JOURNAL OF CLINICAL MICROBIOLOGY, July 2002, p. 2483–2489 Vol. 40, No. 7
0095-1137/02/$04.00+0 DOI: 10.1128/JCM.40.7.2483–2489.2002

Seminested PCR for Diagnosis of Candidemia: Comparison with Culture, Antigen Detection, and Biochemical Methods for Species Identification

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Received 2 January 2002/Returned for modification 9 March 2002/Accepted 15 April 2002

The rapid detection and identification of *Candida* species in clinical laboratories are extremely important for the management of patients with hematogenous candidiasis. The presently available culture and biochemical methods for detection and species identification of *Candida* are time-consuming and lack the required sensitivity and specificity. In this study, we have established a seminested PCR (snPCR) using universal and species-specific primers for detection of *Candida* species in serum specimens. The universal outer primers amplified the 3′ end of 5.8S ribosomal DNA (rDNA) and the 5′ end of 28S rDNA, including the internally transcribed spacer 2 (ITS2), generating 350- to 410-bp fragments from the four commonly encountered *Candida* species, viz., *C. albicans*, *C. tropicalis*, *C. glabrata*, and *C. parapsilosis*. The species-specific primers, complementary to unique sequences within the ITS2 of each test species, amplified species-specific DNA in the reamplification step of the snPCR. The sensitivity of *Candida* detection by snPCR in spiked serum specimens was close to 1 organism/ml. Evaluation of snPCR for specific identification of *Candida* species with 76 clinical *Candida* isolates showed 99% concordant results with the Vitek and/or ID32C yeast identification system. Further evaluation of snPCR for detection of *Candida* species in sera from culture-proven ($n = 12$), suspected ($n = 16$), and superficially colonized ($n = 10$) patients and healthy subjects ($n = 12$) showed that snPCR results were consistently negative with sera from healthy individuals and colonized patients. In culture-proven candidemia patients, the snPCR results were in full agreement with blood culture results with respect to both positivity and species identity. In addition, snPCR detected candidemia due to two *Candida* species in five patients, compared to three by blood culture. In the category of suspected candidemia with negative blood cultures for *Candida*, nine patients (56%) were positive by snPCR; two of them had dual infection with *C. albicans* and either *C. tropicalis* or *C. glabrata*. In conclusion, the snPCR developed in this study is specific and more sensitive than culture for the detection of *Candida* species in serum specimens. Moreover, the improved detection of cases of candidemia caused by more than one *Candida* species is an additional advantage.

Nosocomial candidiasis is a major fungal infection occurring mostly in patients undergoing prolonged hospitalization due to a variety of underlying conditions (26). Bloodstream infections due to *Candida* are now regarded as the fourth most frequent cause of septicemia, with a mortality rate of about 50% (29). Diagnosis of candidemia or hematogenous candidiasis has been problematic due to the low positivity of blood cultures. Even in patients with autopsy-proven systemic candidiasis, the rate of recovery from blood cultures ranged between 40 and 60% (27). Although various laboratory tests based on detection of *Candida*-specific antibodies, antigens, or metabolites have been developed, they all suffer from lack of specificity and/or sensitivity, besides being time-consuming (36). Moreover, these tests fail to clearly discriminate between the infecting *Candida* species, information that is crucial for initiating specific antifungal therapy since several non-*C. albicans Candida* species are known to be inherently less susceptible to commonly used antifungal drugs (14, 28).

In order to overcome the limitations of conventional diagnostic tests, DNA-based methods have been developed for the detection of *Candida* species and offer a potentially more sensitive means of diagnosing systemic candidiasis (4, 30). The use of PCR-based tests to detect *Candida* DNA in body fluids has produced encouraging results (5, 13, 15, 16, 23). However, detection of *Candida* species by PCR lacks sensitivity when the test is performed with blood or serum specimens (5, 10). DNA amplification with universal fungal primers followed by detection using species-specific probes greatly improved the sensitivity of *Candida* detection (7, 10, 28, 31, 35), but probing methods involved the use of radioactivity and/or laborious and time-consuming additional steps.

