Detection and Identification of Fungal Pathogens by PCR and by ITS2 and 5.8S Ribosomal DNA Typing in Ocular Infections

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The goal of this study was to determine whether sequence analysis of internal transcribed spacer/5.8S ribosomal DNA (rDNA) can be used to detect fungal pathogens in patients with ocular infections (endophthalmitis and keratitis). Internal transcribed spacer 1 (ITS1) and ITS2 and 5.8S rDNA were amplified by PCR and seminested PCR to detect fungal DNA. Fifty strains of 12 fungal species (yeasts and molds) were used to test the selected primers and conditions of the PCR. PCR and seminested PCR of this region were carried out to evaluate the sensitivity and specificity of the method. It proved possible to amplify the ITS2/5.8S region of all the fungal strains by this PCR method. All negative controls (human and bacterial DNA) were PCR negative. The sensitivity of the seminested PCR amplification reaction by DNA dilutions was 1 organism per PCR, and the sensitivity by cell dilutions was fewer than 10 organisms per PCR. Intraocular sampling or corneal scraping was undertaken for all patients with suspected infectious endophthalmitis or keratitis (nonherpetic), respectively, between November 1999 and February 2001. PCRs were subsequently performed with 11 ocular samples. The amplified DNA was sequenced, and aligned against sequences in GenBank at the National Institutes of Health. The results were PCR positive for fungal primers for three corneal scratchings, one aqueous sample, and one vitreous sample; one of them was negative by culture. Molecular fungal identification was successful in all cases. Bacterial detection by PCR was positive for three aqueous samples and one vitreous sample; one of these was negative by culture. Amplification of ITS2/5.8S rDNA and molecular typing shows potential as a rapid technique for identifying fungi in ocular samples.

The microbiological spectrum of infectious endophthalmitis shows that the percentage of isolates that are fungi is 8 to 18.5% (2, 7, 12, 22, 23) and in keratitis the rate is 16 to 35.9% (8, 42). Clinical diagnosis of these ocular infections is confirmed by obtaining intraocular (aqueous or vitreous) specimens or corneal scratchings. However, standard microbiological tests are positive in only 54 to 69% of endophthalmitis cases (13, 22, 23) (by culture) and 80% (8) of keratitis cases (by Gram and Giemsa stains and culture). In fungal infections, even when positive, results usually take longer than a week because these organisms are difficult to identify and/or are slow-growing. Early diagnosis and rapid intervention is a critical element for an effective treatment of ocular infections. This has led to the development of culture-independent diagnostic tests such as PCR. PCR-based detection methods with universal primers for bacterial DNA in ocular samples (5, 16, 20, 21, 26, 27, 34, 36, 40) have recently been developed. For detection of fungal pathogens, multicyclic targets have been evaluated for increasing the sensitivity (33, 39) and universal fungal PCR primers have been developed for broadening the range of detectable fungi (9, 14, 18, 31, 37). Studies on fungal DNA detection in ocular samples have been performed (3, 15, 17, 35); the small number of conidia in the samples, the difficulty of DNA extraction (25, 43) (some filamentous fungi have a sturdy cell wall which is resistant to standard DNA extraction procedures for yeast and bacteria), and the presence of PCR inhibitors in human specimens (45) are some of the difficulties with fungal detection in ocular samples. The ideal marker to detect a fungal infection should be present in all fungal genera (but should contain enough internal variation in its sequence to define a given species) and should be a multi-copy gene to maximize the sensitivity of the detection method. The rRNA genes are good candidates, since they are present in high copy number and the sensitivity of their detection may be dramatically increased by the use of nested PCR. The transcriptional unit is composed of 18S, 5.8S, and 28S rRNA genes. Between the 18S and 5.8S and between the 5.8S and 28S ribosomal DNA (rDNA) gene subunits are intergenic transcribed spacer regions (ITS1 and ITS2) that are not translated into rRNA. Although rRNA genes are highly conserved the ITS regions are divergent and distinctive (1, 6, 10, 29, 30, 41, 46). This report describes the application of molecular techniques (sequence analysis of PCR-amplified ITS2/5.8S rDNA) for fungal detection in two sets of samples: serial dilutions of different fungal strains and clinical samples obtained from patients with delayed postoperative endophthalmitis or keratitis. The aim of this technique is to reduce the time required for mycological diagnosis, increase the number of ocular samples from which a confirmed diagnosis is made, and identify the causative fungal agent.

