



Research Paper

Bayesian analysis of two methods MALDI-TOF-MS system and culture test in otomycosis infection

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KEYWORDS

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Sensitivity;
Specificity

Abstract *Objective:* Identification of otomycotic fungi using matrix-assisted laser desorption ionization (MALDI) time of flight (TOF) mass spectroscopy (MS) and to quantify pervasive errors with Bayes rule; values of sensitivity and specificity of culture test and MALDI-TOF-MS method are quantified.

Method: Fungi cultured ear discharge samples were identified with culture test and MALDI-TOF-MS system. Minimum inhibitory concentration (MIC) or MEC (minimum effective concentration) for 6 antifungals were determined by antifungal susceptibility testing *in vitro*. With Bayes rule, sensitivity and specificity of both MALDI-TOF MS and culture test methods were computed.

Results: Cultures yielded 42 fungal isolates which were confirmed as species (specified against each species) belonging to 8 genera, *A. niger* 22, *Candida* sp. 7, *A. fumigatus* 3, *A. flavus* 3, mixed *Aspergillus* sp. 3, *Mucor* sp. 2, *Rhizopus* sp. 1, and *Scopulariopsis* sp.1; and MALDI-TOF-MS system also confirmed those isolates. *In vitro* antifungal susceptibility testing with terms of MIC 50 and MIC 90, isolates fungi were highly susceptible to 6 antifungals; and caspofungin was the most active antifungal. The high value of specificity 84.6%, suggested a limited loss of confidence on the culture test at the absence of an infection, in comparison to

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MALDI-TOF-MS. Sensitivity of the culture test was 87.5%; this high figure strongly approves the culture test to be the dependable method for the otomycosis diagnosis, when the infection is stable.

Conclusion: Based on sensitivity and specificity together the culture test could be done in face of the gold-standard MALDI-TOF-MS system the estimating both methods.

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Introduction

Otomycosis is a superficial acute/sub-acute mycotic-infection of the external auditory canal, characterized by inflammation, pruritus, scaling, feeling of fullness and severe discomfort.¹ The prevalence of otomycosis varies from 9% to 30.4% of cases of otitis externa, with symptoms of otitis or inflammatory conditions occurring worldwide being influenced by a number of predisposing factors such as, local climate, extremely moist and hot environments, chronic bacterial otitis external, swimming, dermatomycoses, insertion of foreign bodies and wearing head clothes; but it is more common in tropical and subtropical zones.²⁻⁴ Several predisposing factors are involved with otomycosis, bacterial infection, hearing aid or a hearing prosthesis, self-inflicted trauma, swimming in contaminated pools, broad spectrum antibiotic therapy, steroids and cytostatic medication, neoplasia and immune disorders, changes in the coating epithelium, pH and quantity and quality of the ear wax.⁵ Obviously, treatment recommendations include mainly the germ termination or controlling the predisposing factors, but the local debridement using suction with some topical/systemic antimicrobial agents is common.⁶ The use of 1% antifungal solution, bifonazole and derivatives were followed in the 1980s with an efficacy of up to 100%.⁷ However, the extensive and sometimes unnecessary use of antibiotic ear-drops for the treatment of otitis media and otitis externa has been linked to its increased prevalence. Moreover, secondary overgrowth of fungi is a well-recognized complication of the use of broad-spectrum quinolone antibiotics.⁵

The development of soft ionization technique matrix-assisted laser desorption ionization (MALDI) has made the analysis of large biomolecules such as, intact proteins possible.^{8,9} For the first time in 1996, methods of MALDI-time of flight (TOF) spectral fingerprints could be initiated from whole bacterial cells by mass spectroscopy (MS) analysis.¹⁰ Thus, the MALDI-TOF MS procedure has emerged as a rapid, accurate and sensitive tool for microbial characterization and identification of infecting bacteria, fungi and viruses.

