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YEAST PANEL multiplex PCR for identification of clinically important yeast species: stepwise diagnostic strategy, useful for developing countries

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ABSTRACT

Identification of opportunistic yeasts in developing countries is mainly performed by phenotypic assays, which are time-consuming and prone to errors. Wrong species identification may result in suboptimal treatment and inaccurate epidemiological data. To improve rapidity and accuracy of species identification, a diagnostic strategy using a stepwise "YEAST PANEL multiplex PCR assays" targeting 21 clinically important yeast species of *Candida*, *Trichosporon*, *Rhodotorula*, *Cryptococcus*, and *Geotrichum* was designed. Four hundred CBS reference strains were used for optimization and specificity testing. Eight hundred clinical species were prepared in blinded sets for multiplex polymerase chain reaction (PCR) and matrix-assisted laser desorption time of flight mass spectrophotometry (MALDI-TOF MS) investigation. Results obtained from YEAST PANEL multiplex PCR assay were 100% consistent with those of MALDI-TOF MS. Utilization of pure colony testing showed distinct amplicons for each species, thus eliminating the need for DNA extraction. The targeted yeast species of this assay are responsible for 95% of the yeast infections. In conclusion, due to the high accuracy and coverage of a broad range of yeasts, this assay could be useful for identification in routine laboratories and epidemiological studies.

1. Introduction

The number of patients with invasive fungal infections (IFIs) continues to rise (Pfaller and Diekema, 2007), and the majority of the infections are attributable to members of yeast genera, e.g., *Candida*, *Trichosporon, Rhodotorula, Cryptococcus,* and *Geotrichum* (Pappas et al., 2010; Pfaller and Diekema, 2007). *Candida* species account for the majority of candidiasis in patients with a broad range of underlying conditions (Miceli et al., 2011). *Trichosporon* species are the second leading cause of fungemia among patients with hematological disorders (Miceli et al., 2011). *Rhodotorula* species are linked to catheter-related infections (Miceli et al., 2011), while *Cryptococcus* species are more likely to be found in HIV/AIDS-infected patients (Park et al., 2009). *Geotrichum* species are associated with infections in severely immunocompromised patients (Miceli et al., 2011). Depending on the species, mortality of invasive yeast infections ranges from 15 to 80% (Pfaller and Diekema, 2007; Richardson and Lass-Flörl, 2008). Additionally, the growing number of non-*albicans Candida* species that are less susceptible to fluconazole is worrisome (Cleveland et al., 2015; Vallabhaneni et al., 2016).

Historically, phenotypic and biochemical assays are frequently used in developing countries for species identification (Posteraro et al., 2015). However, these assays are time-consuming and prone to error (Kathuria et al., 2015). Despite the rising popularity of matrix-assisted laser desorption time of flight mass spectrophotometry (MALDI-TOF

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MS), this technique is suffering from the limited availability of reference spectra for rare *Candida* species and opportunistic yeast species in general, and the database should be updated regularly (Clark et al., 2013; Posteraro et al., 2013, 2015). Sanger sequencing of so-called DNA barcodes is the gold standard approach in medical mycology; however, the application in developing countries is limited (Clark et al., 2013; Criseo et al., 2015; Posteraro et al., 2013, 2015). Limited usage of MALDI-TOF MS and Sanger sequencing in developing countries is due to high costs (Azim et al., 2015; Criseo et al., 2015; Mathur et al., 2014).

Nowadays, polymerase chain reaction (PCR) is regarded as a standard platform in many clinical laboratories even in developing countries due to its affordability and reproducibility (Ragheb and Jimenez, 2014; World Health Organization, 2016). Herein, we developed a panel of PCR assays, the YEAST PANEL multiplex PCR assay, which identifies the most clinically important yeast species, including the most important species of *Candida*, *Cryptococcus* spp., *Trichosporon asahii*, *Rhodotorula mucilaginosa*, and *Geotrichum* spp.

2. Materials and methods

2.1. Isolates and growth conditions

For the primary validation, our multiplex PCR assay was evaluated using 405 CBS reference strains. In order to optimize and test the specificity of the YEAST PANEL multiplex PCR, 121 CBS reference strains (Supplementary Table A1), including 100 strains of clinically important nontarget fungal species and 21 yeast target species (pooled from the validation test set), were used. Upon optimization and proving specificity, 305 CBS reference strains containing 21 target species were employed for the validation test set. Finally, in order to assess the reproducibility of the multiplex PCR assay, 804 clinical isolates from Austria, Iran, and China were used (Fig. 1). Isolates were grown on Glucose-Yeast Extract-Peptone-Agar (GYPA) media for 48 h at 25 °C. In order to detect mixed colonies and presumptively identify target species, the same copy of strains alternatively was grown on CHROMagar (Chromogenic Technology, Paris, France). Afterward, single pure colonies were subjected to the second round of culture on GYPA media for 48 h at 25 °C.

