Comparison of PCR-RFLP with 21-plex PCR and rDNA Sequencing for Identification of Clinical Yeast Isolates

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Abstract  Non-

albicans Candida species and other rare yeasts have emerged as major opportunistic pathogens in fungal infections. Identification of opportunistic yeasts in developing countries is mainly performed by phenotypic assay, which are time-consuming and prone to errors. The aim of the present study was to evaluate PCR-RFLP as a routinely used identification technique for the most clinically important Candida species in Iran and make a comparison with a novel multiplex PCR, called 21-plex PCR. One hundred and seventy-three yeast isolates from clinical sources were selected and identified with sequence analysis of the D1/D2 domains of rDNA (LSU rDNA) sequencing as the gold standard method. The results were compared with those obtained by PCR-RFLP using MspI restriction enzyme and the 21-plex PCR. PCR-RFLP correctly identified 93.4% of common pathogenic Candida species (C. albicans, C. glabrata, C. parapsilosis, C. tropicalis, and P. kudriavsevii (= C. krusei)) and was able to identify 45.5% of isolates of the uncommon yeast species compared to the D1/D2 rDNA sequencing. Compared with PCR-RFLP, all common Candida species and 72.7% of uncommon yeast species were correctly identified by the 21-plex PCR. The application of the 21-plex PCR assay as a non-sequence-based molecular method for the identification of common and rare yeasts can reduce turnaround time and costs for the identification of clinically important yeasts and can be applied in resource-limited settings.

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Introduction

The increasing frequency of non-albicans Candida (NAC) species over Candida albicans as the main cause of candidiasis results in accelerated clinical failure rates in Candida infections specially candidemia cases, largely due to their insensitivity to the few antifungal drugs available to treat these infections [1–3]. The intrinsic and acquired resistance of these yeasts to antifungal drugs and their consequential epidemiological and clinical issues dictate the needs for highly accurate means of fungal species-specific identification [4]. Therefore, rapid and accurate means of species-level identification of yeasts is essential for effective diagnostic practices in clinical mycology [5]. Despite technological advancements that improved cheap and rapid diagnostic methods, many clinicians in developing countries face challenges in the correct identification of human fungal pathogens, including yeasts [6]. Although rDNA sequencing is regarded to be the gold standard tool for fungal species identification, the technique is less affordable and inaccessible to many laboratories in the developing world. Recently, MALDI-TOF MS emerged as an alternative to rDNA sequencing for the identification pathogenic fungi [1, 7, 8], but this technique also requires expensive equipment. Several studies confirmed that the PCR-RFLP assay is a simple, rapid, inexpensive, and highly valuable tool to differentiate between yeast species [9]. PCR-RFLP is widely used in Iran for yeast identification [10–12] and is accurate and easy to use for the identification of common yeast species. However, it has some limitations for the identification of rare and emerging species, such as C. auris and related species [13, 14]. Recently, Arastehfar et al. developed a 21-plex PCR assay identifying the most clinically important yeast species, including species of Candida, Trichosporon, Rhodotorula, Cryptococcus, and Geotrichum. This 21-plex PCR uses basic PCR and does not require post-PCR digestion and additional gel electrophoresis [5]. The application of the 21-plex PCR assay for the identification of common and rare yeasts can reduce turnaround times and costs, and thus, the technique will be useful for application in developing countries. In the present study, we evaluated the PCR-RFLP with the 21-plex PCR assay and compared the outcome with that obtained by rDNA sequencing.

Materials and Methods

Yeast Isolates

Yeast isolates were selected from the TMML collection (Tehran Medical Mycology Laboratory). One hundred and seventy-three yeast isolates containing pathogenic Candida species, including C. albicans (n = 94), C. glabrata (n = 36), C. parapsilosis (n = 11), C. tropicalis (n = 7), Kluyveromyces marxianus (= C. kefyr) (n = 5), C. orthopsilosis (n = 5), C. dubliniensis (n = 3), Pichia kudriavzevii (= C. krusei) (n = 3), Clavispora lusitaniae (= C. lusitaniae) (n = 3), Meyerozyma guilliermondii (= C. guilliermondii) (n = 2), and non-Candida pathogenic yeasts, i.e., Cryptococcus neoformans (n = 2), Saccharomyces cerevisiae (n = 1), and Trichosporon asahii (n = 1), were all identified by rDNA sequencing. The D1/D2 domains of rDNA (LSU rDNA) were chosen for sequencing pursuant to a protocol by Leaw et al. [15]. Sequence data obtained were compared with those deposited in GenBank using the Nucleotide BLAST algorithm (http://blast.ncbi.nlm.nih.gov) and the local database at the Westerdijk Fungal Biodiversity Institute (Utrecht, The Netherlands, http://www.westerdijkinstitute.nl/Collections/). This study was approved by the local ethical committee of Tehran University of Medical Science (IR.TUMS.SPH.REC.1399.086).