MATERIALS AND METHODS

Standard fungal isolates. (i) Strains. Clinical and standard isolates of Aspergillus, Candida, Fusarium, Scedosporium, Alternaria, and Cryptococcus were used in this study (Table 1). Strains were cultured on Sabouraud dextrose broth (2%...
TABLE 1. Strains and source of ocular isolates analyzed by PCR amplification of rDNA

<table>
<thead>
<tr>
<th>Strain</th>
<th>Predicted size (bp)</th>
<th>Primer(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alternaria alternata IOA-K3</td>
<td>570</td>
<td>ITS1- ITS4</td>
</tr>
<tr>
<td>Aspergillus flavus CECT 2684</td>
<td>595</td>
<td>ITS4</td>
</tr>
<tr>
<td>Aspergillus fumigatus CECT 2071/IOA-K1</td>
<td>596</td>
<td>ITS4</td>
</tr>
<tr>
<td>Aspergillus niger CECT 2574/IOA-K2</td>
<td>599</td>
<td>ITS4</td>
</tr>
<tr>
<td>Aspergillus terreus CECT 2748</td>
<td>608</td>
<td>ITS4</td>
</tr>
<tr>
<td>Candida albicans CECT 1472</td>
<td>556</td>
<td>ITS4</td>
</tr>
<tr>
<td>Candida parapsilosis ATCC 22019/IOA-E1, IOA-E2</td>
<td>520</td>
<td>ITS4</td>
</tr>
<tr>
<td>Candida tropicalis CECT 1005</td>
<td>524</td>
<td>ITS4</td>
</tr>
<tr>
<td>Candida glabrata CECT 1448</td>
<td>820</td>
<td>ITS4</td>
</tr>
<tr>
<td>Candida krusei ATCC 6258</td>
<td>510</td>
<td>ITS4</td>
</tr>
<tr>
<td>Fusarium oxysporum CECT 2154</td>
<td>564</td>
<td>ITS4</td>
</tr>
<tr>
<td>Fusarium solani CECT 2199</td>
<td>569</td>
<td>ITS4</td>
</tr>
<tr>
<td>Cryptococcus neoformans var. neoformans SCNC SP1</td>
<td>556</td>
<td>ITS4</td>
</tr>
<tr>
<td>var. gattii SCNC C48</td>
<td>556</td>
<td>ITS4</td>
</tr>
<tr>
<td>Scedosporium apiospermum HMM-K1</td>
<td>611</td>
<td>ITS4</td>
</tr>
</tbody>
</table>

* IOA, ocular isolates obtained from the Instituto Oftalmológico de Alicante, Alicante, Spain; HMM, Ocular isolates obtained from Móstoles Hospital, Madrid, Spain; SCNC, Clinical isolates obtained from Spanish Cryptococcus neoformans Collection, Mycology Laboratory, Universidad Miguel Hernández, Alicante, Spain; CECT, Spanish Type Culture Collection, Valencia, Spain; ATCC, American Type Culture Collection, Rockville, Md.

[wt/vol] glucose, 1% [wt/vol] peptone supplemented with chloramphenicol (1 mg liter⁻¹), subcultured onto Sabouraud dextrose agar slants, and kept at 4°C.