2.2. Primer design

Target loci for primer design were retrieved from NCBI database (https://www.ncbi.nlm.nih.gov/nucleotide/). Selection criteria for primers were as follows: 1) primers should not cross-react with the other species, 2) compatibility of amplicon sizes of one target species with the rest of target species in the same multiplex PCR, 3) melting temperature compatibility of primers within the same multiplex PCR, 4) LOCATION of primers in the most stable segment of target loci, and 5) in order to prevent cross-reactivity with nontarget species, the gaps and mismatches were positioned in the 3′ end of primers. Online free software of Integrated DNA Technology was used to calculate Tm and Delta G of primers (https://eu.idtdna.com/calc/analyzer). Primers were constructed and shipped by IDT Company (Leuven, Belgium). All employed primers in this study are listed in Supplementary Tables A2-A4.

2.3. DNA extraction

DNA samples were extracted by QIAGEN DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) with slight modifications. One full loop of pure colonies (with the volume of 10 μL) was mixed with 200

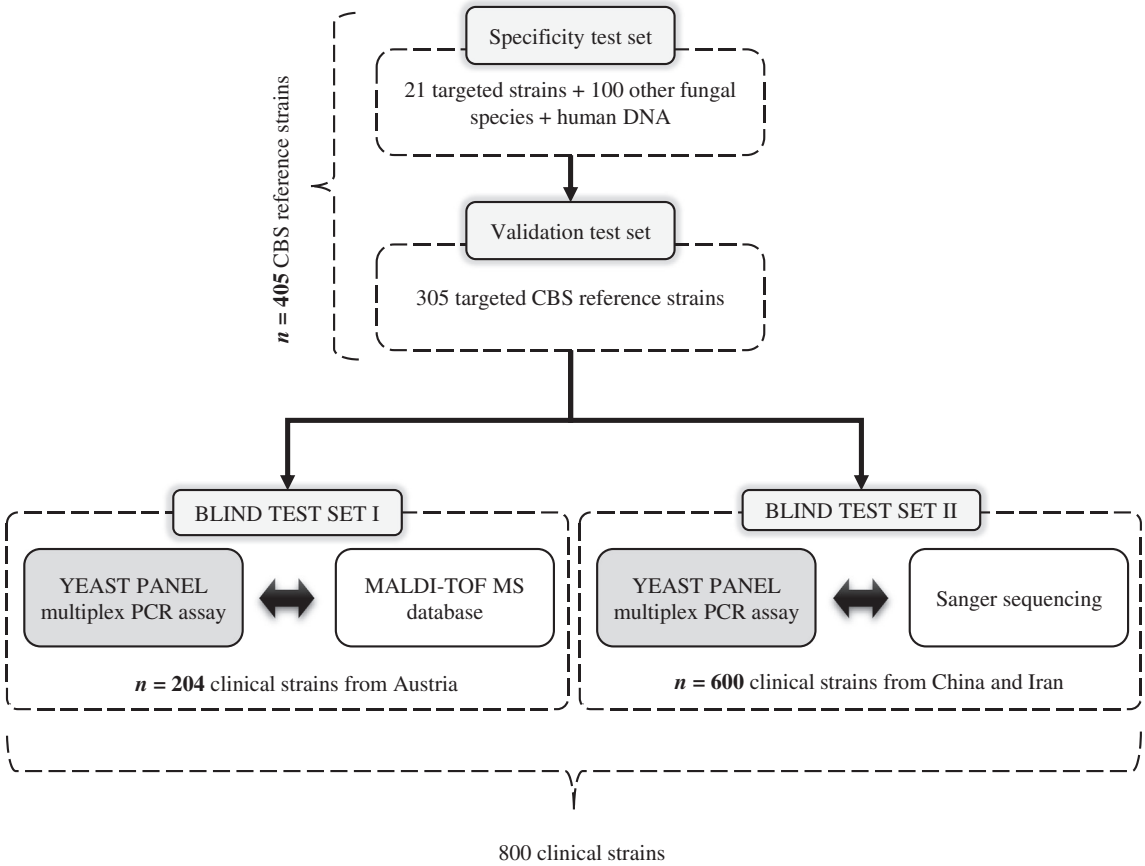

Fig. 1. Workflow chart utilized in this study. After initial optimization, specificity, and validation using CBS reference strains, YEAST PANEL multiplex PCR assay was subjected to reproducibility testing using two blinded test sets containing clinical isolates (204 isolates from Austria and 600 isolates from Iran and China).

μL of ATL buffer and 20 μL of proteinase K (QIAGEN, Hilden, Germany) and incubated at 56 °C for 30 min. Cell suspensions were bead-beaten for 3 min with the frequency of 30,000 oscillations/min. Subsequently, samples were mixed with 200 μL of AL buffer and incubated at 56 °C for 30 min. The rest of DNA extraction was performed as instructed by manufacturer. Quantity and quality of DNA samples were measured by QuBit dsDNA BR Assay Kit (Thermo Fisher Scientific corporation, Waltham, MA, USA) and NanoDrop™ 2000 (Thermo Fisher Scientific, Waltham, MA), respectively. DNA samples were adjusted at 1 ng/μL, and 1 μL of DNA samples was used as PCR template.