DNA Extraction

Yeast isolates were cultured on Sabouraud dextrose agar (Merck, Germany) for 48 h. DNA was extracted using a commercial extraction kit (DNA EXTRACTION Kit DNP, Sinacolon, Iran), based on binding of the DNA to silica columns, in accordance with the manufacturer’s instructions.

PCR-RFLP

PCR-RFLP was performed based on a standard method described by Mirhendi et al. [12]. The
universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used to amplify the ITS1-5.8S-ITS2 regions. PCR amplification was carried out in a final volume of 25 μl. Each reaction comprised 2 μl template DNA (2 ng), 1 μM (0.5 μl) of the primers, 10 μl of Taq DNA Polymerase 2 × Master Mix RED (Amplicon, Denmark), and 12 μl of DW. PCR process involves an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, then the annealing step at 58 °C for 45 s with an extension of 72 °C for 1 min, and at last a final extension step at 72 °C for 5 min. The PCR products were digested with the restriction enzyme MspI (Thermo Fisher Scientific, USA) at 37 °C for 10 min. The amplified products and RFLP products were electrophoresed on 2% agarose gel in TBE buffer (90 mMTris, 90 mM boric acid, 2 mM EDTA) at 90 V for 60 min.

The molecular sizes and electrophoretic patterns resulting from the PCR-RFLP method are shown in Table 1.

### Table 1 Yeast species and related ITS, PCR–RFLP, and 21-plex PCR fragment sizes used in this study

| Yeast species                        | PCR-RFLP [12, 24] | 21-plex PCR [16] |
|--------------------------------------|-------------------|-----------------|
|                                       | ITS size | Msp I-RFLP Fragments size | Step 1 | Step 2 | Step 3 |
| Candia albicans                      | 537      | 239, 298         | 606    | –     | –     |
| Candia dubliniensis                 | 537a     | 239, 298         | 718    | –     | –     |
| Candia glabrata                     | 881      | 320, 561         | 212    | –     | –     |
| Candia nivariensis                  | 760      | 205, 236, 319    | –      | –     | –     |
| Candia bracarenseis                 | 805      | 253, 552         | –      | –     | –     |
| Candia tropicalis                   | 526      | 186, 340         | 126    | –     | –     |
| Pichia kudriavzevii                 | 510      | 250, 260         | 1159   | –     | –     |
| Candia parapsilosis                 | 530      | 530              | 490b   | –     | –     |
| Candia orthopsilosis                | 510      | 510              | 490    | –     | –     |
| Candia metapsilosis                 | 531      | 531              | 490    | –     | –     |
| Candia auris                        | –        | –                | 331    | –     | –     |
| Debaryomyces Hansenii               | 639      | 639              | –      | 818   | –     |
| Diutina rugosa                      | 399      | 121, 278         | –      | 689   | –     |
| Pichia norvegensis                  | 493      | 8, 227,258       | –      | 536   | –     |
| Clavispora lusitaniae               | 382      | 118, 264         | –      | 377   | –     |
| Meyerozyma guillermondii            | 607      | 82, 155, 370     | –      | 302   | –     |
| Kluyveromyces marxianus             | 720      | 720              | –      | 203   | –     |
| Yarrowia lipolytica                 | –        | –                | –      | 149   | –     |
| Trichosporon asahii                 | –        | –                | –      | –     | 483   |
| Trichosporon lactis                 | –        | –                | –      | –     | 480   |
| Cryptococcus neoformans             | 555      | 127, 428         | –      | –     | 392   |
| Cryptococcus deneiformans           | –        | –                | –      | –     | 235   |
| Cryptococcus gattii                 | –        | –                | –      | –     | 184   |
| Geotrichum candidum                 | –        | –                | –      | –     | 299   |
| Rhodotorula mucilaginosa            | –        | –                | –      | –     | 111   |
| Candida viswanathii                 | 484      | 25, 153, 306     | –      | –     | –     |
| Candida intermedia                  | 389      | 122, 267         | –      | –     | –     |
| Saccharomyces cerevisiae             | 840      | 124, 716         | –      | –     | –     |
| Candida inconspicua                 | 455      | 8, 208, 239      | –      | –     | –     |

a Candia dubliniensis was identified as Candia albicans (537 bp)
b Candia orthopsilosis and Candia metapsilosis were identified as Candia parapsilosis (490 bp)