(ii) Fungal DNA extraction. DNA extraction, preparation of the PCR mixture, and post-PCR analysis were carried out in separate rooms using equipment designated for each area to minimize the possibility of specimen contamination. The type strains (Table 1) were inoculated in 1.5-ml Eppendorf tubes containing 0.5 ml of Sabouraud dextrose broth supplemented with chloramphenicol and incubated overnight in an orbital shaker at 150 rpm and 30°C. Thereafter, fungal cultures were adjusted photometrically (absorbance at 530 nm; McFarland 0.5 standard) to a concentration of 1 × 10⁵ to 5 × 10⁶ cells/ml. In the case of filamentous fungi, conidia were separated from the rest of the mycelium by filtration through sterile glass wool (20). Tenfold serial dilutions of Candida albicans and Aspergillus fumigatus (10⁶ to 10⁸ cells) were prepared to test the sensitivity and specificity of the assay. The fungal suspensions with predetermined concentrations were centrifuged at 5,000 × g, and then the pellet was frozen at −20°C for 1 h and incubated at 65°C for 1 h in 0.5 ml of extraction buffer (50 mM Tris-HCl, 50 mM EDTA, 3% sodium dodecyl sulfate, 1% 2-mercaptoethanol). The lysate was extracted with phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol). Then, 65 μl of 3 M sodium acetate and 75 μl of 1 M NaCl were added to 350 μl of the supernatant and the resulting volume was incubated at 4°C for 30 min. DNA was recovered by isopropanol precipitation and washed with 70% (vol/vol) ethanol. The concentration was measured by monitoring the UV absorbance at 260 nm (Gene Quant System; Pharmacia, LKB Biocrom). Serial aqueous dilutions of DNA from C. albicans and A. fumigatus were prepared to different concentrations (10 ng to 10 μg) and stored at −20°C.

(iii) Negative controls. (a) Extraction of DNA from human leukocytes. Human DNA from whole blood was isolated by using the InstaGene Matrix (Bio-Rad Laboratories, Hercules, Calif.) as specified by the manufacturer.

(b) Extraction from bacteria. A variety of bacterial organisms capable of producing ocular infections were used to determine the specificity of the fungal primers: Staphylococcus epidermidis, Pseudomonas aeruginosa, Escherichia coli, and Streptococcus pneumoniae from the Spanish Type Culture Collection. Bacterial DNA was isolated by using the InstaGene Matrix.

(iv) PCR assay. Extracted DNA was amplified using a RoboCycler 96 temperature cycles (Stratagene, La Jolla, Calif.). The primers and PCR conditions used are specified below. PCR amplification was carried out in two steps.

(a) First-round amplification. The universal primers used for fungal amplification were ITS1 (5′-TCC GGT GGT GAA CCT GGG G 3′), which hybridizes at the end of 18S rDNA, and ITS4 (5′-TCC TCT TAT TAT TAT GC 3′), which hybridizes at the beginning of 28S rDNA (44) (Life Technologies, Barcelona, Spain). The 50-μl PCR mixture contained 10 μl of DNA template, 6 μl of 25 mM MgCl₂, 5 μl of PCR buffer without MgCl₂, 200 μM each deoxynucleoside triphosphate, 25 pmol of each primer, and 1 U of Taq DNA polymerase (Biotools B&H Labs, S.A., Madrid, Spain). Reactions involved 1 cycle at 95°C for 5 min, followed by 35 cycles with a denaturation step at 95°C for 30 s, an annealing step at 65°C for 1 min, and an extension step at 72°C for 1 min, followed by 1 cycle at 72°C for 6 min.

(b) Seminested amplification. For the second amplification, the primers were ITS46 (5′-GTT AAT CAT CAG GTA TTT TGA CAA C 3′), which hybridizes with the 5.8S rDNA region (29), and ITS4 (Life Technologies, Barcelona, Spain). Seminested PCR amplification mixtures contained 1 μl of first-round product in 50 μl of PCR reaction mixture (6 μl of 25 mM MgCl₂, 5 μl of PCR buffer without MgCl₂, 0.5 μl each of dNTP, 30 pmol of primer ITS4, and 100 pmol of primer ITS86, and 1 U of Taq DNA polymerase (Biotools B&H Labs). Reactions involved 1 cycle at 95°C for 5 min, followed by 30 cycles with a denaturation step at 95°C for 30 s, an annealing step at 55°C for 30 s, and an extension step at 72°C for 30 s, followed by 1 cycle at 72°C for 6 min.

