry, overlaid with 1 μL of HCCA matrix (saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 2.5% trifluoroacetic acid; Bruker Daltonics) and allowed to dry again.

Measurement was performed with the MALDI BioTyper 2.0 (library version 3.0) and FlexControl software on a Microflex LT20 mass spectrometer (20-Hz nitrogen laser), using a bacterial test standard (Bruker Daltonics) as a molecular mass standard. Spectra were detected in positive linear mode, with a mass range of 2–20 kDa. The intensity of the laser was controlled by the FlexControl software driven in automatic mode, at the settings recommended by the manufacturer. Only species identifications with scores >2.000 were accepted, and proposed identifications at the genus level only were rejected.

MALDI-TOF MS yeast identification with the AnagnosTec Saramis system

For yeast identification with the Saramis system (Spectral Archive and Microbial Identification System; AnagnosTec), cells from a single colony on a Sabouraud agar plate were directly applied onto the steel carrier, dried for a short time (approximately 2 min) and lysed by suspension in 0.5 μL of 25% formic acid. The sample was allowed to air-dry at room temperature, overlaid with 1 μL of HCCA matrix (saturated solution of α-cyano-4-hydroxycinnamic acid in acetonitrile/ethanol/water 1 : 1 : 1 acidified with 3% v/v trifluoroacetic acid) (AnagnosTec) and again allowed to air-dry.

Measurement was performed on an AXIMA Assurance platform (Shimadzu Biotech, Duisburg, Germany) in positive linear mode, with a mass range of 2–20 kDa, using *Escherichia coli* strain CCUG 10979 as a molecular mass standard. The intensity of the 50-Hz nitrogen laser was under the control of the acquisition software, at the settings recommended by

the manufacturer. Only hits within the Superspectra database (Saramis Premium, version 3.3.1) with scores >80% were accepted, and identifications proposed from the single-spectrum database were excluded.

rDNA sequencing

For sequencing of the ITS2 rDNA region, fungal DNA from a single large colony was isolated with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The ITS2 rDNA region was amplified [18], and the product was sequenced (SeqLab, Göttingen, Germany) and identified in the CBS yeast sequence database (http://www.cbs.knaw.nl).

Results

Identification procedure

In total, for the 1192 isolates examined, the overall success rate was >96% for each of the three individual methods (Table 3a). For 1134 isolates, all three procedures led to a correct species identification (95.1%), leaving 58 strains for which at least one of the methods repeatedly gave either a wrong or no identification (Table 2). All isolates of *C. dubliniensis* (*n* = 8), *C. glabrata* (*n* = 272), *Candida tropicalis* (*n* = 88), *Candida parapsilosis* (*n* = 105), *Cryptococcus neoformans* (*n* = 7), *Pichia anomala* (*n* = 5), *P. norvegica* (*n* = 5), *Pichia jadinii* (*n* = 1), *Trichosporon asahii* (*n* = 4), and *Yarrowia lipolytica* (*n* = 1) were recognized correctly by all methods. With our approach, one isolate could not be identified at the species level, and three further isolates showed slight nucleotide differences from other known species and therefore could not be fully classified. They were treated as separate species in the context of this study.

For a more detailed data analysis, the tested isolates were separated into several distinct subsets on the basis of their representation status in the databases of the respective method, e.g. if the species was listed on the code list for API 20 C AUX/ID 32 C identification, or reference spectra (BioTyper 2.0)/'superspectra' (Saramis) were present in the MALDI-TOF MS databases. Consequently, an 'unknown' result could be considered to be a correct identification for isolates not present in a database, and any other identification could be considered to be a false identification for that particular species.

