

# Detection and Identification of *Candida* Species in Experimentally Infected Tissue and Human Blood by rRNA-Specific Fluorescent In Situ Hybridization

AXEL LISCHEWSKI,<sup>1</sup> MARIANNE KRETSCHMAR,<sup>2</sup> HERBERT HOF,<sup>2</sup> RUDOLF AMANN,<sup>3</sup>  
JÖRG HACKER,<sup>1</sup> AND JOACHIM MORSCHHÄUSER<sup>1\*</sup>

Zentrum für Infektionsforschung, Universität Würzburg, D-97070 Würzburg,<sup>1</sup> Institut für Medizinische Mikrobiologie und Hygiene, Klinikum der Stadt Mannheim, D-68135 Mannheim,<sup>2</sup> and Max-Planck-Institut für Marine Mikrobiologie, D-28359 Bremen,<sup>3</sup> Germany

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**Two 18S rRNA-targeted oligonucleotide probes specific for *Candida albicans* and *Candida parapsilosis* were used to detect and identify by fluorescent in situ hybridization these medically important *Candida* species in deep organs of mice after experimental systemic infection. The *C. albicans*-specific probe detected fungal cells in kidney, spleen, and brain sections of a mouse infected with *C. albicans* but not in a mouse infected with the closely related species *C. parapsilosis*. Conversely, the *C. parapsilosis*-specific probe detected fungal cells in the deep organs of a mouse infected with *C. parapsilosis* but not in the deep organs of a *C. albicans*-infected mouse. In addition, the *C. albicans*-specific probe was used to detect this species in human blood spiked with yeast cells by a lysis-filtration assay and subsequent fluorescent in situ hybridization. By this assay, as few as three yeast cells per 0.5 ml of blood were consistently detected. Our results demonstrate that fluorescent in situ hybridization with species-specific rRNA-targeted oligonucleotide probes provides a novel, culture-independent method for the sensitive detection and identification of *Candida* species in clinically relevant material.**

The opportunistic fungal pathogen *Candida albicans* and other species of the genus *Candida* are able to cause superficial and often fatal disseminated infections in immunocompromised patients. A clear-cut diagnosis is often difficult to obtain, and deep-seated candidiasis is in most cases only recognized postmortem. Disseminated candidiasis is usually diagnosed by blood culture, although blood cultures from patients with histologically proven disseminated candidiasis are often negative. In addition, even with an optimal blood culture system such as the lysis-centrifugation method, it takes a minimum of 1 to 2 days for detection and even longer for species identification (8). To reduce mortality in patients suffering from invasive candidiasis, early initiation of proper antifungal drug therapy is critical (2, 4). Because *Candida* species differ in their susceptibilities to widely used systemic antifungal agents, such as azoles (20), identification of the infecting species is important for ensuring effective therapeutic strategies. Direct histologic detection of the fungus in biopsy material from deep tissue offers the relatively best diagnostic assurance (11). The infecting species, however, cannot be identified by microscopic examination alone. Among other nonculture methods, like the less reliable detection of antigens or specific antibodies (19), PCR-based approaches for the detection of *Candida* infections have also been developed. Identification of the *Candida* species, however, relies on the application of one of a number of techniques which in most cases require additional steps after PCR (6, 9, 14–16, 21, 24, 26). The diagnostic value of these methods, however, remains to be established (19).

Fluorescent in situ hybridization of whole cells with rRNA-targeted oligonucleotide probes has increasingly become a valuable tool for the specific detection of microorganisms with-

out cultivation (1, 3). Using a probe that binds to a target region in the 18S rRNA of *C. albicans* and *Candida tropicalis*, we have previously demonstrated that this method is applicable for the detection of different *Candida* species, irrespective of the morphological growth form (12). Under suitable hybridization conditions, the target organisms *C. albicans* and *C. tropicalis* were discriminated from all other clinically relevant *Candida* species, even the closely related species *Candida parapsilosis*, which contains a similar probe target region with only two nucleotide mismatches. However, to develop this technique for a diagnostic application, it was necessary to demonstrate its utility with clinically relevant material. Therefore, this study was undertaken to evaluate the sensitivity and specificity of fluorescent in situ hybridization for the detection and identification of *Candida* species in infected tissue and blood.