2.4. PCR conditions

The PCR was optimized in a final volume of 50 μL as follows: 5 μL 10× buffer (10× NH4, No $MgCl_2$), 1.5 mM $MgCl_2$, 0.2 mM of mixed dNTP (dNTP mix, 100 Mm, Biolab), 2.5 U of *Taq* enzyme (Bio Taq DNA Polymerase, Biolab), and 1 μL of DNA, and MilliQ water was used to adjust the volume to 50 μL. The quantity of each primer used for each reaction is mentioned in Supplementary Tables A2–A4.

PCR was carried out on Applied Biosystem 2720 Thermal Cycler (Thermo Fisher Scientific, Walham, MA). PCR conditions and programs are mentioned in Supplementary Tables A2–A4. Upon PCR, amplicons were run on a 2% agarose gel for 75 min (8 V/cm), stained with GelRed (BioTium, USA), and visualized using gel documentation (Gel Doc XR^+, BioRad, USA). Species identification was achieved by discrimination of amplicon sizes (Fig. 3).

2.5. Specificity testing and optimization

A test set encompassing closely and distantly related yeast species (CBS reference strains), filamentous fungi (CBS reference strains), and human genomic DNA (Promega Corporation, Madison, WI) was prepared by a first technician in a blinded fashion. Isolates were sequentially ordered from 1 to 121 (Supplementary Table A1). The second technician carried out the PCR as indicated in the workflow (Fig. 2). The results obtained from YEAST PANEL multiplex PCR assay were compared to the species identity as designated by Westerdijk Institute, Utrecht, Netherlands (http://www.westerdijkinstitute.nl/Collections/). For ease of identification and prevention of misidentifications, the following tips are essential: 1) running respective controls along with unidentified isolates to monitor presence of inhibitors or appropriate condition of master mix (inactivity or lack of PCR components) and 2) instead of regular commercial ladders, 15 μL of mixture of PCR products of target species was utilized, which contained 10 μL of PCR product from each target species (70 μL), 10 μL of deionized water, and 16 μL of 10× loading dye.

2.6. Validation of YEAST Panel Multiplex PCR Assay using pure colonies of CBS reference strains

In order to optimize each PCR reactions of YEAST Panel multiplex PCR assay, 305 CBS reference strains were utilized. In this stage, in order to shorten hands-on time, instead of DNA samples, the pure