TABLE 2. Isolates with at least one false or missing identification

Species	*n*	Classical ID result	Biotyper 2.0 ID	Saramis ID
Candida albicans	1	missed	✔	✔
Saccharomyces cerevisiae	3	missed	✔	✔
Issatchenkia orientalis	2	missed	✔	✔
Pichia guilliermondii	2	✔	missed	✔
	1	✔	missed	missed
Pichia farinosa	1	✔	✔	missed
Pichia fermentans	1	✔	✔	missed
Pichia membranefaciens	1	✔	✔	missed
Pichia cactophila	2	✔	*Pichia norvegica*	✔
	2	*Pichia norvegica*	*Pichia norvegica*	missed
	1	✔	*Pichia norvegica*	missed
Rhodotorula mucilaginosa	1	✔	*Rhodotorula glutinis*	✔
Candida rugosa	1	✔	missed	unknown[a]
	7	✔	✔	unknown[a]
Kodamaea ohmeri	1	✔	missed	unknown[a]
	1	✔	✔	unknown[a]
Galactomyces geotrichum	1	*Geotrichum* sp.[b]	unknown[a]	✔
Blastoschizomyces capitatus	1	✔	unknown[a]	unknown[a]
Geotrichum clavatum	3	*Geotrichum* sp.[b]	unknown[a]	unknown[a]
Candida metapsilosis	1	*Candida parapsilosis*[a]	✔	*Candida parapsilosis*[a]
Candida orthopsilosis	8	*Candida parapsilosis*[a]	✔	unknown[a]
Candida pararugosa	1	*Candida boidinii*[a]	✔	unknown[a]
	2	*Candida rugosa*[a]	✔	unknown[a]
Pichia fabianii	1	*Candida glabrata*[a]	unknown[a]	unknown[a]
	2	*Candida albicans*[a]	unknown[a]	unknown[a]
	2	*Pichia anomala*[a]	unknown[a]	unknown[a]
	2	*Pichia jadinii*[a]	unknown[a]	unknown[a]
Candida bracarensis	1	*Candida glabrata*[a]	unknown[a]	unknown[a]
Candida viswanathii	1	*Candida tropicalis*[a]	unknown[a]	unknown[a]
Uncharacterized basidiomycete	1	*Cryptococcus humiculus*[a]	unknown[a]	unknown[a]
Candida orthopsilosis-like	1	*Candida parapsilosis*[a]	unknown[a]	unknown[a]
Issatchenkia orientalis-like	1	*Pichia cactophila*[a]	*Issatchenkia orientalis*[a]	*Issatchenkia orientalis*[a]
Candida glabrata-like	1	unknown[a]	*Candida glabrata*[a]	unknown[a]

✔, isolates correctly identified; 'missed', isolates contained in the database but not recognized; 'unknown', isolates not recognized and not in the database. Species names indicate misidentifications.
[a]Not in database.
[b]Genus level only.

TABLE 3. Frequencies of classification, misclassification and non-recognition

	Subset description			Classification results			
	Number of species	Number of isolates	% of total isolates	Correctly identified	Isolates misclassified	Unknown	Success rate (%)
(a) Performance, total							
Classical	36	1192	100.0	1155	30	7	96.9
Biotyper 2.0	36	1192	100.0	1163	8	21	97.6
Saramis	36	1192	100.0	1146	2	44	96.1
(b) Performance only on species in respective method database							
Classical	25	1163	97.4	1155	2	6	99.3
Biotyper 2.0	27	1175	98.4	1163	6	5	99.0
Saramis	22	1152	96.6	1145	0	7	99.4
(c) Performance only on species contained in all three databases							
Classical	21	1148	96.5	1140	2	6	99.3
Biotyper 2.0	21	1148	96.5	1138	6	4	99.1
Saramis	21	1148	96.5	1141	0	7	99.4
(d) Performance, total (including 'unknown' as the correct identification for isolates absent from the database)							
Classical	36	1192	100.0	1156	30	6	97.0
Biotyper 2.0	36	1192	100.0	1180	8	5	99.0
Saramis	36	1192	100.0	1186	2	7	99.5

Success rates were calculated from: (a) all isolates; (b) all isolates identifiable with the respective method; (c) isolates commonly identifiable with all three methods; and (d) all isolates, including 'unknown' as true identifications for isolates not represented in the database.

Isolates misclassified or not recognized but present in the databases

The success rate for classification was >99% by all three methods when only those isolates were considered that were represented in the respective database (Table 3b). Twenty-one species, representing 1148 isolates (96%), should have been identifiable with all of the three methods. The overall sensitivity in this subgroup was again comparable, at >99%, for all three methods (Table 3c).

Several individual isolates could not be identified by at least one of the methods (six by the classical methods, five by the BioTyper and seven by the Saramis), and a few individual isolates were misidentified by the classical approach as well as the BioTyper. Among the latter, the most notable were false identifications of *P. cactophila* as *P. norvegica*, which are also difficult to resolve biochemically [21]. No misclassifications were found within the Saramis results among species contained in its database (Table 2).

Classification of isolates not contained in the respective databases

Both MALDI-TOF MS methods each misidentified only two single isolates absent from the respective database as a wrong species (Table 2). In contrast, the biochemical approach misidentified 30 isolates as a wrong species (including four *Galactomyces geotrichum* and *Geotrichum clavatum* isolates, which were classified at the genus level in the ID 32 C test), instead of reporting them as 'unknown'.

Success rates for the MALDI-TOF MS systems were 99.0% and 99.2%, respectively (Table 3d), but that for classical identification was only 96.7% (Table 3d).

Strikingly, isolates of *Pichia fabianii* were (correctly) not recognized by either MALDI-TOF MS system, and were misclassified as four different species biochemically (Table 2).

Discussion

In this study, we tested the suitability of the two commercially available MALDI-TOF MS databases BioTyper 2.0 and Saramis for rapid species identification of yeasts in a clinical diagnostic approach. Both systems were compared with a conventional differentiation scheme on a set of 1192 clinical isolates.

Independently of the geographical origin of the isolates (data not shown), both MALDI-TOF MS systems demonstrated an overall species identification rate (97.6% and 96.1%, respectively) that was comparable to the one obtained with the biochemical tests (96.9%, Table 3a). A recent study on bacterial isolates showed similar results [22].

