Specific detection of *Candida albicans* and *Candida tropicalis* by fluorescent *in situ* hybridization with an 18S rRNA-targeted oligonucleotide probe

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***In situ* hybridization of whole cells with rRNA-targeted, fluorescently labelled oligonucleotide probes is a powerful method to specifically detect micro-organisms in their natural habitat without cultivation and subsequent identification by phenotypic characterization. To examine the use of this method for the specific detection of pathogenic *Candida* species, we have designed an oligonucleotide probe which binds to the 18S rRNA of *C. albicans* and *C. tropicalis*, the two most important pathogenic *Candida* species, and differentiates them from other clinically relevant species. After establishing suitable hybridization conditions, we confirmed the specificity of our probe 020 in RNA dot blot hybridizations with a series of reference strains and clinical isolates of medically important *Candida* species. All *C. albicans* and *C. tropicalis* strains hybridized with the probe, whereas all strains of *C. parapsilosis*, *C. glabrata*, *C. krusei*, *C. guilliermondii*, *C. kefyr* and *C. lusitanae* did not. When we used the fluorescently labelled probe 020 to specifically detect single cells of the two target species by *in situ* hybridization, both *C. albicans* and *C. tropicalis* reacted strongly with the probe and could be clearly differentiated from *C. krusei* and *C. parapsilosis*, although the latter organism contains only two nucleotide mismatches in the probe target region. This discrimination capacity was also seen when mixed suspensions of *C. albicans* and *C. parapsilosis* were hybridized with the probe. After infection of a human endothelial cell line with *C. albicans* and *C. krusei*, *C. albicans* cells adhering to the endothelial cells were easily distinguishable from the *C. krusei* cells by fluorescent *in situ* hybridization with probe 020. In addition, germ tubes and hyphae of *C. albicans* were also efficiently labelled. The application of fluorescently labelled rRNA-targeted oligonucleotide probes therefore appears to be a valuable tool for the specific detection and identification of different members of the genus *Candida*, which does not require any cultivation.**

Keywords: *Candida*, 18S rRNA, oligonucleotide probe, fluorescence, *in situ* hybridization

INTRODUCTION

The opportunistic fungal pathogen *Candida albicans* and other species of the genus *Candida* are able to cause superficial and disseminated infections in immunocompromised patients. As *Candida* species differ in their

susceptibility to widely utilized systemic antifungal agents, such as azoles (Rex *et al.*, 1995), identification of the infecting species is important to ensure effective therapy strategies. The diagnosis of systemic *Candida* infections is a major problem because the clinical presentation is usually non-specific (Jones, 1990). Disseminated candidiasis is in most cases diagnosed by positive blood culture. However, blood cultures from patients with histologically proven disseminated candi-

Abbreviation: FLUOS, 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester.

diasis are often negative. In addition, even with an optimal blood culture system like the lysis centrifugation method, it takes a minimum of 2 d for detection and even longer for species identification (Jones, 1990). To reduce mortality in patients suffering from invasive candidiasis, however, early initiation of proper antifungal drug therapy is critical (Anaissie, 1992; Fraser *et al.*, 1992).

Direct histologic detection of the fungus in biopsy material from deep tissue offers the best diagnostic assurance and eliminates the need for culture. The infecting species, however, can not solely be identified by microscopic examination, and *Candida* pseudohyphae may even be erroneously diagnosed as *Aspergillus* hyphae, for example in liver biopsy (Kwon-Chung & Bennett, 1992). Other non-culture methods for the detection of *Candida* infections, such as measurement of serum D-arabinitol, detection of specific antigens or antibodies, or amplification of fungal DNA from blood and urine by PCR, have also been developed, but their diagnostic value is doubtful or, for the latter, remains to be established (for a review, see Reiss & Morrisson, 1993).

