HHS Public Access

Author manuscript

J Pak Med Assoc. Author manuscript; available in PMC 2015 October 28.

Published in final edited form as:

J Pak Med Assoc. 2012 October ; 62(10): 995–998.

Species identification of invasive yeasts including Candida in Pakistan: limitations of phenotypic methods

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Abstract

Objective—To compare phenotypic and genotypic methods of yeast identification.

Methods—The in-vitro cross-sectional study was conducted from January 2006 to May 2009. Invasive yeasts isolated at the clinical microbiology laboratory at the Aga Khan University (AKU), Karachi, Pakistan, were identified. Speciation by phenotypic and molecular methods was compared. All yeasts isolated during the study period from blood and other invasive sites were identified using standard methods. Isolates were shipped to Mycotic Diseases Branch, Centres for Disease Control and Prevention, Atlanta, Georgia, USA, for identification by Luminex flow cytometric multianalyte profiling (xMAP) system. Ribosomal ITS2 DNA sequencing was performed on isolates not identified by Luminex.

Result—Of the 214 invasive yeasts evaluated, Candida species were 209 (97.7%) while the frequency of non-Candida species was 5 (2.3%). Overall agreement between phenotypic and molecular identification was 81.3%, 90.3% amongst the more common Candida species, and only 38.8% amongst the uncommon yeasts.

Conclusion—Phenotypic methods of identification proved adequate for common Candida species, but were deficient in recognising rare Candida and non-Candida yeasts, highlighting the importance of molecular methods for identification.

Keywords

Invasive yeasts; Candida; Rhodotorula; Cryptococcus; Luminex MAP

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Introduction

Invasive fungal infections are increasingly becoming important infectious agents in this era of advancing medical technology.[1,2] Invasive yeast infections can cause severe illness with high mortality, and with the lowest survival rates seen in cancer patients.[3] These infections increase the overall cost of management and prolong hospital stay.[4] Appropriate and timely therapy can significantly reduce mortality.[3] Accurate identification of these yeasts is crucial for the initiation of appropriate anti-fungal chemotherapy as some species are known to be intrinsically resistant to certain anti-fungals.[5,6]

DNA-based methods are considered authoritative for the identification of fungal isolates.[7-9] The Luminex xMAP (Luminex Corp, Austin, TX USA) is a rapid multiplex system that utilises polystyrene beads coupled to oligonucleotide probes directed at the ribosomal ITS2 regions of major Candida species to detect and differentiate each species in a single microtiter well plate by flow cytometry.[10] Various forms of this technology have been validated as reliable molecular techniques for the identification of various medically important fungal species,[11] and can be used to speciate Candida isolates as long as their specific oligonucleotide probes are available. Although nucleotide sequence analysis is still the most accurate molecular method because known species, as well as undescribed species, are quickly recognized,[12] but the use of the Luminex method with oligonucleotide probes for detecting the well-conserved ITS2 region has the advantage of detecting multiple species even in mixed cultures if the correct species-specific probes are present.[12]

However, clinical laboratories in resource-constrained countries like Pakistan have limited access to expensive molecular techniques and rely mainly on phenotypic methods for the identification of yeasts.[13] Phenotypic identification by experienced personnel is effective for the identification of most commonly encountered pathogenic yeasts. The identification of less frequently encountered species may become problematic or common yeasts may manifest strain variation.[9] Determining biochemical assimilation profiles of yeasts on API® 20 C AUX (bioMérieux,SA, Lyon France) alone may be considered the primary phenotypic method for yeast identification as it has been reported to have a correct identification rate of more than 90% at 72 hours for most clinically relevant species.[8,14,15]

This study aimed to assess the agreement between conventional phenotypic methods with either Luminex xMAP system or DNA sequencing for the identification of invasive yeasts isolated from clinical samples in Pakistan.

Materials and Methods

The in-vitro cross-sectional study was conducted between January 2006 and May 2009 at the clinical microbiology laboratory, Aga Khan University (AKU), Karachi, Pakistan, and the Mycotic Diseases Branch, Centres for Disease Control and Prevention, Atlanta, GA, USA. The study included 214 yeasts isolated from 185 patients' sterile sites: 189 from blood; 12 from pleural, peritoneal, bile and synovial fluid; 6 from cerebrospinal fluid; and 7 from the tips of central venous catheters or endoventricular drains. Standard phenotypic identification was based on the production of germ tube, inhibition by cycloheximide, urease

production, colour and morphology on BiGGY Agar (BD BBL™, USA), microscopic morphology on cornmeal-Tween 80 agar and identification profile generated on API 20C AUX (bioMérieux, SA, Lyon France) at 72 hours, interpreted according to API 20C codebook (1988). Of the supplemental tests suggested by the codebook, bile-esculin test was performed for identification of C. lusitaniae. Isolates were saved in glycerol phosphate buffer at −80°C, revived and shipped to the Mycotic Diseases Branch, Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, USA.