(c) Negative controls. Two negative controls were included in the first amplification: a reagent control (sterile water) and a sample extraction control. The sample extraction control consisted of sterile MilliQ water subjected to the same extraction procedures as the specimens. In the seminested PCR, 1 μl each of the two negative control samples from the first-round amplification and a third negative control of sterile water were included.

(v) Detection of the amplified products. Aliquots (10 μl) of each amplified product were electrophoretically separated in a 1% agarose gel in 1× Tris-borate-EDTA buffer and visualized using ethidium bromide under UV illumination. Molecular weight ladders were included in each run (pBR322 DNA/BsuRI or Gene Ruler 100-bp DNA Ladder Plus [MBI Fermentas, Vilnius, Lithuania]).

Ocular samples. (i) Patient selection. Intraocular sampling or corneal scrapings were undertaken for all patients with suspected infectious endophthalmitis or keratitis (nonherpetic), respectively, between November 1999 and February 2001. Before sampling, informed consent was obtained from all patients. The protocol for collection of aqueous samples, vitreous samples, and corneal scrapings was approved by the Institutional Review Board at the Instituto Oftalmológico de Alicante, Alicante, Spain. This research followed the tenets of the Declaration of Helsinki at all times.

(ii) Sample collection and culture. (a) Procedure for endophthalmitis cases. The extracocular environment was sterilized with 5% povidone iodine solution before surgery. Approximately 100 to 200 μl of aqueous fluid was withdrawn using a 30-gauge needle with a limbal paracentesis. Vitreous samples (200 μl) were taken at the time of three-port pars plana vitrectomy. The samples were divided into two aliquots and transported to the microbiology laboratory and to the molecular biology laboratory at 4°C. One portion was immediately examined by conventional microbiological diagnostic tests, and the other was frozen at −20°C until processed by PCR.

For the microbiological diagnostic test, 50 μl of aqueous humor or 50 μl of vitreous was cultured at 30°C in Sabouraud’s dextrose agar or at 37°C in thioglycolate broth, blood agar, chocolate agar, CLED agar, or MacConkey agar. Bacteria were identified by the API Staph and API 20A systems (Biomeriux, bioMérieux Sa, Marcy L’Etoile, France). Yeasts were identified by the Auxacolor system (Sanofi Diagnostics Pasteur, Inc, Marnes-la-Coquette, France), and filamentous fungi were differentiated by isolation in Sabouraud dextrose agar plus chloramphenicol and morphological examination of a macroscopic and microscopic characteristics.

(b) Procedure for keratitis cases. Upon completion of the ocular examination and after instillation of topical anesthetic, a sterile Kimura spatula was used to scrape the area of infection. Scrapings were inoculated into thioglycolate broth, Roiron broth, and Löwenstein-Jensen medium and were placed onto glass slides for staining with Gram and Giemsa stains. The PCR sample was obtained by scraping and stirring the spatula for a few seconds in 100 μl of sterile water in a 1.5-ml sterile Eppendorf tube. Two aliquots of 50 μl were taken from each sample and stored at −20°C.

(iii) Fungal DNA extraction. A 50-μl volume of each ocular sample was frozen for at least 1 h at −20°C. The DNA extraction was performed as described for the standard fungi isolates. DNA was diluted in 10 μl of sterile water.

(iv) PCR assay. The PCR for fungal DNA detection was performed as described for the standard fungal isolates. The bacterial PCR and specific Pseudomonas aeruginosa PCR amplification with ocular samples were performed as described by Hykin et al. (16).
DNA was extracted as described above, and PCR was carried out.

Database.

provides a list of matching organisms, ranked in order of similarity between the GenBank database (National Institutes of Health). The computer alignment

ment of the selected sequences, fragment sizes were manually calculated. ITS2/5.8S rDNA sequences were analyzed by using the BLAST alignment program of

primers hybridization using this program. PCGENE facilitates the positive or

which the rDNA sequence is available in GenBank were assayed for selected
members of the method, including a large number of fungi. Ocular pathogenic fungi for

Biosystems Warrington, United Kingdom). The primers used were ITS4 and ITS86.

an ABI Prism automated DNA sequencer (model 377, version 2.1.1; Applied

manufacturer and directly cycle sequenced in both directions using the BigDye terminators Ready Reaction Kit (PE Applied Biosystems, Foster City, Calif.) on