Fluorescent *in situ* hybridization of whole cells with rRNA-targeted oligonucleotide probes has become a highly valuable tool for the specific detection of micro-organisms without cultivation (DeLong *et al.*, 1989; Amann *et al.*, 1995). Non-culturable bacteria have been identified in their natural environment, e.g. in activated sludge, in the mammalian gut, or as endosymbionts (Amann *et al.*, 1991; Poulsen *et al.*, 1994; Wagner *et al.*, 1994). The use of rRNA as the target for hybridization has several advantages. Firstly, the sequence of the rRNA, especially the small subunit (ss) rRNA, is known for many organisms and consists of evolutionarily more or less conserved regions. Consequently, target sites can be selected that are specific for a certain species or for groups of related organisms. Secondly, the target molecule is abundant in the cells, resulting in a strong hybridization signal that enables the detection of single cells. Thirdly, in contrast to immunological methods that rely on the expression of specific markers, which may not be constant, phenotypic variation does not pose a problem when rRNA is used as the target. For these reasons, we intended to establish the method of fluorescent *in situ* hybridization for the specific detection of *Candida* species. One aim was to design a probe that detects *C. albicans*, the most important pathogenic species. To examine whether this technique is generally applicable for other *Candida* species, we chose a target region that is identical in *C. albicans* and *C. tropicalis* but different in other clinically important *Candida* species. Our results demonstrate that fluorescently labelled, rRNA-targeted oligonucleotide probes can be used to specifically detect *Candida* species by *in situ* hybridization.

METHODS

Strain maintenance and culture conditions. All fungal strains used in this study are listed in Table 1. *Candida* reference strains were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA), from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig,

Table 1. Fungal strains used in this study

Strain no.	Species	Strain
1	<i>C. albicans</i>	ATCC 44808
2	<i>C. albicans</i>	ATCC 44807
3	<i>C. albicans</i>	ATCC 38696
4	<i>C. albicans</i>	DSM 1386
5	<i>C. albicans</i>	CBS 562
6	<i>C. albicans</i>	CBS 5983
7	<i>C. albicans</i>	Clinical isolate 95
8	<i>C. albicans</i>	Clinical isolate 99
9	<i>C. albicans</i>	Clinical isolate 100
10	<i>C. albicans</i>	Clinical isolate 101
11	<i>C. albicans</i>	Clinical isolate 102
12	<i>C. albicans</i>	Clinical isolate 103
13	<i>C. albicans</i>	Clinical isolate 104
14	<i>C. albicans</i>	Clinical isolate 105
15	<i>C. albicans</i>	Clinical isolate 106
16	<i>C. albicans</i>	Clinical isolate 107
17	<i>C. tropicalis</i>	DSM 4238
18	<i>C. tropicalis</i>	Clinical isolate GR1
19	<i>C. tropicalis</i>	Clinical isolate GR2
20	<i>C. tropicalis</i>	Clinical isolate GR3
21	<i>C. tropicalis</i>	Clinical isolate GR4
22	<i>C. parapsilosis</i>	DSM 70126
23	<i>C. parapsilosis</i>	Clinical isolate GR33
24	<i>C. parapsilosis</i>	Clinical isolate GR34
25	<i>C. parapsilosis</i>	Clinical isolate GR36
26	<i>C. glabrata</i>	DSM 70614
27	<i>C. glabrata</i>	Clinical isolate GR10
28	<i>C. glabrata</i>	Clinical isolate GR11
29	<i>C. glabrata</i>	Clinical isolate GR12
30	<i>C. glabrata</i>	Clinical isolate GR13
31	<i>C. krusei</i>	DSM 70079
32	<i>C. krusei</i>	Clinical isolate GR20
33	<i>C. krusei</i>	Clinical isolate GR21
34	<i>C. krusei</i>	Clinical isolate GR22
35	<i>C. krusei</i>	Clinical isolate GR23
36	<i>C. guilliermondii</i>	Clinical isolate GR30
37	<i>C. guilliermondii</i>	Clinical isolate GR31
38	<i>C. guilliermondii</i>	Clinical isolate GR32
39	<i>C. kefyr</i>	Clinical isolate GR38
40	<i>C. kefyr</i>	Clinical isolate GR39
41	<i>C. kefyr</i>	Clinical isolate GR40
42	<i>C. kefyr</i>	Clinical isolate GR41
43	<i>C. lusitaniae</i>	Clinical isolate GR42
44	<i>C. lusitaniae</i>	Clinical isolate GR43
45	<i>C. lusitaniae</i>	Clinical isolate GR44
46	<i>C. lusitaniae</i>	Clinical isolate GR45
47	<i>S. cerevisiae</i>	Clinical isolate

Germany) and from the Central Bureau voor Schimmelcultures (CBS, Baarn, The Netherlands). Clinical isolates were obtained from various hospitals in Greifswald as well as Würzburg, Germany, and were identified using the Vitek Automated Microbiology System (Vitek Systems Inc.). The identity of *C. albicans* isolates was also confirmed by a positive germ tube test. Immediately upon arrival, the strains were stored at -80°C in Sabouraud medium (4%, w/v, glucose, 1%, w/v, peptone),

containing 15% (v/v) glycerol. Strains were cultured on Sabouraud plates, containing 1.5% (w/v) agar at 30 °C for 1–2 d and then stored at 4 °C.

