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One-Step Multiplex PCR for Simultaneous Detection and Identification of Eight Medically Important *Candida* Species

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Abstract

Recently, the incidence of *Candida* infections has substantially increased. Conventional identification methods for *Candida* species are technically difficult to conduct and cannot accurately distinguish each species. The purpose of the present study was to design primers to identify and detect simultaneously eight medically important *Candida* species using one-step multiplex PCR. PCR primers were designed based on partial sequences of intergenic spacer (IGS) and internal transcribed spacer (ITS) genes of eight medically important *Candida* species. These primers were able to distinguish each *Candida* species and did not display cross-reactivity with representative *Candida* species other than the eight *Candida* species. Moreover, our developed one-step multiplex PCR method is accurate, specific, cost-effective, time-saving, and worked without requiring DNA extraction.

Keywords

Candida, *Candida albicans*, One-Step Multiplex PCR, PCR Method

1. Introduction

The genus *Candida* belongs to the kingdom Fungi, class Deuteromycetes, and comprises 150 - 200 species. Recently, distinct shifts in the distribution of *Can-*

dida species isolated from nosocomial infections have been reported. Although *Candida albicans* remains the most frequent cause of candidemia and hematogenously disseminated candidiasis, an increasing number of hospital-acquired infections caused by other *Candida* species, so-called non-*albicans Candida* species, is being observed [1] [2] [3] [4] [5]. Candidemia is often associated with human immunodeficiency virus (HIV) or advanced medical and surgical interventions that compromise patient immunity, e.g., bone marrow or solid organ transplant, aggressive chemotherapy, and broad application of antifungal agents [6]. In fact, nosocomial fungal bloodstream infections are an increasingly significant cause of morbidity, with an estimated mortality of 25% - 38% [7]. *C. albicans* is the most common and clinically relevant pathogen of the genus. However, there has been a significant upward trend in the emergence of non-*albicans Candida* species, especially *Candida glabrata*, *Candida parapsilosis*, and *Candida tropicalis* [8] [9] [10]. In addition, because several non-*C. albicans Candida* species are frequently resistant to common antifungal agents, accurate identification methods are essential for the establishment of appropriate antifungal therapy [11].

The basis for laboratory detection of bloodstream fungal infections, including candidemia, remains direct examination and conventional blood culture. However, these methods are of limited clinical value because there are negative outcomes in as high as 50% of autopsy-confirmed cases of candidemia. In addition, cultures may only become positive late in the infection [12]. Furthermore, most phenotypic methods of identification used in clinical laboratories are often time-consuming and may lead to inconclusive results. For example, phenotypic tests such as VITEK and API ID32C systems need several days before biochemical reactions can be interpreted [13]. In contrast, molecular approaches have the potential to detect candidemia swiftly with increased sensitivity and specificity. Buchman *et al.* demonstrated first that detection of *C. albicans* in clinical specimens was possible by PCR amplification of the lanosterol-alpha-demethylasegene [14]. Other PCR-based techniques have been developed using amplification of target DNA, providing alternative strategies for the diagnosis and identification of fungal pathogens [15] [16] [17] [18]. Fungal ribosomal genes are common DNA targets in PCR-based procedures for the identification of fungi at the species level. The highly variable sequences of internal transcribed spacer (ITS) regions ITS1 and ITS2 flanked by the relatively conserved coding regions of 18S, 5.8S, and 28S nuclear rRNA genes have been used in various PCR-based approaches for the identification of medically important yeasts [19] [20] [21]. Although molecular techniques such as the conventional PCR method are highly sensitive and specific, they are also expensive, laborious, and a little time-consuming. Thus, a simple and more reliable assay for identifying *Candida* species is desired.

The purpose of the present study was to develop a one-step multiplex PCR system for identifying and detecting simultaneouslyeight medically important *Candida* species, *i.e.*, *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *Candida dubliniensis*, *Candida guilliermondii* (currently *Pichia guilliermondii*), *Can-*

dida krusei (currently *Issatchenkia orientalis*), and *Candida lusitaniae* (currently *Clavispora lusitaniae*).