Herein, routine identification was performed by positioning a small quantity of fungal cells on a target plate, which are overlaid with a matrix containing a solution of α -cyano-4-hydroxycinnamic acid in a mixture of organic solvents. The sample is then air-dried, and during this process all the molecules become embedded in the matrix, and then the target plate is positioned in the mass spectrometer for automated measurement. A defined mass spectrum

is obtained, usually encompassing peaks in the 1000 to 30,000 m/z range. Raw spectra from the test sample are acquired and compared to a library of reference spectra already listed in the instrument database. Based on this comparison, different algorithms provide identification Results, expressed with score values and the interpretative criteria for naming a given species are provided by the different manufacturers. For example, using MALDI Biotyper automation control and the Bruker Biotyper 3.1 software and library the identification scores are as follows: a value ≥ 2.000 indicates species-level identification, scores between 1.700 and 1.999 refer to genus-level identification, and scores below 1.700 are taken as unreliable identification.¹¹

Advantages of MALDI-TOF MS ionization: this is a soft ionization technique that allows ionization and vaporization of large non-volatile biomolecules such as, intact proteins.¹² It generates mostly single-charged ions ($z = 1$) so that the mass-to-charge ratio (m/z) of the analyte corresponds to its mass value. Each MALDI-TOF MS system is composed of 3 principal units. First, the ion source makes ionization possible and transfers sample molecule ions into a gas phase. Second, the mass analyzer allows ion separation according to mass-to-charge ratio and the last unit is the detection device for monitoring separated ions.

Samples are prepared by mixing the analyte with a matrix made of small acid molecules that possesses a strong optical absorption in the range of the wavelength used by the laser device; 2,5-dihydroxybenzoic acid and α -cyano-4-hydroxycinnamic acid are optimal matrices for the detection of lower mass ions. After co-crystallization of the sample and matrix, the latter absorbs energy from the laser, leading to desorption and ionization of analytes in the gas phase. Ions are further accelerated through an electrostatic field, created by a potential of about 20 kV into the high vacuum flight tube until those reach the detector, with smaller ions travelling faster than larger ones. Thus, TOF required to reach the detector is dependent on the mass and charge of the bio-analyte, resulting in a spectral profile unique for a given species, composed of peaks ranging usually from 2 to 20 kDa. With housekeeping functions, each conserved protein generates a unique MALDI-TOF MS spectrum. Detected biomolecules correspond mostly to ribosomal proteins that are abundant, basic and medium hydrophobicity; but biochemical traits favour an efficient ionization of a protein.¹³ During the MALDI process, structural proteins, DNA or RNA binding proteins, ribosome modulation factors, carbon storage regulators, cold-shock proteins and translation initiation factors are

identified.^{14,15} These biomarkers generate spectral fingerprints that vary between individual microorganisms and have peaks specific to genus, species and subspecies. A comparison to protein peaks of used organism as positive control with those of the query organism facilitates identification of the later from biomarkers. Eventually, MALDI-TOF MS system is efficient in accurately typifying taxa.

