JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 1995, p. 2476–2479 Vol. 33, No. 9
0095-1137/95/$04.00+0

Identification of *Candida* Species by PCR and Restriction Fragment Length Polymorphism Analysis of Intergenic Spacer Regions of Ribosomal DNA

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Received 10 February 1995/Returned for modification 17 April 1995/Accepted 20 June 1995

The PCR was used to amplify a targeted region of the ribosomal DNA from 84 *Candida* isolates. Unique product sizes were obtained for *Candida guilliermondii*, *Candida* (*Torulopsis*) *glabrata*, and *Candida pseudotropicalis*. Isolates of *Candida albicans*, *Candida tropicalis*, *Candida stellatoidea*, *Candida parapsilosis*, and *Candida krusei* could be identified following restriction digestion of the PCR products.

Candidoses comprise a range of human opportunistic infections which may occur in either acute or chronic forms. Candida infections frequently arise on the mucosal surfaces of the mouth or vagina. Chronic hyperplastic candidosis of the oral mucosa is of particular importance since it has been associated with the development of squamous cell carcinoma (3). In addition to superficial lesions, deeper candida infections, such as esophagitis and endocarditis, may occur, particularly in immunocompromised individuals (9, 10).

In the past, many studies of candidosis have not identified candidal isolates to species level. Indeed, in the case of chronic hyperplastic candidosis, reporting is usually limited to the presence of structures consistent with candidal hyphae following histological examination of lesional tissue. However, it is becoming increasingly recognized that both species and subspecies of *Candida* differ in their ability to cause disease (1, 2, 4). Traditional methods used for the identification and typing of clinical isolates of *Candida* include morphological and biochemical analysis (13, 24), colony morphotyping (22), resistogram typing (21), and serotyping (6). These techniques are time-consuming, and their reliance on phenotypic expression makes them potentially unreliable. An alternative method of identification would be one based on genotypic properties.

Genotypic methods have been used extensively for the detection and typing of *Candida* strains (5, 8, 10, 11, 16) but have been used less frequently for differentiation of species. Recently, a combination of molecular techniques was used to analyze single isolates of eight species of *Candida*, and this allowed the identification of four species (17). In addition, identification of *Candida albicans*, *Candida stellatoidea*, *Candida tropicalis*, and *Candida guilliermondii* has also been achieved by restriction fragment length polymorphism (RFLP) analysis of the ribosomal DNA (rDNA) (genes encoding rRNA) repeat of *Candida* species (14).

The aim of the present study was to evaluate species identification of eight species of *Candida* on the basis of size and primary structural variation of rDNA intergenic spacer regions. It was our intention to determine the reliability of the technique by examining a large number of isolates from a range of sources.

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In total, 84 candidal isolates comprising 8 species were examined. These included *Candida* isolates cultured from oral infections and asymptomatic carriers, in addition to type strains (Table 1). Identification of test isolates was established by germ tube formation and use of the API-20C system (bioMérieux, Basingstoke, United Kingdom). Prior to extraction of the DNA, test isolates were grown in yeast nitrogen base (Difco, East Molesey, United Kingdom) medium overnight at 37°C. The DNA was extracted from all the isolates by the method described by Scherer and Stevens (20), with one modification of the protocol: the diethyl pyrocarbonate was omitted from the lysis buffer.

The DNA extracted from each *Candida* isolate was subjected to PCR amplification (18). The PCR was designed to amplify intergenic spacer regions of the rDNA with established primers ITS1 (5′-TCC GTA GGT GAA CCT GCG G 3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC 3′) (23). Initial experiments determined the optimum PCR conditions to be as follows: a reaction volume of 100 μl contained 0.2 mM each deoxynucleoside triphosphate, 1.5 mM magnesium chloride, 0.5 μM each primer, *Taq* buffer and 2.5 U of *Taq* polymerase (Promega, Southampton, United Kingdom), and 0.5 μg of candidal DNA as template. Negative controls were performed with sterile deionized water in place of the template DNA. Reaction mixtures were overlaid with 120 μl of light mineral oil (Sigma, Poole, United Kingdom) and subjected to 35 cycles of the following incubations: denaturation at 95°C for 1 min, primer annealing at 55°C for 1 min, and extension at 72°C for

