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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 sub-optimal 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 PCR and MALDI-TOF MS investigation. Results obtained from YEAST PANEL multiplex PCR assay was 100% consistent with that 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.

Key words: Developing countries, yeast infection, multiplex PCR, molecular identification, developing countries

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* (Pfaller and Diekema 2007; Pappas et al. 2010). *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 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; Posteraro et al. 2013, 2015; Criseo et al. 2015). Limited usage of MALDI-TOF MS and Sanger sequencing in developing countries is due to high costs (Mathur et al. 2014; Azim et al. 2015; Criseo et al. 2015).

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.

Materials and methods

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 non-target 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 (Figure 1). Isolates were grown on Glucose-Yeast Extract-Peptone-Agar (GYPA) media, for 48 hours at 25 ℃. In order to detect mixed colonies and presumptively identify target species, the same copy of strains alternatively were grown on CHROMagar (Chromogenic Technology, Paris, France). Afterward, single pure colonies were subjected to

the second round of culture on GYPA media for 48 hours at 25

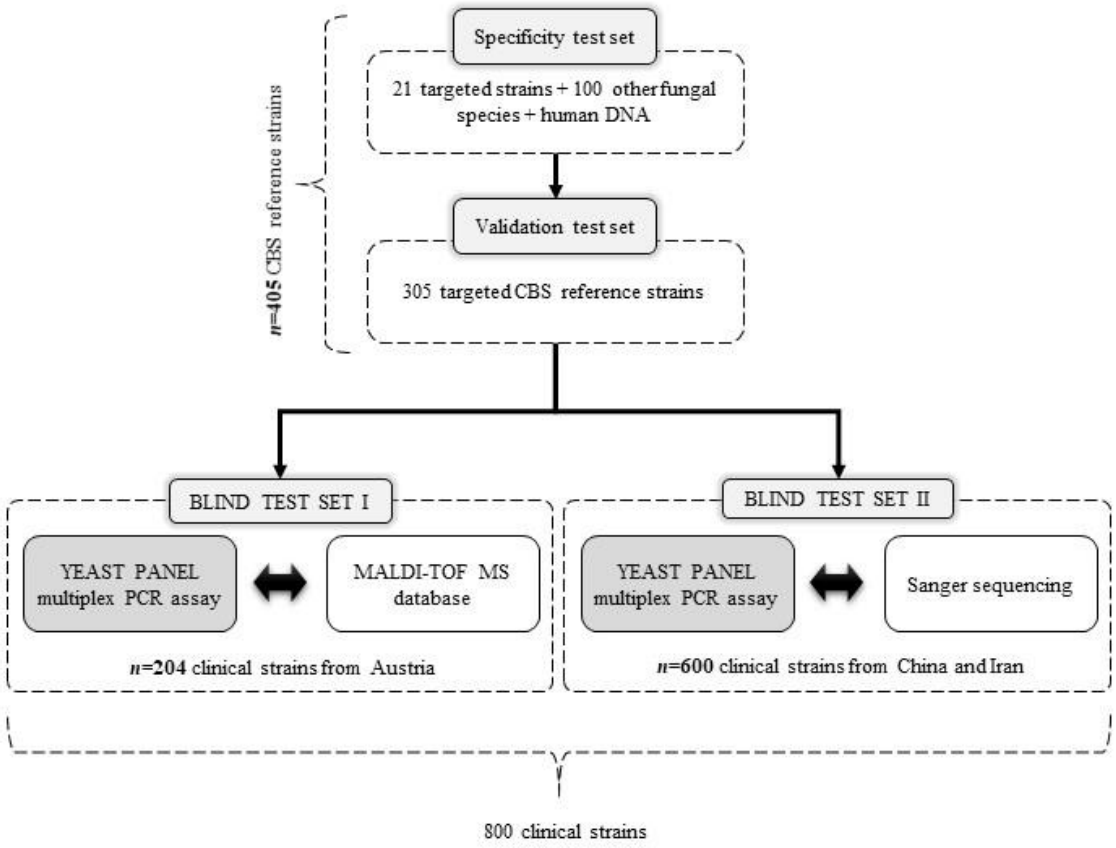

Figure 1. Workflow chart utilized in this study. After Initial optimization, specificity and validation using CBS reference strains, YEAST PANEL multiplex PCR assay was subjected to reproducibility testing using two blinded test set containing clinical isolates (204 isolates from Austria and 600 isolates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

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 following; 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 non-

target 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 Table A2-A4.

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 10µl) were mixed with 200µl of ATL buffer and 20 µl of proteinase K (QIAGEN, Hilden, Germany) and incubated at 56 °C for 30 minutes. Cell suspensions were bead-beaten for 3 minutes with the frequency of 30,000 oscillations/minute. Subsequently, samples were mixed with 200µl of AL buffer and incubated at 56 °C for 30 minutes. 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, Massachusetts, USA) and NanoDrop™ 2000 (Thermo Fisher Scientific, Waltham, Massachusetts, USA), respectively. DNA samples were adjusted at 1ng/µl and 1µl of DNA samples were used as PCR template.

