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Multiplex PCR Using Internal Transcribed Spacer 1 and 2 Regions for Rapid Detection and Identification of Yeast Strains

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Multiplex PCR amplification followed by either agarose gel electrophoresis (PCR-AGE) or microchip electrophoresis (PCR-ME) was used to test a total of 120 fungal strains. The internal transcribed spacer 1 (ITS1) and ITS2 regions and the 5.8S ribosomal DNA (rDNA) region of the fungi were amplified by using universal primers ITS1 and ITS4. The ITS2 region was simultaneously amplified by using universal primers ITS3 and ITS4. Since *Trichosporon asahi* and *T. asteroides* showed similar lengths for two amplicons, 29 different gel patterns were demonstrated for 30 yeast species tested on the basis of differences in the lengths of one or two amplicons. Of 75 yeast isolates from clinical materials, 5 isolates (6.8%) which were incompletely identified or not identified by the phenotypic method were identified with our PCR-based method (2 isolates as *Candida guilliermondii*, 2 as *C. krusei*, and 1 as *C. zeylanoides*). No differences in discriminating power or sensitivity were observed between the PCR-AGE method and the PCR-ME method. These methods, prospectively applied to 24 yeast-positive blood culture bottles (16 patients), resulted in the correct detection of 24 yeast strains. In conclusion, multiplex PCR followed by electrophoresis seems to be a promising tool for the rapid identification of common and uncommon yeast strains from culture colonies and from yeast-positive blood culture bottles (5.5 h for the PCR-AGE method and 3 h for the PCR-ME method).

The virulence of *Candida* isolates differs according to the species, with *Candida albicans* being most virulent, followed by *C. tropicalis* (23); thus, the prompt and accurate detection and identification of yeast species are very important. Detection and identification have been made even more important because of the emergence of candidemia caused by innately fluconazole-resistant non-*C. albicans Candida* species (12). Various commercial systems that can identify these pathogens within 4 to 72 h have been developed (2). Although correct identification of clinically relevant yeast strains can be achieved with these systems, incomplete or incorrect identification may occur when certain new and emerging yeast strains are tested (2). Moreover, culture-based phenotypic identification of *Candida* species from clinical materials requires at least 1 day to obtain a pure culture.

PCR-based methods for the rapid detection and identification of *Candida* species have also been described. These include restriction fragment length polymorphism analysis (3), PCR-restriction fragment length polymorphism analysis (7, 11), PCR with species-specific probes (14, 15), and random amplification of polymorphic DNA analysis (16). These methods have already shown great promise in the field of species identification and strain delineation. However, none of these methods has yet been used in the clinical laboratory.

PCR with fungus-specific primers, targeting the conserved sequences of 5.8S and 28S ribosomal DNAs (rDNAs) as well as those of 18S and 28S rDNAs, results in the respective amplification of the species-specific internal transcribed spacer (ITS) 2 (ITS2) region and of the ITS1 and ITS2 regions, which vary in amplicon length and sequence according to species (4, 6). Recently, Turenne et al. reported that differences in the sizes of the ITS2 regions of fungi were useful for the rapid identification of clinically important fungi (20). However, they found only a three-nucleotide band difference when comparing the lengths of *C. albicans* and *C. krusei* ITS2 amplicons. We used the variability in the lengths of both ITS1 and ITS2 regions to achieve the specific identification of fungal strains, and we determined the lengths of PCR fragments by using both agarose gel electrophoresis (AGE) and microchip electrophoresis (ME).

MATERIALS AND METHODS

Organisms. The 45 strains used in this study as control strains are listed in Table 1. These included 40 strains obtained from the Institute of Fermentation (IFO), Osaka, Japan; 4 strains obtained from the Japan Collection of Microorganisms (JCM), Institute of Physical and Chemical Research, Wako, Japan; and 1 strain (given the prefix K) which has been identified and maintained in our laboratory.

Seventy-five clinical isolates were obtained from patients at the Kanazawa University Hospital. Presumptive identification of these isolates was made by means of colony color on CHROMagar Candida medium, and the isolates were definitively identified with an API 20C kit (bioMerieux-Vitek, Hazelwood, Mo.). When necessary, their abilities to produce germ tubes and chlamydospores and to grow at 42°C were tested. These isolates comprised *C. albicans* (n = 23), *C. tropicalis* (n = 12), *C. parapsilosis* (n = 6), *C. guilliermondii* (n = 9), *C. glabrata* (n = 8), *C. krusei* (n = 6), *Cryptococcus neoformans* (n = 6), and incompletely identified or unidentified strains (n = 5).