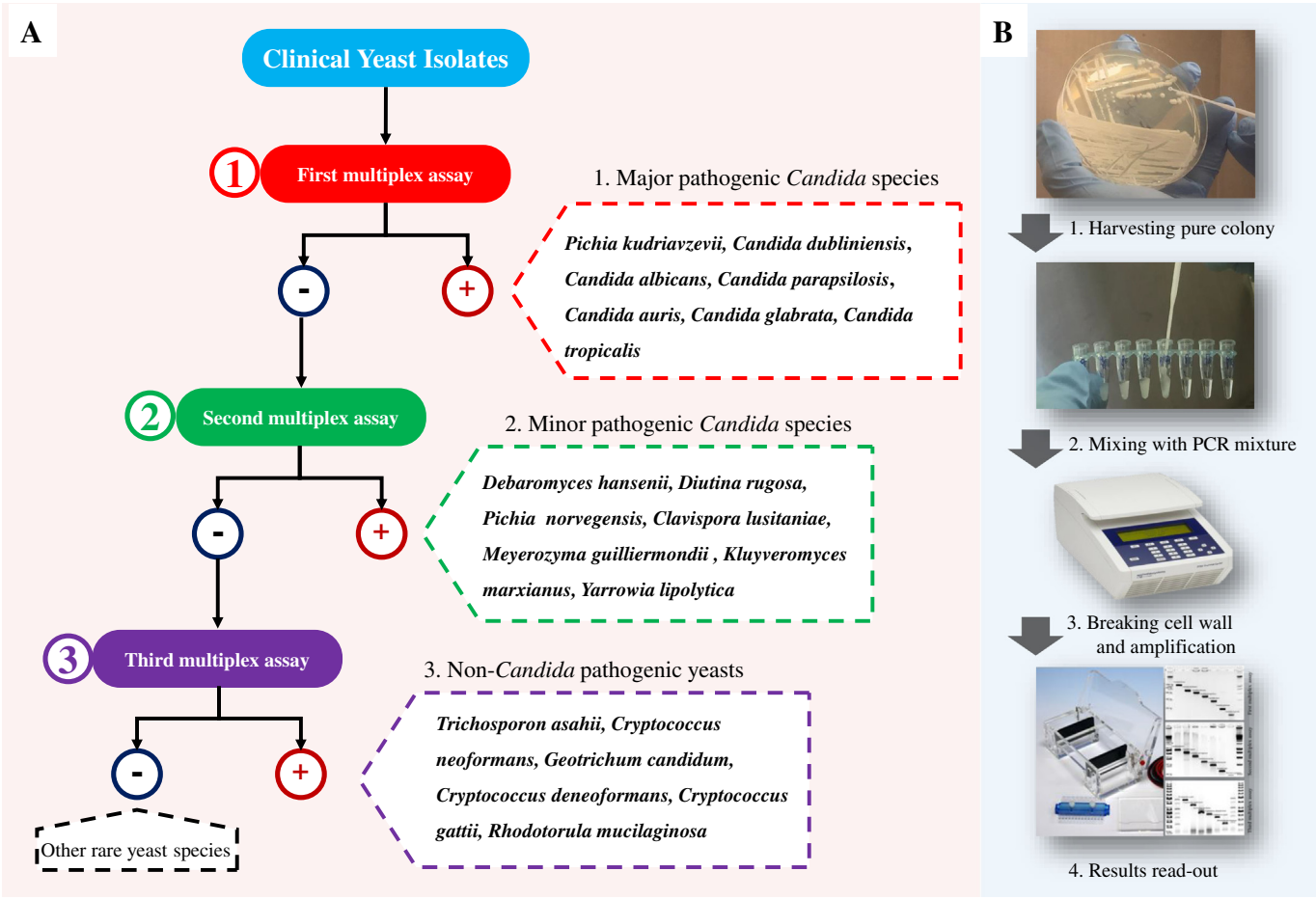

Fig. 2. The workflow of YEAST PANEL. A) The identification strategy used. If the first multiplex PCR is negative, the yeast colony is subjected to the second multiplex PCR. If the result from the second multiplex PCR is negative, a third multiplex PCR is carried out. If the third multiplex PCR is negative, it could be another yeast species not covered by our multiplex PCR. B) Two to 3 small colonies are mixed with the PCR master mix followed by running PCR program. Results are visualized on the gel, and the banding patterns are compared with the respective controls.

colonies of each target species were utilized. Briefly, one full loop of colonies (1 μL volume, around 1–3 small colonies) was used as the PCR template, and by turning the loop, colonies were completely mixed inside the PCR master mixes (Table 1 and Fig. 1).

2.7. Identification strategy

The identification strategy of YEAST PANEL multiplex PCR assay is summarized in Fig. 2. Initially, pure yeast colonies are obtained, and they were subjected to the first multiplex PCR assay targeting the most clinically encountered *Candida* species. In case of negative results, the pure colonies were employed in the second multiplex PCR targeting less common *Candida* species. If strain identification was not successful, the pure colonies were mixed in the third tube, targeting common basidiomycetous yeast species and *Geotrichum* spp. If identification through the final multiplex was not possible, the yeast species is not present in the panel, and it might be regarded as a rare yeast species.

2.8. Evaluation of YEAST Panel Multiplex PCR assay through MALDI-TOF MS-identified clinical strains (blind test set I)

In order to validate the reproducibility of YEAST Panel multiplex PCR assay, one blind test set from Hygiene and Medical Microbiology Division, Innsbruck, Austria (n=204) was prepared. These isolates were identified by MALDI-TOF MS, and they were serially coded from 1 to 204. Blind test set was prepared as mentioned before. Results obtained from the PCR were compared with those of MALDI-TOF MS. In this experiment, pure colonies were used as template.

2.9. MALDI-TOF MS

Identification by MALDI-TOF MS (Bruker Biotyper, MicroFlex, LT, BRUKER DALTONIOS, Bremen, Germany) was performed by full extraction method (Cassagne et al., 2013).

2.10. Evaluation of YEAST Panel Multiplex PCR assay through Sanger sequencing-identified clinical strains (blind test set II)

In order to validate the reproducibility of the YEAST Panel multiplex PCR assay, a second blind test set from Invasive Fungal Research Center, Sari, Iran, and Shanghai Key Laboratory Molecular Medical Microbiology, Shanghai, China (n=600), was prepared. These isolates were identified by dideoxy-chain termination sequencing using large subunit ribosomal DNA primers and internal transcribed spacer (Stielow et al., 2015). The blind test set was prepared as mentioned before. Results obtained from the PCR were compared with those of Sanger sequencing. In this experiment, except for *Rhodotorula mucilaginosa* (appeared as red colonies), for the rest of isolates, pure colonies were used as PCR template.

3. Results

3.1. Specificity testing

Specificity testing using a wide range of yeast species, 4 filamentous fungi (*Aspergillus* spp.), and human DNA showed no cross-reactivity for the first and third multiplex PCR assay. In the second multiplex PCR, *Meyerozyma caribbica* was identified as *Meyerozyma guilliermondii*. Additionally, in the same reaction, *Candida zeylanoides* was misidentified as *M. guiliermondii*.

3.2. YEAST PANEL Multiplex PCR assay resolution for species identification

Subjecting 305 CBS reference strains to the YEAST PANEL multiplex PCR assay resulted in unequivocal identification of each target species as designated by the Westerdijk Institute (Supplementary Table A1, Fig. 3). Additionally, members of the cryptic species complex of *C. albicans* and *C. africana* were identified as *C. albicans* species complex. *C. parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis* were identified as

Table 1
CBS reference strains utilized for validation of YEAST Panel Multiplex PCR Assay. Species identification was assigned based on distinct PCR products sizes. Identification for all of strains was performed on pure colonies, while for *R. mucilaginosa*, DNA samples extracted were used as PCR template.

