Journal of Microbiological Methods 132 (2017) 160–165

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Rapid identification of *Candida* spp. frequently involved in invasive mycoses by using flow-through hybridization and Gene Chip (FHGC) technology

Chen Li [a,d], Xiurong Ding [b], Zhizhong Liu [a,d,*], Juanjuan Zhu [c]

[a] School of Rehabilitation Medicine, Capital Medical University, China
[b] Departments of Clinical Laboratory, Beijing Youan Hospital, Capital Medical University, Beijing 100069, China
[c] Chaozhou Hybribio Limited Corporation, Chaozhou 521011, China
[d] Department of Clinical Laboratory, China Rehabilitation Research Center, Beijing 100068, China

ARTICLE INFO

Article history:
Received 11 September 2016
Received in revised form 28 November 2016
Accepted 28 November 2016
Available online 29 November 2016

Keywords:
Candida spp.
Flow-through hybridization and gene chip
Identification
Microarray
Rapid

ABSTRACT

The incidence of invasive fungal infections in immunocompromised patients has increased in recent decades. Rapid and accurate identification of these pathogenic fungi is crucial for initiating a timely, safe, and effective antifungal therapy. Here we developed a microarray based on flow-through hybridization gene chip technology. The microarray was tested for its specificity using a panel of reference and blinded clinical isolates. The results proved that this microarray was highly discriminative, leading to the unequivocal identification of each species, including *Candida famata* and the highly related species *Candida parapsilosis*, *Candida orthopsilosis*, and *Candida metapsilosis*. This new system represents a reliable method that is of potential use in clinical laboratories for the simultaneous detection and identification of the most common pathogenic fungi.

1. Introduction

Invasive fungal infections (IFIs) represent a major cause of increased morbidity and mortality in critically ill patients. *Candida* spp. are responsible for approximately 80% of nosocomial fungal infections, and they are the fourth and seventh most common systemic pathogens in America and Europe, respectively (Wisplinghoff et al., 2004; Hidron et al., 2008; Leroy et al., 2009; Marchetti et al., 2004). Although, *Candida albicans* causes the majority of life-threatening candidemia cases, an increase in the proportion of non-albicans (NAC) *Candida* spp., which have distinctive patterns of antifungal susceptibility, has been observed worldwide (Pfaller and Diekema, 2007; Montagna et al., 2013). Thus, the eradication of candidemia caused by NAC *Candida* spp. is likely to require higher doses of fluconazole or other effective agents (e.g., echinocandin or amphotericin B) (Serefhanoglu et al., 2012; Redding et al., 2000). Therefore, identifying fungal pathogens to the species level is the most important step in selecting an adequate antifungal agent, which improves the efficacy of antibiotic therapy (Rex and Pfaller, 2002).

Currently, suspected candidemia is diagnosed using blood cultures, subsequent Gram staining, and various morphological and biochemical identification methods. These methods, although continuously improving, are still time-consuming, operator -dependent, and costly, and they are often inadequate for ensuring early and accurate therapy, especially for unusual fungal species, which leads to avoidable increased mortality (Kumar et al., 2006).

Molecular methods have been used to facilitate the early diagnosis of IFIs caused by common and less common clinically relevant fungi, and some of them take advantage of a combination of the polymerase chain reaction (PCR) and microarray hybridization (Leinberger et al., 2005; Leaw et al., 2007; Campa et al., 2008; Aittakorpi et al., 2012). Although these methods have shown enhanced sensitivities and specificities for microbial pathogens, general methods for the hybridization of PCR products on a microarray chip are time-consuming and labor-intensive.

In view of the increasing need for clinically applicable, technical approaches for the rapid identification of fungal species, we have established a high-throughput assay that facilitates the reliable recognition of a range of pathogenic *Candida* spp. that are relevant in the context of invasive infections. Our approach is based on flow-through hybridization gene chip (FHGC) technology, which, using simple equipment, can significantly improve the efficiency of hybridization and reduce hands-on time and human errors, thus improving precision, reproducibility, and traceability. Recently, this technology has been used successfully to detect 21 human papillomavirus HPV subtypes, and it has been used widely in clinic diagnoses in several European and Asian countries (Liu et al., 2010; Shigehara et al., 2011).