DNA Ladder Plus (500 bp, black triangle); C−, negative control (ddH2O). (B) Sensitivity of the seminested PCR (ITS4-ITS86 primer pair) performed with 1 μl of the first-round product of C. albicans PCR. M, ladder marker pBR322 DNA/BsuRI (434 bp, white triangle; 267 bp, black triangle); C−, negative control (ddH2O). (C) Sensitivity of the first PCR (ITS1-ITS4 primer pair) with A. fumigatus genomic DNA. M, ladder marker GeneRuler 100bp DNA Ladder Plus (500 bp, black triangle); C−, negative control (ddH2O). (D) Sensitivity of the seminested PCR (ITS4-ITS86 primer pair) performed with 1 μl of the first-round product of A. fumigatus PCR. M, ladder marker pBR322 DNA/BsuRI (434 bp, white triangle; 267 bp, black triangle); C−, negative control (ddH2O).

FIG. 2. (A) Sensitivity of the first PCR (ITS1-ITS4 primer pair) with C. albicans genomic DNA. M, ladder marker GeneRuler 100bp DNA Ladder Plus (500 bp, black triangle); C−, negative control (ddH2O). (B) Sensitivity of the seminested PCR (ITS4-ITS86 primer pair) performed with 1 μl of the first-round product of C. albicans PCR. M, ladder marker pBR322 DNA/BsuRI (434 bp, white triangle; 267 bp, black triangle); C−, negative control (ddH2O). (C) Sensitivity of the first PCR (ITS1-ITS4 primer pair) with A. fumigatus genomic DNA. M, ladder marker GeneRuler 100bp DNA Ladder Plus (500 bp, black triangle); C−, negative control (ddH2O). (D) Sensitivity of the seminested PCR (ITS4-ITS86 primer pair) performed with 1 μl of the first-round product of A. fumigatus PCR. M, ladder marker pBR322 DNA/BsuRI (434 bp, white triangle; 267 bp, black triangle); C−, negative control (ddH2O).

spiked with 1 μl of C. albicans culture (10 cells) as an internal positive control.

DNA was extracted as described above, and PCR was carried out.

(v) DNA sequencing of PCR products. Amplified DNA from PCR was purified using the GeneClean II kit (Bio 101, Inc., Carlsbad, Calif.) as specified by the manufacturer and directly cycle sequenced in both directions using the BigDye terminators Ready Reaction Kit (PE Applied Biosystems, Foster City, Calif.) on an ABI Prism automated DNA sequencer (model 377, version 2.1.1; Applied Biosystems Warrington, United Kingdom). The primers used were ITS4 and ITS86.

(vi) Data analysis. The PCGENE program was used to ascertain the specificity of the method, including a large number of fungi. Ocular pathogenic fungi for which the rDNA sequence is available in GenBank were assayed for selected primers hybridization using this program. PCGENE facilitates the positive or negative theoretical union of primers to the sequence target. After clustal alignment of the selected sequences, fragment sizes were manually calculated. ITS2/5.8S rDNA sequences were analyzed by using the BLAST alignment program of the GenBank database (National Institutes of Health). The computer alignment provides a list of matching organisms, ranked in order of similarity between the unknown sequence and the sequence of the corresponding organism from the database.

RESULTS

Standard fungal isolates. (i) PCR specificity. The primers used in this study (ITS1 and ITS4 for the first round amplification and ITS86 and ITS4 for the second round) successfully amplified DNA from all the standard fungal strains tested. After the first round of amplification, a product of approximately 550 bp was obtained. After the second round of amplification, the fragment obtained was about 280 bp (Fig. 1).

No amplification products were detected by using the ITS1-

ITS4 and ITS86-ITS4 primer pairs with genomic DNA isolated from human leukocytes or from any of the following bacteria: S. epidermidis, E. coli, S. aureus, P. aeruginosa, and S. pneumoniae (data not shown).

Other fungi reported in the reference list as ocular pathogens were tested with the PCGENE program to ascertain the specificity of this method (Table 1). The sizes of the fragments obtained were in agreement with those obtained by PCR.