2. Materials and Methods

2.1. Fungal Strains and Culture Conditions

All microorganisms were obtained from Japan Collection of Microorganisms (JCM; Japan). The following fungal strains were used in the present study: *C. albicans* JCM 1537, *C. glabrata* JCM 1539, *C. tropicalis* JCM 1541, *C. parapsilosis* JCM 1612, *C. dubliniensis* IFM 54605, *C. guilliermondii* JCM 1539, *C. krusei* JCM 1609, *C. lusitaniae* JCM 1814, *Candida kruisii* JCM 1779, *Candida orthopsilosis*, *Candida kefyr* JCM 9556, *Candida aaseri* JCM 1689, *Candida inconspicua* JCM 9555, *Candida melibiosica* JCM 9558, and *Candida norvegica* JCM 8897. These strains were maintained by cultivation on Bacto™ Brain Heart Infusion (BHI; Becton Dickinson and Co., Sparks, MD, USA) and 1.5% agar (BHI agar). The organisms were cultured overnight at 30˚C under aerobic condition.

2.2. Design of Species-Specific Primers for Eight Species

Design of species-specific primers for eight species was performed as described previously [22]. Briefly, the intergenic spacer (IGS) gene sequences of *C. albicans* (accession no. FN554375), *C. glabrata* (FN554379), *C. tropicalis* (FN554382), *C. parapsilosis* (FN554241), *C. dubliniensis* (FN554377), and *C. guilliermondii* (AM992960) and internal transcribed spacer (ITS) gene sequences of *C. albicans* (accession No. AF217609), *C. glabrata* (KJ546151), *C. tropicalis* (KY495750), *C. parapsilosis* (KY685084), *C. dubliniensis* (KX231794), *C. guilliermondii* (AF022717), *C. lusitaniae* (AF009215), and *C. krusei* (AF246989) were obtained from the DNA Data Bank of Japan (DDBJ; https://www.ddbj.nig.ac.jp/services.html, Mishima, Japan), and a multiple sequence alignment analysis was performed using the CLUSTAL W program; *i.e.*, IGS gene sequences of six *Candida* species and ITS gene sequences of eight *Candida* species were aligned and analyzed. Homology among the primers selected for each *Candida* species and their respective IGS and ITS gene sequences was confirmed by a BLAST search.

2.3. Development of a One-Step Multiplex PCR Method Using Designed Primers

Fungal cells were cultured in BHI supplemented with 0.5% yeast extract for 24 h, and 1-ml samples were then collected in microcentrifuge tubes and resuspended at a density of 1.0 McFarland standard [approximately 10^7 colony-forming units (CFU)/ml] in 1 ml of sterile distilled water. A total of 3.6 µl of the suspension was then used as the PCR template. The detection limit of PCR was assessed by serially diluting known numbers of fungal cells in sterile distilled water and then subjecting each suspension to PCR. The multiplex PCR mixture contained 0.2 µM of each primer, 10 µl of 2× MightyAmp Buffer Ver.3 (Takara Bio Inc., Shiga, Japan), 0.4 µl of MightyAmp DNA Polymerase (Takara), and 5 µl of the template

in a final volume of 20 μl. PCRs were performed in a DNA thermal cycler (Applied Biosystems 2720 Thermal Cycler; Applied Biosystems, Foster City, CA, USA). PCR conditions included an initial denaturation step at 98˚C for 2 min, followed by 30 cycles consisting of 98˚C for 10 s and 68˚C for 1 min. PCR products were analyzed by 2.0% agarose gel electrophoresis and then visualized by electrophoresis in 1× Tris-borate-EDTA on a 2% agarose gel stained with ethidium bromide. A 100-bp DNA ladder (Takara Biomed, Shiga, Japan) was used as a molecular size marker. All experiments were performed in triplicate.