## MATERIALS AND METHODS

**Strains and culture conditions.** The reference strains *C. albicans* ATCC 44808 and *C. parapsilosis* DSM 70126 were used throughout this study. Additional strains from different *Candida* species which were used for RNA dot blot hybridization experiments have been described elsewhere (12). Strains were maintained on YPD agar plates (1% [wt/vol] yeast extract, 2% [wt/vol] peptone, 2% [wt/vol] glucose, 1.5% [wt/vol] agar). The strains were grown in YPD liquid medium at 30°C.

**Oligonucleotide probes.** The oligonucleotides used in this study were synthesized by MWG Biotech, Ebersberg, Germany. Oligonucleotide O20 (5'-CCCCCTTTCCTAAACCAATCCGGA-3') is complementary to a sequence in the 18S rRNA of *C. albicans* and *C. tropicalis* and has been described previously (12). Oligonucleotide Cpara (5'-CCCCCTTTCCTAAACCAATCCGGA-3') contains two nucleotides (highlighted in boldface letters) different from those highlighted in oligonucleotide O20, resulting in complementarity to the corresponding 18S rRNA region of *C. parapsilosis*. For RNA dot blot hybridizations the oligonucleotide probes were labeled with digoxigenin by using 3' oligonucleotide labeling and nucleic acid labeling kits according to the instructions of the manufacturer (Boehringer Mannheim, Mannheim, Germany). Fluorescein-labeled oligonucleotides for in situ hybridization were purchased from MWG Biotech.

**RNA dot blot hybridization.** RNA extraction and dot blot hybridizations were performed as described previously (12) by using a hybridization temperature of 43°C in a buffer containing 45% (vol/vol) formamide, which resulted in the specific binding of the oligonucleotide probes to their respective target regions in the 18S rRNA.

\* Corresponding author. Mailing address: Zentrum für Infektionsforschung, Universität Würzburg, Röntgenring 11, D-97070 Würzburg, Germany. Phone: 49-931-31 26 47. Fax: +49-931-31 25 78. E-mail: joachim.morschhaeuser@mail.uni-wuerzburg.de.

**Fluorescent in situ hybridization.** *Candida* yeast cultures were grown overnight in YPD medium at 30°C, harvested, and washed with phosphate-buffered saline (1× PBS; pH 7.2). A mixture of *C. albicans* and *C. parapsilosis* was prepared by mixing equal volumes of the yeast suspensions. Cells from pure cultures or the mixture of both species were immobilized on glass slides, washed with 1× PBS (pH 7.2), fixed for 20 min by adding 100% (vol/vol) methanol, air dried, and dehydrated by subsequent immersion in 50, 80 and 96% (vol/vol) ethanol for 3 min at each concentration. For in situ hybridization, 8 μl of hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl [pH 7.5], 0.01% [wt/vol] sodium dodecyl sulfate [SDS], 45% [vol/vol] formamide) containing 50 ng of the fluorescein-labeled probes O20 or Cpara was applied per hybridization. The microscope slides were placed in a moisture chamber (25) to ensure comparable vapor pressure, and hybridization was performed at 43°C for 2 h. The slides were gently rinsed with water and immersed in a prewarmed washing solution (40 mM NaCl, 20 mM Tris-HCl [pH 7.5], 0.01% [wt/vol] SDS, 5 mM EDTA) at 45°C for 20 min. The slides were rinsed with water, dried in a light-tight box, and mounted with Citifluor. Fluorescence was detected with a Zeiss Axiolab microscope (Carl Zeiss, Jena, Germany), equipped for epifluorescence microscopy with a 50-W mercury high-pressure bulb and Zeiss fluorescein-specific filter set 09. Photographs were taken on slide film without any subsequent digital image processing.