Oligonucleotide probes and fluorescence dye labelling. The oligonucleotides used in this study were synthesized by MWG Biotech, Munich, Germany. Oligonucleotide O20 (5' CCCCC-TTTCCTAAACCAATCCGGA 3') is complementary to a sequence in the *C. albicans* ss rRNA corresponding to positions 1674–1697 of the *Saccharomyces cerevisiae* 18S rRNA (Mankin *et al.*, 1986, see Results). Oligonucleotide EUK516 (5' ACCAG-ACTTGCCCTCC 3') is complementary to positions 548–563 of the *S. cerevisiae* 18S rRNA, a sequence which is identical in all eukaryotic organisms (Amann *et al.*, 1990a). For *in situ* hybridization, the oligonucleotide O20 was coupled with a C6-TFA aminolinker [6-(trifluoroacetyl-amino)-hexyl-(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidite] at the 5' end (MWG Biotech). Subsequent labelling with 5(6)-carboxy-fluorescein-*N*-hydroxysuccinimide ester (FLUOS, Boehringer Mannheim) was carried out as described previously (Manz *et al.*, 1992). The labelled probe was purified by exclusion chromatography, PAGE and reversed-phase chromatography as described by Amann *et al.* (1990b) and stored at –20 °C.

RNA extraction and dot blot hybridization. Total RNA was extracted from *Candida* strains grown to exponential phase in Sabouraud medium at 30 °C, by the hot acidic phenol method (Golemis *et al.*, 1993). After spectrophotometric quantification, the quality of the RNA was controlled by agarose gel electrophoresis and staining with ethidium bromide. RNA samples were stored at –20 °C. For dot blot hybridization experiments, 10 µg RNA of each strain were transferred onto a Biotodyne B nylon membrane, using a minifold I dot blot apparatus (Schleicher & Schüll), and immobilized by UV treatment. Labelling of oligonucleotide probes with digoxigenin, hybridization, washing of the membranes and signal detection with the substrate 3-(4-methoxyspiro{1,2-dioxetan-3,2'-(5'-chloro)tricyclo-[3,3,1.1^{3,7}]decan}-4-yl)phenyl phosphate (CSPD) were performed using the 3' oligonucleotide labelling and nucleic acid labelling kits provided by Boehringer Mannheim. The instructions of the manufacturer were modified as follows. Hybridization stringency was controlled by varying the formamide content of the hybridization buffer and/or the hybridization temperature (see Results). The relationship between hybridization temperature and formamide content of the buffer was calculated by assuming a decrease of about 0.7 °C in hybridization temperature for every 1% increase in the concentration of formamide (McConaughy *et al.*, 1969). Washing was performed at slightly elevated temperatures compared to hybridization (4 °C increase for O20, 3 °C increase for EUK516). For rehybridization, filters were rinsed with water, treated twice with 0.2 M NaOH/0.1% (w/v) SDS at 37 °C for 15 min to remove the succinimide probe, and subsequently washed with 2 × SSC (300 mM NaCl, 30 mM Na citrate).

***In situ* hybridization.** *Candida* yeast cultures were grown in Sabouraud medium at 30 °C to late exponential phase, harvested and washed with PBS (pH 7.2). For preparing mixtures of *C. albicans* and *C. parapsilosis*, the cells were counted using a Neubauer haemocytometer, adjusted to equal density and mixed at different relative concentrations. To immobilize the cells on glass slides, 40 µl of the cell suspensions was applied onto the slides and incubated for 1 h at room temperature. After washing with PBS (pH 7.2), the cells were fixed for 20 min by adding 100% (v/v) methanol, air-dried and dehydrated by subsequent immersion for 3 min each in 50, 80 and 96% (v/v) ethanol.