DNA extraction. The Zymolyase-based method used to extract DNA has been described previously (3). In brief, yeast isolates were grown on CHROMagar Candida medium for 24 to 48 h at 35°C. Single colonies were inoculated into 1 ml of Tris-EDTA (TE) buffer at a McFarland standard of 0.5. *Aspergillus* strains were grown on Sabouraud dextrose agar for 3 days, and the conidia were inoculated into TE buffer. After centrifugation (5,000 × g, 3 min) in an Eppendorf microcentrifuge, the sediments were resuspended in 0.1 ml of 1 M sorbitol–1 M EDTA (pH 7.5) containing 5 μl of β-mercaptoethanol and 1 to 2 mg of Zymolyase; the mixture was incubated for 15 min at 37°C. For filamentous fungi, 1 to

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TABLE 1. Summary of sizes of PCR-amplified fragments by species

Organism	Strain(s)	Fragment size, in bp, found by PCR with the following primer pairs[a]:	
		ITS1-ITS4	ITS3-ITS4
Candida albicans	IFO 1398, IFO 1974, IFO 1061	529–535 (532)	335–342 (339)
Candida catenulata	IFO 0745	391–393 (392)	262–266 (264)
Candida famata	IFO 0664, IFO 856	632–634 (633)	369–370 (370)
Candida glabrata	IFO 0005, IFO 0622	869–879 (874)	412–418 (415)
Candida guilliermondii	IFO 10279, IFO 1972, IFO 0566	598–607 (603)	375–380 (378)
Candida holmii	IFO 1629	717–717 (717)	431–432 (432)
Candida inconspicua	IFO 0621	447–449 (448)	298–299 (299)
Candida intermedia	IFO 0761	384–388 (386)	250–256 (253)
Candida kefyr	IFO 0586, IFO 10287, IFO 1065	720–723 (722)	422–427 (425)
Candida krusei	IFO 0584	496–503 (500)	332–338 (335)
Candida lambica	IFO 10289	432–434 (434)	301–304 (303)
Candida lipolytica	IFO 1549, IFO 1548	358–359 (359)	233–239 (236)
Candida lusitaniae	IFO 10059, IFO 10058	373–378 (375)	245–254 (250)
Candida norvegensis	IFO 1020	480–483 (482)	314–315 (315)
Candida parapsilosis	IFO 1396	511–521 (516)	308–314 (311)
Candida rugosa	IFO 1364	416–419 (418)	268–269 (269)
Candida saitoana	IFO 0768	611–614 (613)	365–368 (367)
Candida tropicalis	IFO 1400, IFO 10241	517–524 (521)	326–331 (329)
Candida utilis	IFO 10707	553–555 (554)	354–357 (356)
Candida zoylanoides	IFO 10326	620–622 (621)	367–369 (368)
Cryptococcus neoformans	K615	547–555 (550)	370–375 (372)
Cryptococcus humicolus	IFO 10251	520–525 (522)	335–339 (337)
Dipodascus capitatus	IFO 1197	454–457 (456)	296–297 (297)
Pichia anomala	IFO 10213	614–615 (615)	373–376 (375)
Pichia chambardii	IFO 1274	629–630 (630)	380–382 (381)
Saccharomyces cerevisiae	IFO 0565	835–839 (837)	414–417 (416)
Stephanoascus ciferrii	IFO 1854, JCM 9551	571–572 (572)	354–356 (355)
Trichosporon asahii	JCM 2466	535–537 (536)	352–356 (354)
Trichosporon asteroides	JCM 10016	537–540 (539)	354–357 (356)
Trichosporon cutaneum	JCM 1462	521–523 (522)	348–349 (349)
Aspergillus flavus	IFO 5839	581–585 (583)	340–349 (345)
Aspergillus fumigatus	IFO 7839	580–588 (584)	337–347 (342)
Aspergillus niger	IFO 4414	586–590 (588)	342–350 (346)

[a] Range (mean) from three separate experiments.