**In vivo experiments.** Overnight cultures of *C. albicans* ATCC 44808 and *C. parapsilosis* DSM 70126 were washed and resuspended in 1× PBS, and the cells were counted with a Neubauer hemocytometer. Two pairs of 6-month-old female BALB/c mice were infected with either  $1.28 \times 10^6$  cells of *C. albicans* or  $1.36 \times 10^6$  cells of *C. parapsilosis* by injection into the lateral tail vein. Animals were sacrificed 24 h after infection. The kidneys, spleen, and brain of one of each pair of mice were homogenized, and the fungal loads in the organs were determined by counting the numbers of CFU after plating serial dilutions. The organs of the second animal were perfused intracardially with 0.9 M NaCl, frozen in 2-methylbutane (Sigma-Aldrich, Steinheim, Germany), and, after the organs were embedded in Tissue-Tec OTC compound (Miles Scientific, Naperville, Ill.), stored at -80°C.

**Tissue in situ hybridization.** Cryosections (6 μm) of infected organs were cut at -16°C with a cryostat HM 500 OM (Micom, Walldorf, Germany), carefully melted on siliconized glass slides, and dried at room temperature for 1 h. After fixing the tissue sections in 1× PBS (pH 7.2) containing 4% (wt/vol) paraformaldehyde for 20 min, the slides were immersed in 3× PBS (pH 7.2) for 5 min, followed by two immersions in 1× PBS for 5 min. The tissue sections were subsequently dehydrated by immersion in 50, 80, and 96% ethanol for 3 min at each concentration and were dried at room temperature. In situ hybridization of the tissue sections with the fluorescein-labeled probes O20 and Cpara was performed as described above for the immobilized yeast cells. Depending on the size of the tissue section, 10 to 30 μl of prewarmed hybridization mixture containing 5 ng of labeled oligonucleotide per μl was applied.

**Blood lysis-filtration assay.** A total of 10 ml of a culture of *C. albicans* ATCC 44808 grown for 10 h at 30°C in YPD medium was harvested by centrifugation, washed in 1× PBS, and suspended in 1 ml of 1× PBS. The viability of the cells exceeded 99%, as determined by trypan blue exclusion. A total of 100 μl of cells diluted  $10^{-6}$ ,  $10^{-7}$ , or  $10^{-8}$  (between 2 and 300 yeast cells; see Table 2) was mixed with 0.5 ml of whole blood from a healthy human donor. In parallel, 100 μl of the dilutions was plated onto YPD plates to determine the yeast cell number. The blood samples, spiked with different amounts of *C. albicans* cells, were treated with 1% (vol/vol) Triton X-100 at 37°C for 5 min to lyse the blood cells and were subsequently incubated for 30 min at 37°C with 5 mg of proteinase K per ml to reduce the protein content of the samples. The resulting clear lysates were diluted in 2 ml of 1× PBS, transferred into a 10-ml syringe, and filtered onto a polycarbonate filter (13 mm in diameter) with 2-μm pores (Millipore, Eschborn, Germany) by using a Swinnex filter-holding device (Millipore). The filters were washed twice with 5 ml of 1× PBS, fixed by treatment with 100% methanol for 30 min, and air dried on glass slides. In situ hybridization was performed as described above with 60 μl of hybridization mixture (40% [vol/vol] formamide) containing 5 ng of labeled probe O20 per μl.

## RESULTS

**Design of an oligonucleotide probe specific for *C. parapsilosis*.** To assess the specificity of detection of *Candida* species by fluorescent in situ hybridization in clinically relevant material, we designed an oligonucleotide probe in which the two mismatched bases of the previously described probe O20 were exchanged such that the resulting probe Cpara was complementary to the 18S rRNA of *C. parapsilosis* instead of to those of *C. albicans* and *C. tropicalis* (see Materials and Methods). The specificity of probe Cpara for *C. parapsilosis* strains was tested in a dot blot hybridization experiment with the digoxigenin-labeled probe Cpara and total RNA from a series of reference strains and clinical isolates of medically important *Candida* species. All *C. parapsilosis* strains but none of the