Species	PCR ID		PCR results		
		Amplicon Size (bp)	First tube	Second tube	Third tube
Candida albicans (n=38)	*Candida albicans*	606 bp	38/38	0/38	0/38
C. africana (n=1)	*Candida albicans*	606 bp	1/1	NA	NA
C. dubliniensis (n=15)	*C. dubliniensis*	718 bp	15/15	0/15	0/15
C. glabrata (n=20)	*C. glabrata*	212 bp	40/40	0/40	0/40
C. nivarensis (n=1)	NA	NA	0/1	0/1	0/1
C. bracarensis (n=1)	NA	NA	0/1	0/1	0/1
C. parapsilosis (n=26)	*C. parapsilosis* complex	490 bp	44/44	0/44	0/44
C. orthopsilosis (n=1)	*C. parapsilosis* complex	490 bp	1/1	NA	NA
C. metapsilosis (n=1)	*C. parapsilosis* complex	490 bp	1/1	NA	NA
C. auris (n=35)	*C. auris*	331 bp	35/35	0/35	0/35
Pichia kudriavzevii (n=15)	*Pichia kudriavzevii*	1159 bp	15/15	0/15	0/15
C. tropicalis (n=15)	*C. tropicalis*	126 bp	15/15	0/15	0/15
Debaromyces hansenii (n=5)	*Debaromyces hansenii*	818 bp	0/5	5/5	0/5
Meyerozyma guilliermondii (n=8)	*Meyerozyma guilliermondii*	302 bp	0/8	8/8	0/8
Kluyveromyces marxianus (n=2)	*Kluyveromyces marxianus*	203 bp	0/2	2/2	0/2
Yarrowia lipolytica (n=8)	*Yarrowia lipolytica*	149 bp	0/8	8/8	0/8
Clavispora lusitaniae (n=12)	*Clavispora lusitaniae*	377 bp	0/12	12/12	0/12
Pichia norvegensis (n=5)	*Pichia norvegensis*	536 bp	0/5	5/5	0/5
Diutina rugosa (n=13)	*Diutina rugosa*	689 bp	0/13	13/13	0/13
Cryptococcus deneoformans (n=19)	*Cryptococcus deneoformans*	235 bp	0/19	0/19	19/19
Cryptococcus neoformans (n=30)	*Cryptococcus neoformans*	392 bp	0/30	0/30	30/30
Cryptococcus gattii (n=22)	*Cryptococcus gattii*	184 bp	0/22	0/22	22/22
Geotrichum candidum (n=6)	*Geotrichum* spp.	299 bp	0/6	0/6	6/6
Rhodotorula mucilaginosa (n=6)	*Rhodotorula mucilaginosa*	111 bp	0/6	0/6	6/6
Trichosporon asahii (n=10)	*Trichosporon* spp.	483 bp	0/10	0/10	10/10
Trichosporon lactis (n=7)	*Trichosporon* spp.	480 bp	0/7	0/7	7/7

Candida africana was identified as *Candida albicans* (606 bp), *Candida orthopsilsis* and *Candida metapsilosis* were identified as *Candida parapsilosis* (490 bp), and *Candida zeylanoides* was misidentified as *Candida guilliermondii* (302 bp).
NA = not amplified.