(ii) PCR sensitivity. The sensitivity was estimated using two kinds of samples: DNA dilutions (DNA was extracted from a culture and subsequently diluted) and culture dilutions (serial dilutions of cells were prepared and DNA extracted from each culture).

For C. albicans DNA dilutions, the sensitivity of the first PCR was found routinely (more than three times) to be 1 to 10 fg (Fig. 2A). The seminested PCR, performed with 1 μl from the first PCR, was positive in all the DNA dilutions (Fig. 2B). The sensitivity for the mold A. fumigatus was found to be 10 to 100 fg (Fig. 2C). The second round of PCR markedly improved this sensitivity to 1 fg, similar to the results obtained with C. albicans (Fig. 2D).

Using cell dilutions, the sensitivity of the PCR was routinely found to be 1 to 10 organisms (Fig. 3A and B). However, for A. fumigatus the sensitivity was lower; the first PCR was positive only when carried out with samples containing 10 to 10^2 organisms, but with semnested PCR the sensitivity improved to less than 10 organisms (Fig. 3C and D).
(iii) Detection of PCR inhibitors in ocular samples. The expected amplification products were obtained indicating that no PCR inhibitors were present in the clinical samples (aqueous humor and vitreous) after DNA extraction.

Ocular samples. Six cases of endophthalmitis and three cases of keratitis were analyzed by molecular and culture methods. In the six cases of endophthalmitis, six aqueous samples and two vitreous samples were taken. Table 2 shows the results of Gram’s stain, culture and PCR of all ocular samples. Samples from patients 2, 5, and 8 were PCR negative with fungal primers and positive with bacterial primers. The sample from patient 2 was culture positive for coagulase-negative staphylococci; the patient was successfully treated and responded well to antibiotic therapy. The sample from patient 5 was PCR positive with bacterial and \textit{P. acnes} primers and culture positive for \textit{P. acnes}. The patient underwent anterior vitrectomy with intravitreal injection of antibiotics. Clinical and visual improvement was rapid. Patient 8 is still under antibiotic treatment. The sample from patient 4 was negative for both PCR and culture analysis. The patient is under clinical observation, and the case is being reviewed every 3 months. The samples from patients 3 and 7 were bacterial PCR negative and fungal PCR positive (Fig. 4). The sequence analysis and the culture showed a \textit{C. parapsilopsis} infection. Patient 3 successfully finished the antifungal treatment (fluconazole), and patient 7 is still under antifungal treatment with fluconazole and amphotericin B. Patient 10 had keratitis and was being treated at Móstoles Hospital (Madrid). Microscopic visualization and conventional culture were positive for fungi, and \textit{Scedosporium apiospermum} was identified (E. Amor, personal communication). The DNA was extracted from culture, and its ITS/5.8S rDNA sequence confirmed the identification as \textit{S. apiospermum}. Although DNA extraction from corneal samples was not done, this case was also considered interesting for the assessment of the technique.

Microscopic fungal visualization (Gram stains) was negative for three of the six ocular samples (Table 2). Only the corneal sample from patient 6 and the aqueous sample from patient 8 were negative by culture and positive by PCR. The other samples showed the same results by culture and by PCR. However, cultures needed an average of 6 to 7 days to grow, while the PCR results were obtained in 6 to 8 h.
DNA sequencing. DNA database comparison of the DNA sequences obtained with the full-sequence ITS2 and partial-sequence 5.8S rDNA from the ocular samples demonstrated that they were derived from the fungal ITS regions. Two of them were identical to the *C. parapsilosis* ITS/5.8S rDNA region, and one each were identical to the *A. niger*, *A. fumigatus*, *Alternaria alternata*, and *Scedosporium apiospermum* ITS2/5.8S rDNA region (Table 2).

### DISCUSSION

In this report, an optimized rapid technique for the detection and identification of fungi in ocular samples is presented. It consists of an inexpensive and rapid method of DNA extraction and a sensitive and precise method of identification of fungal pathogens based on amplification of ITS2/5.8S rDNA and molecular typing.