In this study, we have used the species-specific primers described previously (10) for species-specific detection of PCR-amplified ribosomal DNAs (rDNAs) of four commonly encountered *Candida* species, i.e., *C. albicans*, *C. parapsilosis*, *C. tropicalis*, and *C. glabrata*, by Southern hybridization and enzyme-linked immunosorbent assay, to develop a seminested PCR (snPCR). By employing universal fungal primers, a portion of the 5.8S and 28S rDNAs including the intervening internally transcribed spacer (ITS2) was amplified. The amplicon so obtained was reamplified in an snPCR using species-specific primers complementary to unique sequences within the ITS2 together with a generic fungal primer. The snPCR thus developed was evaluated for its specificity and sensitivity by using clinical *Candida* isolates and sera of patients with

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TABLE 1. *Candida* isolates used

Source of isolation	No. of isolates	No. of isolates of the following *Candida* species as identified by Vitek:			
		C. albicans	*C. parapsilosis*	*C. tropicalis*	*C. glabrata*
Blood culture	33	26	4	3	0
Mouth swab	9[a]	2	1	3	2
Urine	8	1	2	2	3
ET aspirate	6	0	2	3	1
Skin swab	13	2	5	1	5
Rectal swab	7	1	2	1	3

[a] One isolate was unidentified.

suspected and proven candidemia in comparison with conventional diagnostic and identification methods.

MATERIALS AND METHODS

Reference organisms. The reference strains used in the study were *C. albicans* ATCC 76615, *C. parapsilosis* ATCC 10233, *C. tropicalis* ATCC 750, *C. glabrata* ATCC 15545, *C. dubliniensis* type strain CD 36, a *C. krusei* clinical isolate, and a *C. lusitaniae* clinical isolate. In addition, bacterial strains of *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Salmonella enterica* serovar Typhimurium (clinical isolate), and *Legionella pneumophila* (ATCC 33152) were also included in the study. All strains, including clinical isolates, were stored at −20°C in sterile distilled water.

Clinical *Candida* isolates. To test the specificity of snPCR, 76 clinical *Candida* isolates identified to species level with the Vitek commercial yeast identification system (bioMerieux, Marcy l'Etoile, France) were evaluated by snPCR. The sources of these *Candida* isolates are presented in Table 1.

Subjects. Thirty-eight patients admitted to the intensive care unit and other wards of Mubarak Al-Kabeer Hospital, Jabriya, Kuwait, were included in the study. For the purpose of these investigations, they were broadly divided into three groups. (i) Colonized patients ($n = 10$) were those yielding *Candida* species from one or more anatomic sites with no clinical suspicion of *Candida* infection. (ii) Patients with suspected candidiasis ($n = 16$) were those with three or more of the following conditions: having an extended period of hospitalization (>2 weeks), yielding *Candida* from one or more anatomic sites, having an inadequate response to broad-spectrum antibiotics, and having *Candida* antigen titers of ≥1:2 and blood cultures negative for *Candida* species. (iii) Candidemic patients ($n = 12$) were clinically suspected patients with blood cultures yielding *Candida* species on one or more occasions. In addition, 12 apparently healthy individuals with no complaints of oral or vaginal *Candida* infection were included as controls.

Culture. Clinical specimens, such as sputum, bronchoalveolar lavage (BAL), endotracheal aspirate (ET), and urine specimens, were processed for the isolation of *Candida* species according to standard procedures (21). All cultures were made on Sabouraud glucose agar (SGA) (glucose [15 g], peptone [10 g], and agar [158 g] in 1 liter of distilled water, pH 6.8) at 30°C. Blood cultures were processed either with the BACTEC 9240 system (Becton Dickinson, Paramus, N.J.) or with a lysis centrifugation system (Isostat; Wampole Laboratories, Cranbury, N.J.) and the growth so obtained was subcultured on Sabouraud glucose agar plates (SGA) for further identification.

Identification. All of the yeast isolates were examined by wet mount and tested for germ tube formation. The germ tube-positive isolates were provisionally identified as *C. albicans*, and their identities were further confirmed with the Vitek and ID32C yeast identification systems (bioMerieux). Likewise, all non-*C. albicans Candida* species were identified by both systems.

Serum samples. Fifty serum samples, 38 from patients and 12 from healthy volunteers, were analyzed by the snPCR for the detection of *Candida* DNA. Blood samples were obtained in plain tubes, and sera were separated by centrifugation and stored frozen at −20°C until used.

Antigen detection. Cand-Tec (Ramco Inc., Houston, Tex.), a latex agglutination test based on rabbit antibodies to a heat-labile antigen, was used for the detection of *Candida* antigen in the sera. The test was performed according to the manufacturer's instructions.

Extraction of *Candida* DNA from cultures and sera. DNA was extracted from broth cultures by the method of Lee (18) with an additional step of DNA purification by extraction in phenol-chloroform (24:1). DNA from serum was extracted by the method of Sandhu et al. (31). To remove PCR inhibitors, the samples were heated with Chelex-100 (Sigma) (30 mg/ml) before precipitation of the DNA.