PCR conditions

The PCR reaction was optimized in a final volume of 50 µl as follows, 5 µl 10X buffer (10X NH4, No $MgCl_2$), 1.5mM $MgCl_2$, 0.2mM of mixed dNTP (dNTP mix, 100Mm, Biolab), 2.5 units of *Taq* enzyme (Bio Taq DNA Polymerase, Biolab), 1 µl of DNA, and MiliQ water was used to adjusted the volume to 50 µl. The quantity of each primer used for each reaction mentioned in Supplementary Tables 2-4.

PCR was carried out on Applied Biosystem 2720 Thermal Cycler (Thermo Fisher Scientific, Walham, Massachusstts, USA). PCR conditions and programs are mentioned in the Supplementary Tables 2-4. Upon PCR, amplicons were run on a 2% agarose gel for 75 minutes (8 volt/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 (Figure 3).

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, USA), were prepared by a first technician in a blinded fashion. Isolates were sequentially ordered from 1-121 (Supplementary Table 1). The second technician carried out the PCR as indicated in the workflow (Figure 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 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), 2) Instead of regular commercial ladders, 15µl of mixture of PCR products of target species were utilized, which contained 10 µl of PCR product from each target species (70 µl), 10µl of deionized water, and 16 µl of 10X loading dye.

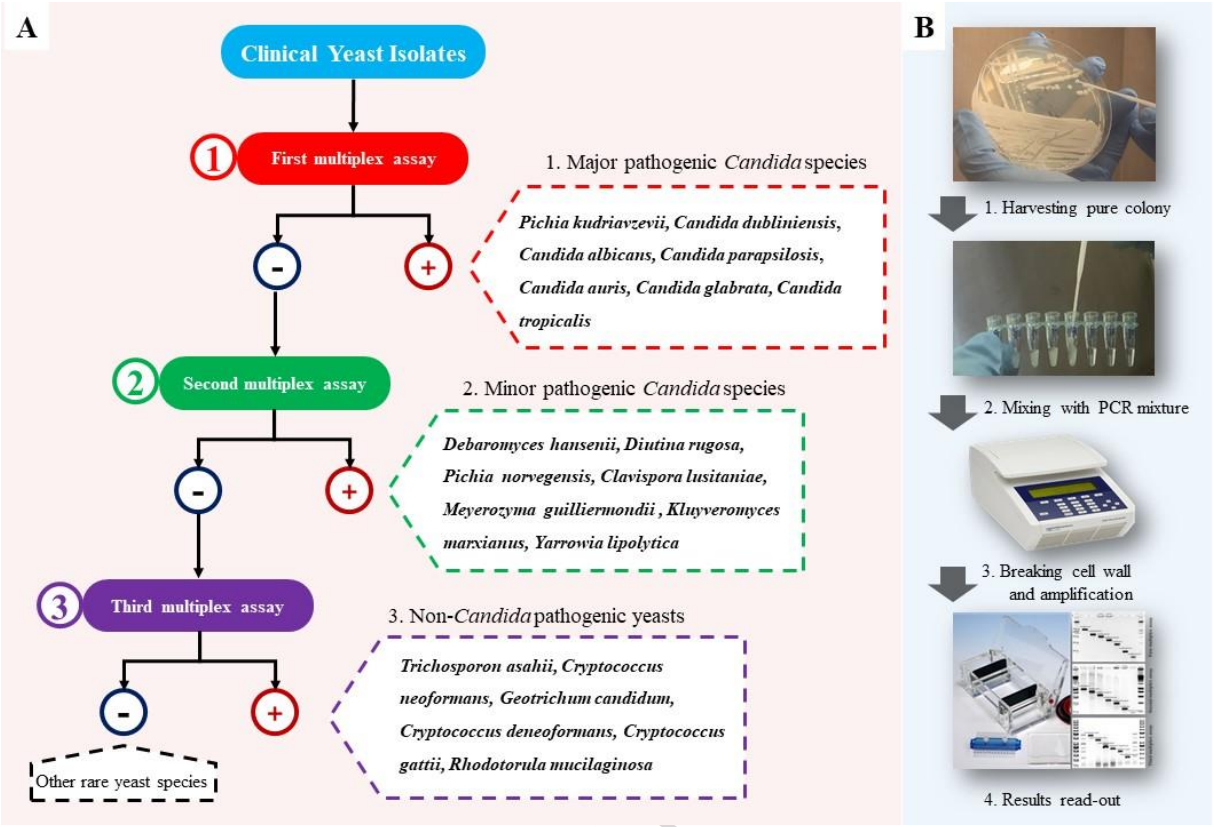

Figure 2. The workflow of YEAST PANEL. A) shows the identification strategy used. If the first multiplex PCR is negative, the yeast colony is subjected to the second multiplex PCR. If the results from the second multiplex PCR is negative, a third multiplex PCR is carried out. If the third multiplex PCR is negative, it could be another yeast species not covered by our multiplex PCR. B), 2-3 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.

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 colonies of each target species were utilized. Briefly, one full loop of colonies (1µl Volume, around 1-3 small colonies) were used as the PCR template and by turning the loop, colonies were completely mixed inside the PCR master mixes (Table 1 and Figure 1).