3. Results

3.1. Primer Design

Sixteen specific primers covering the upstream regions of IGS and ITS gene sequences of eight medically important *Candida* species were designed in the present study (**Figure 1** and **Figure 2**). The specific forward primers were designated as CLF1 for *C. lusitaniae*, PKF1 for *C. krusei*, CGLF1 for *C. glabrata*, CTF1 for *C. tropicalis*, CPF1 for *C. parapsilosis*, CDF1 for *C. dubliniensis*, CGUF1 for *C. guilliermondii*, and CAF1 for *C. albicans*. The specific reverse primers were designated as CLF2 for *C. lusitaniae*, PKF2 for *C. krusei*, CGLF2 for *C. glabrata*, CTF2 for *C. tropicalis*, and CPF2 for *C. parapsilosis*, CDF2 for *C. dubliniensis*, CGUF2 for *C. guilliermondii*, and CAF2 for *C. albicans*. Amplicon sizes of *C. lusitaniae*, *C. krusei*, *C. glabrata*, *C. tropicalis*, and *C. parapsilosis*, *C. dubliniensis*, *C. guilliermondii*, and *C. albicans* were 125 bp, 230 bp, 331 bp, 424 bp, 507 bp, 684 bp, 816 bp, and 1009 bp, respectively.

3.2. Multiplex PCR

3.2.1. Detection Limit

Our multiplex PCR method for identifying and detecting eight medically important *Candida* species, *i.e.*, *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. dubliniensis*, *C. guilliermondii*, *C. krusei*, and *C. lusitaniae*, successfully amplified DNA fragments of the expected size for each species (**Figure 3**). The detection limit was assessed in the presence of titrated fungal cells, and the sensitivity of the PCR assay was 5 - 50 CFU per PCR template (5.0 μl) for the *C. lusitaniae*-specific primer set with strain JCM 1814, the *C. krusei*-specific primer set with strain JCM 1609, the *C. glabrata*-specific primer set with strain JCM 1539, the *C. tropicalis*-specific primer set with strain JCM 1541, the *C. parapsilosis*-specific primer set with strain JCM 1612, the *C. dubliniensis*-specific primer set with strain IFM 54605, the *C. guilliermondii*-specific primer set with strain JCM 1539, and the *C. albicans*-specific primer set with strain JCM 1537 (**Figure 4** and **Figure 5**).

3.2.2. Assay of Representative *Candida* Species Other than the Eight Medically Important Species

As representative *Candida* species other than the eight species targeted in this study, *C. kruisii*, *C. orthopsilosis*, *C. kefyr*, *C. aaseri*, *C. inconspicua*, *C. melibiosica*,