PCR primers. A 22-bp forward primer, CTSF (5′-TCGCATCGATGAAGA ACGCAGC-3′), and a 25-bp reverse primer, CTSR (5′-TCTTTTCCTCCGCT TATTGATATGC-3′), capable of amplifying the 3′ end of 5.8S rDNA and the 5′ end of 28S rDNA, including the intervening spacer region, were synthesized by Genemed Synthesis, Inc., San Francisco, Calif. Species-specific oligonucleotide primers for snPCR were derived from the ITS2 regions of *C. albicans* (CADET, 5′-ATTGCTTGCGGCGGTAACGTCC-3′), *C. parapsilosis* (CPDET, 5′-ACAA ACTCCAAAACTTCTTCCA-3′), *C. tropicalis* (CTDET, 5′-AACGCTTATTTT GCTAGTGGCC-3′), and *C. glabrata* (CGDET, 5′-TAGGTTTTACCAACTCG GTGTT-3′) (10).

DNA amplification and detection. Amplification of target DNA was carried out in thin-walled 0.2-ml PCR tubes in a total volume of 50 μl containing 1× AmpliTaq PCR buffer I, 1 U of AmpliTaq DNA polymerase, 10 pmol each of CTSF and CTSR primers, 1 μl of DNA extracted from culture or 5 μl of DNA extracted from serum, and 0.1 mM each deoxynucleoside triphosphate. After amplification in the first step, 1 μl of the product was further amplified using the initial reverse primer (CTS1R) and a species-specific forward primer in four separate tubes corresponding to each of the *Candida* species to be detected. For snPCR, the reaction mixture consisted of 1× AmpliTaq PCR buffer I; 1 U of AmpliTaq DNA polymerase; 5 pmol of CTSR together with 5 pmol of CADET, CPDET, CGDET, or CTDET; 1 μl of the first PCR product; and 0.1 mM each deoxynucleoside triphosphate. All reagents except primers were obtained from Perkin-Elmer Corp., Norwalk, Conn. PCR cycling was carried out in a Perkin-Elmer cycler (GeneAmp PCR system 2400) under the following conditions: denaturation at 94°C for 1 min, annealing at 60°C for 30 s, and extension at 72°C for 1 min. An initial denaturation step at 94°C for 3 min and a final extension step at 72°C for 10 min were also included. Optimum amplification was determined to be obtained with 30 cycles of the first PCR followed by 20 cycles of the snPCR for DNA extracted from broth cultures and with 35 cycles followed by 25 cycles for DNA extracted from sera. To avoid the risk of contamination of PCR samples, the precautions and guidelines advocated by Kwok and Higuchi (17) were followed. The area where the PCR mixtures were prepared was physically separated from the laboratory where DNA extraction was performed. Amplicon carryover was prevented by using aerosol-guarded pipette tips. Appropriate negative controls were included in each test run, including controls omitting the DNA template during PCR assays.

To detect amplified DNA fragments, agarose gel electrophoresis was performed as described previously (16). The gels were exposed to UV light and photographed. The sizes of amplified DNA fragments were identified by comparison with molecular size marker DNA (100-bp DNA ladder).

RESULTS

Standardization of snPCR. The PCR amplification of rDNAs from the four *Candida* species, viz., *C. albicans*, *C. parapsilosis*, *C. tropicalis*, and *C. glabrata*, using universal fungal primers (CTSF and CTSR) resulted in amplification of a single DNA fragment of the expected size (Fig. 1) (2, 10). Similar results were obtained when genomic DNAs prepared from *C. krusei*, *C. lusitaniae*, and *C. dubliniensis* were used as templates (data not shown). Reamplification of the product of the first PCR with CTSR and the species-specific primers corresponding to the ITS2 sequences from *C. albicans*, *C. parapsilosis*, *C. tropicalis*, and *C. glabrata* resulted in specific amplification of single DNA products of the expected sizes (Fig. 2). For example, CTSR and CGDET amplified a ~140-bp product in snPCR only when the first PCR was performed with template DNA from *C. glabrata* and not when it was performed with DNAs from *C. albicans*, *C. parapsilosis*, and *C. tropicalis* (Fig. 3), as well as *C. krusei*, *C. lusitaniae*, and *C. dubliniensis* (data not shown). Similar results were obtained with other primer combinations for specific detection of *C. albicans*, *C. parapsilosis*, and *C. tropicalis* (data not shown). No amplification was detected with genomic DNAs from *E. coli*, *S. aureus*, *L. pneumophila*, *S. enterica* serovar Typhimurium, and a human cell