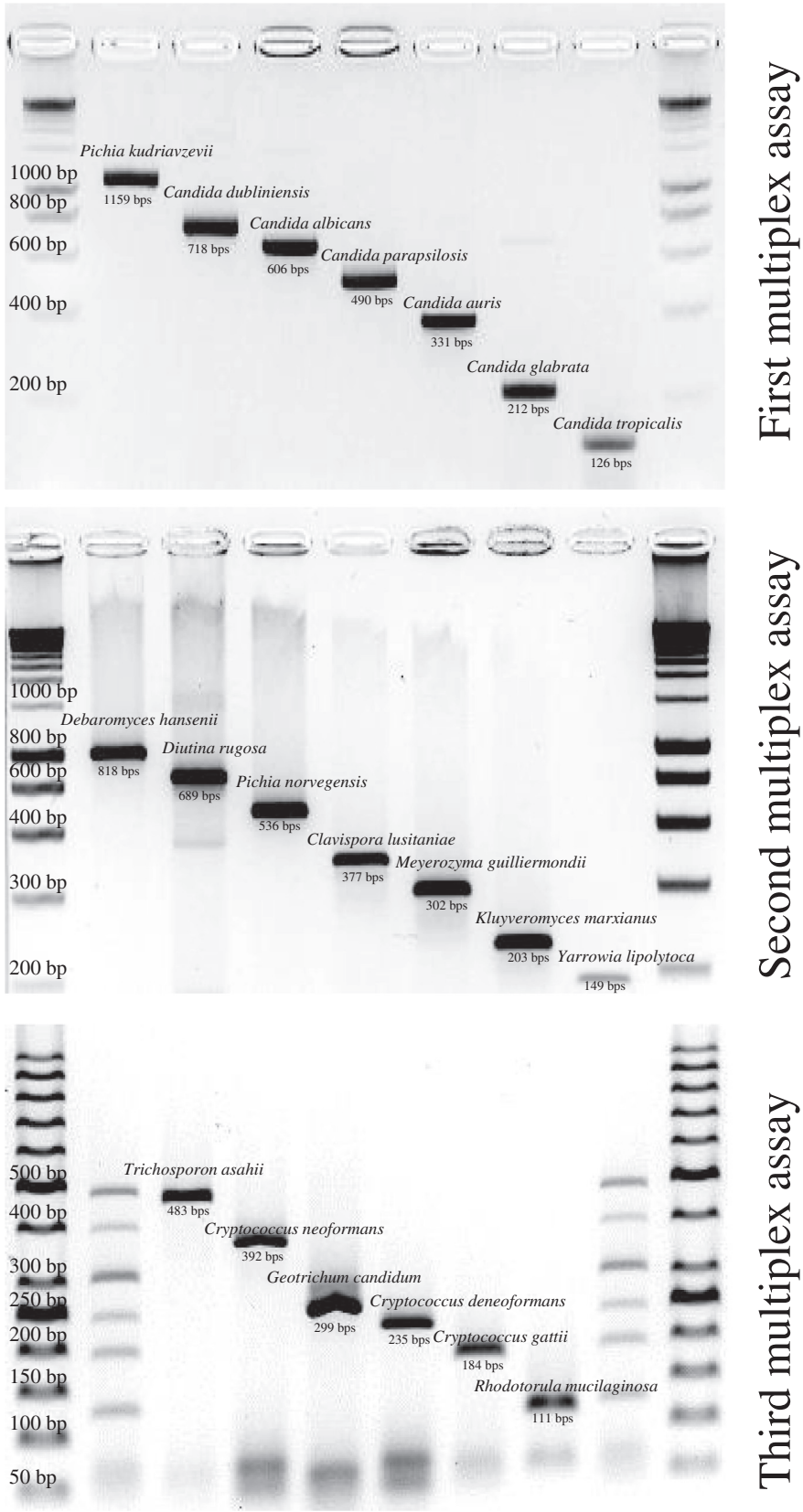

Fig. 3. Banding patterns and the strategy of discrimination of opportunistic yeast species in each multiplex PCR are depicted. Species identification was assigned based on distinct PCR products sizes.

C. parapsilosis. However, the specific primers of *C. glabrata* did not amplify *C. nivariensis* and *C. bracarensis*. Subjecting mixtures of cells of *C. albicans* and *C. glabrata*, *C. albicans* and *C. parapsilosis*, *C. albicans* and *C. tropicalis*, *C. glabrata* and *C. tropicalis*, and *C. glabrata* and *C. parapsilosis* resulted in 2 separate bands in 1 lane corresponding to target species (Supplementary Fig. 1).

3.3. Comparison of MALDI-TOF MS with YEAST PANEL Multiplex PCR assay

Two hundred isolates from Austria were prepared in a blinded way and identified by MALDI-TOF MS (Table 2). YEAST PANEL multiplex PCR showed 100% consistency with results obtained from MALDI-TOF MS. Nontarget yeast species were not amplified, leading to further proof of specificity.

3.4. Comparison of sequencing with YEAST PANEL Multiplex PCR assay

Testing 600 clinical isolates from Iran and China with the YEAST Panel multiplex PCR assay and its comparison with results obtained from the sequencing of D1/D2 large subunit of rDNA and internal transcribed spacer were fully concordant (Table 3).

3.5. Pure colony testing

The YEAST PANEL multiplex PCR assay, except for *Rhodotorula mucilaginosa*, showed full compatibility with pure colony testing. Of 12 clinical isolates of *Rhodotorula mucilaginosa*, none were amplified using pure colony testing; hence, DNA samples were extracted for these isolates. Subsequently, 12 DNA samples corresponding to 12 clinical isolates of *Rhodotorula mucilaginosa* were all successfully amplified. Failure in amplification of direct colonies of *Rhodotorula mucilaginosa* is linked to its pigments that hamper PCR amplification.

4. Discussion

Due to the worldwide growing incidence of non-*albicans Candida* species and their association with difficulty in treatment (Richardson and Lass-Flörl, 2008), identification to the species level is of great importance. However, hospitals and clinics in developing countries due to limited financial supports are deprived of precise but expensive means of identifications, i.e., DNA sequencing and MALDI-TOF MS (Azim et al., 2015; Mathur et al., 2014), leading to poor infection and mortality control in these regions (Herwaldt et al., 1996). However, because of the affordability of the PCR device even for developing countries (Ragheb and Jimenez, 2014; World Health Organization, 2016), this technique can be successfully employed as a useful identification tool. Hence, we present a YEAST PANEL multiplex PCR assay that can identify and distinguish the most clinically important opportunistic yeast species.