The procedure for the *in situ* hybridization of whole cells with FLUOS-labelled oligonucleotide probes was described pre-

viously (Manz *et al.*, 1992) and was followed with minor modifications. Hybridization was performed at 43 °C using a formamide concentration of 45% (v/v) in the hybridization buffer. Labelled O20 probe (50 ng) in 8 µl hybridization mix was applied per hybridization and the microscope slide was placed in a moisture chamber (Wilcox, 1993) to ensure a comparable vapour pressure. The slides were gently washed with water and immersed in a prewarmed washing solution (40 mM NaCl, 20 mM Tris/HCl, pH 7.5, 0.01% 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 using a Zeiss Axiolab microscope (Zeiss), equipped for epifluorescence microscopy with a 50 W mercury high pressure bulb and the Zeiss fluorescein-specific filter set 09. Colour micrographs were taken on Fujichrome 400 or Agfachrome 100 slide film without any subsequent digital image processing. Exposure times were 0.1–1.5 s for phase contrast and 5–60 s for epifluorescence micrographs. The *in situ* hybridization experiments were carried out at least in duplicate to ensure reproducibility.

Induction of germ tubes and hyphae formation. *C. albicans* ATCC 44808 yeast cells were grown overnight in Soll's medium (Soll, 1986) at 25 °C (pH 4.5). For hyphae induction, the cells were diluted 1:10 in Soll's medium (pH 6.8) and incubated at 37 °C. After 2 h of incubation most cells had formed germ tubes, and after 4 h growth prolonged hyphae were visible. Germ tubes and hyphae were washed in PBS (pH 7.2) and processed for *in situ* hybridization as described above.

Adherence assay. The human umbilical vein endothelial cell line EA.hy 926 (Edgell *et al.*, 1983) was used in this study. The cells were cultured in RPMI 1640 tissue culture medium, containing 10% (v/v) foetal calf serum (FCS) and 2 mM glutamine, at 37 °C in the presence of 5% (v/v) CO₂, and routinely passaged every 4–5 d. For the adherence assays, the endothelial cells were seeded onto coverslips (13 mm diam.) in 24-well plates (Becton Dickinson) and grown to confluence. *C. albicans* ATCC 44808 and *C. krusei* DSM 70079 were grown overnight in Sabouraud medium at 30 °C, washed in cell culture medium and counted using a Neubauer haemocytometer. The viability of the cells was routinely inspected by trypan blue exclusion and consistently exceeded 99%. Cells were diluted to a density of 10⁷ cells ml⁻¹ and the *C. albicans* and *C. krusei* suspensions were mixed at equal concentrations. One-hundred microlitres of this mixed suspension (5 × 10⁵ cells of each strain) were inoculated into the wells containing the endothelial cells and the plate was incubated for 4 h (37 °C, 5% CO₂) to allow adherence of the yeasts to the endothelial cells. Under these conditions (cell numbers of 5 × 10⁵ per well and above, Ollert *et al.*, 1993) the formation of germ tubes in *C. albicans* is repressed. After brief washing in PBS (pH 7.2) the cover slips were fixed and processed for *in situ* hybridization as described above.

Sequence analysis. For sequence comparisons, the PILEUP and FASTA programs of the Genetics Computer Group Wisconsin Package (Version 8, September 1994) were used.

RESULTS

Selection of an oligonucleotide probe specific for *C. albicans* and *C. tropicalis*

To establish the method of fluorescent *in situ* hybridization for the specific detection of different species of the genus *Candida*, we decided to use a probe which was directed against a target region in the 18S rRNA which is identical in *C. albicans* and *C. tropicalis* but differs from those of