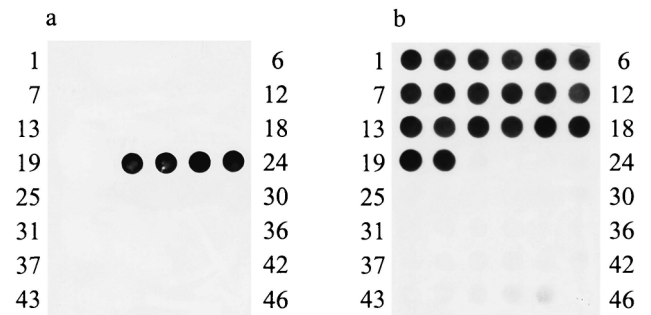


FIG. 1. Species specificity of probes Cpara and O20. Dot blot hybridizations with RNA from strains of medically important *Candida* species and the digoxigenin-labeled probes Cpara (a) and O20 (b) are shown. Dots 1 to 16, *C. albicans*; dots 17 to 20, *C. tropicalis*; dots 21 to 24, *C. parapsilosis*; dots 26 to 29, *Candida glabrata*; dots 30 to 34, *C. krusei*; dots 35 to 37, *Candida guilliermondii*; dots 38 to 41, *Candida kefyr*; dots 42 to 45, *C. lusitanae*; dot 46, *Saccharomyces cerevisiae*.

other *Candida* species reacted with the Cpara probe (Fig. 1a). In a control experiment the filter was rehybridized with probe O20 (Fig. 1b). As demonstrated previously (12), all *C. albicans* and *C. tropicalis* strains, but not the *C. parapsilosis* strains and isolates from other *Candida* species, give a positive signal with this probe.

The fluorescence-labeled probe Cpara was then used to specifically detect *C. parapsilosis* by whole-cell in situ hybridization. *C. parapsilosis* cells were sensitively detected by the Cpara probe (Fig. 2a and b), in contrast, no *C. albicans* cells were stained (Fig. 2c and d). The discrimination capacity was further demonstrated by hybridizing a mixture of *C. albicans* and *C. parapsilosis* with probe Cpara. As expected, only some of the cells were labeled (Fig. 2e and f).

**Specific detection of *C. albicans* and *C. parapsilosis* in deep organs of experimentally infected mice.** To evaluate the use of probes O20 and Cpara for detection of *C. albicans* and *C. parapsilosis* in infected organs, mice were infected intravenously with a lethal dose of either *C. albicans* or *C. parapsilosis* which resulted in rapid dissemination of the fungi (for details, see Materials and Methods). Different internal organs were recovered for the preparation of cryosections and subsequent hybridization with the fluorescence-labeled probes. The fungal burdens in the different organs were determined by counting the numbers of CFU after plating dilutions of homogenized tissue from mice infected in parallel experiments. *C. albicans* exhibited the known preference for dissemination into the kidneys, but cells were also recovered from the brain and spleen (Table 1). Lower cell numbers were found for *C. parapsilosis* in the brain and especially in the kidneys, whereas in the spleen even more *C. parapsilosis* than *C. albicans* cells were detected after plating (Table 1).

Figure 3 presents the results obtained after in situ hybridization of the corresponding organ cryosections with the two oligonucleotide probes. In the kidney from the mouse infected with *C. albicans*, yeast cells and pseudohyphal elements were easily detected by the *C. albicans*-specific probe O20 because of the high number of fungal cells residing in this organ (Fig. 3a and b). The *C. albicans* cells were not stained by the probe Cpara, demonstrating the specificity of detection (Fig. 3c and d). Conversely, in the kidney from the mouse infected with *C. parapsilosis*, yeast cells were stained only by the *C. parapsilosis*-specific probe Cpara (Fig. 3g and h) but not by the probe O20 (Fig. 3e, f). It should be stressed that in this latter case it was not easy to demonstrate the negative result for probe O20