2 mg of lysing enzymes (Sigma Chemical Co., St. Louis, Mo.) was added to the tube, and the mixture was incubated for a further 15 min at 37°C. Finally, 5 μl of proteinase K solution (20 mg/ml) was added to the tube, and the mixture was incubated at 55°C for 10 min and in a boiling water bath for 5 min.

Blood cultures. A total of 4 to 8 ml of blood drawn from each patient was inoculated into aerobic and anaerobic blood culture bottles (Organon Teknika Corp., Durham, N.C.). All bottles were incubated in a BACTEC 9240 automated system. When microbial growth was detected by the system, microscopic examinations using Gram staining and subcultures were performed. Yeast-positive bottles were immediately processed for DNA extraction, and a prospective identification of the blood isolate was made on the basis of results obtained with the commercial system and PCR analysis. Ten randomely selected samples from patients with bacteremia were also tested as negative controls.

Processing of blood samples for PCR. About 0.2 ml of blood culture fluid from each bottle was inoculated into a tube containing 1 ml of 0.17% sodium dodecyl sulfate, and this mixture was diluted with 8 to 10 ml of sterile distilled water. After centrifugation (3,000×*g*, 10 min), the pellets were placed in 1 ml of TE buffer and processed with the same method as that described above. Purified DNA was obtained with a MagExtract DNA extraction kit (Toyobo Co. Ltd., Osaka, Japan); about 1 h was required to disrupt and isolate DNA from positive blood culture bottles.

PCR procedure. Primers ITS1 (5′-TCCGTAGGTGAACCTGCG-3′), ITS3 (5′-GCATCGATGAAGAACGCAGC-3′), and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), targeting the conserved regions of 18S, 5.8S, and 28S rDNAs (22), respectively, were used for amplification. The ITS1-ITS4 primer pair was used to amplify the intervening 5.8S rDNA and the adjacent ITS1 and ITS2 regions, and the ITS3-ITS4 primer pair was used to amplify a large portion of the 5.8S rDNA and the adjacent ITS2 region (Fig. 1). PCR amplification was performed with a volume of 50 μl. Two microliters of each sample was added to the PCR master mixture, which consisted of 5 μl of 10× PCR buffer, 4 μl of a deoxynucleoside triphosphate mixture (0.1 mM each dNTP), 0.8 μl of each primer (40 pmol of each primer), and 0.4 μl (2.0 U) of Ex*Taq* DNA polymerase (Takara Biomedicals, Osaka, Japan), with the remaining volume consisting of distilled water. Amplification consisted of an initial denaturation at 94°C for 4 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min; and a final extension at 72°C for 4 min; a GeneAmp PCR system 9600 thermal cycler (Perkin-Elmer Corp., Emeryville, Calif.) was used. Negative control reactions without any template DNA were carried out simultaneously.

Electrophoresis. Gel electrophoresis with 1.5% agarose gels was conducted with 1× TBE buffer (0.1 M Tris, 0.09 M boric acid, 1 mM EDTA) at 4.8 V/cm for 2 h. A 100-bp DNA ladder (Promega Corp., Madison, Wis.) was run concurrently with amplicons for sizing of the bands. Gels were stained with ethidium bromide-TBE solution for 20 min and then photographed. The computer program Diversity Database (PDI Inc., New York, N.Y.) was used to calculate molecular sizes. Fragment sizes of control strains were measured three times for each strain on different gels. In addition to AGE, PCR products were analyzed with an ME instrument (model SV1100; Hitachi Electronics Engineering Co. Ltd., Tokyo, Japan). The microchip had three sample wells, and wells were

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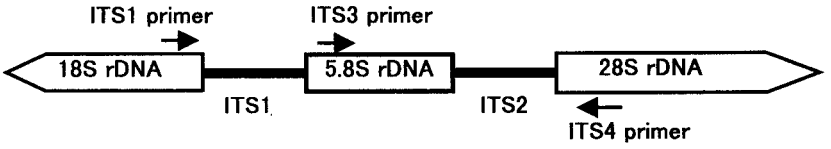

FIG. 1. Schematic representation of the fungal ribosomal genes containing the primer target areas used in this study.

connected with a cross- channel 0.1 mm wide, 0.03 mm deep, and 45 mm long. After the channel was filled with 0.6% hydroxypropyl methylcellulose containing ethidium bromide, a mixture of 9 μl of PCR products and 1 μl of loading buffer containing the internal standards (100 and 800 bp) was loaded into one of the sample wells of the microchip; the program was run at 300 V for 1 min (injection time) and at 750 V for 3 min (separation time). Accurate fragment size analysis based on the electrophoretic mobility of the sample relative to the internal standards was achieved by using DNA size analysis software.