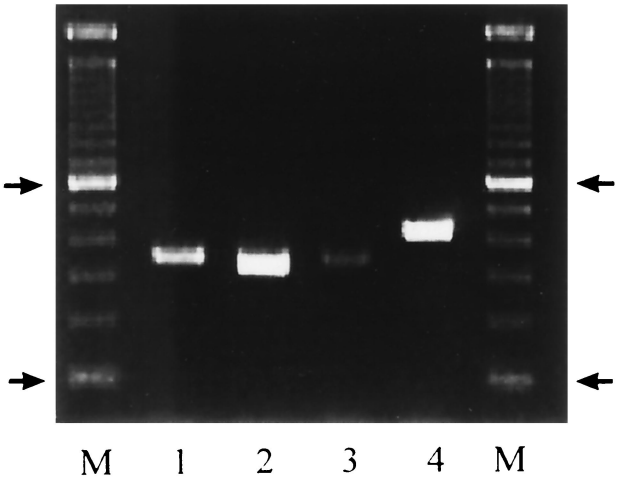

FIG. 1. PCR amplification of genomic DNAs of *C. albicans* (lane1), *C. parapsilosis* (lane 2), *C. tropicalis* (lane 3), and *C. glabrata* (lane 4) with universal fungal primers. Lanes M, 100-bp molecular size marker. Arrows indicate positions of 100 and 600 bp in ascending order.

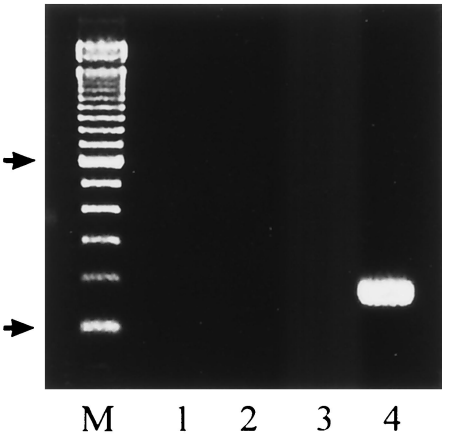

FIG. 3. Lanes 1 to 4, snPCR amplification using primers CTSR and CGDET and DNAs from *C. albicans*, *C. parapsilosis*, *C. tropicalis*, and *C. glabrata*, respectively. Lane M, 100-bp molecular size marker. Arrows indicate positions of 100 and 600 bp in ascending order.

line in the first as well as the second step of snPCR (data not shown). The results of these experiments established the species specificity of the snPCR.

To determine the sensitivity of snPCR for detection of *Candida* in clinical specimens, experiments were performed with total DNA isolated from serum specimens from a healthy individual spiked with different concentrations of *C. albicans* DNA. The results showed that snPCR was positive in specimens spiked with 4 fg of *C. albicans* DNA isolated from 200-μl spiked specimens (Fig. 4). The sensitivity of snPCR was therefore 20 fg of DNA/ml, which is equivalent to one *C. albicans* genome per milliliter of serum.

Species identification of *Candida* isolates by snPCR. The specificity of the snPCR was further investigated in species identification with 76 clinical *Candida* isolates. These isolates were first identified to species level with the Vitek and ID32C identification systems (Tables 1 and 2). The results of snPCR in species identification were concordant with those of the Vitek and ID32C systems for 68 of 76 (89%) and 71 of 76 (93%) isolates, respectively. The identification results for the 13 isolates showing discrepancy between snPCR and one or both biochemical tests are listed in Table 3. The results further showed that compared to snPCR, the discordant results in the identification of *C. albicans* were more marked between Vitek and ID32C (Table 2). It may be noted that the species identification of only one isolate was different by snPCR from that of both the Vitek and ID32C (Tables 3 and 4). Thus, the overall concordance of the snPCR compared to the two biochemical tests together in the identification of *Candida* species can be taken to be 75 of 76 or ~99%.

Comparison of blood culture versus snPCR. The comparative results of blood culture and the snPCR for the detection of

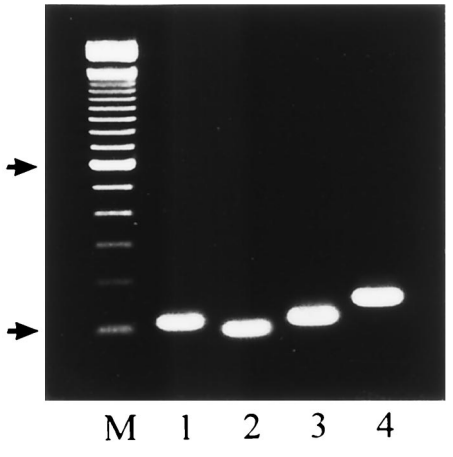

FIG. 2. Lanes 1 to 4, snPCR amplification of DNAs from *C. albicans*, *C. parapsilosis*, *C. tropicalis*, and *C. glabrata*, respectively, using primer CTSR with primers CADET, CPDET, CTDET, and CGDET, respectively. Lane M, 100-bp molecular size marker. Arrows indicate positions of 100 and 600 bp in ascending order.