To quantify pervasive errors, as sensitivity and specificity of both culture and MALDI-TOF-MS tests, the statistical method Bayes rule was applied on the obtained data.¹⁶ Consequently, time and resources for treating false-positives (FP; culture test positive, MALDI-TOF-MS test negative) cases with the staggering antifungal therapy would not be necessary and complications of non-target use of antifungals could be blithely avoided. Moreover, false-negatives (FN; culture test negative, MALDI-TOF-MS test positive) cases should not be neglected. An intransigent attitude regarding FN cases should be taken as an unredeemable medical infraction, as that would promote a commotion in public health. However, true-positives (TP; culture test positive, MALDI-TOF-MS test positive) and true-negatives (TN; culture test negative, MALDI-TOF-MS test negative) are the sought-after cases of the prognosis. Moreover, both tests are independent by themselves, but are critical in determining the status of the culture test, since the former is the gold standard test. There are several associated test statistics: the sensitivity (true positive rate), which is the portion of the people with the disease who will have positive culture test Results, computed by $[TP/(TP + FN)]$, and the specificity (true negative rate), which is the portion of the people without the disease who will have negative culture test results, computed by $[TN/(FP + TN)]$; these two test statistics are based on the Bayesian analysis. Furthermore, the false positive rate, which is the probability of errors of the MALDI-TOF-MS test, computed by $[FP/(FP + TN)]$, and the false negative rate, which is the probability of errors of the culture test, computed by $[FN/(TP + FN)]$ are important in assessing the performance level of each test. And the positive predictivity, which is the post-test probability of the fungal infection that gave a positive test result, or the portion of the otomycosis with positive test results of patients, who actually have the disease, computed by $[TP/(TP + FP)]$, predicts positivity by the culture test. Moreover, the negative predictivity, which is the post-test probability of the infection that gave a negative test result, or the portion of patients with negative test results, who actually do not have the infection, computed by $[TN/(FN + TN)]$, predicts negativity by the culture test. Herein, a comparison between cultures of ear swabs from non-infecteds and infecteds was done to assess susceptibility profiles of pathogenic fungi for aiding to in appropriate management.

Materials and methods

Isolation of fungi

Collected clinical samples as ear-swabs were cultured on Sabouraud dextrose agar (SDA) for 48 h at 37 °C. Fungal species were isolated and identified with conventional methods such as, germ tube test.^{16,17} Surveillance of an

ENT outpatient department in one year for otomycotic fungi was undertaken.

Identification of fungi with the use of MALDI-TOF MS

Plates incubated for 24 h (alternately, 48 h if colonies were not present or when the purity of fungicolonies could not be verified) at 30 °C on SDA plates and colonies were transferred into 1.5 ml screw cap tubes and mixed thoroughly in an aliquot of 0.3 ml of double-distilled water; an aliquot of 0.9 ml ethanol was added to tubes, and after vortexing contents were centrifuged at $11,200 \times g$ for 2 min. The pellet was mixed thoroughly with 50 μ l 70% aqueous formic acid. After further addition an aliquot of 50 μ l acetonitrile, the mixture was centrifuged at $11,200 \times g$ for 2 min. An aliquot of 1 μ l supernatant of a fungus extract was placed onto the polished steel MALDI target plate in duplicates, and plates were allowed to dry at room temperature. An aliquot of 2 μ l matrix solution consisting saturated α -cyano-4-hydroxycinnamic acid in 50%, acetonitrile 2.5% and trifluoroacetic acid was used to overlay each sample, and the plates were air dried at room temperature. Each plate was loaded into a Bruker Autoflex III MALDI-TOF mass spectrometer (Systronics, India), and the analysis was performed. Spectra were automatically recorded in the linear positive ion mode with delayed extraction at a laser frequency of 20 Hz within a mass range from 4000 to 10,000 Da; and 600 satisfactory shots in 100-shot steps from the sampling area of the target spot were obtained for each spectrum. Each run included fungal test standard, microbial type culture collections (MTCC) with a characteristic peptide and protein profile, provided by Bruker for calibration; a negative extraction control; and the reference strains.¹⁸ Failures were repeated using fresh colonies with the same methods.

Antifungal susceptibility testing

In vitro antifungal susceptibility testing, involving determination of minimum inhibitory concentration (MIC) and minimum effective concentrations (MEC, for caspofungin only), of six antifungal agents amphotericin B, caspofungin, griseoflavin, itraconazole, posaconazole and voriconazole were performed according to recommendations stated in the Clinical and Laboratory Standards Institute M38-A2 document.¹⁹

Data analysis

Obtained mass spectra were evaluated with the Flex analysis software version 3.0. The automated data analysis was processed with MALDI Biotyper software version 2.0. The list of higher peaks of the spectrum was created automatically by the software after smoothing, normalization, and baseline subtraction. The obtained spectra were analyzed by standard pattern-matching algorithm, which compared the raw spectra with the spectra of the Bruker library by using the standard setting, and the Results were listed in a ranking table. The results were expressed as log (score) values, which ranged from 0 to 3 as recommended by the

manufacturer. Score values of N1.7 generally indicated relationships at the genus level, and values of N2.0 generally indicated relationships at the species level. The highest score was used for the identification of species. With principles of Bayes rule, sensitivity and specificity of both MALDI-TOF MS and culture test methods were computed.¹⁶