RESULTS

To determine the sensitivity of the PCR followed by electrophoresis, 0.5-ml aliquots of TE buffer were seeded with 0, 10^1, 10^2, 10^3, 10^4, or 10^5 *C. albicans* blastoconidia. The sensitivity of PCR-AGE and PCR-ME was 10^3 cells per 0.5 ml of buffer, or 20 cells per 2 μl of sample.

For the isolation of yeast DNA from culture colonies, 40 min was required; in comparison, 1 h is required for DNA isolation from yeast-positive blood culture bottles. For the PCR-AGE method, 2 h was required for PCR amplification, 2 h was required for AGE (4 min for ME), and 30 min was required for staining and fragment analysis. Therefore, the times from yeast-positive blood culture bottles to genus or species identification by our PCR-based method were about 5.5 h for the PCR-AGE method and about 3 h for the PCR-ME method.

Amplification of all fungi tested with ITS1 and ITS4 primers yielded fragments 350 to 880 bp long, while ITS3 and ITS4 primers yielded fragments 233 to 432 bp long. The sizes of the PCR products amplified with the former were nearly equal for some species: *C. parapsilosis* and *C. tropicalis*, about 520 bp; *C. utilis* and *C. neoformans*, about 554 bp; and *C. saitoana* and *Pichia anomala*, about 614 bp. However, these organisms showed species-specific differences in the sizes of the PCR products amplified with ITS3 and ITS4 primers. In contrast, the sizes of the PCR products amplified with ITS3 and ITS4 primers were identical for *C. albicans*, *C. krusei*, and *C. humicolus* and for *C. famata*, *C. neoformans*, and *P. anomala*. The PCR products amplified with ITS3 and ITS4 primers were about 356 bp long for *C. utilis* and *Stephanoascus ciferrii*. However, these organisms showed species-specific differences in the sizes of the PCR products amplified with ITS1 and ITS4 primers. On the other hand, the lengths of two amplicons of *Trichosporon asahi* and *T. asteroides* were almost equal. Three *Aspergillus* species tested also demonstrated fragments of equal or nearly equal lengths. Therefore, 30 genus- or species-specific patterns were recognized among the 33 fungal species on the basis of differences in the lengths of one or two fragments of amplicons (Table 1). No intraspecies variability was detected.

Seventy-five isolates identified with routine phenotypic identification methods were correctly identified by the species-specific patterns shown in Table 1. The results of electrophoresis of PCR products from eight *Candida* species are shown in Fig. 2. ME analysis of 120 fungal strains showed that the lengths of two amplicon were in agreement with the results obtained with AGE analysis. The results obtained with the ME method for three *Candida* species are shown in Fig. 3. The standard deviations (SDs) of calculated molecular sizes ranged from 1.20 to 3.36 with the AGE method and from 1.97 to 4.61 with the ME method (Table 2). Fragments having molecular sizes with more than 2 SDs were considered different, a value of about <7 bp in the fragment size range of 300 to 500 bp. The documented resolution of the ME instrument provided by the manufacturer was specified as 10 bp. We concluded that the AGE and ME methods could delineate strains which showed differences of 10 bp or more in the mean lengths of either one or two PCR products.

Five strains could not be identified with the API 20C kit, and the code numbers of these strains were 6000004 (*T. capitatum*, *C. krusei*, and *C. lipolytica*) for K10320, 6556377 (*C. famata* and *C. guilliermondii*) for K7058, 6000104 (*C. lipolytica* and *C. krusei*) for K447, 6116333 (no code) for K10585, and 6402224 (no code) for K5701. The PCR-based method identified strains K10320 and K447 as *C. krusei*, strains K7058 and K10585 as *C. guilliermondii*, and strain K5701 as *C. zeylanoides*.