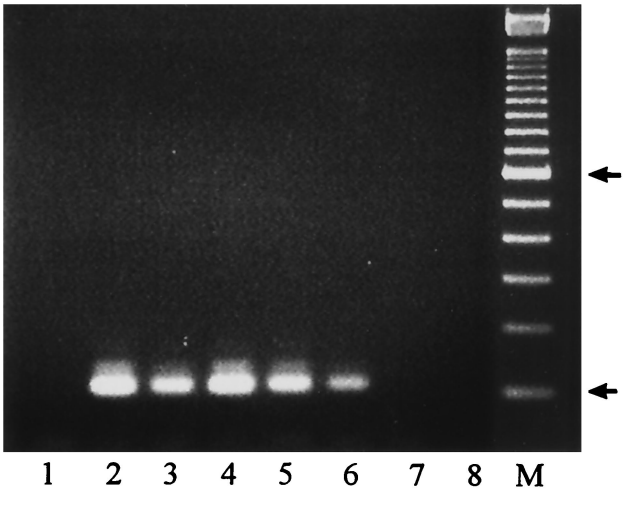

FIG. 4. Lanes 2 to 8, snPCR using DNA extracted from serum spiked with 40 and 4 pg and 400, 40, 4, 0.4, and 0.04 fg of *C. albicans* rDNA, respectively. Lane 1, negative control with water in place of template DNA. Lane M, 100-bp molecular size marker. Arrows indicate positions of 100 and 600 bp in ascending order.

TABLE 2. Identification of clinical *Candida* isolates by commercial assimilation methods and agreement with snPCR

Candida species	No. of isolates identified by:			% Agreement with PCR[a]	
	PCR	Vitek	ID32C	Vitek	ID32C
C. albicans	27	32	23	84	85
C. parapsilosis	20	17	20	85	100
C. tropicalis	14	13	13	93	93
C. glabrata	15	13	15	87	100

[a] Percent agreement = (number positive by PCR/number positive by Vitek-ID32C) × 100. However, if the number of isolates positive by Vitek or ID32C exceeded the number of PCR-positive isolates, percent agreement = number positive by Vitek or ID32C/number of PCR-positive isolates × 100.

TABLE 4. Agreement between snPCR and biochemical test results

Candida species	No. of clinical isolates identified by:		% Agreement
	snPCR	Vitek and ID32C	
C. albicans	27	27	100
C. parapsilosis	19	20[a]	95
C. tropicalis	15[a]	14	93
C. glabrata	15	15	100

[a] One isolate was identified as *C. tropicalis* by snPCR and as *C. parapsilosis* by both biochemical tests.

Candida species are presented in Table 5. None of the blood or serum samples from 12 healthy volunteers were positive for *Candida* by culture or snPCR. Similarly, culture and snPCR from sera were negative in all 10 patients colonized with *Candida* species (Table 5). Among 16 clinically suspected cases of candidemia or hematogenous candidiasis, 9 (~56%) yielded positive results by snPCR, while blood cultures remained negative for all of them. Six of the positive snPCR results were due to *C. albicans*, one was due to *C. parapsilosis*, and two were due to dual infection, i.e., *C. albicans* with *C. tropicalis* and *C. tropicalis* with *C. glabrata* (Table 5). All of the snPCR-positive patients had the corresponding *Candida* species isolated from one or more specimens other than blood (Table 5). Ten of the 16 patients demonstrated *Candida* antigen titers ranging from 1:2 to 1:16. Of the nine snPCR-positive patients, one demonstrated antigen titers of 1:16, two had antigen titers of 1:8, one each had antigen titers of 1:4 and 1:2, and four were negative (Table 5).