The isolates were then refreshed on Sabouraud dextrose agar to be identified by Luminex flow cytometric multianalyte profiling (xMAP) system according to the method of Deak.[15] The principle is based on hybridisation of ribosomal ITS2 region DNA amplicons to Candida species-specific oligonucleotide capture probes covalently bound to polystyrene beads internally labelled with different ratios of red and infrared dyes so they can be distinguished from one another. The target region of interest, in this case the ITS2 region, was amplified and concurrently labelled with biotin on the 5′ end of the bead-coupled polymerase chain reaction (PCR) amplicon. After hybridisation, the capture probe that binds the biotinylated amplicon of the complementary species was then detected by streptavidin-phycoerythrin (PE) detection buffer which binds to the biotin label. A reporter laser excited the PE and a fluorescent signal was detected, processed, and recorded by the multianalyte profiling system. The classification laser revealed the unique spectral address of each bead (distinguishing one species from another), and the identity laser allowed the system to detect specific amplicon binding to the bead. Those isolates which were not identified by the Luminex underwent ITS2 gene sequencing for their final identification.[16]

The study was approved by the Ethical Research Committee of Aga Khan University. SPSS version 19.0 was used for data entry and analysis. Male-to-female ratio, mean and standard deviation for age of patients was calculated. Agreement of phenotypic and molecular methods of yeast identification was calculated using Kappa scores. The following standards were used for the strength of agreement for the Kappa coefficient: 0 - 0.0099 as poor; 0.01-0.20 as slight; 0.21- 0.40 as fair; 0.41- 0.60 as moderate; 0.61- 0.80 as substantial; and 0.81-1 as almost perfect.

Results

Out of the total of 185 patients whose samples were taken, the male-to-female ratio was 1.57 and the mean age was 30.2 ± 26.97 years. The most common species isolated amongst the 214 invasive yeasts were C.tropicalis (n=69; 32.2%), C.albicans (n=43; 20%), C.parapsilosis (n=30; 14.0%), and C.glabrata (n=23; 10.7%), respectively (Table-1). Less common yeasts isolated constituted 22.9% (44/214) of the total (Table-2). Five (2.3%) non-Candida isolates were identified, four of which were Rhodotorula mucilaginosa from a parenteral nutrition outbreak, and one was Cryptococcus neoformans.

Use of phenotypic methods alone showed that correct identification of Candida species, making up 97.7% (n=209) of the collection, was found to be inadequate with a Kappa score of only 0.13 ± 0.06 compared to DNA-based identification. However, excluding less commonly encountered species brought agreement rates up to 0.557±0.07. Phenotypic and

molecular identification agreement rates were particularly low for C.guilliermondii, C.metapsilosis, C.orthopsilosis, C.viswanathii, C.utilis, C.fabianii and novel Candida species and non-Candida isolates, all except 5 C.guilliermondii identified only on DNA-based studies. All four R.mucilaginosa isolates were categorised as R.rubra by API 20C AUX. Though the identification was low discrimination category (55% R.rubra and 45% R.glutinis), it was excellent identification till genus level. However, the supplemental nitrate assimilation test, suggested by the API codebook, could not be performed due to the unavailability of media. The error thus can be considered minor. One C.neoformans isolate was identified correctly by the phenotypic test battery.

Discussion

The epidemiology of invasive yeast infections has globally identified C. albicans as the most common species.[17] Other prevalent yeasts causing invasive disease are C.tropicalis, C.parapsilosis, C.glabrata and C.krusei.[17,18] Epidemiology and distribution of invasive yeasts show geographical variability, and depend on a variety of risk factors. Most of these common Candida species can reliably be identified using phenotypic methods. Thus, yeast identification for most surveillance purposes is performed using assimilation tests.[19,20] However, it has been reported before that genotypic identification methods are superior to conventional biochemical tests.[11,21] In one study, the accuracy of identification has been calculated to be 77% for API 20C AUX.[21] Our results (81.3%) showed only a minimal increase in the efficiency of identification with the addition of microscopic and gross morphology, urease and cycloheximide tolerance. Agreement in conventional and genotypic identification amongst commonly encountered Candida species was higher than among those species less frequently isolated mostly because many of the less frequent species were not included in the API codebook. This finding is supported by other studies comparing conventional and molecular identification.[9,20]

The technologist can be faced with the problem of low discriminative identification even while using microscopy as a supplementary test.[22] This presents difficulty in identifying them by conventional methods.[20,21] Although such strains are not frequently encountered in the clinical laboratory, their mis-identification can have serious consequences on therapeutic decisions as several of these rare species may be resistant to fluconazole or amphotericin B, the primary drugs used for treating serious fungal infections.[9] In cases where definitive phenotypic identification is not possible or the microscopic and biochemical features do not match, molecular identification can help resolve the conflict.[9]

Certain yeasts cannot be satisfactorily identified using conventional methods and will require molecular identification techniques. These include C.metapsilosis, C.orthopsilosis, C.viswanathii, C.fabianii and novel isolates because their profile numbers are not yet included in the chemical profile databases.[20,23]

An important finding highlighting the merits of the Luminex xMAP system is the detection of mixed cultures that are missed by conventional methods. Two of our C.albicans isolates, both from the same patient, were thought to be pure. When xMAP was performed on the isolate, it showed a mixed identification of C.albicans and C.glabrata for both specimens.