Twenty-four blood culture specimens obtained from 16 patients were positive for yeasts, as identified by primary Gram stain. These blood isolates were identified as *C. albicans* (12 patients, 14 specimens), *C. parapsilosis* (1 patient, 1 specimen), *C. guilliermondii* (1 patient, 4 specimens), *C. glabrata* (1 patient, 2 specimens), and *C. neoformans* (1 patient, 3 specimens) with the conventional phenotypic identification method. The results of this identification method were in agreement with the results of the PCR-based identification method.

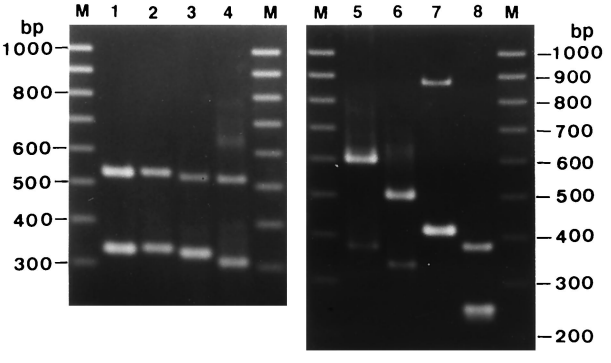

FIG. 2. Gels showing amplification by PCR and length variations in the ITS regions of medically important *Candida* isolates. Lane 1, *C. albicans*; lane 2, *C. dubliniensis*; lane 3, *C. tropicalis*; lane 4, *C. parapsilosis*; lane 5, *C. guilliermondii*; lane 6, *C. krusei*; lane 7, *C. glabrata*; lane 8, *C. lusitaniae*; lanes M, 100-bp DNA ladder.

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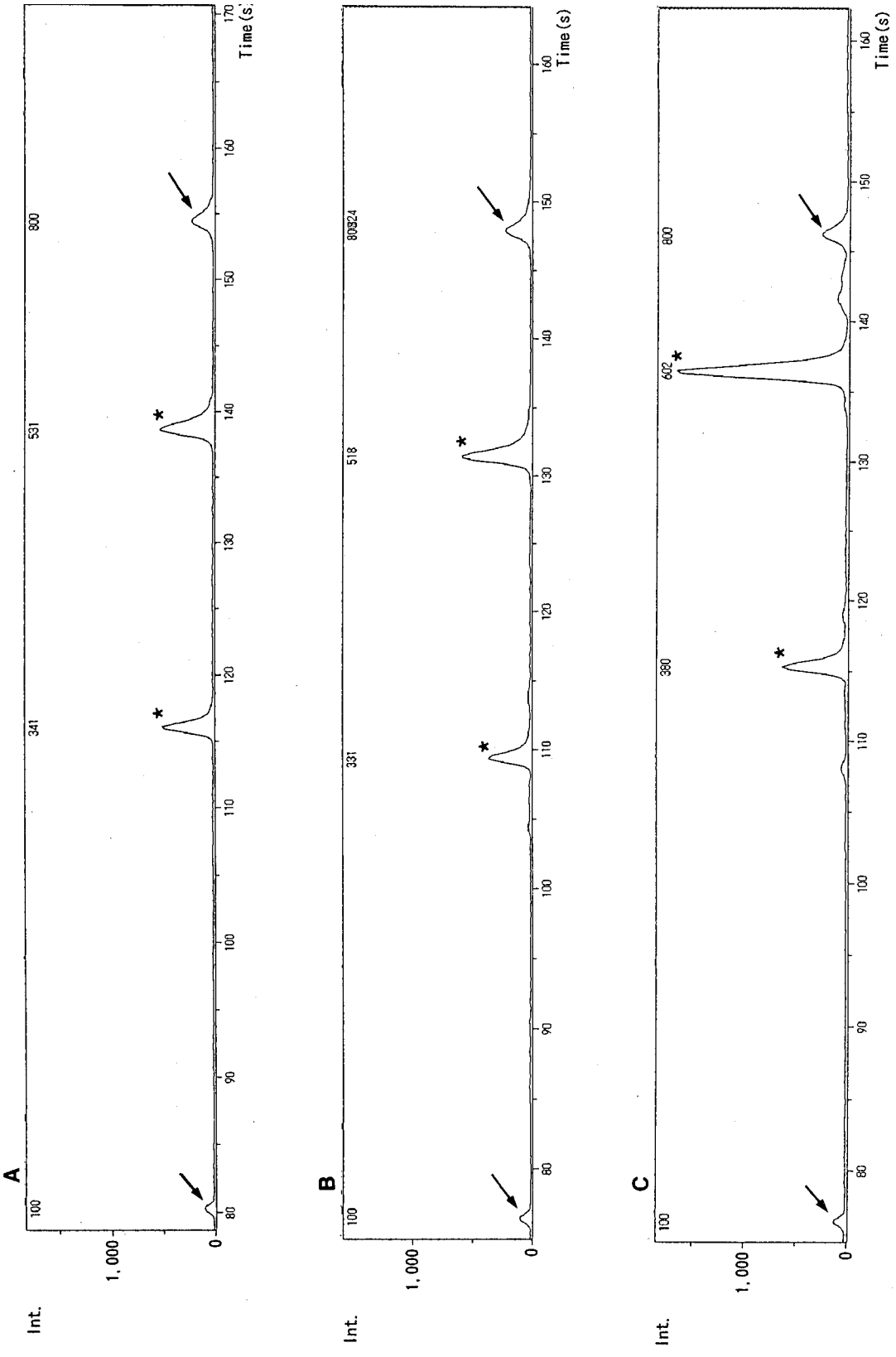