Results

The study was carried out with 110 patients presenting to the ENT outpatient department; 51 patients were suspected to have otomycosis, after fulfilling sampling smearing and culturing, and 59 were the non-infecteds, but from them only 2 *Candida* sp. samples were isolated. Otomycosis was confirmed with 42 isolates, belonging to eight genera of fungi as follows, *A. niger* 22, *Candida* sp. 7, *A. fumigatus* 3, *A. flavus* 3, mixed *Aspergillus* sp. 3, *Mucor* sp. 2, *Rhizopus* sp. 1, and *Scopulariopsis* sp.1 (Fig. 1).

Of 42 clinical fungal isolates were cultured with isolated and identified with the use of MALDI-TOF-MS system. The MALDI-TOF-MS system was able to identify the fungus species isolates accurately (Fig. 2). In isolated *Candida* sp., *C. albicans* strain, one of the duplicate spots was identified as another *Candida* sp. such as, *C. guilliermondii*, *C. glabrata* and *C. utilis*, with lower scores of MALDI-TOF-MS. Therefore, these isolates were retested in duplicates repeatedly for accuracy in species identification and the 4 strains were identified as *C. albicans* only. Similarly, the confusion of identification of *C. glabrata*, which was detected in one spot, was addressed as a colony of *C. guilliermondii* and as one colony of *C. krusei*, which was finally identified as *C. tropicalis*. With the advanced system of identification,

these strains were identified correctly as *C. glabrata* and *C. krusei*. Altogether, MALDI-TOF-MS yielded the correct species identification for 7 *Candida* isolates.

Clearly, the test Results of all samples were grouped to 4 categories: 14 samples were TP, and 22 were TN; the rest samples comprised both 04 FP (type I errors) and 02 FN (type II errors) samples. In other words, there was a mismatching of culture test and MALDI-TOF-MS test results for 36 samples (Table 1). The total of 16 MALDI-TOF-MS test was positive (TP + FN) cases; the prevalence of the infection was 38% (0.38) in the targeted population of 42 patients. Indeed, two important test statistics, sensitivity and specificity are conditional on either having or not having the otomycosis infection with a sample-donor and both are not affected by the prevalence value. The sensitivity of the culture test was 87.5%; consequently, this high figure strongly approves the culture test to be the dependable method for the otomycosis diagnosis, when the infection is stable. A high moderate value of the specificity (84.6%), however, suggests a limited loss of confidence on the culture test at the absence of an infection, confirmed by MALDI-TOF-MS. Furthermore, to provide an unbiased evaluation of the culture test would be to use the known correction, the cumulative value, based on sensitivity and specificity together (Table 1).

In vitro antifungal susceptibility testing of the antifungal agents observed the widest range (Fig. 3). The highest MIC with respect to amphotericin B ranging between 0.25-8 and 0.25–4 µg/ml, respectively. Posaconazole and caspofungin had potent activity against all isolates, while the highest MIC were consistently observed for itraconazole, voriconazole, and amphotericin B. Results showed that, in terms of MIC50 and MIC90, isolates fungi were highly susceptible to