The assay presented herein permits the rapid identification of 12 *Candida* species, representing pathogens that are involved in invasive infections and frequently exhibit a drug-resistant phenotype, such as

* Corresponding author.
E-mail addresses: lilycrrc@sina.com (C. Li), Dingxr586@126.com (X. Ding), lzzlab@126.com (Z. Liu), jjzhu@hybribio.cn (J. Zhu).

http://dx.doi.org/10.1016/j.mimet.2016.11.019
0167-7012/

C. glabrata and *C. krusei*, emerging fungal pathogens, such as *C. famata* and *C. lusitaniae*, and the newly defined species *C. orthopsilosis* and *C. metapsilosis*. Because of feasibility concerns, other fungi were not included. This method can be used to accurately and rapidly identify common clinical fungal pathogens.

2. Materials and methods

2.1. Yeast strains and clinical specimens

For the development and optimization of the assay, 12 reference strains and 171 clinical isolates were used (Tables 1 and 2). The yeast reference isolates used in this study were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) (Table 1). A selected panel of 171 clinical fungal isolates, which were identified previously using conventional laboratory tests (including macro- and micromorphologies, germ tube formation, growth characteristics at different temperatures, and the VITEK 2 Compact system [bioMérieux, Marcy-l'Etoile, France] with yeast identification (YST ID) cards), were provided by the Beijing Friendship Hospital and Beijing Youan Hospital. The isolates (Table 2) were blindly submitted to the DNA microarray identification system. All the isolates were maintained on Sabouraud agar for the duration of the study.

2.2. Preparation of oligonucleotide probes for the microarray chip

To maximize the sensitivities and specificities of the species- or genus-specific primers and probes, sequences from the most variable regions within the 18S to 5.8S internal transcribed sequence (ITS) were selected by multiple alignment using the ClustalW program (http://www.cmbi.kun.nl/bioinf/tools/clustalw.shtml). The specific primers and probes used for the species-level identifications of *Candida* spp. are listed in Table 1. All the probes were designed to display an optimal length of 21 nucleotides (range, 18 to 25) and a similar G + C content to facilitate multiplex hybridization under uniform conditions. The consensus hybridization temperature of ≥54 °C was determined using the software program Primer3. To predict the potential cross-reactivities of the hybridization probes, additional database searches were performed using the BLASTN program (www.ncbi.nlm.nih.gov/).

Table 1
Fungal species tested, primers and probes used in this study.

Species	Nucleotide sequence (5′-3′)
Primer	
Fungus	
Fung-F	TTCTCGCATCGATGAAGARCGCA (R: A/G)
Fung-R	TCCTCCGCTTATTGATATGC
Candida parapsilosis	
CP-F	GTAACAAGGTTTCCGTAGGT
CP-R	GTTGAAAGTTTTGACTATTA
Internal control	
IC-F	GCAGGCTGCCTATCAGAAAGTG
IC-R	GGACTTAGGGAACAAAGGAACCTT
Probe	
C. albicans ATCC90028	ATTGCTTGCGGCGGTAACGTCC
C. glabrata ATCC38326	TAGGTTTTACCAACTCGGTGTT
C. tropicalis ATCC14506	AACGCTTATTTTGCTAGTGGCC
C. krusei ATCC6258	GGCCGAGCGAACTAGACTTTT
C. parapsilosis ATCC22019	ACAAACTCCAAAACTTCTTCCA
C. parapsilosis ATCC22019	TGGCAGGCCCCATATAGAA
C. orthopsilosis DSM 24508	TGACTATTAGTTAATCAGTTGACTT
C. metapsilosis ATCC96144	ATTAGTTAATCAAGTTGACAATTAA
C. norvegensis ATCC22977	GTACTTCGCTCAGTCCCG
C. lusitaniae ATCC34449	TATTTCGGAGCAACGCCT
C. dubliniensis DSM 13268	AAGGCGGTCTCTGGCGTCGCCC
C. guilliermondii DSM 11947	AACAATACCAGAAATATCC
C. famata DSM70590	CCTAGAATACCGAGAAATATA
Internal control	CACAAGTATCACTAAGCTCG