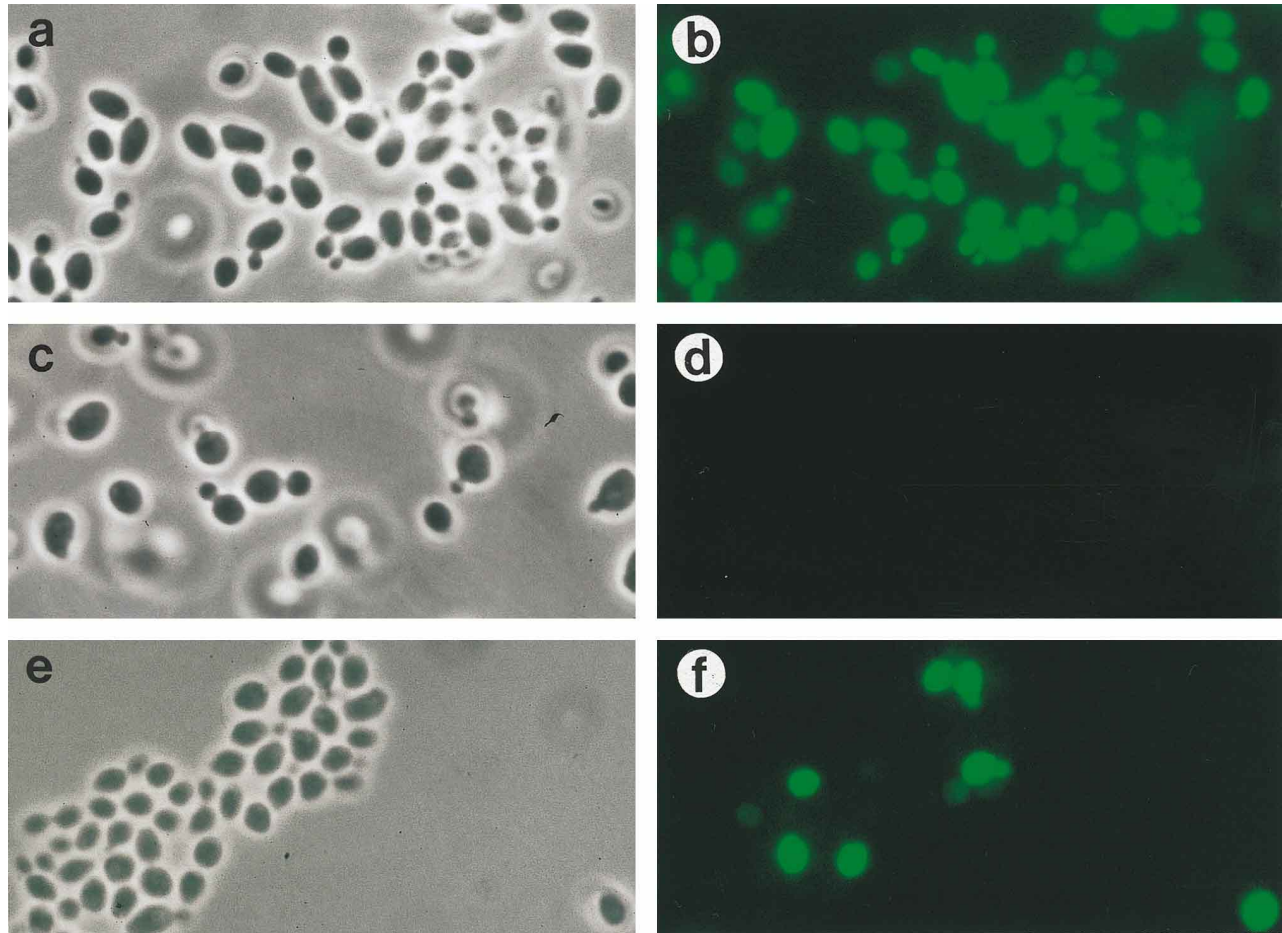


FIG. 2. Identification of *C. parapsilosis* cells by the fluorescence-labeled probe Cpara. Phase-contrast (a, c, and e) and epifluorescence (b, d, and f) micrographs of *C. parapsilosis* (a and b) and *C. albicans* (c and d) or a mixture of both species (e and f) after hybridization with the fluorescence-labeled probe Cpara. The same microscopic fields are shown in all respective phase-contrast and fluorescence micrographs. Magnifications,  $\times 1,000$ .

because the few *C. parapsilosis* cells located in the kidney were hardly detectable without staining (Fig. 3e). In contrast, the species-specific fluorescence labeling by probe Cpara easily facilitated the detection of the fungi in the infected tissue (Fig. 3h). Similarly, both *Candida* species in the other organs were detected by their specific probes, as shown for *C. albicans* in the brain (Fig. 3i) of the *C. albicans*-infected mouse and for *C. parapsilosis* in the spleen of the animal infected with this species (Fig. 3j). In no case were any *C. albicans* cells stained by the probe Cpara, nor were *C. parapsilosis* cells labeled by the probe O20.