<i>C. albicans</i>	GTGAGGCC	TCCGGATTGGTTTAGGAAAGGGGG	CAACTCCA
<i>C. tropicalis</i>	GTGAGGCT	TCCGGATTGGTTTAGGAAAGGGGG	CAACTCCA
<i>C. viswanathii</i>	GTGAGGCT	TCCGGATTGGTTTAGGAAAGGGGG	CNWTCCA
<i>C. maltosa</i>	GTGAGGCC	TCCGGATTGGTTTAGGAAAGGGGG	CAACTCCA
<i>C. parapsilosis</i>	GTGAGGCT	TCCGGATTGGTTTAGAGAAGGGGG	CWACTCCA
<i>D. hansenii</i>	GTGAGGCC	TCCGGATTGGTTTAAAGAAGGGGG	CAACTCCA
<i>C. guilliermondii</i>	GTGAGGCC	TCCGGATTGGTTTAAAGTAGGGGG	CWWCTCCA
<i>H. polymorpha</i>	GTGAGGCT	TCAGGATTGGTTTCAGAGAAGGGGG	CAACTCCA
<i>C. kefyr</i>	GTGAGGCC	TCAGGATTGGTTTAGAGAAGGGGG	CAWCTCCA
<i>K. lactis</i>	GTGAGGCC	TCAGGATTGGTTTAGAGAAGGGGG	CAACTCCA
<i>C. glabrata</i>	GTGAGGCC	TCAGGATCTGCTTAGAAGAGGGGG	CNACTCCA
<i>S. cerevisiae</i> 1666	GTGAGGCC	TCAGGATCTGCTTAGAGAAGGGGG	CAACTCCA 1705
<i>C. lusitaniae</i>	GTGAGGCC	TCCGGATTGTCTA.AGCCGAGGG	CNATCCTT
<i>A. fumigatus</i>	GTGAGGCC	TTCCGACTGGCTCAGGGGAGTTGG	CAACGACT
<i>C. krusei</i>	GTGAGGCT	TCAAGATTGGCGCCGCGGAGGGGG	CAACTTTC

Fig. 1. Sequence alignment of the region in the ss rRNA genes of *Candida* spp. and other fungi corresponding to nucleotides 1666–1705 of the *S. cerevisiae* 18S rRNA. The target region of the oligonucleotide probe O20 from 1674 to 1697 is boxed; nucleotides different from the *C. albicans* sequence are shown in bold. The sequences were obtained from the EMBL data bank using the following accession numbers: X53497 (*C. albicans*), M55527 (*C. tropicalis*), M60309 (*C. viswanathii*), D14593 (*C. maltosa*), M60307 (*C. parapsilosis*), X62649 (*Debaryomyces hansenii* = *C. famata*), M60304 (*C. guilliermondii*), M60310 (*Hansenula polymorpha*), M60303 (*C. kefyr*), X51830 (*Kluyveromyces lactis*), M60311 (*C. glabrata*), M27607 (*S. cerevisiae*), M60306 (*C. lusitaniae*), M60301 (*Aspergillus fumigatus*), M60305 (*C. krusei*). W denotes A or T; N denotes A, C, G or T.

Fig. 2. RNA dot blot hybridizations using RNA from *C. albicans* ATCC 44807, *C. tropicalis* DSM 4238 and *C. parapsilosis* GR 34, and the digoxigenin-labelled oligonucleotide O20 as probe. Hybridizations were performed as described in Methods at a temperature of 51 °C with the indicated formamide concentrations (% v/v) in the hybridization buffer.

other pathogenic *Candida* species. Fig. 1 shows the alignment of a region in known 18S rRNA sequences of *Candida* species and some other fungi corresponding to positions 1666–1705 in the *S. cerevisiae* 18S rRNA. This

sequence is located in the variable region V9 of ss rRNAs (Neefs *et al.*, 1993). A 24mer oligonucleotide named O20, complementary to positions 1674–1697 (see Methods and Fig. 1), was chosen as a probe because this region is identical in the two target species (and in the clinically irrelevant species *C. viswanathii* and *C. maltosa*), but different to various degrees in other medically important *Candida* species. Comparison of the selected target sequence with all accessible rRNA sequences in the data bank confirmed its uniqueness in the 18S rRNA of the four species.

Confirmation of probe specificity

One of the advantages of oligonucleotide hybridizations is that by stringently controlling assay conditions, even sequences with only one nucleotide mismatch can be discriminated (Amann *et al.*, 1990b). To establish suitable hybridization conditions for the specific detection of *C. albicans* and *C. tropicalis*, dot blot hybridizations with total RNA from these two organisms and the next related species, *C. parapsilosis*, which contains only two mismatches in the target region, were performed using the digoxigenin-labelled probe O20. Hybridization stringency was varied by changing the formamide content of the hybridization buffer. A hybridization temperature of 51 °C allowed the use of a wide range of formamide concentrations. At this temperature, a formamide concentration of 35 % was found to be sufficient to distinguish *C. albicans* and *C. tropicalis* from *C. parapsilosis* (Fig. 2).