TABLE 1. Number and source of *Candida* isolates analyzed by PCR amplification of rDNA

Candida sp.	No. and source of *Candida* isolates			
	Oral candidosis	HIV[a] oral isolate	Type strain	Asymptomatic carrier
C. albicans	29	7	2	3
C. (Torulopsis) glabrata	7	6	0	0
C. krusei	3	5	2	0
C. tropicalis	4	2	0	0
C. pseudotropicalis	1	4	0	0
C. parapsilosis	4	0	1	0
C. guilliermondii	1	2	0	0
C. stellatoidea	0	0	1	0

[a] HIV, human immunodeficiency virus.

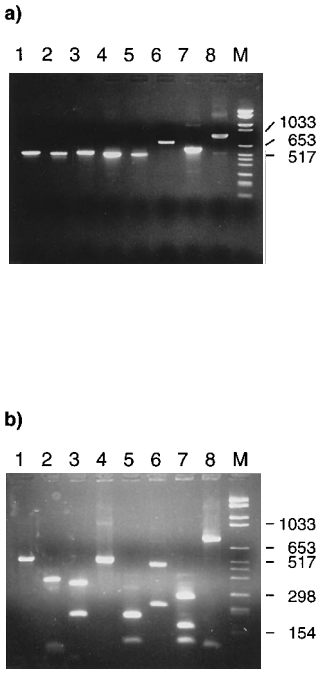

FIG. 1. (a) PCR amplification of candidal rDNA with ITS1 and ITS4 primers. (b) Restriction digestion of PCR products with the enzyme *Bfa*I. Lanes 1 to 8 contain DNA from *C. albicans*, *C. tropicalis*, *C. stellatoidea*, *C. parapsilosis*, *C. krusei*, *C. pseudotropicalis*, *C. guilliermondii*, and *C.* (*Torulopsis*) *glabrata*, respectively. Lanes M contain a molecular weight marker; the sizes of the bands are indicated in base pairs.

2 min. A Hybaid thermal cycler was used in all experiments (Hybaid Corp., Cambridge, United Kingdom). The size and purity of the PCR products were confirmed by electrophoresis of 10% of the product on a 0.75% agarose and 0.75% NuSieve (Flowgen Ltd., Sittingbourne, United Kingdom) gel. DNA was visualized after staining with ethidium bromide by illumination with UV light.

PCR products were purified with a commercial purification system (Wizard PCR Preps, Promega) in accordance with the manufacturer's recommended protocol. Total purified product was digested individually with 10 U of the restriction enzymes *Hae*III, *Bfa*I, and *Dde*I (New England Biolabs, Hitchin, United Kingdom) by overnight incubation at 37°C. The resulting restriction fragments were analyzed by agarose gel electrophoresis.

The intergenic spacer region was successfully amplified from all isolates tested, and a distinct product size was consistently obtained for all isolates of a given species. All isolates identified as *Candida* (*Torulopsis*) *glabrata* yielded a unique product size of approximately 800 bp. Similarly, all isolates of *C. guilliermondii*, and *Candida pseudotropicalis* yielded product sizes of 600 and 650 bp, respectively (Fig. 1a). A product of approximately 520 bp was obtained with the remaining isolates. These isolates were studied further by RFLP analysis following digestion of the PCR product by the restriction enzymes *Hae*III, *Dde*I, and *Bfa*I. Figure 1b shows a typical gel electrophoresis of PCR products obtained from eight *Candida* species and digested with *Bfa*I restriction enzyme. The restriction profiles produced by these enzymes are shown schematically in Fig. 2. Thirty isolates were analyzed with three restriction enzymes, and the restriction profile obtained with each enzyme for each isolate is summarized in Table 2. The other isolates were tested with one or two restriction enzymes, and the resulting profiles correlated with those obtained for the 30 isolates shown in Table 2. Repeated testing of a given isolate was found to yield consistent results. The enzyme *Hae*III was found to produce seven RFLP profiles, while seven and eight RFLP profiles were obtained with *Dde*I and *Bfa*I, respectively.