All of the 12 patients with culture-proven candidemia yielded positive results by snPCR for the corresponding *Candida* species, with 100% concordance (Table 5). The infecting species identified by blood culture included *C. albicans* in nine patients, *C. parapsilosis* and *C. tropicalis* in two patients each and *C. glabrata* in one patient. The results of snPCR revealed that five of the patients were infected with more than one *Candida* species, as against three detected by blood culture. Only 7 of the 12 patients had demonstrable antigen titers: 1:8 in 2 patients, 1:4 in 1 patient, and 1:2 in 4 patients (Table 5). In case 34, Vitek identified the *Candida* isolate as *C. albicans* whereas the snPCR with serum identified the species as *C. parapsilosis*. Further identification of the blood culture isolate by ID32C and snPCR (using genomic DNA purified from the culture) established the species to be *C. parapsilosis* (Table 5).

DISCUSSION

In the present study, snPCR assays targeting species-specific sequences in the rDNA have been established for the specific detection of four clinically important *Candida* species by using reference strains. These snPCRs were evaluated for *Candida* species identification with clinical isolates and for direct detection as well as specific identification of *Candida* species in serum samples from patients. The target for snPCR amplification was rDNA. Although PCR assays with several other target sequences have been reported in the literature (22, 30), the use of rDNA for sensitive detection of *Candida* was considered to be most suitable because it is present in multiple copies (50 to 100 copies) per *Candida* genome (30), and PCR assays with multiple-copy targets are usually more sensitive than those with single-copy targets (22, 30). Moreover, between the highly conserved rDNA subunits are the internally transcribed spacers, which contain sequences unique to each *Candida* species, and thus the use of primers corresponding to these regions facilitates species identification. Although sev-

TABLE 3. *Candida* isolates identified differently by snPCR and by the Vitek and ID32C methods

Isolate	Identification by:			Germ tube formation
	PCR	ID32C[a]	Vitek[b]	
431/98[c]	*C. tropicalis*	*C. parapsilosis*	*C. parapsilosis*	−
82/97 (626)	*C. albicans*	*C. pelliculosa*	*C. albicans*	+
82/97 (660)	*C. albicans*	*C. pelliculosa*	*C. albicans*	+
602/99	*C. albicans*	Unidentified	*C. albicans*	+
548/98	*C. tropicalis*	*C. tropicalis*	*C. albicans*	−
829/98	*C. parapsilosis*	*C. parapsilosis*	*C. albicans*	−
576/99	*C. albicans*	Unidentified	*C. albicans*	+
706/2k	*C. parapsilosis*	*C. famata*	*C. parapsilosis*	−
P1R(3)	*C. parapsilosis*	*C. parapsilosis*	*C. albicans*	−
P1M(5)	*C. parapsilosis*	*C. parapsilosis*	*C. albicans*	−
P1G(3)	*C. parapsilosis*	*C. parapsilosis*	*C. albicans*	−
P26U(7)B	*C. glabrata*	*C. glabrata*	*C. tropicalis*	−
P34M(1)B	*C. glabrata*	*C. glabrata*	Unidentified	−

[a] Read at 48 h.
[b] Read at 24 and 48 h.
[c] The identification was reconfirmed as *C. tropicalis* by PCR with species-specific primers amplifying the gene encoding P_{450} lanosterol-α-demethylase (16).