To confirm the ability of probe O20 to specifically detect *C. albicans* and *C. tropicalis* strains in general, dot blot experiments with RNA from a series of reference strains and clinical isolates of *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, *C. krusei*, *C. guilliermondii*, *C. kefyr*, *C. lusitaniae* and *S. cerevisiae* (Table 1) were performed. A hybridization temperature of 43 °C was used because this temperature was found to be suitable also for *in situ* hybridization (see below). The decrease in temperature

Fig. 3. RNA dot blot hybridizations using RNA from strains listed in Table 1 and the digoxigenin-labelled oligonucleotide O20 as probe. Hybridizations were performed as described in Methods at 43 °C in a buffer containing 45% (probe O20) or 0% (probe EUK516) formamide. (a) Strain number (as in Table 1), (b) hybridization with probe O20, (c) hybridization with probe EUK516.

was compensated for by increasing the formamide concentration in the hybridization buffer to 45%, resulting in comparable stringency conditions as determined in the previous experiment (35% formamide, 51 °C, see Methods). The results confirmed the general applicability of probe O20 to distinguish *C. albicans* and *C. tropicalis* from other, even closely related, yeast species. All 16 *C. albicans* and the four *C. tropicalis* isolates tested gave a positive signal, whereas no hybridization to RNA from other species was detected (Fig. 3b). In a control experiment, the filter was stripped and rehybridized with the oligonucleotide probe EUK516, which binds to RNA from all eukaryotic organisms, applying published stringency conditions (Amann *et al.*, 1990a). Fig. 3(c) shows that all strains reacted with this probe, confirming that comparable amounts of RNA had been immobilized on the filter. These results demonstrated probe O20 to be suitable for the specific detection of the selected pathogenic target species *C. albicans* and *C. tropicalis*.

Specific detection of *C. albicans* and *C. tropicalis* by fluorescent *in situ* hybridization

To investigate if specific detection of both target species was also possible by fluorescent *in situ* hybridization, yeast cells of *C. albicans* and *C. tropicalis* and of two other *Candida* species having either a similar (*C. parapsilosis*) or a more divergent (*C. krusei*) target sequence, were grown to exponential phase and processed for *in situ* hybridization with the FLUOS-labelled oligonucleotide O20 as described in Methods. All cells of *C. albicans* and *C. tropicalis* were brightly stained (Fig. 4a–d), whereas no fluorescence of *C. parapsilosis* and *C. krusei* was detectable (Fig. 4e–h). Thus, the difference of only two nucleotides in the target sequence of probe O20 in *C. parapsilosis* was sufficient to distinguish this organism from *C. albicans* and

C. tropicalis also in *in situ* hybridization experiments. To present additional evidence for this discrimination capacity, suspensions of the morphologically indistinguishable *C. albicans* and *C. parapsilosis* were mixed at different relative concentrations and hybridized to the FLUOS-labelled probe O20 (Fig. 4i–o). Only a part of the cells seen in the phase contrast micrographs were labelled by the probe and the percentage of fluorescent cells in all cases corresponded well with the relative proportion of *C. albicans* cells in the mixtures. In some cases, a weak background fluorescence of *C. parapsilosis* cells was detected; however, this was not comparable with the specific staining of the *C. albicans* cells.

C. albicans does not only grow as a yeast but also in a mycelial form, which is often encountered in infected tissue. It was therefore investigated whether, in addition to blastoconidia, *C. albicans* hyphae can also be detected by *in situ* hybridization. The formation of germ tubes and hyphae in *C. albicans* ATCC 44808 was induced by growth in Soll's medium at pH 6.8 and 37 °C (see Methods). Following hybridization to the FLUOS-labelled probe O20, *C. albicans* germ tubes (Fig. 4p, q) and also longer hyphal elements (Fig. 4r, s) were efficiently labelled, although somewhat weaker than the mother blastoconidia, which is probably due to a reduced ribosome concentration in the vacuole-containing hyphae (Odds, 1988). The different cell wall of these morphological forms thus did not interfere with probe permeability.

Specific detection of *C. albicans* in *in vitro* cell adhesion models

Adherence of *C. albicans* to epithelial and endothelial cells is important for colonization as well as for invasion of the host tissue by the fungus (Calderone & Braun, 1991;

Fig. 4. For legend see facing page.