Identification strategy

The identification strategy of YEAST PANEL multiplex PCR assay is summarized in Figure 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.

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 one to 204. Blind test set was prepared as mentioned before. Results obtained from the PCR were compared with that of MALDI-TOF MS. In this experiment pure colonies were used as template.

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).

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 that 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.

Results

Specificity testing

Specificity testing using a wide range of yeast species, four 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. guiiliermondii*.

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 (Table A1, Figure 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 *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*, *C. glabrata* and *C. parapsilosis* resulted in two separate bands in one lane corresponding to target species (Supplementary Figure 1).

Species	PCR ID	Amplicon Size (bps)	PCR Results		
			First Tube	Second Tube	Third Tube
Candida albicans (*n*=38)	*Candida albicans*	606 bps	38/38	0/38	0/38
C. africana (*n*=1)	*Candida albicans*	606 bps	1/1	NA	NA
C. dubliniensis (*n*=15)	*C. dubliniensis*	718 bps	15/15	0/15	0/15
C. glabrata (*n*=20)	*C. glabrata*	212 bps	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 bps	44/44	0/44	0/44
C. orthopsilosis (*n*=1)	*C. parapsilosis* complex	490 bps	1/1	NA	NA
C. metapsilosis (*n*=1)	*C. parapsilosis* complex	490 bps	1/1	NA	NA
C. auris (*n*=35)	*C. auris*	331 bps	35/35	0/35	0/35
Pichia kudriavzevii (*n*=15)	*Pichia kudriavzevii*	1159 bps	15/15	0/15	0/15
C. tropicalis (*n*=15)	*C. tropicalis*	126 bps	15/15	0/15	0/15
Debaromyces hansenii (*n*=5)	*Debaromyces hansenii*	818 bps	0/5	5/5	0/5
Meyerozyma guilliermondii (*n*=8)	*Meyerozyma guilliermondii*	302 bps	0/8	8/8	0/8
Kluyveromyces	*Kluyveromyces*	203 bps	0/2	2/2	0/2

marxianus (*n*=2)	*marxianus*				
Yarrowia lipolytica (*n*=8)	*Yarrowia lipolytica*	149 bps	0/8	8/8	0/8
Clavispora lusitaniae (*n*=12)	*Clavispora lusitaniae*	377 bps	0/12	12/12	0/12
Pichia norvegensis (*n*=5)	*Pichia norvegensis*	536 bps	0/5	5/5	0/5
Diutina rugosa (*n*=13)	*Diutina rugosa*	689 bps	0/13	13/13	0/13
Cryptococcus deneoformans (*n*=19)	*Cryptococcus deneoformans*	235 bps	0/19	0/19	19/19
Cryptococcus neoformans (*n*=30)	*Cryptococcus neoformans*	392 bps	0/30	0/30	30/30
Cryptococcus gattii (*n*=22)	*Cryptococcus gattii*	184 bps	0/22	0/22	22/22
Geotrichum candidum (*n*=6)	*Geotrichum* spp.	299 bps	0/6	0/6	6/6
Rhodotorula mucilaginosa (*n*=6)	*Rhodotorula mucilaginosa*	111 bps	0/6	0/6	6/6
Trichosporon asahii (*n*=10)	*Trichosporon* spp.	483 bps	0/10	0/10	10/10
Trichosporon lactis (*n*=7)	*Trichosporon* spp.	480 bps	0/7	0/7	7/7

NA. Not amplified.

Candida africana was identified as *Candida albicans* (606 bps), *Candida orthopsilsis* and *Candida metapsilosis* were identified as *Candida parapsilosis* (490 bps) and *Candida zeylanoides* was misidentified as *Candida guilliermondii* (302 bps).

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 were performed on pure colonies, while for *R. mucilaginosa* DNA samples extracted were used as PCR template.