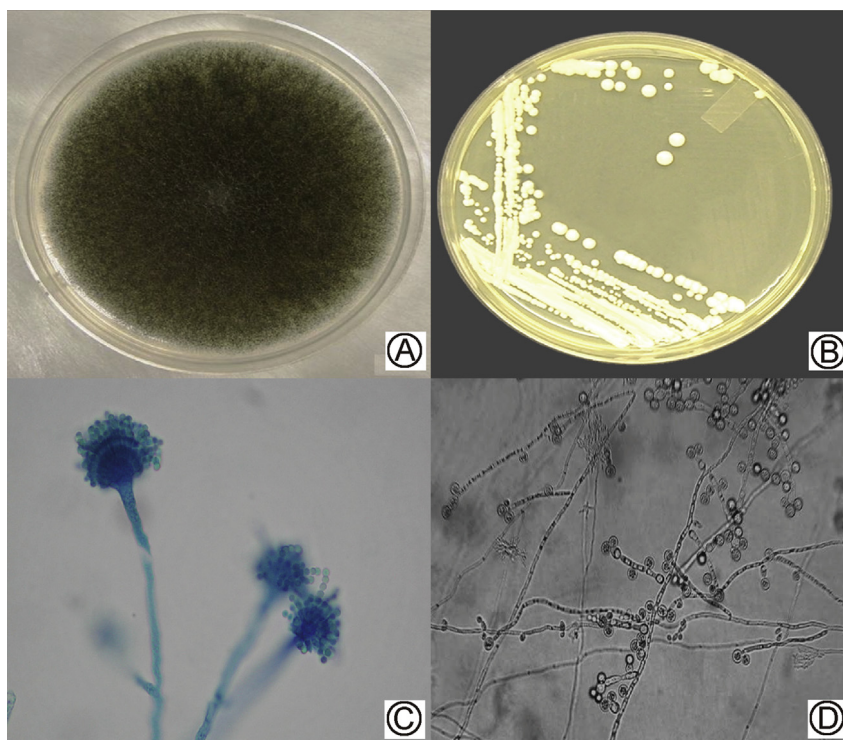


Fig. 1 Identification of *A. niger* (A) and *C. albicans* (B), in SDA plate isolates from ear-swab samples of otomycosis as well as *A. niger* (C) and *C. albicans* (D) in a microscope.