Identification of yeasts with 21-plex PCR was performed in three multiplex PCRs as described previously [16]. The first step identifies the major pathogen

**21-Plex PCR**

Identification of yeasts with 21-plex PCR was performed in three multiplex PCRs as described previously. The first step identifies the major pathogen.
Table 2  Summary of yeast isolates used in this study with their source of isolation

| Species (number)                      | Sources of isolation (number)                                                                 |
|---------------------------------------|---------------------------------------------------------------------------------------------|
| Candida albicans (94)                 | Vagina \((n = 2)\), Oropharyngeal \((n = 51)\), Bronchoalveolar lavage \((n = 20)\), Stool \((n = 1)\), Hand and Nail \((n = 9)\), Urine \((n = 1)\), Groin \((n = 9)\), Cerebrospinal fluid \((n = 1)\), |
| Candida glabrata (36)                 | Oropharyngeal \((n = 25)\), Bronchoalveolar lavage \((n = 8)\), Stool \((n = 1)\), Urine \((n = 1)\), Pleural fluid \((n = 1)\) |
| Candida parapsilosis (11)             | Blood \((n = 4)\), Bronchoalveolar lavage \((n = 1)\), Hand & Nail \((n = 5)\), Groin \((n = 1)\) |
| Candida tropicalis (7)                | Blood \((n = 1)\), Oropharyngeal \((n = 2)\), Bronchoalveolar lavage \((n = 1)\), Hand & Nail \((n = 3)\) |
| Kluyveromyces marxianus (= Candida kefyr) (5) | Oropharyngeal \((n = 5)\) |
| Pichia kudriavzevii (= Candida krusei) (3) | Blood \((n = 1)\), Stool \((n = 1)\), Pleural fluid \((n = 1)\), |
| Clavispora lusitaniae (= Candida lusitaniae) (3) | Oropharyngeal \((n = 1)\), Hand and Nail \((n = 2)\) |
| Candida orthopsilosis (5)             | Vagina \((n = 1)\), Hand and Nail \((n = 3)\), Groin \((n = 1)\) |
| Candida dubliniensis (3)              | Vagina \((n = 3)\) |
| Meyerozyma guilliermondii (= Candida guilliermondii) (2) | Blood \((n = 2)\) |
| Cryptococcus neoformans (2)           | Cerebrospinal fluid \((n = 2)\) |
| Saccharomyces cerevisiae (1)           | Hand and Nail \((n = 1)\) |
| Trichosporon asahii (1)               | Urine \((n = 1)\) |
| Total (173)                           |                                                                                             |

*Comparison of the Results of rDNA Sequencing and PCR-RFLP*

In this study, the PCR-RFLP method correctly identified 151 (87.3%) yeast isolates and misidentified 22 (12.7%) isolates compared to the D1/D2 LSU rDNA sequence analysis. PCR-RFLP correctly identified 93.4% of isolates of the common yeast pathogens (i.e., *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *P. kudriavzevii*) and was able to identify 45.5% (10/22; 45.5%) of the uncommon opportunistic yeast species compared to the D1/D2 sequencing (Table 3). The PCR-RFLP method misidentified *C. orthopsilosis* (5) as a *C. parapsilosis* (4/5) and *C. albicans* (1/5). *C. dubliniensis* was misidentified as *C. albicans*, *Cl. lusitaniae* as a *Diutina rugosa* (= *C. rugosa*)/*C. intermedia*, and *T. asahii* as a *C. tropicalis*.

*Results*  

**Distribution of Yeast Species**

One hundred and seventy-three yeast isolates were obtained from various clinical sources, including blood \((n = 8)\), vagina \((n = 6)\), pleural fluid \((n = 2)\), oropharyngeal \((n = 84)\), cerebrospinal fluid \((n = 3)\), urine \((n = 3)\), nail \((n = 23)\), bronchoalveolar lavage \((n = 30)\), stool \((n = 3)\), and groin \((n = 11)\). The sources of these 173 isolates are shown in Table 2.
main Candida species, including C. albicans, C. glabrata, C. parapsilosis, C. tropicalis and P. kudriavzevii, and 72.7% (16/22; 72.7%) of the isolates belonging to uncommon yeast species (Cl. lusitaniae, C. dubliniensis, M. guilliermondii, Cr. neoformans and T. asahii), were correctly identified by 21-plex PCR. However, five C. orthopsilosis isolates were misidentified as C. parapsilosis and S. cerevisiae could not be identified. Table 4 summarizes the misidentified isolates using the 21-plex and PCR–RFLP compared to LSU rDNA sequencing as the gold standard method.