FIG. 3. ME patterns of *C. albicans* IFO 0745 (A), *C. tropicalis* IFO 1400 (B), and *C. guilliermondii* IFO 0566 (C). Arrows indicate DNA size markers of 100 bp (left) and 800 bp (right), and asterisks indicate PCR products (A, 341 and 531 bp; B, 329 and 518 bp; and C, 380 and 602 bp). Int., intensity.

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TABLE 2. PCR fragment lengths of medically important *Candida* species

Organism (no. of strains)	Base pair determination (mean ± SD) of PCR fragments by:	
	AGE	ME
C. albicans (36)	337 ± 2.77 533 ± 2.73	338 ± 3.04 531 ± 3.09
C. tropicalis (14)	327 ± 264 521 ± 2.33	328 ± 2.79 520 ± 3.09
C. parapsilosis (8)	309 ± 1.20 519 ± 3.31	309 ± 2.79 518 ± 3.21
C. guilliermondii (13)	376 ± 1.85 602 ± 2.64	377 ± 2.89 601 ± 3.02
C. krusei (7)	333 ± 2.96 499 ± 3.01	335 ± 3.02 499 ± 3.12
C. glabrata (11)	414 ± 2.54 874 ± 3.36	416 ± 1.97 873 ± 4.61

DISCUSSION

The prompt and accurate identification of yeasts is becoming increasingly important because new antifungal agents with different activities against various species are being developed and because *Candida* species differ in their virulence characteristics (5, 23). Although yeast identification kits such as the RapID yeast system, API 20C kit, and ID 32C allow for simple identification of yeast isolates, the ability to correctly identify isolates at the species level differs significantly among the commercial systems (21). For the API 20C kit, 5 to 10% incomplete identification (i.e., supplemental tests were required for correct identification) has been reported for *C. albicans*, *C. tropicalis*, and *C. glabrata* strains (21). In our study, five *Candida* strains incompletely identified or not identified with the API 20C kit were correctly identified with the PCR-based method described here. Thanos et al. (19) have reported that three *Candida* isolates incompletely identified with the ID 32C system were correctly identified by PCR fingerprints (one each of *C. albicans*, *C. guilliermondii*, and *C. parapsilosis*).

Various molecular approaches have been used for the detection of fungi from clinical samples. Targets for the detection of fungi at the genus or species level have included 18S rDNA (9), mitochondrial DNA (4), intergenic spacer regions (13), and ITS regions (7). The sizes of the 18S, 5.8S, and 26S rRNA genes are essentially identical in all species, while the lengths of the ITS regions depend on the species. The ITS region is located between the 18S and 26S rRNA genes and is subdivided into the ITS1 region, between the 18S and 5.8S rRNA genes, and the ITS2 region, between the 5.8S and 26S rRNA genes. Interspecies differences in fragment sizes or sequences of the ITS1-5.8S rDNA-ITS2 regions (6, 7), ITS1 region (10), and ITS2 region (8, 20) have been used to detect and identify fungi. We utilized the differences in the lengths of both ITS1 and ITS2 regions to achieve specific identification of pathogenic fungi from culture colonies.