Our assay presents a novel stepwise strategy for identification of the clinically most important yeasts belonging to Ascomycota and Basidiomycota. The ARTEMIS-DISK study has shown that *Candida* and basidiomyceteous yeast species account for 95.8% and 4.2% of episodes of yeast infections, respectively (Pfaller et al., 2009). Although *Geotrichum* spp. was not reported in the ARTEMIS study, other studies conducted in the United States, France, and Denmark showed that this opportunistic pathogen accounts for 4.5% to 10.1% of episodes of non-*Candida* yeast infections (Arendrup et al., 2014). The YEAST PANEL multiplex PCR assay can identify 99.6% of the causative agents of candidiasis, and 95% of infections caused by all yeast pathogens (Pfaller et al., 2009). Additionally, as *C. auris* caused fatal outbreak in 5 continents (Chowdhary et al., 2017) and constitutes 5.2% of nosocomial infections in India (Chakrabarti et al., 2014), this globally emerging multidrug-resistant yeast was included in our multiplex PCR assay.

The consistency between YEAST PANEL multiplex PCR assay and MALDI-TOF MS was 100%. Although MALDI-TOF MS bears a high degree of specificity, misidentification of important *Candida* and other yeast species due to insufficient number of spectra is considered as a deficiency of this platform (Jamal et al., 2014; Kim et al., 2016; Ling et al., 2014). Additionally, despite lower experimental costs of MALDI-TOF MS than conventional phenotypic assays, the purchase and maintenance of a MALDI-TOF MS device are cost-prohibitive, driving developing countries to rely on nonspecific conventional and phenotypic methods (Kim et al., 2016; Mathur et al., 2014; Xiao et al., 2016).

Subjecting a wide range of yeast species to the YEAST PANEL multiplex PCR assay and its comparison with sequencing resulted in 100% agreement with all clinical target strains (n=600). Traditionally, Sanger sequencing is considered as the gold standard approach in medical mycology (Pryce et al., 2003). However, the inevitable requirement for highly trained technicians to run the device, apart from being more time-consuming and expensive, is a deterrent factor for its popularity in routine laboratories (Criseo et al., 2015). However, YEAST PANEL multiplex PCR assay is less expensive and requires less hands-on time when compared to sequencing.

Despite the widespread usage of phenotypic and biochemical assays in routine laboratories, especially in developing countries (Kathuria et al., 2015), a multitude of studies have revealed that commercially routine biochemical assays misidentify uncommon yeast species (de

Table 2
Clinical strains from Austria utilized for validation of YEAST Panel Multiplex PCR assay and its comparison with MALDI-TOF MS. Species identification was assigned based on distinct PCR products sizes.

Species	PCR ID	Amplicon size	PCR results			Concordance with MALDI-TOF MS
			First tube	Second tube	Third tube	
C. albicans (n=84)	*C. albicans*	606 bp	84/84	NA	NA	84/84
C. dubliniensis (n=3)	*C. dubliniensis*	718 bp	3/3	NA	NA	3/3
C. glabrata (n=33)	*C. glabrata*	212 bp	33/33	NA	NA	33/33
C. parapsilosis (n=26)	*C. parapsilosis* complex	490 bp	26/26	NA	NA	26/26
Pichia kudriavzevii (n=17)	*Pichia kudriavzevii*	1159 bp	17/17	NA	NA	17/17
C. tropicalis (n=15)	*C. tropicalis* (n=15)	126 bp	15/15	NA	NA	15/15
Meyerozyma guilliermondii (n=3)	*Meyerozyma guilliermondii*	302 bp	0/3	3/3	NA	3/3
Kluyveromyces marxianus (n=2)	*Kluyveromyces marxianus*	203 bp	0/2	2/2	NA	2/2
Clavispora lusitaniae (n=5)	*Clavispora lusitaniae*	377 bp	0/5	5/5	NA	5/5
Diutina pararugosa (n=5)	NA	NA	0/5	0/5	0/5	0/5
Diutina rugosa (n=1)	*Diutina rugosa*	689 bp	0/1	1/1	NA	1/1
Pichia inconspicua (n=5)	NA	NA	0/5	0/5	0/5	0/5
Cryptococcus neoformans (n=2)	*Cryptococcus neoformans*	392 bp	0/2	0/2	2/2	2/2
Trichosporon asahii (n=1)	*Trichosporon* spp.	483 bp	0/1	0/1	1/1	1/1
Stephanoascus ciferrii (n=1)	NA	NA	0/1	0/1	0/1	0/1
Saccharomyces cerevisiae (n=1)	NA	NA	0/1	0/1	0/1	0/1
Total number (n=204)[a]	n= 192 (94%)		n=178 (100%)	n=11 (100%)	n=3 (100%)	n=192 (94%)

NA = not amplified.
[a] Among total 204 isolates, 12 are nontarget yeast species, while the rest of 192 isolates are among target species.