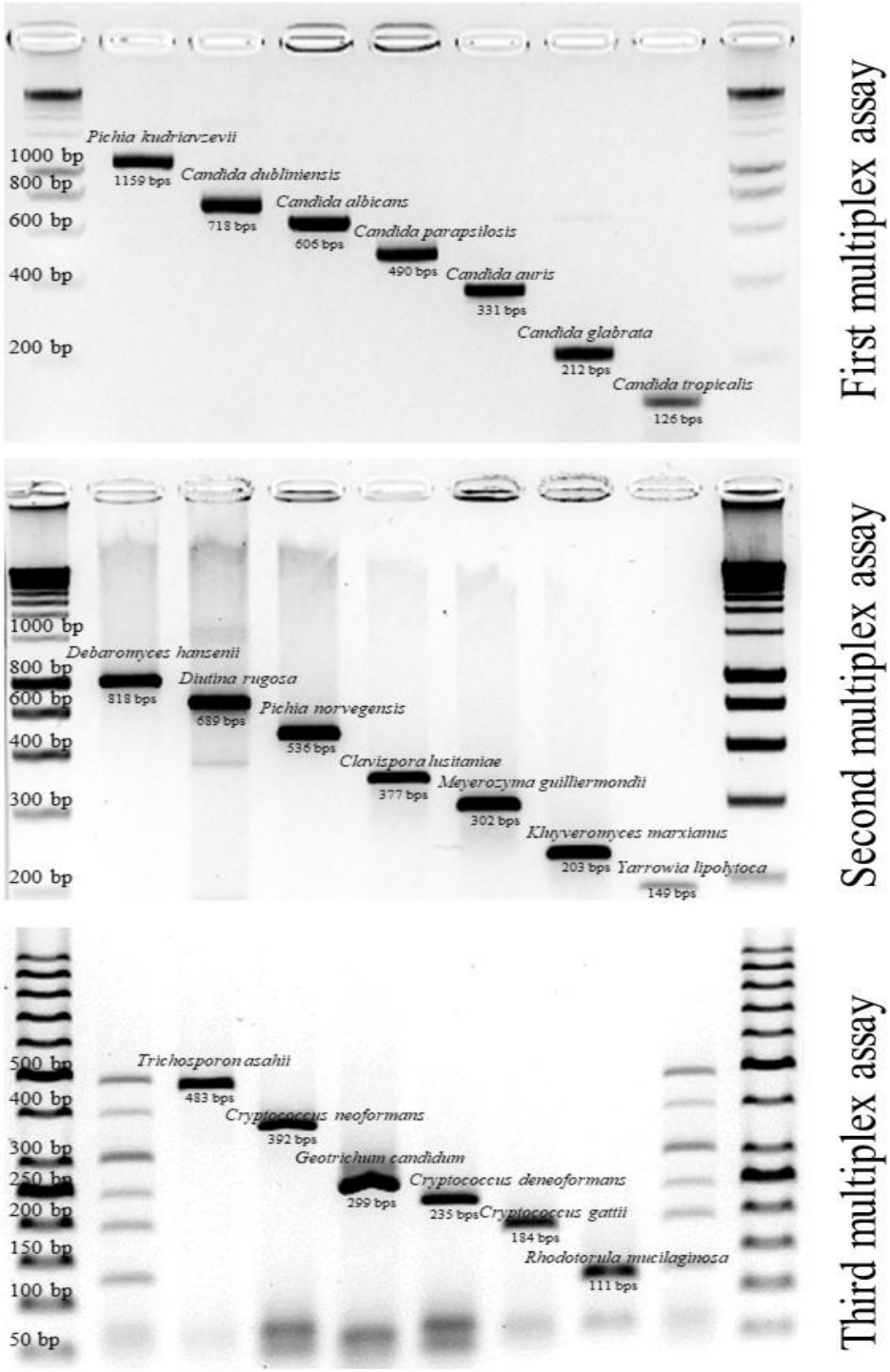

Figure 3. Banding patterns and the strategy of discrimination of opportunistic yeast species in each multiplex PCR are depicted. Species identification was assigned based on distinct PCR products sizes.

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. Non-target yeast species were not amplified, leading to further proof of specificity.

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 bps	84/84	NA	NA	84/84
C. dubliniensis (*n*=3)	*C. dubliniensis*	718 bps	3/3	NA	NA	3/3
C. glabrata (*n*=33)	*C. glabrata*	212 bps	33/33	NA	NA	33/33
C. parapsilosis (*n*=26)	*C. parapsilosis* complex	490 bps	26/26	NA	NA	26/26
Pichia kudriavzevii (*n*=17)	*Pichia kudriavzevii*	1159 bps	17/17	NA	NA	17/17
C. tropicalis (*n*=15)	*C. tropicalis* (*n*=15)	126 bps	15/15	NA	NA	15/15
Meyerozyma guilliermondii (*n*=3)	*Meyerozyma guilliermondii*	302 bps	0/3	3/3	NA	3/3
Kluyveromyces marxianus (*n*=2)	*Kluyveromyces marxianus*	203 bps	0/2	2/2	NA	2/2
Clavispora lusitaniae (*n*=5)	*Clavispora lusitaniae*	377 bps	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 bps	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 bps	0/2	0/2	2/2	2/2
Trichosporon asahii (*n*=1)	*Trichosporon* spp.	483 bps	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)*	*n*= 192 (94%)		*n*=178 (100%)	*n*=11 (100%)	*n*=3 (100%)	*n*=192 (94%)

*NA. Not amplified

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.

*Among total 204 isolates 12 are non-target yeast species, while the rest of 192 isolates are amongst target species.

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).

Species	PCR ID	Amplicon Size	PCR Results			Concordance with Sanger sequencing
			First Tube	Second Tube	Third Tube	
C. albicans (*n*=156)	*C. albicans*	606 bps	156/156	NA	NA	156/156
C. dubliniensis (*n*=8)	*C. dubliniensis*	718 bps	8/8	NA	NA	8/8
C. glabrata (*n*=140)	*C. glabrata*	212 bps	140/140	NA	NA	140/140
Pichia kudriavzevii (*n*=25)	*Pichia kudriavzevii*	1159 bps	25/25	NA	NA	25/25
C. parapsilosis complex* (*n*=80)	*C. parapsilosis* complex	490 bps	80/80	NA	NA	80/80
C. tropicalis (*n*=65)	*C. tropicalis*	126 bps	65/65	NA	NA	65/65
Debaromyces hansenii (*n*=19)	*Debaromyces hansenii*	818 bps	0/19	19/19	NA	19/19
Meyerozyma guilliermondii (*n*=40)	*Meyerozyma guilliermondii*	302 bps	0/40	40/40	NA	40/40
Kluyveromyces marxianus	*Kluyveromyces*	203 bps	0/17	17/17	NA	17/17