The traditional methods of identifying *Candida* species are often based on examination of phenotypic characteristics. This approach can be time-consuming, and the reliance on the variable expression of phenotypic characteristics can lead to inconsistent results. The phenotypic switching of *Candida* species has been well documented (19, 22), and consequently, genotype-based approaches may be preferable. Genotypic techniques have previously been used for the analysis of *Candida* species, but these have often concentrated on subspecies typing or detection of individual species (5, 7, 8, 16).

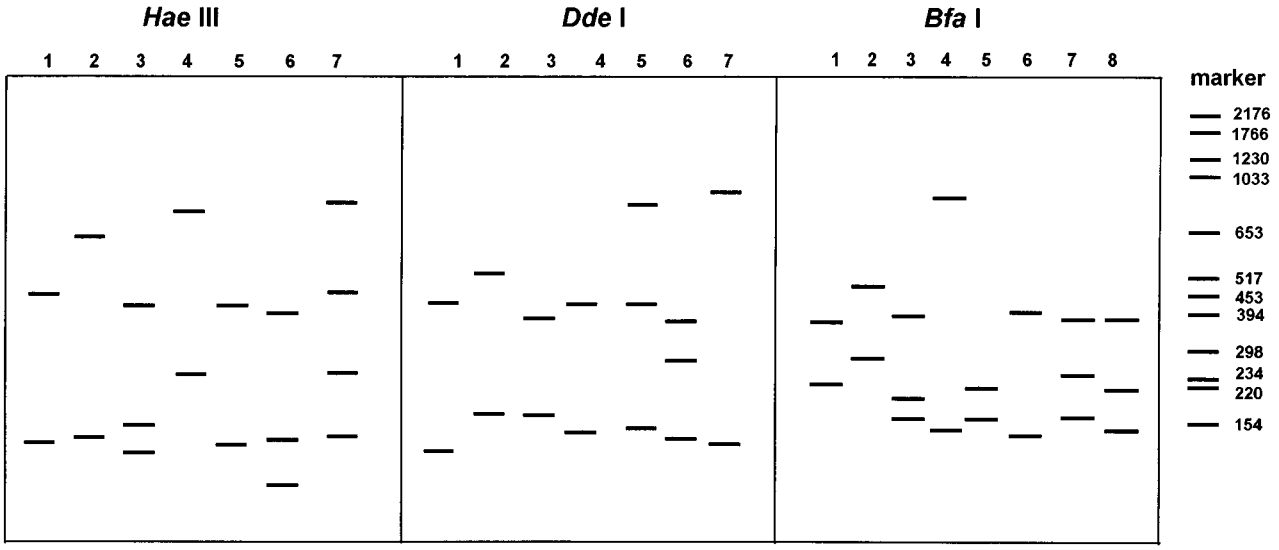

FIG. 2. RFLP profiles of candidal PCR products obtained with the restriction enzymes *Hae*III, *Dde*I, and *Bfa*I. The figures above each restriction pattern correspond to the RFLP profiles in Table 2. The sizes of the marker bands are indicated in base pairs.

The present study has shown that eight medically important *Candida* species can be distinguished on the basis of size and primary structural differences in the rDNA spacer regions. Isolates of *C.* (*Torulopsis*) *glabrata*, *C. guilliermondii*, and *C. pseudotropicalis* could be discriminated on the basis of PCR product size alone. Characteristic profiles, obtained following restriction digestion of PCR products, allowed the discrimination of the remaining isolates studied, with the restriction enzyme *Bfa*I providing the greatest level of species discrimination. A possible protocol for identifying *Candida* species would therefore involve electrophoretic analysis of the amplified product for the immediate discrimination of *C.* (*torulopsis*) *glabrata*, *C. guilliermondii*, and *C. pseudotropicalis*. Digestion of the amplified PCR product with a combination of *Bfa*I and *Dde*I restriction enzymes would subsequently allow identification of *C. albicans*, *Candida parapsilosis*, *Candida krusei*, *C. tropicalis*, and *C. stellatoidea*. The third enzyme, *Hae*III, could be used for further confirmation of identity.