A good DNA extraction method is critical for PCR detection to avoid the possibilities of false-negative results. We tested some commercial kits (Instagene; Bio-Rad Laboratories, Hercules, Calif., and Mo-Bio Laboratories, Inc., Solana Beach, Calif.) for rapid extraction of DNA, but the result was not satisfactory for all the tested fungal strains (*A. niger* or *C. neoformans*). Similar results with the use of other commercial kits, except for a QIAmp Tissue kit, for fungal DNA extraction have been previously described (25). This protocol applied to vitreous ocular samples provides high-quality DNA that is devoid of PCR-inhibiting substances. The DNA loss is minimal because we have been able to amplify DNA from 1 to 10 microorganisms (*C. albicans*) and from 10 to 100 microorganisms (*A. fumigatus*). Additionally, this procedure is rapid, technically simple, broadly applicable, and inexpensive.

rRNA genes are highly conserved in all fungal species tested to date. The use of rRNA genes for identification of fungal species is based on the detection of conserved sequences in the rDNA genes. Our results showed high fungal specificity with the selected primers. All fungal strains were PCR positive, and all negative controls (human and bacterial DNA) were PCR negative. This same protocol of extraction and amplification of DNA from ocular samples was performed in an animal fungal infection model (11). In this work, the fungal infection was induced in one eye of each of five rabbits (New Zealand) with *C. albicans*, *C. parapsilosis*, *A. niger*, *A. fumigatus*, and *F. oxyosporum*, The DNA sequence target was detected in all infection samples studied.

The primers used for PCR amplification were checked and found to be specific for fungal rDNA; they did not target prokaryotic rDNA sequences. Moreover, they targeted all the rDNA sequences from ocular pathogenic fungi available in database.

As discussed above, the sensitivity for the first amplification of the ITS/5.8S rDNA region was 1 fg for *C. albicans* and 10 fg for *A. fumigatus* in water dilutions of DNA. Assuming a total DNA content of 37 fg per organism (38), this amount is equivalent to less than one *C. albicans* cell. This is in agreement with the fact that rRNA genes are multiple-copy genes, with 100 or more copies within the fungal genome (32), making them ideal targets for PCR amplification and permitting the amplification from a very small number of microorganisms.

When culture dilutions were used as targets for amplification, 1 to 10 organisms of *C. albicans* and fewer than 100 organisms of *A. fumigatus* could be detected. As shown in Fig. 2 and 3, the intensity of the band corresponding to the PCR carried out with 10 to 100 fg of genomic *C. albicans* DNA is similar to the intensity of the band corresponding to the PCR with 1 to 10 microorganisms (the total DNA content is of 37 fg per organism) (38). For *A. fumigatus*, the intensity of the band corresponding to the PCR performed with 100 fg of DNA was similar to that obtained with 1 to 10 microorganisms (total DNA of this mold could be estimated at approximately 35 Mb [~100 fg]) (24). This means that there was no significant DNA loss during the DNA extraction. Nevertheless, to make sure, a large number of experiments would be necessary because the range given for the DNA amount and the range given for cell numbers in these preparations were not exactly the same.

Infectious agents were detected by PCR in eight of nine clinical ocular infections; five of them were positive by amplification with fungal primers, and three were positive by amplification with bacterial primers. In seven of the cases, the pathogen could also be retrieved by cultivation (two bacteria and five fungi). However, while the PCR result was obtained in a few hours, cultures needed 5 to 6 days to grow and 2 to 3 days for identification. When PCR was followed by sequencing of the PCR product, the total identification time was 24 h, still significantly shorter than that needed for cultured-based identification.