(*n*=17)	*marxianus*					
Yarrowia lipolytica (*n*=3)	*Yarrowia lipolytica*	149 bps	0/3	3/3	NA	5/5
Clavispora lusitaniae (*n*=1)	*Clavispora lusitaniae*	377 bps	0/1	1/1	NA	1/1
Pichia norvegensis (*n*=3)	*Pichia norvegensis*	536 bps	0/3	3/3	NA	5/5
Diutina rugosa (*n*=5)	*Diutina rugosa*	689 bps	0/5	5/5	NA	5/5
Cryptococcus neoformans (*n*=10)	*Cryptococcus neoformans*	392 bps	0/10	0/10	10/10	10/10
Geotrichum silvicola (*n*=9)	*Geotrichum spp.*	299 bps	0/9	0/9	9/9**	9/9
Trichosporon asahii (*n*=7)	*Trichosporon* spp.	483 bps	0/7	0/7	7/7	7/7
Rhodotorula mucilaginosa (*n*=12)	*Rhodotorula mucilaginosa*	111 bps	0/12	0/12	12/12	12/12
Total Number (*n*=600)			*n*=474 (100%)	*n*=88 (100%)	*n*=38 (100%)	*n*=600 (100%)

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 were performed on pure colonies, while for *R. mucilaginosa* DNA samples extracted were used as PCR template.

* *Candida parapsilosis* complex contained two strains of *Candida orthopsilosis* and two strains of *Candida metapsilosis* and were identified as *Candida parapsilosis* (490bps).

** *Geotrichum silvicola* is the synonym of *Galactomyces candidum*

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.

Discussion

Due to the worldwide growing incidence of non-*albicans Candida* species and their association with difficulty in treatment (Richardson and Lass-Flörl 2008; Pfaller et al. 2010), 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 (Mathur et al. 2014; Azim et al. 2015) 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, 2010). Although, *Geotrichum* spp. was not reported in the ARTEMIS study, other studies conducted in the US, 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 (Pfaller et al. 2010), and 95% of infections caused by all yeast pathogens (Pfaller et al. 2009, 2010). Additionally, as *C. auris*

caused fatal outbreak in five continents (Chowdhary et al. 2017) and constitutes 5.2% of nosocomial infections in India (Chakrabarti et al. 2014), hence, 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; Ling et al. 2014; Kim et al. 2016). Additionally, despite of lower experimental costs of MALDI-TOF MS than conventional phenotypic assays, the purchase and maintenance of a MALDI-TOF MS device is cost - prohibitive, driving developing countries to rely on non-specific conventional and phenotypic methods (Mathur et al. 2014; Kim et al. 2016; 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, inevitable requirement for highly trained technicians to run the device, apart from being more time-consuming and expensive, are deterrent factors 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 Almeida Júnior et al. 2014), 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 turn-around-time for identification, another inherent obstacle of conventional biochemical assays (up to 48 hours), was improved with the YEAST PANEL multiplex PCR assay, since identification was achieved in 3-6 hours.

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 (Pfaller et al. 2010). In the same tube, *Meyerozyma caribbica* was misidentified as *Meyerozyma guilliermondii*, which can be explained by their close genetic similarity (Pfaller et al. 2010). 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 amongst the other limitations to our study. As *Rhodotorula mucilaginosa* contains red-colored pigments, DNA extraction and purifications is imperative to obtain successful PCR amplification and subsequently establish the identity of this species.