TABLE 1. Fungal load in organs of mice infected with *C. albicans* or *C. parapsilosis*<sup>a</sup>

Mouse infection	No. of CFU in the following organ		
	Kidney	Spleen	Brain
<i>C. albicans</i>	327,600	11,200	33,600
<i>C. parapsilosis</i>	4,340	20,160	10,360

<sup>a</sup> The kidneys (data are averages for left and right organs), spleens, and brains of infected mice were recovered 1 day after intravenous infection, homogenized, and plated onto agar plates to determine the numbers of CFU. Cell numbers were determined from one animal in each experiment.

**Detection of *C. albicans* in human blood.** We further tested if *C. albicans* could be detected by probe O20 in human blood. Blood from a healthy donor was spiked with different amounts of *C. albicans* cells and, after lysis of the blood cells, filtered through a membrane which held back the intact yeast cells. The filter was subsequently processed for in situ hybridization with the fluorescence-labeled probe O20, and the presence of *C. albicans* cells was detected by fluorescence microscopy. Yeast cells could readily be distinguished from background fluorescent material by their typical morphology, which was retained during the whole process (Fig. 4). The sensitivity of detection was evaluated by comparing the number of yeast cells detected by visual inspection of the filter with the number of cells inoculated into the blood samples (Table 2). In repeat experiments, as few as two to three *C. albicans* cells inoculated into 0.5 ml of blood could reliably be detected. On average, about 75% of the fungal cells were recovered on the filter and were detected after fluorescent in situ hybridization.

## DISCUSSION

The diagnosis of disseminated candidiasis requires rapid and sensitive detection of the fungi in relevant body sites like deep tissue and blood. Because *Candida* species other than *C. albicans* have been isolated with increasing frequency during the