Table 3
Clinical strains from Iran and China utilized for validation of YEAST Panel Multiplex PCR assay and its comparison with Sanger sequencing. Species identification was assigned based on distinct PCR products sizes. Identification for all of strains was performed on pure colonies, while for *R. mucilaginosa*, DNA samples extracted were used as PCR template.*a, **b

Species	PCR ID	Amplicon size	PCR results			Concordance with Sanger sequencing
			First tube	Second tube	Third tube	
C. albicans (*n*=156)	*C. albicans*	606 bp	156/156	NA	NA	156/156
C. dubliniensis (*n*=8)	*C. dubliniensis*	718 bp	8/8	NA	NA	8/8
C. glabrata (*n*=140)	*C. glabrata*	212 bp	140/140	NA	NA	140/140
Pichia kudriavzevii (*n*=25)	*Pichia kudriavzevii*	1159 bp	25/25	NA	NA	25/25
C. parapsilosis complex[a] (*n*=80)	*C. parapsilosis* complex	490 bp	80/80	NA	NA	80/80
C. tropicalis (*n*=65)	*C. tropicalis*	126 bp	65/65	NA	NA	65/65
Debaromyces hansenii (*n*=19)	*Debaromyces hansenii*	818 bp	0/19	19/19	NA	19/19
Meyerozyma guilliermondii (*n*=40)	*Meyerozyma guilliermondii*	302 bp	0/40	40/40	NA	40/40
Kluyveromyces marxianus (*n*=17)	*Kluyveromyces marxianus*	203 bp	0/17	17/17	NA	17/17
Yarrowia lipolytica (*n*=3)	*Yarrowia lipolytica*	149 bp	0/3	3/3	NA	5/5
Clavispora lusitaniae (*n*=1)	*Clavispora lusitaniae*	377 bp	0/1	1/1	NA	1/1
Pichia norvegensis (*n*=3)	*Pichia norvegensis*	536 bp	0/3	3/3	NA	5/5
Diutina rugosa (*n*=5)	*Diutina rugosa*	689 bp	0/5	5/5	NA	5/5
Cryptococcus neoformans (*n*=10)	*Cryptococcus neoformans*	392 bp	0/10	0/10	10/10	10/10
Geotrichum silvicola (*n*=9)	*Geotrichum spp.*	299 bp	0/9	0/9	9/9[b]	9/9
Trichosporon asahii (*n*=7)	*Trichosporon* spp.	483 bp	0/7	0/7	7/7	7/7
Rhodotorula mucilaginosa (*n*=12)	*Rhodotorula mucilaginosa*	111 bp	0/12	0/12	12/12	12/12
Total Number (*n*=600)			*n*=474 (100%)	*n*=88 (100%)	*n*=38 (100%)	*n*=600 (100%)

[a] *Candida parapsilosis* complex contained 2 strains of *Candida orthopsilosis* and 2 strains of *Candida metapsilosis* and were identified as *Candida parapsilosis* (490 bp).
[b] *Geotrichum silvicola* is the synonym of *Galactomyces candidum*

Almeida Júnior et al., 2014) and a broad variety of *Candida* species (Keçeli et al., 2016), especially *M. guilliermondii* and *C. auris* (Castanheira et al., 2013; Kathuria et al., 2015). Additionally, delayed and inappropriate identifications are associated with a higher mortality rate and hospitalization costs (Morrell et al., 2005). The turnaround time for identification, another inherent obstacle of conventional biochemical assays (up to 48 h), was improved with the YEAST PANEL multiplex PCR assay since identification was achieved in 3–6 h.

Misidentification of *C. zeylanoides* as *M. guilliermondii* in the second multiplex PCR reaction was the major limitation of our study. However, literatures data revealed that the incidence of *C. zeylanoides* is extremely low (0.01–0.04%) globally. In the same tube, *Meyerozyma caribbica* was misidentified as *Meyerozyma guilliermondii*, which can be explained by their close genetic similarity. Although *Saccharomyces cerevisiae*, *C. nivariensis*, and *C. bracarensis* have been implicated in human infections, they were not included in our multiplex PCR assay (Enache-Angoulvant and Hennequin, 2005; Hou et al., 2017). Direct colony and gel electrophoresis dependence and lack of identification of clinically important molds were among the other limitations to our study. As *Rhodotorula mucilaginosa* contains red-colored pigments, DNA extraction and purification are imperative to obtain successful PCR amplification and subsequently establish the identity of this species.

5. Conclusion

Due to the shortages of systematic epidemiological data in developing countries and the lack of financial supports for high-cost and precise means of identification, such as Sanger sequencing and MALDI-TOF MS, these countries might face difficulties in the establishment of empiric therapies, which cause a higher rate of mortality. However, because of the affordability of PCR devices, the YEAST PANEL multiplex PCR assay has the potential to be integrated in large-scale epidemiological studies. On the other hand, routine laboratories in developing countries can take the advantage of supplementation of biochemical assays with YEAST PANEL multiplex PCR assay.

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