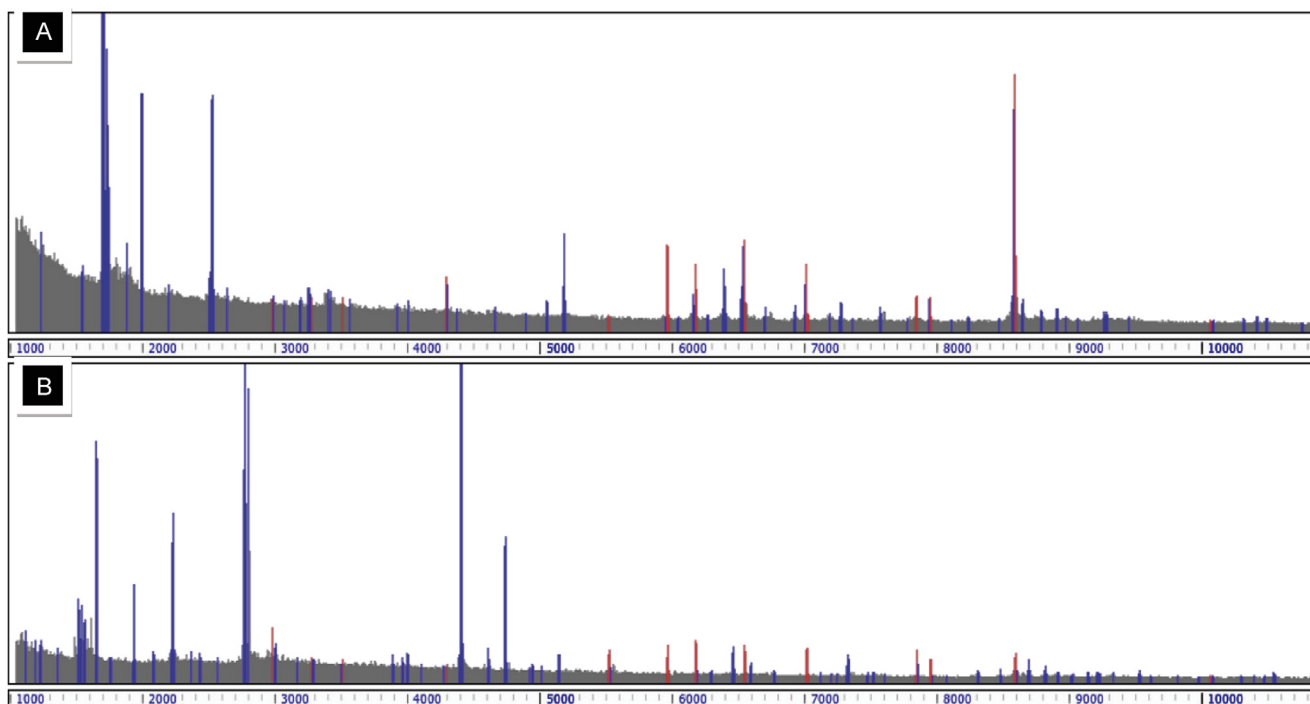


Fig. 2 A mass spectrometric peptide/protein profiles of *A. niger* (A) and *C. albicans* (B), as graphical windows evaluated by the software Biospean. Blue peaks are detected ions and red peaks are the matching peak-positions between both spectra.

Table 1 The generic 2×2 table with number of cases based on MALDI-TOF-MS test and culture test Results during diagnosis with sensitivity and specificity rates of both tests of otomycosis.

Culture test	MALDI-TOF-MS test		Total	Sensitivity (true positive rate)	Specificity (true negative rate)
	Positive	Negative			
Positive	TP = 14 (0.33)	FP = 04 (0.095)	(TP + FP) = 18 (0.43)	TP/(TP + FN)	TN/(FP + TN)
Negative	FN = 02 (0.095)	TN = 22 (0.48)	(FN + TN) = 24 (0.57)		
Total	(TP + FN) = 16 (0.38)	(FP + TN) = 26 (0.62)	N = 42 (1.0)	0.875	0.846

TP = 14 cases were true-positives (MALDI-TOF-MS test-positive, culture test-positive); FP = 04 cases were false-positives (culture test-positive, MALDI-TOF-MS test-negative); FN = 02 cases were false-negatives (MALDI-TOF-MS test-positive, culture test-negative); and TN = 22 samples were true-negatives (MALDI-TOF-MS test-negative, culture test-negative); N = population size or total number of cases. Corresponding fraction values are given in parentheses. FP cases are the type I errors, while FN cases are the type II errors.

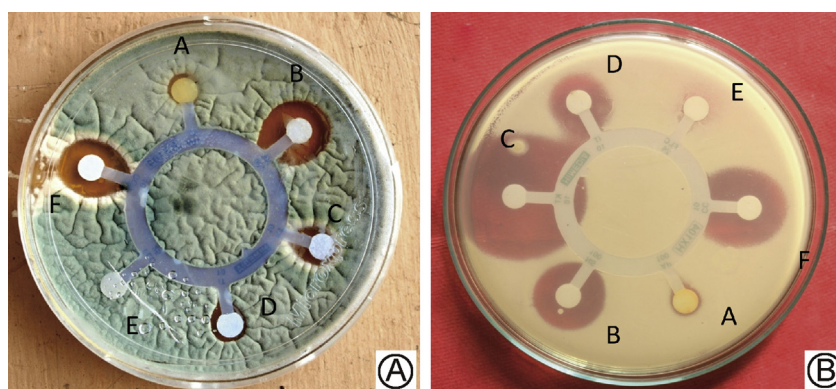


Fig. 3 *A. niger* in (A) and *C. albicans* in (B), *In vitro* susceptibilities of clinical isolates fungus to six antifungal agents: A: Amphotericin B; B: Itraconazole; C: Voriconazole; D: Posaconazole; E: Caspofungin; F: Griseoflavin.