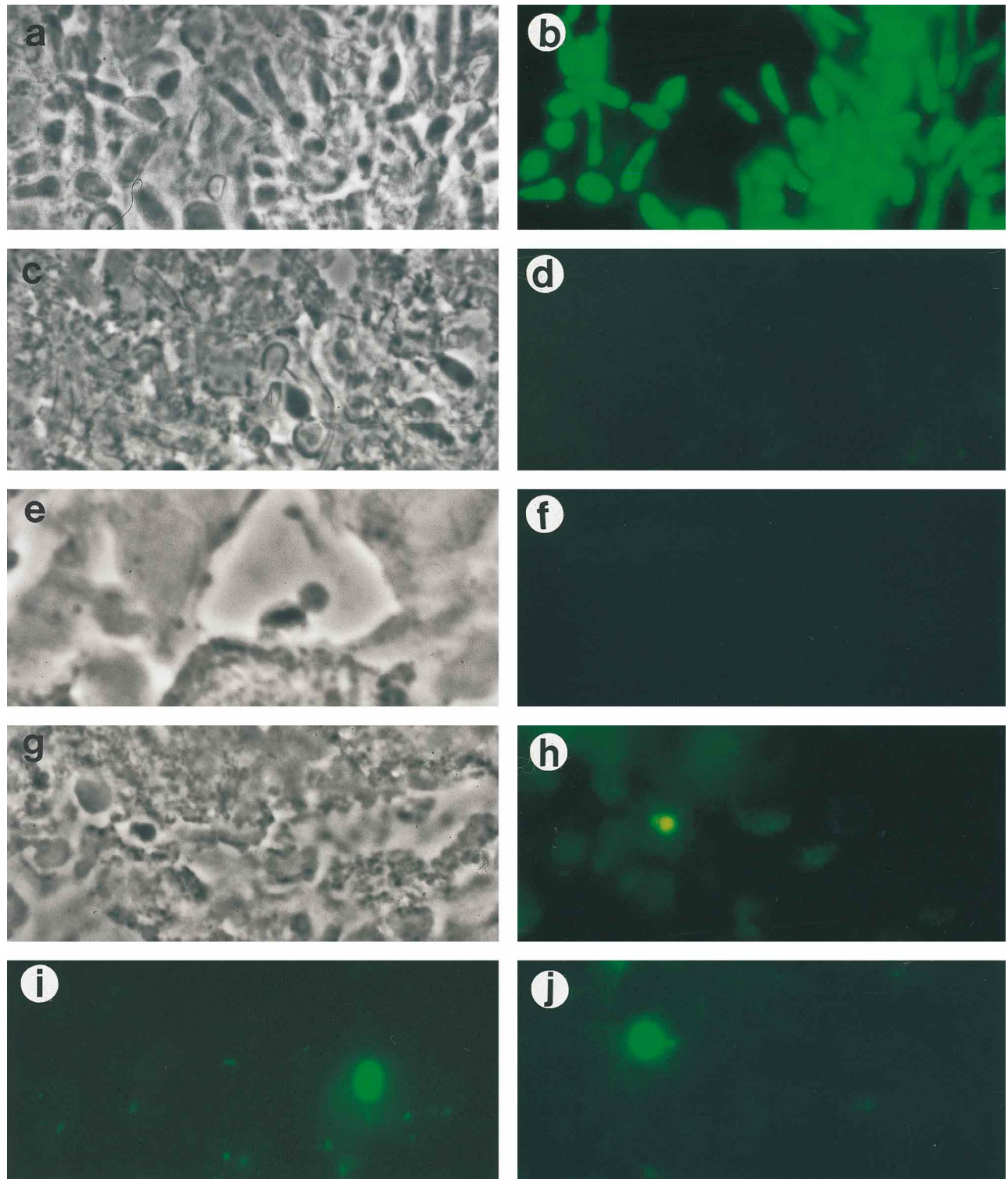


FIG. 3. Specific detection of *C. albicans* and *C. parapsilosis* in experimentally infected tissue by fluorescent in situ hybridization. Phase-contrast (a, c, e, and g) and epifluorescence (b, d, f, h, i, and j) micrographs of tissue sections from kidney (a to h), brain (i), and spleen (j) of mice infected with *C. albicans* (a to d and i) or *C. parapsilosis* (e to h and j) after hybridization with the fluorescence-labeled probes O20 (a, b, e, f, and i) or Cpara (c, d, g, h, and j). For a to h the same area is shown in the corresponding phase-contrast and fluorescence micrographs. (a to d and g to j) Magnifications,  $\times 1,000$ ; (e and f) magnifications,  $\times 2,000$ .

last few years and because they differ in their susceptibilities to antifungal agents (27, 28), identification of the infecting species is important. Recently, we have demonstrated that in situ hybridization of whole cells with a fluorescence-labeled, 18S

rRNA-targeted oligonucleotide probe can be used for the sensitive detection and identification of *Candida* species without the need for culture (12). In those pilot studies a probe that recognizes two medically important *Candida* species, *C. albi-*

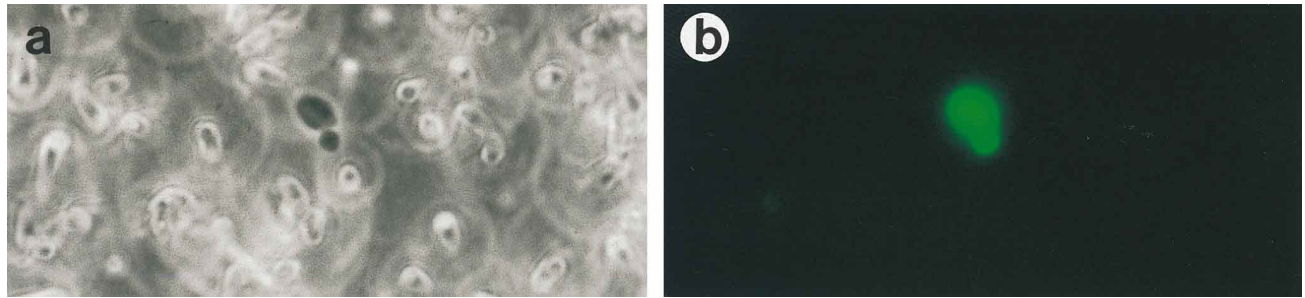


FIG. 4. Detection of *C. albicans* in human blood by a lysis-filtration assay and subsequent in situ hybridization with the fluorescence-labeled probe O20. (a) Phase-contrast micrograph; (b) the corresponding fluorescence micrograph of an area where a budding *C. albicans* cell was detected by probe O20. Magnification,  $\times 1,000$ .