In this study, most yeast strains were correctly identified at the species level by means of gel electrophoresis, but *C. albicans* and *C. dubliniensis* could not be differentiated because of their nearly identical amplicon sizes (data not shown). *C. dubliniensis* is a recently reported species of chlamydospore- and germ tube-positive yeast which has been isolated from the oral cavities of human immunodeficiency virus-infected individuals. Despite the many phenotypic similarities between *C. dubliniensis* and *C. albicans*, pronounced genetic differences between the two species have been determined by DNA fingerprinting, karyotype analysis, and DNA sequence analysis of rDNA (18). However, no significant differences in fragment sizes were observed with our PCR-based method. Therefore, we routinely plate yeast isolates on CHROMagar Candida medium, and colonies which have turned dark green after 48 h of incubation are tested for their ability to grow at 42°C on Sabouraud agar medium. Isolates which fail to grow or which grow poorly at 42°C are tentatively identified as *C. dubliniensis*. On the other hand, five incompletely identified or unidentified strains were identified as *C. guilliermondii* (two strains), *C. krusei* (two strains), and *C. zeylanoides* (one strain) based on the characteristic lengths of two amplicons. The multiplex PCR-based method described here and used by us may thus be effective for the preliminary identification of clinically uncommon or emerging yeast isolates.

Sequences of the ITS1 and ITS2 regions of *Trichosporon* species have been reported by Sugita et al. (17). They found that the ITS1 and ITS2 regions of *T. asahi* and *T. asteroides* are about 298 bp long and that those of *T. cutaneum* and *T. mucoides* are about 285 or 286 bp long. In addition, the lengths of the PCR products, including the complete ITS1-5.8S rDNA-ITS2 regions and portions of the 18S (30 bp) and 28S (59 bp) rRNA genes, were identified as 595 bp for *Aspergillus flavus*, 596 to 598 bp for *A. fumigatus*, 599 bp for *A. niger*, and 609 to 613 bp for *A. terreus* (6). These previously reported results, which are similar to ours, indicate that differences in the lengths of the ITS regions of *Trichosporon* spp. and *Aspergillus* spp. are of little value for species identification.

As for rapid sizing of PCR products, Turenne et al. (20) used a capillary electrophoresis system which needs less than 30 min for analysis. Recently, electrophoretic analysis with a microchip was developed (1). To determine the lengths of PCR products more rapidly, we used an ME instrument. This instrument can detect PCR products within 4 min, whereas AGE used in our study needed 2 h to determine the lengths of amplicons. The former has enabled us to generate a profile of the expected amplicon sizes for a broad range of yeasts, thus allowing for their rapid identification. The advantages of using such an instrument include speed and the elimination of slab gels and staining steps as well as of species-specific probes or DNA sequencing. Furthermore, minimal manual labor is required, thus making the fragment detection system accurate and cost-effective. To the best of our knowledge, this is the first study in which the ME analysis system was used in a clinical microbiology laboratory for determining the lengths of PCR products.

Because our PCR-based method with universal primers amplified all kinds of genomic DNA from fungal strains, its use may be limited to culture colonies on agar plates. The major limitation of our PCR-based method for the direct detection of fungi from clinical samples is mixed flora. Mixed candidemia, which was not observed during our study, has a reported fre-

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quency of 3.3% (14 of 427 patients) (12). In our preliminary study, electrophoresis of PCR products obtained from a mixture of *C. albicans* and *C. glabrata* cultures showed four fragments, indicating mixed flora. Therefore, when four or more fragments are detected with the PCR-AGE or PCR-ME method, the presence of mixed flora should be taken into consideration and the identification of fungi may be difficult.

In summary, we have developed a method for the comprehensive identification of clinically relevant fungal isolates which is sensitive, rapid, and specific for all yeast organisms tested. Using this PCR-ME analysis system, we are now processing yeast-positive blood culture bottles as well as identifying problematic isolates of yeasts in routine laboratory work.

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