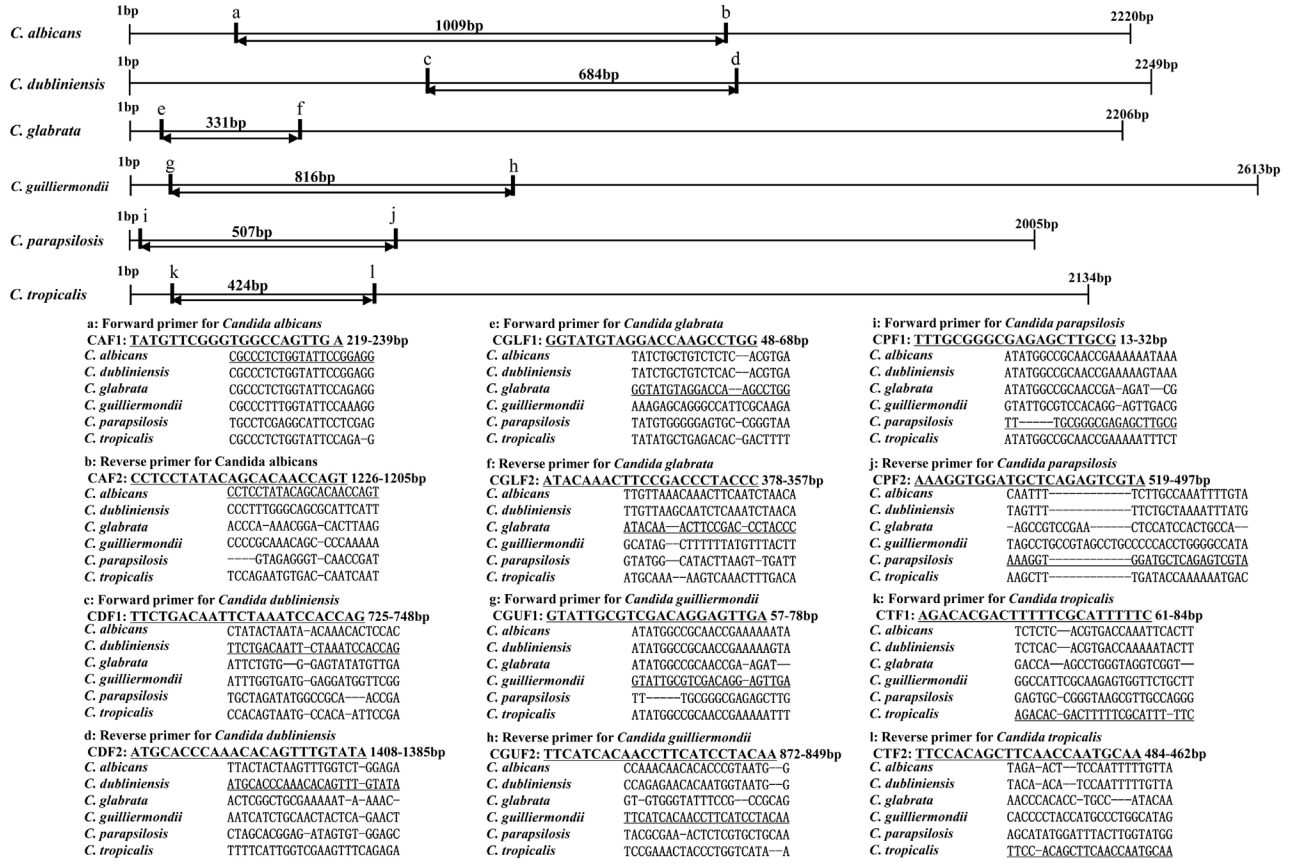

Figure 1. Locations and sequences of species-specific primers for IGS genes of *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. guilliermondii*, *C. parapsilosis*, and *C. tropicalis*. The nucleotide sequence of each primer is underlined.