2.3. DNA extraction

Prior to DNA extraction, fungal cultures were grown on Sabouraud agar for 48 h at 30 °C. Then, one microbial colony was picked from the Sabouraud agar and suspended in 2 mL of NucliSENS lysis buffer (bioMérieux). DNA extraction from fungal cultures was performed with the QIAGEN DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

2.4. PCR amplification

Reaction mixtures (40 μL) contained 2 μL of fungal DNA, 1 μL of universal primers (10 μM, Invitrogen, Carlsbad, CA, USA), 1 μL of type-specific primers of *C. parapsilosis* (10 μM, Invitrogen), 0.6 μL of internal control primers (10 μM, Invitrogen), 0.5 μL of a deoxynucleotide triphosphate mix (25 mM, TaKaRa, Shiga, Japan), 4 μL of magnesium-free buffer (10×; TaKaRa), 7 μL of $MgCl_2$ (25 mM; Solis BioDyne), and 0.5 μL of Hot Start *Taq* DNA polymerase (5 U/μL; Roche, Basel, Switzerland); sterile distilled water was added to give a final volume of 40 μL. A negative control, in which an equal volume of water replaced the DNA template, was included in all the PCR experiments. The PCRs were performed according to the following protocol: 95 °C for 9 min (hot start), followed by 40 cycles of denaturation at 95 °C (20 s), annealing at 55 °C (30 s), and elongation at 72 °C (50 s), followed by a final extension step at 72 °C (5 min).

2.5. Hybridization of PCR products on the chip, and signal detection

Flow-through hybridization was performed on a prewarmed instrument at 45 °C, into which the DNA microarray membrane was placed. Twelve *Candida* spp. probes, including those for *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. krusei*, *C. lusitaniae*, *C. norvegensis*, *C. dubliniensis*, *C. guilliermondii*, *C. famata*, *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis*, were immobilized on the membrane, as shown in Fig. 1-A. The number of samples tested in a batch could be adjusted from 1 to 15 as required. The PCR products (25 μL) were denatured at 95 °C for 5 min just before hybridization, and then they were chilled on ice for at least 2 min. We mixed the PCR products with 0.5 mL of hybridization solution (2× saline-sodium citrate SSC/0.1% sodium dodecyl sulfate SDS, 45 °C) and then added the mixture to the sample wells to proceed with the flow-through hybridization for approximately 5–10 min. After draining the mixture, the membrane was washed three times with hybridization solution, and then the membrane was blocked for 5 min at 25 °C using a solution comprising 0.25% skimmed milk powder and 0.25% thiomersalate. After draining the solution, we labeled the membrane with an alkaline phosphatase-conjugated streptavidin solution for 5 min. Then, the membrane was washed four times with 0.8 mL of solution A (Tris-buffered saline, 0.1% Tween-20, and 0.05% sodium azide). After a stringent wash with hybridization solution, we added an NBT/BCIP (nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) solution to display the results; a positive result was indicated by a clearly visible indigo dot. Reagents were provided with a kit from the Chaozhou Hybribio Limited Corporation (Chaozhou, China). After hybridization, the presence of a positive result for both the "internal control" and "biotin" dots within the membrane indicated that the isolated DNA was of good quality, the enzyme conjugate was valid, and the hybridization process was proper. The identification results of *Candida* spp. were determined according to the position of the *Candida* spp. species-species probes on the microarray chip. Multiple dots indicated multiple infections. The PCR control for the assay was a low-copy-number the *Homo sapiens* hemoglobin subunit beta (HBB) (nonbacterial/fungal) DNA fragment, which was added to every PCR to verify and validate the amplification conditions. Hybridization and reagent controls confirmed