Discussion

Accurate species-level identification of the etiologic agent is important in the management of patients suffering from fungal infections caused by emerging opportunistic yeast species. However, precise technique such as DNA sequencing and MALDI-TOF MS are generally unaffordable and inaccessible in many developing countries [17]. Although many studies have demonstrated the importance of conventional methods in fungal identification, these techniques fail to identify uncommon yeasts, such as M. guilliermondii and C. auris [18–20]. The application of a 21-plex PCR assay for the identification of common and rare yeasts can reduce turnaround times and costs, and thus can be applied in developing countries. The results of this study showed that the 21-plex PCR showed superior performance over PCR-RFLP as a routine technique for the identification of the most clinically important Candida species. The 21-plex

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**Table 3** Species identification of clinically obtained yeast species using three approaches, PCR–RFLP, 21–plex, and sequencing of the D1/D2 domains of ribosomal DNA

| Species (Number) | Correctly identified | Misidentified | Not identified |
|------------------|----------------------|---------------|---------------|
|                  | PCR–RFLP (%)         | 21–plex PCR (%) | PCR–RFLP (%) | 21–plex PCR (%) | PCR–RFLP (%) | 21–plex PCR (%) |
| Candida albicans (n = 94) | 92 (97.9) | 94 (100) | 2 (2.1) | – | – | – |
| Candida glabrata (n = 36) | 32 (88.9) | 36 (100) | 4 (11.1) | – | – | – |
| Candida parapsilosis (n = 11) | 9 (81.8) | 11 (100) | 2 (18.2) | – | – | – |
| Candida tropicalis (n = 7) | 6 (85.7) | 7 (100) | 1 (14.3) | – | – | – |
| Kluyveromyces marxianus (n = 5) | 5 (100) | 5 (100) | – | – | – | – |
| Pichia kudriavzevii (n = 3) | 2 (66.7) | 3 (100) | 1 (33.3) | – | – | – |
| Clavispora lusitaniae (n = 3) | – | 3 (100) | 3 (100) | – | – | – |
| Candida orthopsilosis (n = 5) | – | – | 5 (100) | 5 (100) | – | – |
| Candida dubliniensis (n = 3) | – | 3 (100) | 3 (100) | – | – | – |
| Meyerozyma guilliermondii (n = 2) | 2 (100) | 2 (100) | – | – | – | – |
| Cryptococcus neoformans (n = 2) | 2 (100) | 2 (100) | – | – | – | – |
| Saccharomyces cerevisiae (n = 1) | 1 (100) | – | – | – | – | 1 (100) |
| Trichosporon asahii (n = 1) | – | 1 (100) | 1 (100) | – | – | – |
| Total (n = 173) | 151 (87.3) | 167 (96.5) | 22 (12.7) | 5 (2.9) | 0 | 1 (0.57) |
PCR correctly identified 96.5% of all yeast species included, 100% of most prevalent Candida species, and 72.7% of uncommon yeast species. Our results corroborated those of Arastehfar et al. who showed that the 21-plex PCR accurately identified 87.3% of yeast isolates, all top five Candida species, and 72% of rare yeast species [5]. As this assay was not intended for the identification of other rare yeast species, such as S. cerevisiae that could be identified with PCR-RFLP, this was not the case with the 21-plex PCR. Other studies found a higher degree of accuracy when the 21-plex PCR, as a first line identification tool, was used in combination with API 20C AUX for isolates that were not identified by the 21-plex PCR [5, 16]. Therefore, for laboratories lacking automated Vitek 2, MALDI-TOF, and biochemical identification assay, the 21-plex PCR may be a useful technique for the identification of yeasts isolated from clinical samples.

PCR-RFLP has been used as a method to identify yeasts isolated from clinical specimens in several studies conducted in Iran [12, 21–23]. Mirhendi et al. successfully identified six clinically important Candida spp. using the PCR-RFLP method [12]. Other studies in Iran have used the same method [9, 11, 21, 22]. Interpreting the results is difficult for some yeast species. In PCR-RFLP, the digestion of the PCR products with restriction enzymes increases the time required to identify yeast species, while the 21-plex PCR does not need post-PCR procedures. Another limitation of RFLP is that similar fragment sizes occur in some uncommon species of Candida. For example, the PCR product size of ITS1-5.8S-ITS2 before and after digestion is similar for C. intermedia, D. rugosa, and Cl. lusitaniae in the electrophoresis patterns [24]. Also, some uncommon yeasts like K. marxianus (= C. kefyr) and D. hansenii (= C. famata) had no restriction enzyme cutting site for Msp I; nevertheless, the PCR product size of ITS1-5.8S-ITS2 was helpful to allow a presumptive identification of the species, but not a reliable one [24].