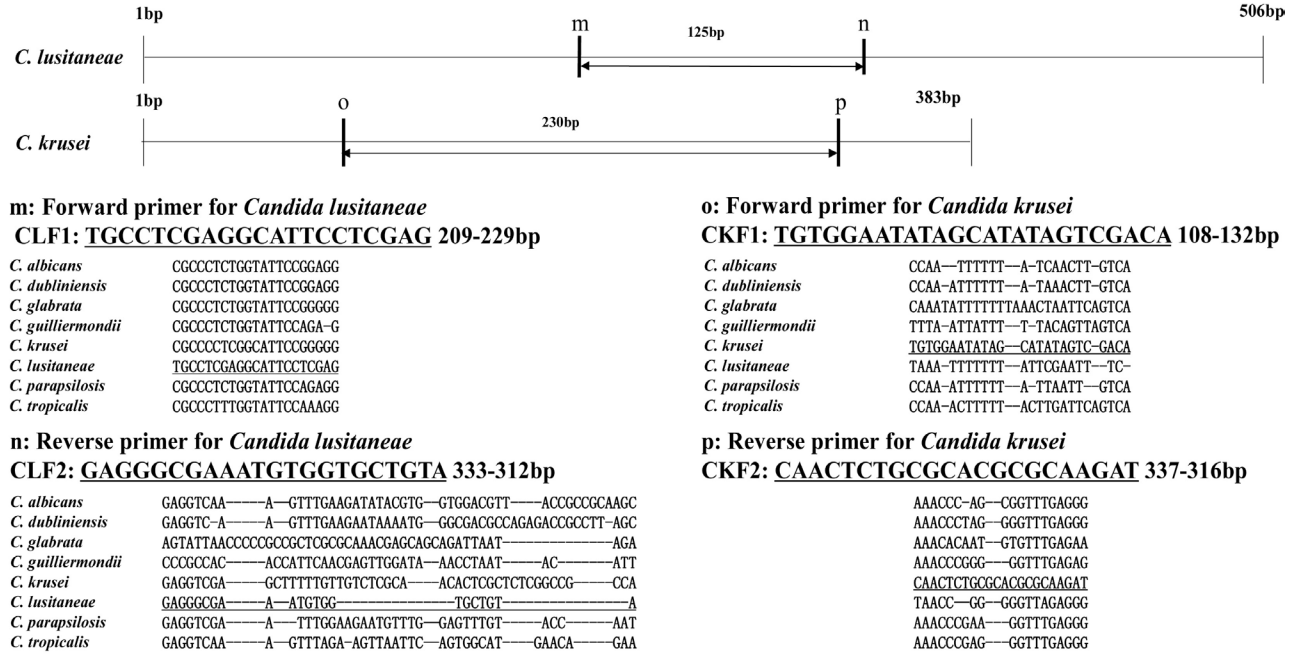

Figure 2. Locations and sequences of species-specific primers for ITS genes of *C. lusitaniae* and *C. krusei*. The nucleotide sequence of each primer is underlined.