The results obtained in the present study were shown to be reproducible and consistent for the large number of isolates tested. In addition, the results were found to be stable after both repeated subculturing and storage of isolates for at least 6 months (results not shown).

The PCR is being increasingly applied within hospital laboratories for the routine detection of human gene mutations and also for HLA typing (15). Since the procedures used in this study are relatively simple to perform and require only standard molecular biology equipment, there is no reason why the use of PCR should be restricted to research-based laboratories.

An additional advantage of this genotypic approach to the identification to species level of *Candida* isolates is its rapidity. Other workers have reported the identification of certain *Candida* species based on molecular methods, but often these studies have included lengthy procedures such as hybridization steps requiring overnight incubations (17). Furthermore, other studies have not tended to examine large numbers of isolates that include all the recognized pathogenic *Candida* species. Identification of three *Candida* species can be achieved within a working day by this procedure, although overnight digestion of PCR products with restriction enzymes does increase the time required to identify other *Candida* species.

In summary, a genotypic method has been evaluated for the identification of *Candida* species. The reliability of the technique has been demonstrated by the examination of a large number of *Candida* isolates from a range of sources.

We thank Guy's and St. Thomas Medical and Dental School, London; Glasgow Dental Hospital and School, Glasgow; Liverpool Dental School, Liverpool; and Pfizer Limited and SmithKline Beecham Pharmaceuticals for supplying some of the isolates used in this study.

D. W. Williams was supported by the Medical Research Council, United Kingdom.

TABLE 2. Grouping of 30 *Candida* isolates (identified by phenotypic criteria) according to RFLP profiles obtained following restriction digestion of PCR products[a]

RFLP profile							
Hae III	1	2	3	4	5	6	7
	C. albicans OC=8; AC=2; HIV=2	*C. stellatoidea* TS=1 *C.tropicalis* OC=1; HIV=1	*C. pseudotropicalis* OC=1; HIV=2	*C. guilliermondii* OC=1; HIV=2	*C. (Torulopsis) glabrata* OC=2	*C. parapsilosis* OC=2; TS=1	*C. krusei* HIV=1	*C. (Torulopsis) glabrata* HIV=1

RFLP profile							
Dde I †	1	2	3	4	5	6	7
	C. stellatoidea TS=1	*C. pseudotropicalis* OC=1; HIV=2	*C. guilliermondii* OC=1; HIV=2	*C. albicans* OC=8; AC=2; HIV=2	*C. tropicalis* OC=1; HIV=1	*C. (Torulopsis) glabrata* HIV=1	*C. krusei* HIV=1	*C. (Torulopsis) glabrata* OC=1; HIV=1

RFLP profile								
Bfa I ‡	1	2	3	4	5	6	7	8
	C. stellatoidea TS=1	*C. pseudotropicalis* OC=1; HIV=2	*C. guilliermondii* OC=1; HIV=1	*C. (Torulopsis) glabrata* OC=1; HIV=1	*C. krusei* OC=2	*C. tropicalis* OC=1; HIV=1	*C. guilliermondii* HIV=1	*C. krusei* HIV=1

[a] TS, type strains; OC, oral candidosis isolates; HIV, isolates obtained from human immunodeficiency virus-infected patients; AC, isolates from asymptomatic carriers. †, additional isolates of *C. krusei* (OC = 2) and *C. parapsilosis* (TS = 1, OC = 2) were not digested by *Dde*I. ‡, isolates of *C. albicans* (OC = 8, AC = 2, HIV = 2), *C. parapsilosis* (TS = 1, OC = 2), and *C. (Torulopsis) glabrata* (HIV = 1) were not digested by *Bfa*I.

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