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 causes 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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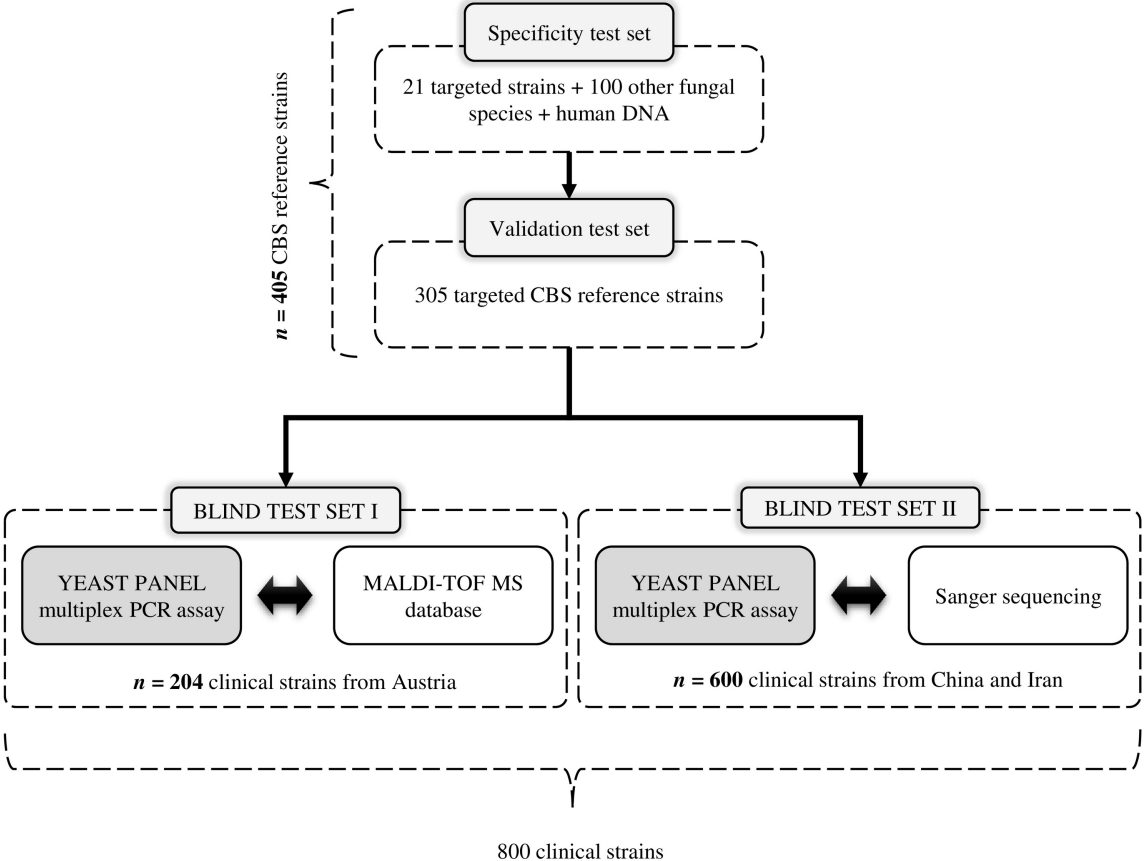

Specificity test set
21 targeted strains + 100 other fungal species + human DNA
Validation test set
305 targeted CBS reference strains
n = 405 CBS reference strains
BLIND TEST SET I
YEAST PANEL multiplex PCR assay
MALDI-TOF MS database
n = 204 clinical strains from Austria
BLIND TEST SET II
YEAST PANEL multiplex PCR assay
Sanger sequencing
n = 600 clinical strains from China and Iran
800 clinical strains