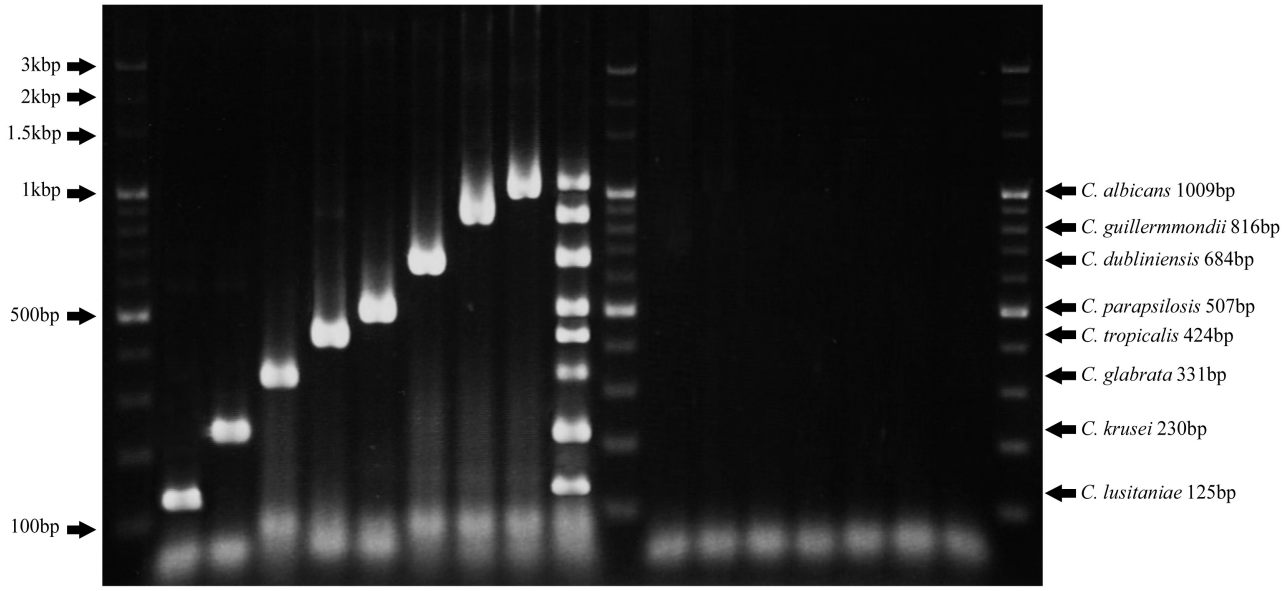

Figure 3. Multiplex PCR assay for detecting eight medically important *Candida* species. The primer mixture contained CLF1, CLF2, CPF1, CPF2, CKF1, CKF2, CGLF1, CGLF2, CDF1, CDF2, CTF1, CTF2, CAF1, CAF2, CGUF1, and CGUF2. Lanes: 1, *Candida lusitaniae* JCM 1814; 2, *Candida krusei* JCM1609; 3, *Candida glabrata* JCM3761; 4, *Candida tropicalis* JCM 1541; 5, *Candida parapsilosis* JCM 1612; 6, *Candida dubliniensis* IFM 54605; 7, *Candida guilliermondii* JCM 1539; 8, *Candida albicans* JCM 1537; 9, Mixture of *C. lusitaniae*, *C. krusei*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. dubliniensis*, *C. guilliermondii*, and *C. albicans*; 10, *Candida kruisii* JCM 1779; 11, *Candida orthopsilosis* JCM 1784; 12, *Candida kefyr* JCM 9556; 13, *Candida aaseri* JCM 1689; 14, *Candida inconspicua* JCM 9555; 15, *Candida melibiosica* JCM 9558; and 16, *Candida norvegica* JCM 8897. M, molecular size marker (100-bp DNA ladder).

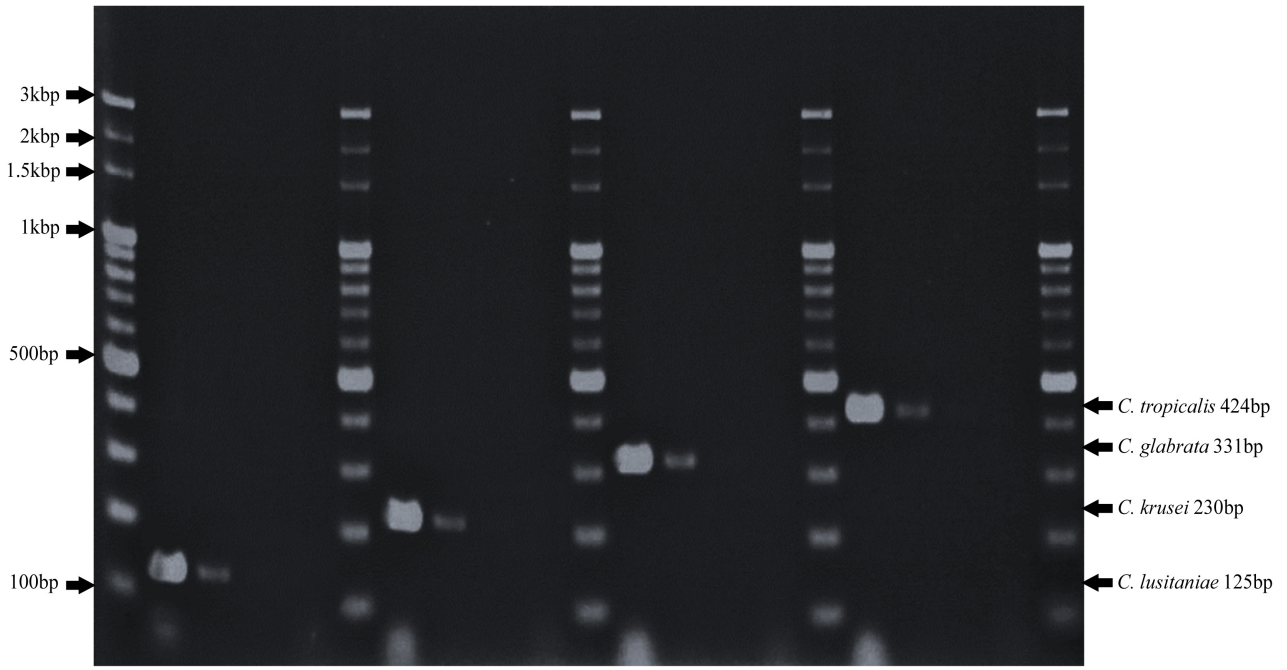

Figure 4. Detection limit of the multiplex PCR assay for detecting *C. lusitaniae*, *C. krusei*, *C. glabrata*, and *C. tropicalis*. The primer mixture contained CLF1, CLF2, CPF1, CPF2, CKF1, CKF2, CGLF1, CGLF2, CDF1, CDF2, CTF1, CTF2, CAF1, CAF2, CGUF1, and CGUF2. Lanes 1 - 4, *Candida lusitaniae* JCM 1814; Lanes 5 - 8, *Candida krusei* JCM1609; Lanes 9 - 12 *Candida glabrata* JCM3761; Lanes 13 - 16 *Candida tropicalis* JCM 1541. The following numbers of cells were added: 5×10^2 (lanes 1, 5, 9, 13), 50 (lanes 2, 6, 10, 14), 5×10 (lanes 3, 7, 11, 15), and 0 (lanes 4, 8, 12, 16). M, molecular size marker (100-bp DNA ladder).