Analysis of the isolates absent from the databases showed that the MALDI-TOF MS systems were better able to recognize these as 'unknown' (Tables 2 and 3), whereas they were generally misidentified as closely related species with the classical approach. Consequently, when we took 'unknown' as a correct prediction for isolates not represented in the database, success rates rose to 99.0% and 99.5% with the MALDI-TOF MS systems (Table 3d), but to only 97.0% with the classical approach. This reduction of false identifications clearly represents a clinically relevant advantage of MALDI-TOF MS over biochemical differentiation.

Among the species contained in the respective databases, each of the methods was unable to identify several individual

isolates. Furthermore, among isolates that were contained in the respective databases, several individual isolates were misidentified by the classical approach, and fewer by the BioTyper; no misclassifications were found within the Saramis results.

Apart from the different hardware, the systems were different in sample preparation (the BioTyper required prior extraction of the yeasts, whereas for the Saramis system, cell lysis was possible on the carrier plate) and the design of the underlying databases: both databases were constructed from sum spectra of multiple readings of single isolates, but the Saramis system introduces an additional layer of 'superspectra', containing only those peaks that are common to different isolates of the same species. Two singular incidences point to the diagnostic consequences resulting from the different approaches: three of seven strains of *P. cactophila* were misclassified as *P. norvegica* by our classical approach. With the BioTyper system, five of these were misclassified in a similar fashion, but were either correctly identified or missed completely with the Saramis system (Table 2). Sequencing of the reference strains used for the BioTyper database entry showed that these had previously been misidentified (Bruker Daltonics, personal communication), and a correction in the database remedied the problem.

The existence of misidentified reference strains was also seen during the differentiation of *Candida rugosa* and *Candida pararugosa* with the Saramis system: here, the spectra of the *C. pararugosa* isolates clustered together with that of the *C. rugosa* reference strain, indicating a previous misidentification. As the 'superspectra' of the Saramis system are based on mass peaks manually selected from non-identical strains of a species, the problem had been noted during the construction of *C. rugosa* and *C. pararugosa* 'superspectra' before, and they had not been included in the database (AnagnosTec, personal communication).

Taken together, our data suggest that the 'superspectra' of the Saramis system allow easier intrinsic quality control than the single-strain spectra of the BioTyper. However, in clinical routine, these differences are negligible, as the all true pathogens were well recognized by both systems.

The increased resolution of MALDI-TOF MS also allowed for separation of several clusters of closely related yeasts that were indistinguishable by classical differentiation. The clinical importance can be illustrated with the *Candida orthopsilosis/metapsilosis/parapsilosis* cluster: here, different drug susceptibility patterns have been observed [23,24]. Also, several isolates of *P. fabianii* were hidden among incorrect identifications by the classical approach, indicating that this species may have been missed frequently in the past (see also [16]). The biochemical misidentification of *P. fabianii* as *P. anomala* has recently also been observed by others [25], and as *P. anomala* is mainly associated with neonates and other paediatric patients [26,27] and instances of drug resistance have been reported [28], a careful re-examination of this cluster seems necessary.

Two factors could be improved for the routine use of both MALDI-TOF MS systems in the future: first (as also in the classical scheme), neither system provides an automated category 'not in database' for good spectra without matches, leaving it to the user to evaluate whether to repeat the identification by MALDI-TOF MS or to use another method (e.g. PCR). Second, both systems have been established using Sabouraud agar: it is not clear how the use of other agars (e.g. CHROMagar) commonly in use for primary yeast cultivation for the identification of mixed yeast infections will influence the outcome of MS-based species identification.

Both MALDI-TOF MS methods allowed more precise species identification of yeasts in a fraction of the time needed by the classical method. This was mainly because of the reduced false identification of isolates not contained in the database, which also presented the major constraint in species identification. As the MALDI-TOF MS spectral databases are more easily updated than biochemical methods, this will lead to a rapid improvement in the underlying data and probably greatly diminish differences in the near future.

As the intrinsic resistance of yeasts to antifungal agents is generally predictable from the species [4], it is likely that the benefit of the substantial time savings of this technology, together with the increased resolution, will improve yeast diagnostics and have a profound impact on patient survival.

Acknowledgements

MALDI-TOF MS systems and reagents were kindly provided by Bruker Daltonics and AnagnosTec GmbH for the duration of the study. Both companies provided expertise, including repetition of testing for several isolates with ambiguous or unclear testing results. F. Odds (University Aberdeen, UK) provided several isolates. Routine biochemical differentiations were performed by S. Kellner, S. Rittmeier and D. Hermann. This work has been presented in part at the DGHM 2010 Conference.

Transparency Declaration

O. Bader and R. Lugert were partially funded by the German National Reference Center for Systemic Mycoses. None of the authors has affiliations to either company.

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