Figure 1

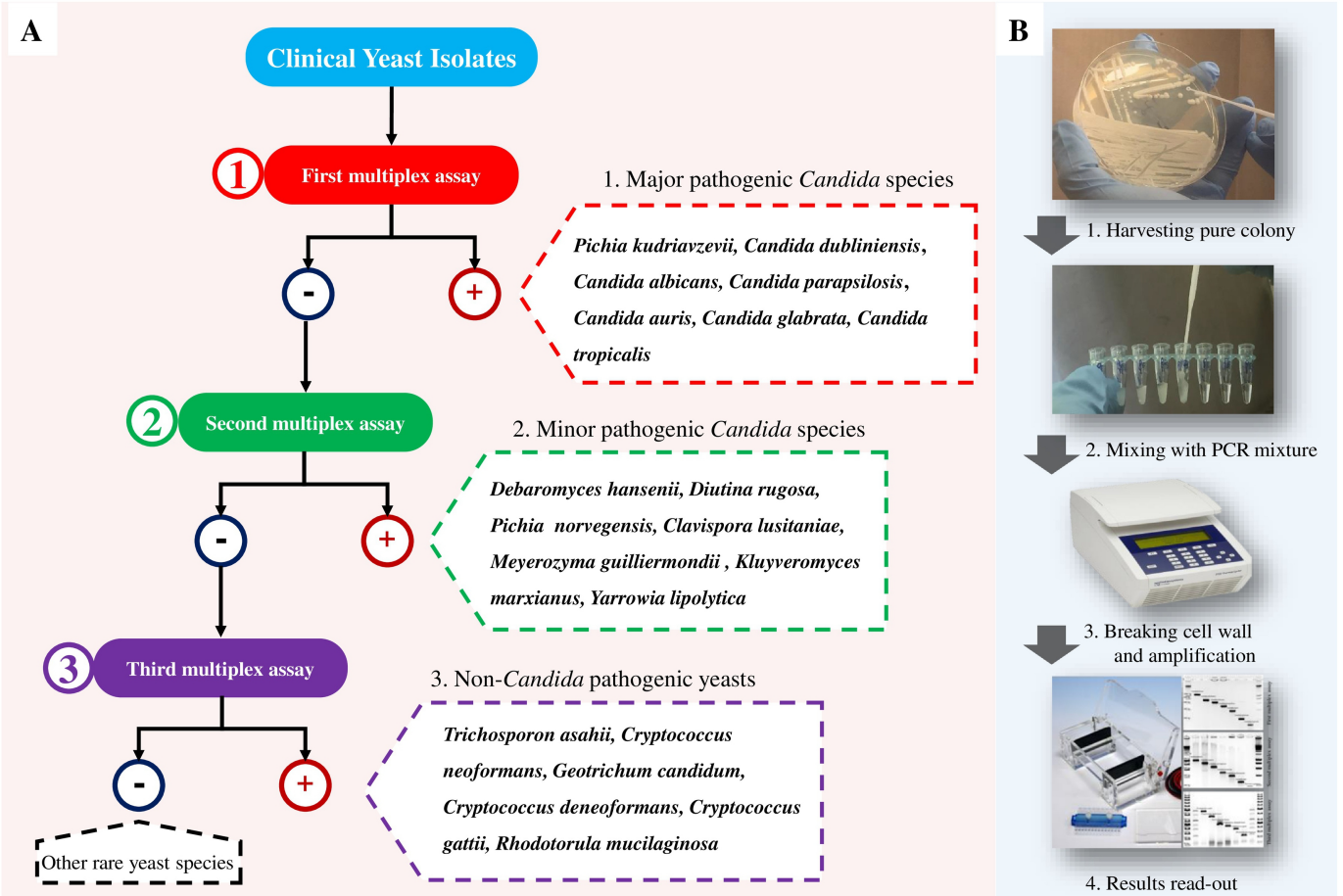
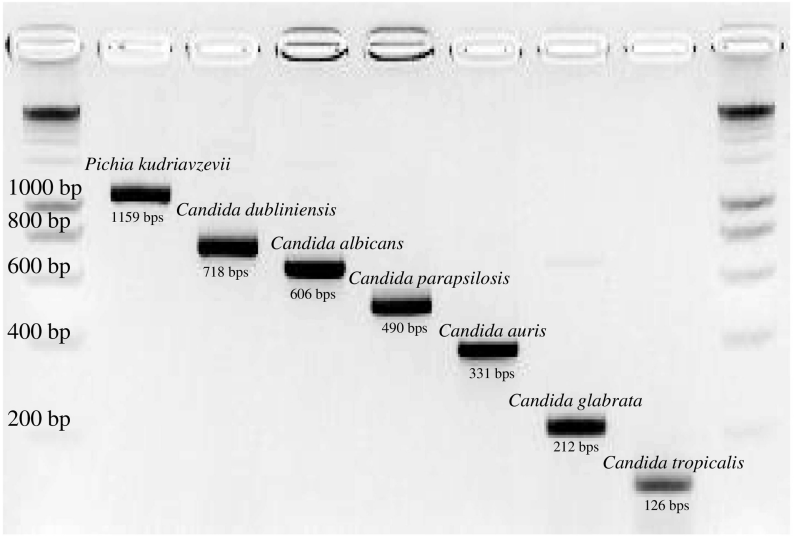
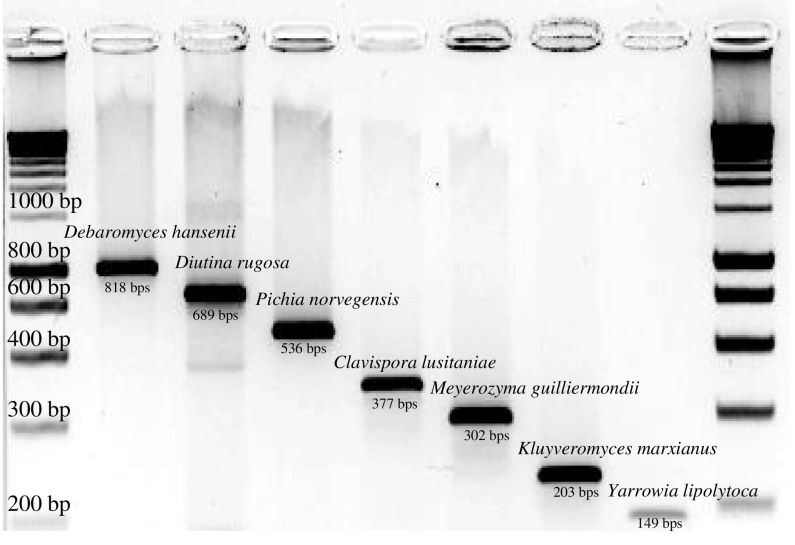

A
Clinical Yeast Isolates
1
First multiplex assay
-
+
1. Major pathogenic Candida species
Pichia kudriavzevii, Candida dubliniensis, Candida albicans, Candida parapsilosis, Candida auris, Candida glabrata, Candida tropicalis
2
Second multiplex assay
-
+
2. Minor pathogenic Candida species
Debaromyces hansenii, Diutina rugosa, Pichia norvegensis, Clavispora lusitaniae, Meyerozyma guilliermondii , Kluyveromyces marxianus, Yarrowia lipolytica
3
Third multiplex assay
-
+
3. Non-Candida pathogenic yeasts
Trichosporon asahii, Cryptococcus neoformans, Geotrichum candidum, Cryptococcus deneoformans, Cryptococcus gattii, Rhodotorula mucilaginosa
Other rare yeast species
B
1. Harvesting pure colony
2. Mixing with PCR mixture
3. Breaking cell wall and amplification
4. Results read-out