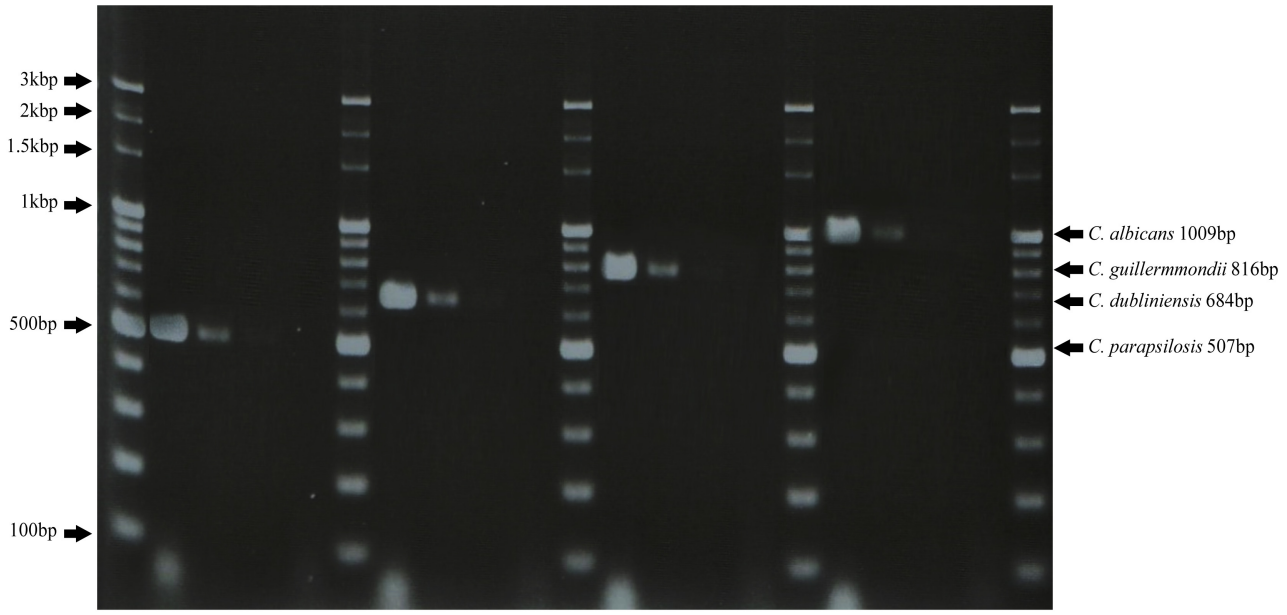

Figure 5. Detection limit of the multiplex PCR assay for detecting *Candida parapsilosis*, *Candida dubliniensis*, *Candida guilliermondii*, and *Candida albicans.* The primer mixture contained CLF1, CLF2, CPF1, CPF2, CKF1, CKF2, CGLF1, CGLF2, CDF1, CDF2, CTF1, CTF2, CAF1, CAF2, CGUF1, and CGUF2. Lanes 1 - 4, *Candida parapsilosis* JCM 1612; Lanes 5 - 8, *Candida dubliniensis* IFM 54605; Lanes 9 - 12 *Candida dubliniensis* IFM 54605; Lanes 13 - 16, *Candida albicans* JCM 1537. The following numbers of cells were added: 5×10^2 (lanes 1, 5, 9, 13), 5×10 (lanes 2, 6, 10, 14), 5×1 (lanes 3, 7, 11, 15), and 0 (lanes 4, 8, 12, 16). M, molecular size marker (100-bp DNA ladder).

and *C. norvegica* were evaluated by PCR using the designed primer sets. However, no amplicons were produced from any of the representative *Candida* species other than the eight medically important species (**Figure 3**).