Table 2 *In vitro* susceptibilities of clinical fungal isolates to six antifungal agents. MIC ranges, geometric mean, as well as values of MIC50 and MIC90 are expressed in $\mu\text{g/ml}$.

Isolated fungus	Antifungal agent	MIC ($\mu\text{g/ml}$)		
		MIC range	MIC50	MIC90
<i>A. niger</i>	Amphotericin B	0.25–8	0.5	1
	Itraconazole	0.031–2	0.25	0.5
	Voriconazole	0.031–4	0.5	0.5
	Posaconazole	0.008–0.5	0.25	0.25
	Caspofungin	0.008–0.25	0.15	0.063
	Griseoflavin	0.008–0.5	0.016	0.063
<i>A. fumigatus</i>	Amphotericin B	0.25–8	0.5	1
	Itraconazole	0.031–2	0.25	0.5
	Voriconazole	0.031–4	0.5	0.5
	Posaconazole	0.008–0.5	0.25	0.25
	Caspofungin	0.008–0.25	0.15	0.063
	Griseoflavin	0.008–0.5	0.016	0.063
<i>A. flavus</i>	Amphotericin B	0.25–8	0.5	1
	Itraconazole	0.031–2	0.25	0.5
	Voriconazole	0.031–2	0.5	0.5
	Posaconazole	0.008–0.5	0.25	0.25
	Caspofungin	0.008–0.25	0.15	0.063
	Griseoflavin	0.008–0.5	0.016	0.063
<i>C. guilliermondii</i>	Amphotericin B	0.25–4	0.5	1
	Itraconazole	0.031–1	0.25	0.5
	Voriconazole	0.031–4	0.5	0.5
	Posaconazole	0.008–0.25	0.25	0.25
	Caspofungin	0.008–0.25	0.15	0.063
	Griseoflavin	0.008–0.5	0.016	0.063
<i>C. glabrata</i>	Amphotericin B	0.25–8	0.5	1
	Itraconazole	0.031–1	0.25	0.5
	Voriconazole	0.031–2	0.5	0.5
	Posaconazole	0.008–0.25	0.25	0.25
	Caspofungin	0.008–0.25	0.15	0.063
	Griseoflavin	0.008–0.5	0.016	0.063
<i>C. utilis</i>	Amphotericin B	0.25–8	0.5	1
	Itraconazole	0.031–2	0.25	0.5
	Voriconazole	0.031–4	0.5	0.5
	Posaconazole	0.008–0.25	0.25	0.25
	Caspofungin	0.008–0.25	0.15	0.063
	Griseoflavin	0.008–0.5	0.016	0.063
<i>Mucor</i> sp.	Amphotericin B	0.25–8	0.5	1
	Itraconazole	0.031–1	0.25	0.5
	Voriconazole	0.031–4	0.5	0.5
	Posaconazole	0.008–0.25	0.25	0.25
	Caspofungin	0.008–0.25	0.15	0.063
	Griseoflavin	0.008–0.5	0.016	0.063
<i>Rhizopus</i> sp.	Amphotericin B	0.25–8	0.5	1
	Itraconazole	0.031–4	0.25	0.5
	Voriconazole	0.031–4	0.5	0.5
	Posaconazole	0.008–0.25	0.25	0.25
	Caspofungin	0.008–0.25	0.15	0.063
	Griseoflavin	0.008–0.5	0.016	0.063
<i>Scopulariopsis</i> sp.	Amphotericin B	0.25–4	0.5	1
	Itraconazole	0.031–1	0.25	0.5
	Voriconazole	0.031–4	0.5	0.5
	Posaconazole	0.008–0.25	0.25	0.25
	Caspofungin	0.008–0.25	0.15	0.063
	Griseoflavin	0.008–0.5	0.016	0.063

MIC: Minimum inhibitory concentration.