TABLE 5. Comparative results of blood cultures and snPCR

Candida status	Subject no.	Age/sex[a]	Underlying condition[b]	Source or site of *Candida* isolation	*Candida* spp. isolated	Antigen titer[c]	Blood culture results[c]	snPCR results[c]
Colonized	1	65/F	Allergy	Oral	*C. albicans*	−	−	−
	2	78/M	CVA	Urine	*C. tropicalis*	−	−	−
	3	88/M	CVA	Urine	*C. tropicalis*	−	−	−
	4	6 mo/F	Chest infection	ET	*C. albicans*	1:2	−	−
	5	70/F	Chest infection	Urine	*C. glabrata*	−	−	−
	6	30/F	UTI	Urine	*C. albicans*	−	−	−
	7	35/F	Vaginitis	Vaginal swab	*C. albicans*	1:2	−	−
	8	42/F	Vaginitis	Vaginal swab	*C. albicans*	−	−	−
	9	29/F	Vaginitis	Vaginal swab	*C. albicans*. *C. glabrata*	−	−	−
	10	38/F	Vaginitis	Vaginal swab	*C. albicans*	−	−	−
Suspected candidiasis	11	35/M	RTA, head trauma	ET	*C. albicans*	−	−	*C. albicans*
	12	17/M	Head trauma, epilepsy	Urine	*C. albicans*	−	−	−
	13	66/M	Chest infection, chronic renal failure	Urine; BAL	*C. tropicalis, C. tropicalis, C. glabrata*	1:4	−	−
	14	44/F	Bronchial asthma	Sputum	*C. albicans*	1:8	−	−
	15	36/M	RTR	Sputum	*C. albicans*	1:4	−	*C. albicans*
	16	45/F	RTR, chest infection	Urine; ET	*C. parapsilosis*, *C. krusei*	1:4	−	−
	17	45/M	RTR, chest infection	Urine; pus swab	*C. tropicalis, C. glabrata, C. albicans*	1:8	−	*C. albicans*
	18	52/M	RTR, pneumonia	Urine	*C. albicans*	1:8	−	−
	19	80/M	Septicemia, fever	Skin swab	*C. albicans*	−	−	*C. albicans, C. tropicalis*
	20	85/F	Fever of unidentified origin	Skin swab	*C. parapsilosis*	−	−	*C. parapsilosis*
	21	52/F	Chest infection	Urine, ET	*C. albicans, C. albicans*	1:8	−	*C. albicans*
	22	45/M	RTR, pneumonia	BAL	*C. albicans*	−	−	−
	23	45/M	RTR	Urine	*C. albicans*	1:4	−	−
	24	41/M	Burns	Skin swab	*C. tropicalis*	1:2	−	*C. tropicalis, C. glabrata*
	25	72/M	Cholecystectomy	ET	*C. albicans*	1:16	−	*C. albicans*
	26	70/F	Pulmonary edema	Urine	*C. albicans*	−	−	*C. albicans*
Candidemic	27	48/M	DM, HTN, CVA	Urine	*C. albicans*	1:8	*C. albicans*	*C. albicans*
	28	77/F	Bronchial asthma, pulmonary embolism	Urine, ET	*C. glabrata, C. glabrata*	1:2	*C. parapsilosis*	*C. parapsilosis, C. glabrata*
	29	7/M	Chest infection, fever	−	−	1:8	*C. albicans, C. parapsilosis*	*C. albicans, C. parapsilosis*
	30	70/F	DM, HTN, LVF	Urine	*C. glabrata*	1:2	*C. glabrata*	*C. glabrata*
	31	64/F	HTN, SAH	Urine	*C. albicans*	1:2	*C. tropicalis, C. albicans*	*C. tropicalis, C. albicans*
	32	1 mo/F	Premature baby	−	−	−	*C. parapsilosis*	*C. parapsilosis*
	33	50/M	Septicemia, fever	Urine	*C. albicans*	1:2	*C. albicans*	*C. albicans*
	34	29/F	Chronic renal failure	−	−	−	*C. albicans* (Vitek), *C. parapsilosis* (ID32C)	*C. parapsilosis*
	35	67/F	Tracheostomy, DM	−	−	−	*C. albicans*	*C. albicans*
	36	60/F	Brain abscess	−	−	−	*C. albicans*	*C. albicans, C. tropicalis*
	37	3 mo/F	Lung infection	−	−	−	*C. albicans*	*C. albicans*
	38	74/F	LVF, CVA, rectal ulcer	Urine	*C. tropicalis*	1:4	*C. albicans, C. tropicalis*	*C. albicans, C. tropicalis*

[a] Ages are in years unless otherwise indicated. F, female; M, male.
[b] RTA, road traffic accident; DM, diabetes mellitus; UTI, urinary tract infection; RTR, renal transplant; LVF, left ventricular failure; CVA, cardiovascular accident; SAH, subarachinoid hemorrhage; HTN, hypertension.
[c] −, negative.

eral PCR assays based on amplification of the rDNA have been recently reported, species identification usually involved further manipulation of the amplified products, i.e., restriction enzyme digestion and analysis (24, 37), use of radioactive and enzyme-labeled probes (2, 10, 15, 20, 23, 32, 33, 35), and DNA sequencing (25). Many of these procedures required prolonged hybridization times and the use of hazardous radioactive materials. Moreover, species identification by the use of a biotinylated probe and detection by enzyme immunoassay showed lower specificity for *C. glabrata* (10). In contrast, the snPCR established in this study has an average processing time of 9 to 10 h, does not require the use of hybridization probes and radioactive substances, and is specific for the detection of all four *Candida* species tested. More recently, multiplex PCR assays, targeting rDNA and spacer regions, to detect *Candida* species have been established (3, 11). However, for a positive result, multiplex PCRs required a minimum of 20 cells, compared to 0.2 cell for snPCR. Thus, the snPCR appears to be at least 100 times more sensitive than the multiplex PCRs. Also, the multiplex PCR assays have not yet been evaluated for their potential use in the diagnosis of *Candida* infection directly with clinical specimens.