4. Discussion

The incidence of *Candida* infections has recently increased due to the widespread use of broad-spectrum antibiotics and growing numbers of HIV-infected and immunocompromised individuals [23] [24] [25]. Despite the predominance of *C. albicans*, non-*albicans Candida* species such as *C. glabrata*, *C. tropicalis*, *C. guilliermondii*, *C. dubliniensis*, *C. parapsilosis*, *C. krusei*, and *C. lusitaniae* are emerging as both colonizers and pathogens that can cause systemic infections [26] [27] [28] [29]. Furthermore, some of these species are naturally more resistant to antifungal agents. *C. glabrata* and *C. krusei* are innately more resistant to the commonly used antifungal agent fluconazole [30]. Moreover, the genome of *C. dubliniensis*, which is mainly sensitive to fluconazole, encodes for multidrug transporters that rapidly mediate fluconazole resistance during clinical therapy [31].

Thus, rapid and accurate identification of disease-causing *Candida* species is crucial for clinical treatment of local or systemic candidiasis. Premature diagnosis of invasive fungal infections is problematic because most clinical signs and symptoms are non-specific, and cultures are often negative or become positive too late for the initiation of effective antifungal therapy. Therefore, several studies have been developed for improvement of new technologies for the diagnosis

of invasive candidiasis. Conventional methodology has long been used as standard identification procedures for *Candida* species. However, these methods are laborious, time-consuming, and not reliable in identifying the broad spectrum of *Candida* species and usually require additional tests. Therefore, several commercial systems have been developed to enable rapid yeast identification within 2 - 72 hours. Although these systems have been extensively used for *Candida* identification, their application is limited, and some species cannot be identified and differentiated. Recently, molecular approaches, such as PCR-based methods, have been used to complement conventional methods and provide more accurate results in less time (2 - 3 hours). Given the high accuracy and speed with which molecular typing techniques can be carried out and rapid advances in technology, most of these methods may improve routine clinical laboratory identification of *Candida* species. However, further studies are needed for the standardization of such technical procedures.

To develop a PCR-based technique more applicable for clinical use than conventional PCR, we established a one-step multiplex PCR system for identifying and detecting simultaneously eight medically important *Candida* species, which uses only one PCR tube per sample. A multiplex PCR method is a rapid tool that allows for the simultaneous amplification of more than one sequence of target DNA in a single reaction, thereby saving time and reagents [32]. The most significant problem with this method is the possibility of hybridization among the different primer sequences. Carvalho *et al.* previously reported a multiplex PCR strategy allowing the identification of eight *Candida* species, similar to the present study [33]. This multiplex PCR was based on the amplification of two fragments from ITS1 and ITS2 regions by combining two yeast-specific and eight species-specific primers in a single PCR. However, according to our pilot study, because this method was too complicated to distinguish PCR fragment patterns, it was difficult to identify accurately each *Candida* species. Moreover, it took more than 4 hours to finish the identification.

In the present study, we designed species-specific primers with the already mentioned means, for the identification and detection of eight *Candida* species using a PCR-based method. These primers were able to distinguish each *Candida* species and did not display cross-reactivity with representative *Candida* species other than the eight species targeted in this study. Moreover, we developed a one-step multiplex PCR method with the ability to identify and differentiate eight medically important *Candida* species (*i.e.*, *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. dubliniensis*, *C. guilliermondii*, *C. krusei*, and *C. lusitaniae*) using only one PCR tube per sample.

Our multiplex PCR method is easy because the use of MightyAmp DNA Polymerase Ver.3 (Takara) means that DNA extraction is not necessary, and species identification and detection using this method only takes approximately 2 hours. Thus, the method described herein will allow the prevalence of the eight medically important *Candida* species and their involvement in various infections to be fully clarified in future studies.

Author's Contributions

Fukatsu A, Suzuki H, Asaka K, Ono Y, Fuchigami M, Kobayashi T, Uchibori S, Takahashi Y, Komine C, Konishi Y, Ogura Y, Omori H and Wakami M corrected the data. Fukatsu A, Tsuzukibashi O, Murakami H and Fukumoto M drafted and wrote the manuscript. The concept of this manuscript was devised by Fukatsu A. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare that there is no conflict of interest.

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