*cans* and *C. tropicalis*, was used. In this report we have shown that *C. albicans* could also be detected in the internal organs of infected mice by fluorescent in situ hybridization with this probe. Specificity was confirmed by using a second probe targeted against *C. parapsilosis*, a closely related organism. Both probes O20 and Cpara detected only the corresponding target species, *C. albicans* or *C. parapsilosis*, respectively, in infected tissue. Because the probe binding regions in the 18S rRNAs of *C. albicans* or *C. parapsilosis* differ by only two nucleotides, these results demonstrate the high discriminatory capacity of the oligonucleotide probes in clinical material. Therefore, it should be possible to design probes specific for all medically relevant *Candida* species which require identification for effective therapy. *Candida krusei*, for example, is intrinsically resistant to the widely used drug fluconazole (22), and *Candida lusitanae* has been associated with amphotericin B resistance (17). Rapid identification of the infecting species would therefore facilitate the selection of a suitable therapeutic regimen.

Detection of *Candida* in blood is the strategy most widely used to diagnose systemic candidiasis. Because blood cultures often fail to detect disseminated candidiasis, PCR assays have been developed. PCR assays are aimed at amplifying *Candida* DNA from blood to improve the sensitivity of detection. Yeast

cell numbers in blood are low, in the range of 1 to 100 CFU/ml or even lower (10). A method that would significantly improve detection by blood culture should be expected to detect 1 to 10 CFU/ml of blood (19). In our assays, we consistently could detect about three *C. albicans* cells in 0.5 ml of blood, which is in the range of sensitivity of or even better than most results obtained by PCR-based detection methods (5, 7, 9, 13, 18, 23). Detection by fluorescent in situ hybridization is rapid; the whole procedure, from the time of blood sampling to the time of microscopic detection, can be performed within 1 working day, in contrast to methods requiring cultivation and phenotypic characterization. Many PCR protocols rely on further manipulations to enhance sensitivity, for example, Southern hybridization (7, 9, 13, 18, 23) or immunoassays (5), which increase the time until a positive result is obtained. Although the lysis-filtration assay and hybridization with fluorescence-labeled oligonucleotide probes require somewhat more hands-on time due to the hybridization and washing steps as well as the visual examination of the filters, the speed of the method is comparable to those of PCR-based methods. In addition, several blood samples can be processed in parallel by using probes of the desired specificity, resulting in the identification of the infecting *Candida* species. We are developing additional probes for other, drug-resistant *Candida* species and are testing their diagnostic value by analyzing blood from patients who are at risk for candidemia and comparing the results with those obtained by conventional diagnostic procedures.

TABLE 2. Sensitivity of detection of *C. albicans* in human blood by fluorescent in situ hybridization<sup>a</sup>

Expt no. and dilution	No. of cells inoculated	No. of cells detected	% Cells detected
Expt 1			
10 <sup>-6</sup>	280	218	78
10 <sup>-7</sup>	51	17	33
10 <sup>-8</sup>	2	1	50
Expt 2			
10 <sup>-6</sup>	250	252	100
10 <sup>-7</sup>	25	21	84
10 <sup>-8</sup>	3	3	100
Expt 3			
10 <sup>-6</sup>	300	244	81
10 <sup>-7</sup>	24	20	83
10 <sup>-8</sup>	3	2	67

<sup>a</sup> The numbers of *C. albicans* cells detected by the lysis-filtration assay and subsequent in situ hybridization with the fluorescence-labeled probe O20 are given. Cell numbers used for inoculation into 0.5 ml of human blood were determined by counting the numbers of CFU after plating aliquots of the same suspensions. The number of yeast cells detected is also given as the percentage of cells used for inoculation. The experiment was performed in triplicate to ensure reproducibility.

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