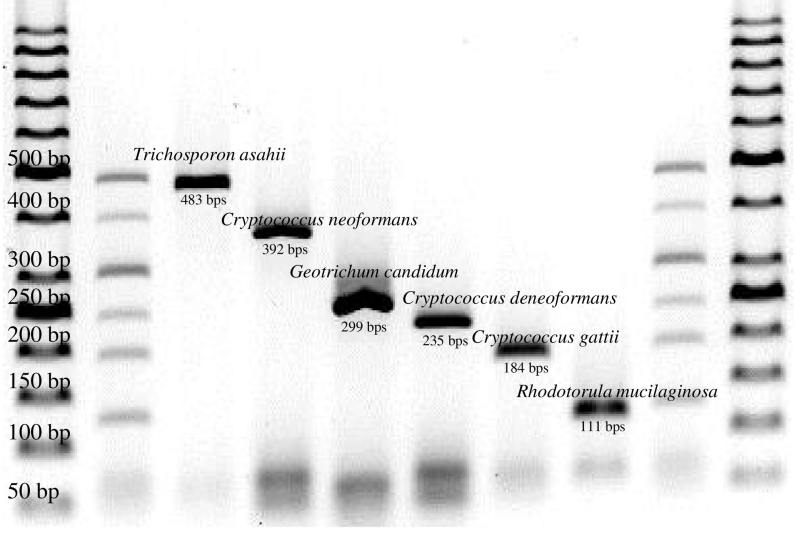
Figure 2



First multiplex assay



Second multiplex assay



Third multiplex assay

Figure 3

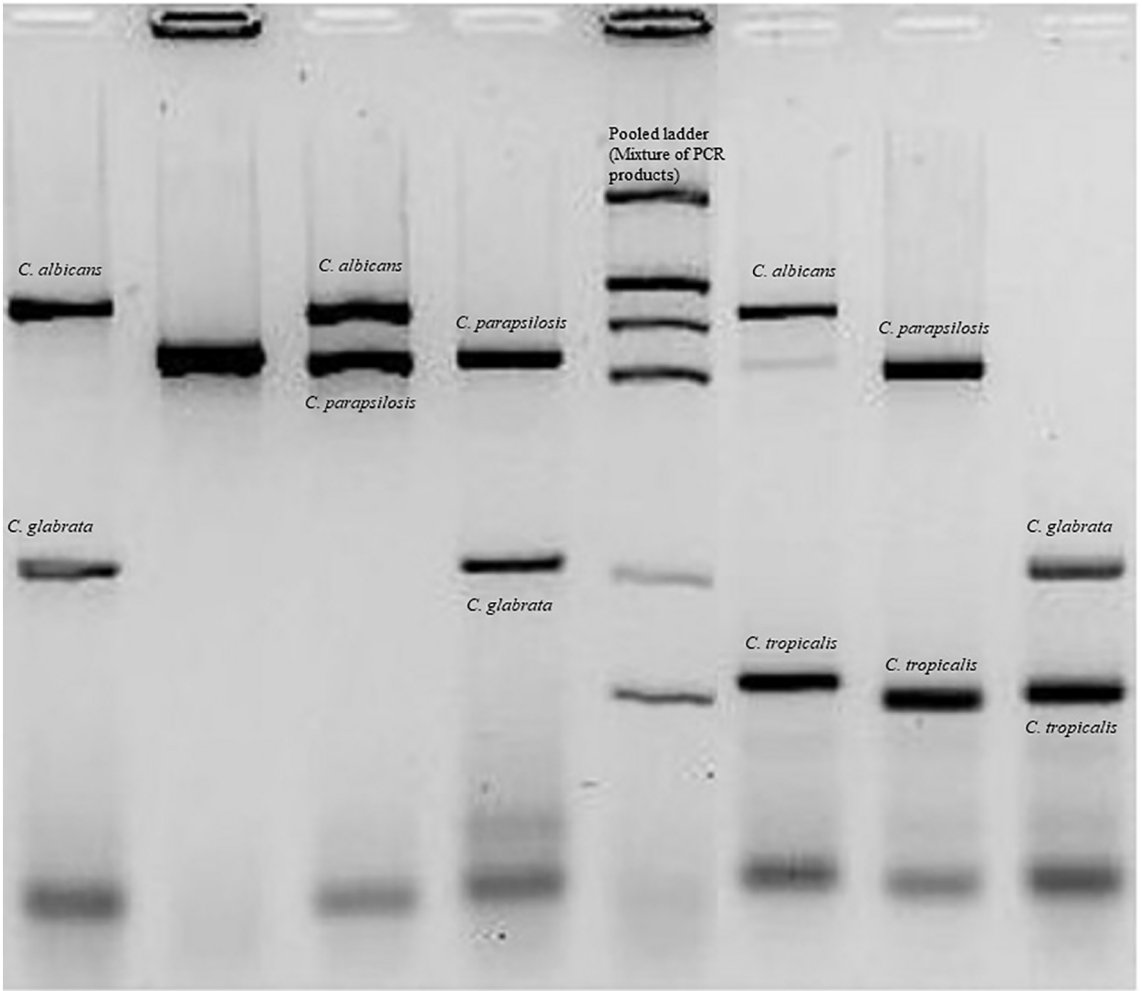

Pooled ladder
(Mixture of PCR
products)
C. albicans
C. albicans
C. parapsilosis
C. parapsilosis
C. albicans
C. parapsilosis
C. glabrata
C. glabrata
C. glabrata
C. tropicalis
C. tropicalis
C. tropicalis

Figure 4