posaconazole and caspofungin, but not voriconazole, itraconazole, and amphotericin B. The difference in the MIC₉₀ between the strains did not differ by more than one dilution. Caspofungin, the lone echinocandin tested, exhibited the best activity among the antifungal agents tested, with MIC that were $> 4\log_2$ and $> 2\log_2$ dilutions more active than the MIC of amphotericin B and azoles, respectively. Caspofungin was the most active drug, followed by posaconazole, itraconazole, voriconazole and amphotericin B. Among the azoles, posaconazole was the most potent, followed by itraconazole and voriconazole. The MIC for posaconazole was 1 µg/ml for all *A. flavus* isolates, whereas 95.9% and 89.4% of the isolates had an MIC of 1 µg/ml for itraconazole and voriconazole, respectively (Table 2). There were no significant differences observed between the susceptibilities of *A. flavus* isolates according to the site of isolation.

Discussion

MALDI based identification and classification of fungal isolates utilize peptide/protein profiles containing characteristic biomarker peaks in the mass-to-charge ratio. Their presence is important for the subsequent database searches and the comparison of experimental data with those for reference strains. For that reason, the future development of MALDI-TOF-MS methodology for fungal analyses will definitely involve a continuous updating of current commercial databases provided by vendors together with the instrumentation as well as, building up new databases for specialized research purposes. Hand in hand with this, improved software solutions will appear for data processing and management. There is still a big gap in linking MALDI-TOF MS peptide/protein profiles with proteomic identification of individual biomarker molecules, which needs to be further expanded. The outer surface proteins, hydrophobins have been recognized as the cause of predominant ions during MALDI-TOF-MS profiling of fungi, *Hypocrea* and *Trichoderma* strains.²⁰ Several methods including colorimetric tests are used for that purpose, but they may provide unsatisfactory correlations with reference data because of subjectivity in reading Results.²¹ Approaches based on this method offer the possibility of identifying correctly the taxon in query with a change in the fingerprint spectrum reflecting detected proteome in related taxa, deciphering contaminations from similar organisms.²² Common fungi such as, *Aspergillus*, *Fusarium*, *Penicillium* or yeasts can be cultured on nutrient-media and clinical samples yield contaminating microbes in cultures; nevertheless, when pure colony material cannot be obtained directly in the first culture, the serial subculture would resolve the issue of due identification microscopically.²³

However, the sample preparation step is most important in the whole analytical procedure herein. For identification and antifungal screening of pathogenic filamentous fungi, the intact cell approach is frequently used, and very effective with respect to the time consumption for analysis of each sample. But, for the analysis of large biomolecules in generating spectral fingerprints that vary between individual fungi, the ion sourced of proteins would makes ionization possible to progress in the described procedure.

Disseminated fungal infections necessitate a rapid and accurate fungal diagnosis in public health dependant on

climate and geographic zone. Species identification by MALDI-TOF-MS represents a reality in the routine clinical laboratory; nevertheless, databases need to be continuously updated to include reference spectra of unusual pathogens and protocols for direct applications from clinical samples. Implementation of MALDI-TOF-MS for intra-specific typing holds a great promise, as it has demonstrated the ability to detect drug resistant phenotypes, biofilm-producing strains and potentially cluster isolates for epidemiological purposes. Though some aspects of biotyping protocols based on MS still require standardization and today these applications seem more feasible to control fungal infections. Moreover, this system is suitable for microbial typification of pathogens, being a novel procedure in clinical microbiology gives, accurate Results of infections to clinicians and patients, as it is with high throughput, precision, accurate.

Conclusion

The sensitivity of the culture test was 0.875%; consequently, this high figure strongly suggests the culture test to be an efficient method for the diagnosis of otomycosis, in presence of stable infection. An exquisite/high value of the specificity (0.846%) too corroborates an absolute dependency on the culture test with the absence of an infection without any proper identification of fungal taxa. Herein, to provide an unbiased estimation of the culture test would be the use of the estimating method based on sensitivity and specificity together, as these two cumulative values, in face of the gold-standard MALDI-TOF-MS system.

Conflicts of interest

The authors have no conflicts of interest to disclose.

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