Hence C.glabrata, a yeast less biochemically active, not forming pseudohyphae and in low numbers, was not detected when mixed with C.albicans. This advantage of Luminex xMAP system has previously been described by other studies.[12,16]

With the advent of new molecular diagnostics, several highly efficient and accurate rapid methods for identification of micro-organisms are now available, such as Luminex xMAP system, matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF) assay, and real-time PCRs.[16,24,25] What remains to be seen is whether investing in any of these advanced techniques will be cost effective for the clinical laboratory.

Conclusion

Conventional identification of yeasts can be considered adequate for identifying species commonly encountered in clinical specimens, though it may require more technological expertise. Molecular identification methods may be more expensive, but are more accurate. Continued surveillance of the spectrum of invasive yeasts can help determine whether it will be worthwhile to invest in molecular identification methods in clinical microbiology laboratories.

Acknowledgement

The study was financially supported by the Joint Pakistan-US Academic and Research Programme HEC/MoST/USAID. We would like to acknowledge the technical expertise and support of Eszter Deak, Joyce Petersen and Lalitha Gade at the Mycotic Diseases Branch, Centres for Disease Control and Prevention, Atlanta Georgia, USA. We are grateful to Syed Iqbal Azam, Assistant Professor, Community Health Sciences, Aga Khan University, Karachi, for his guidance in statistical analysis.

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Table-1

Frequency of invasive yeast species and concordance of phenotypic (API 20C-based) with genotypic (Luminex and DNA sequencing) identification methods.

S. No.	Species	Frequency n (%)	Kappa score (S.E.)	Mis-identified by phenotypic method as:
1	Candida tropicalis	69 (32.2)	0.946 (0.02)	C. glabrata: 2, C. humicola: 1, C. neoformans: 1
2	C. albicans	43 (20.0)	0.956 (0.03)	C. rugosa: 1, C. tropicalis: 1
3	C. parapsilosis	30 (14.0)	0.69 (0.06)	C.guilliermondii: 1
4	C. glabrata	23 (10.7)	0.670 (0.09)	C. humicola: 2, C.parapsilosis: 3, C. albicans: 1, missed: 2
5	C. guilliermondii	13 (6.0)	0.507 (0.14)	C. humicola: 1, C. parapsilosis: 5, C. lusitaniae: 1, C. species:
1				
6	C. krusei	5 (2.3)	0.887 (0.11)	C. lusitaniae: 1
7	C. lusitaniae	4 (1.9)	0.721 (0.15)	-
8	C. metapsilosis	4 (1.9)	-*	All C. parapsilosis
9	C. orthopsilosis	4 (1.9)	-*	C. parapsilosis: 3, C. lusitaniae: 1
10	C. viswanathii	4 (1.9)	-*	C. parapsilosis: 3, C. lusitaniae: 1
11	Rhodotorula mucilaginosa	4 (1.9)	-*	All as R. rubra
12	C. pelliculosa	3 (1.4)	1.00 (0.00)	-
13	C. utilis	2 (0.9)	-*	Both as C. species
14	C. kefyr	1 (0.5)	1.00 (0.00)	-
15	C. rugosa	1 (0.5)	0.665 (0.31)	-
16	C. fabianii	1 (0.5)	-*	C. species
17	Novel Candida spp. MCR C. haemulonii	1 (0.5)	-*	C. species
18	Novel Candida spp.	1 (0.5)	-*	C. parapsilosis
19	Cryptococcus neoformans	1 (0.5)	0.665 (0.31)	-
	Total	214 (100)	-	-

Table-2

Molecular and phenotypic identification agreement rates amongst yeast groups.

Species (n)	Percentage of 214 invasive yeasts studied (n)	Kappa score (S.E.)
Common Candida spp.*	77.1% (170)	0.557 (0.07)
Uncommon yeasts	22.9% (44)	0.237 (0.05)
All Candida species	97.7% (209)	0.13 (0.06)
All non-Candida species	2.3% (5)	0.036 (0.02)
C. glabrata isolates not detected on phenotypic identification	0.9% (2)	-

*Common Candida species include C.tropicalis, C.albicans, C.parapsilosis and C.glabrata.