The specificity of the snPCR was established with 99% accuracy, as it correctly identified 75 of the 76 *Candida* isolates previously identified to species level by conventional biochemical methods. However, a solitary blood culture isolate identified as *C. parapsilosis* by Vitek and ID32C was shown to be *C. tropicalis* by snPCR. Although the precise reason for this discrepancy remains unclear, it could be related to the inadequacy of the presently available commercial yeast identification systems (9). While several investigators have used PCR to identify *Candida* species, their studies have been mostly limited to the use of reference strains and clinical isolates (12, 16, 28). In such studies, there has been no comparison of the identification accuracy between PCR and one or more biochemical methods. This is despite the fact that several studies have shown that commercial yeast identification systems misidentify some percentage of clinical yeast isolates (6, 8, 9, 19, 34). The same is also apparent in the present investigation (Tables 2 and 3).

The snPCR assay was subsequently applied for *Candida* detection and species identification directly in clinical specimens. Consistent with the results of other investigators (15, 35), our results also showed that all of the sera from healthy volunteers were PCR negative. In addition, sera of patients colonized with *Candida* spp., with no suggestive clinical indications for systemic candidiasis, also yielded uniformly negative results. This observation suggests that patients with superficial or mucosal colonization (oral thrush and vaginitis, etc.) may not give rise to detectable levels of *Candida* DNA in the serum, thereby reducing the possibility of false positivity. However, 9 (56%) of the 16 blood culture-negative patients clinically suspected of having invasive candidiasis yielded positive snPCR results. In two of these patients, snPCR was positive for two *Candida* species (Table 5). Interestingly, all of the snPCR-positive patients had the respective *Candida* species isolated from one or more anatomic sites. In addition to supporting the diagnosis of candidiasis in the nine suspected cases, snPCR provided specific information about the incriminating *Candida* species. Considering the detection limit of 0.2 *Candida* genome and the possibility of detecting nonviable cells, the increased sensitivity of the snPCR compared to blood cultures in patients with suspected candidiasis is understandable. This is also consistent with several other studies where PCR has been found to be more sensitive than conventional culture methods in the diagnosis of *Candida* infections (5, 16, 35).

When applied to serum samples from 12 blood culture-proven cases of candidemia, the snPCR showed 100% concordant results and provided evidence of dual infections in 5 patients (41%). The therapeutic implications of this finding are quite apparent, since *Candida* species may have different antifungal susceptibility profiles. It also underscores the fact that the occurrence of candidemia due to more than one species may be more frequent than what is reported in the literature (1). In a study on the epidemiology of hematogenous candidiasis (1), 20 (4%) of the 491 episodes investigated had ≥ 2 species isolated in blood cultures. Besides *C. albicans*, 18 of these patients had coinfection with *C. parapsilosis*, *C. tropicalis*, and *C. glabrata*, while the remaining two had *C. tropicalis* isolated with *C. parapsilosis* or *C. glabrata*. Consistent with these observations, among the five PCR-positive candidemic patients identified in this study, three had concomitant infection with *C. albicans* and *C. tropicalis*, one had concomitant infection with *C. parapsilosis* and *C. glabrata*, and one had concomitant infection with *C. albicans* and *C. parapsilosis*. Since different *Candida* species have similar colonial morphological appearances on routine culture media, such as SGA, cases of candidemia caused by more than one species could be missed, especially if one of the coinfecting species yields only a few colonies. Probably, like polymicrobial (bacterial) septicemias, *Candida* infection involving more than one species may also have its origin from the gastrointestinal tract. Considering the various susceptibilities of *Candida* species to antifungal agents (more so with azoles), the problem of candidemia due to multiple species may be encountered with greater frequency in the future. A further point to note is that there appeared to be no correlation between *Candida* antigen titers and culture or snPCR positivity. Although our results are consistent with a previous study using a murine model of systemic candidiasis (16), the kit used for antigen detection in that study is known to have low sensitivity.

In conclusion, the snPCR that was established and evaluated in this study is a specific and sensitive method for the diagnosis of candidemia or hematogenous candidiasis caused by the four most commonly encountered *Candida* species, i.e., *C. albicans*, *C. parapsilosis*, *C. tropicalis*, and *C. glabrata*. Besides being rapid, the snPCR has the added advantage of identifying patients infected with more than one *Candida* species. This information can facilitate the selection of appropriate therapeutic agents.

ACKNOWLEDGMENTS

We are thankful to T. D. Chugh for his encouragement and to Rachel Chandy and Da'ad Farhat for excellent technical assistance.

This work was supported by Kuwait University grant no. MPI 118.

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