In this study, Cl. lusitaniae was easily identified with the 21-plex PCR, but could not be identified using PCR-RFLP. The importance of this issue is that some strains of Cl. lusitaniae exhibit intrinsic or acquired resistance to amphotericin B [25]. This species is being increasingly isolated from cancer patients on empirical/prophylaxis antifungal therapy [26, 27].

Misidentification of Candida-related species, such as C. albicans and C. dubliniensis, was another limitation of PCR-RFLP. The ambiguous epidemiological distribution of C. dubliniensis, due to its close resemblance to C. albicans, as well as to the

### Table 4 Misidentified isolates using 21-plex PCR, and PCR–RFLP compared to D1/D2 rDNA sequencing as the gold standard method

| Yeast species (misidentified/total) | Misidentified as (number of misidentify) | 21–plex PCR | PCR–RFLP |
|-----------------------------------|------------------------------------------|-------------|-----------|
| Candida albicans (2/94)           |                                          | –           | Candida glabrata (1) |
| Candida glabrata (4/36)           |                                          | –           | Candida tropicalis (1) |
| Candida parapsilosis (2/11)       |                                          | –           | Candida albicans (1) |
| Candida tropicalis (1/7)          |                                          | –           | Candida albicans (1) |
| Pichia kudriavzevii (1/3)         |                                          | –           | Candida parapsilosis (1) |
| Clavispora lusitaniae (3/3)       |                                          | –           | Candida intermedia/ Diutina rugosa (3) |
| Candida orthopsilosis (5/5)       | Candida parapsilosis (5)                 |             | Candida parapsilosis (4) |
| Candida dubliiniensis (3/3)       | –                                        |             | Candida albicans (3) |
| Trichosporon asahii (1/1)         | –                                        |             | Candida tropicalis (1) |
emergence of resistance to fluconazole in C. dubliniensis, urges a simple, reliable, and rapid method to discriminate between these two species. Earlier studies reported that C. dubliniensis isolates were mainly recovered from oral cavity of HIV positive patients. However, recent reports demonstrated that C. dubliniensis have been isolated from non-HIV-positive patients [28, 29]. Nikmanesh et al. used PCR fragment polymorphism of the HWP1 gene for the discrimination of C. albicans from C. dubliniensis and C. africana [30]. In the present study, we used specific primers to differentiate between C. dubliniensis and C. albicans. While PCR-RFLP requires an additional enzyme, which may be expensive (1.3–1.5 Euros/reaction), multiplex PCR is a cost-effective molecular assay (0.75–1 Euros/reaction), which requires only basic and widely used equipment for PCR and electrophoresis. The 21-plex PCR also identified some basidiomycetes yeasts, including Cryptococcus and Trichosporon species. Our results show that PCR-RFLP misidentified Trichosporon as a C. tropicalis. Both Trichosporon and Cryptococcus secrete gluconoxylomannans, the main polysaccharides anti- and phenotypic assay to identify common sequencing, or expensive and time-consuming bio- and expensive equipment, i.e., MALDI-TOF MS and Sanger lack specific and accurate identification tools and diagnostic laboratories in developing countries that PCR can be used as a useful technique in routine fication of rare and emerging species. The 21-plex tion. However, it has some limitations for the identi- and inexpensive method for yeast species identifica- were not present in our clinical specimen collection. In conclusion, PCR-RFLP assay as a simple, rapid, and inexpensive method for yeast species identification. However, it has some limitations for the identi- fication of rare and emerging species. The 21-plex PCR can be used as a useful technique in routine diagnostic laboratories in developing countries that lack specific and accurate identification tools and equipment, i.e., MALDI-TOF MS and Sanger sequencing, or expensive and time-consuming bio- and phenotypic assay to identify common and some uncommon yeast species.

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Author Contributions SK, SJH, RDG, and MK designed the study. AE, SK, MK, and AA collected yeast isolates. MK, MRS, and AA performed molecular identification. SK, SR, TB, RDG, and SJH performed data analysis. MK, SK, MG, and AE prepared the first draft. All authors contributed in revision.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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