**TABLE 2.** Gram stain, culture, and PCR results with samples from 10 patients with a clinical diagnosis of endophthalmitis or keratitis

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sample</th>
<th>Gram stain result</th>
<th>Culture result</th>
<th>Bacterial PCR/P. acnes PCR result</th>
<th>Fungal PCR result/homology sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Corneal scrape</td>
<td>Positive</td>
<td>Positive</td>
<td>ND</td>
<td>Positive/A. fumigatus</td>
</tr>
<tr>
<td>2</td>
<td>Aqueous</td>
<td>ND*</td>
<td>Positive (3 days)</td>
<td>Positive/negative</td>
<td>Negative</td>
</tr>
<tr>
<td>3</td>
<td>Aqueous</td>
<td>ND*</td>
<td>Positive (6 days)</td>
<td>Negative</td>
<td>Positive/C. parapsilosis</td>
</tr>
<tr>
<td>4</td>
<td>Aqueous</td>
<td>ND*</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>5</td>
<td>Aqueous</td>
<td>ND*</td>
<td>Positive (13 days)</td>
<td>Positive/positive</td>
<td>Negative</td>
</tr>
<tr>
<td>6</td>
<td>Vitreous</td>
<td>Positive</td>
<td>Positive (7 days)</td>
<td>Positive/positive</td>
<td>Negative</td>
</tr>
<tr>
<td>7</td>
<td>Vitreous</td>
<td>ND*</td>
<td>Negative</td>
<td>Negative</td>
<td>Positive/C. parapsilosis</td>
</tr>
<tr>
<td>8</td>
<td>Aqueous</td>
<td>ND*</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>9</td>
<td>Aqueous</td>
<td>ND*</td>
<td>Positive (7 days)</td>
<td>Positive/negative</td>
<td>Negative/A. niger</td>
</tr>
<tr>
<td>10</td>
<td>Culture of corneal scrape</td>
<td>Negative</td>
<td>Positive (7 days)</td>
<td>ND</td>
<td>Positive/S. apiospermum</td>
</tr>
</tbody>
</table>

* a, ND, not done.  
* b, insufficient sample.  
* c, Where just one result is shown, it is the result of the bacterial PCR test (the second test was unnecessary).
The advantage of rapidly ascertaining the fungal or bacterial origin of the infection is complemented by the rapid identification of the fungus itself. For example, patient 1 had received intravitreal amphotericin B as the first treatment. The identification of *C. parapsilosis* as responsible for the infection permitted the treatment to be changed from amphotericin B to fluconazole (4, 19).

Analysis of sequences (5.8S/ITS region) from the database confirmed that this method can be used to differentiate fungi at the species level. Some studies show that fungal strains can be distinguished on the basis of the size of the ITS/5.8S fragment (6, 41) and primary structural differences in the rDNA spacer regions (10, 46). However, although yeast demonstrated a higher level of interspecies variability compared to other fungi, size determination based on agarose gel electrophoresis is not precise enough to unmistakably confirm the species identification. Table 1 shows that fragment sizes are very similar and therefore very difficult to differentiate in an agarose gel. If the size is determined by capillary electrophoresis (41), the time required is similar to that needed in sequencing and significantly less information is obtained. Other molecular techniques proposed for fungal identification, such as the use of restriction fragment length polymorphism analysis of ITS/5.8S fragments, hybridization with a specific probe, and the specific nested PCR (10, 35, 46), could be useful to confirm a specific fungal infection, for example in endophthalmitis (frequently produced by *Candida, Aspergillus* and *Fusarium*). However, the range of fungi capable of causing keratitis is significantly wider than that of fungi capable of causing endophthalmitis. Therefore, a large number of species causing infection could remain unidentified by these molecular methods. Specifically, in the course of this study, the identification for patients 9 and 10 (*Alternaria* and *Scedosporium*) would be rather complicated and time-consuming because of the need to consider the possible involvement of these genera as pathogens. In contrast, the amplification and typing of the ITS region eliminates this requirement. In addition, the small size of the fragment permits its sequencing in both directions at once, and the obtained sequence gives enough information to identify the fungal species.

This method proved to be reproducible and very useful for easy and rapid identification and classification of all the species included in the present work. This is the first time that these rDNA-specific primers have been successfully used for fungal detection and identification in ocular samples. This PCR-based method promises to be very effective for the diagnosis of fungal ocular infections in the clinical setting. Compared with standard laboratory techniques, it offers a significant reduction of the time required to establish the diagnosis. However, further studies with a larger number of clinical samples are necessary to assess the efficacy of the method.

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**REFERENCES**