Fig. 4. Phase contrast (a, c, e, g, i, l, n, p, r) and epifluorescence micrographs (b, d, f, h, k, m, o, q, s) of *C. albicans* ATCC 44808 (a, b, p, q, r, s), *C. tropicalis* DSM 4238 (c, d), *C. parapsilosis* DSM 70126 (e, f), *C. krusei* DSM 70079 (g, h), and mixtures of *C. albicans* ATCC 44808 and *C. parapsilosis* DSM 70126 at a relative concentration of 1:9 (i, k), 1:1 (l, m) and 9:1 (n, o), respectively, after hybridization to the FLUOS-labelled probe O20 under stringent conditions (see text). The same section is shown in all respective phase contrast and fluorescence micrographs. In the phase contrast micrograph in (n) the unlabelled *C. parapsilosis* cells are marked by arrows for better illustration. Detection of *C. albicans* ATCC 44808 germ tubes (p, q) and elongated hyphae (r, s) by probe O20 is also demonstrated.

Hostetter, 1994). As adhesion to *in vitro* cultured cells is a widely used model system, human endothelial cells were infected with *C. albicans* and *C. krusei* to investigate if *C.*

albicans can be differentiated from other *Candida* species by fluorescent *in situ* hybridization also on host cell surfaces. *C. krusei* was used as a control because the usually longer,

Fig. 5. Specific detection of *C. albicans* cells adhering to endothelial cells after infection of the human endothelial cell line EA.hy 926 with a mixture of *C. albicans* ATCC 44808 and *C. krusei* DSM 70079. For details see Methods. (a) Phase contrast micrograph showing the round *C. albicans* and the oval-shaped *C. krusei* adhering to the endothelial cell monolayer. (b) The same section viewed by epifluorescence microscopy. (c) Double exposure of the phase contrast and epifluorescence micrographs. Note that only the round *C. albicans* cells, but not the longer *C. krusei* cells are stained by the fluorescently labelled probe O20.

oval-shaped cells (depending on their orientation) of this species could be morphologically distinguished from the round *C. albicans* blastoconidia. A confluent monolayer of the human endothelial cell line EA.hy 926 was incubated for 4 h with a mixture of *C. albicans* and *C. krusei* and, following removal of non-adherent cells by gentle washing, the sample was hybridized to the FLUOS-labelled probe O20. Fig. 5(a) shows the phase contrast micrograph of a section where cells from both *Candida* species adhere to the endothelial cells. Most of the cells with a round morphology were strongly labelled by the probe, whereas the oval-shaped cells were not stained (Fig. 5b). The discrimination between the two *Candida* species is also

demonstrated in the double exposure of the phase contrast and the fluorescence micrograph in Fig. 5(c) where only the round *C. albicans* cells but not the longer *C. krusei* cells are fluorescent.

DISCUSSION

Identification of different species of the genus *Candida* is not possible by morphology alone but relies on cultivation of the organism and subsequent biochemical characterization. To detect and identify *Candida* species by microscopic examination, we have applied the method of fluorescent *in situ* hybridization using an 18S rRNA-targeted oligonucleotide probe. As this method has so far not been used to our knowledge for the detection of *Candida* species, the aim of our study was to evaluate its general applicability for this group of pathogenic fungi. Therefore, a target site in the rRNA of the most important pathogen, *C. albicans*, which is identical in some additional *Candida* species but different in others, was selected. It would have been noticed if structural differences between species, e.g. differences in cell wall permeability, had affected successful detection in spite of a correct target sequence. On the other hand, the various degrees of differences in the target sequence in other species allowed the examination of the discrimination power of the probe. When using suitable hybridization conditions, *C. albicans* and *C. tropicalis* cells, which contain the selected target sequence, could be well discriminated from *C. parapsilosis* following hybridization with the fluorescently labelled probe O20, although the latter species contains only two nucleotide mismatches in the probe target region. This detection is likely to be specific for *C. albicans* and *C. tropicalis* isolates in general as all tested strains of both species reacted with the probe O20 in dot blot hybridizations, whereas no isolate of any other species gave a positive signal. The target sequence of our probe therefore seems to be conserved within the species. Two other *Candida* species, *C. viswanathii* and *C. maltosa*, also contain the identical target sequence, but hybridization of probe O20 to these organisms was not tested in this study as they are not clinically important (Kwon-Chung & Bennett, 1992).

Probe O20 could also distinguish between *C. albicans* and *C. krusei* when a mixed population of both adhered to cultured endothelial cells. Although the adherence of the two species was not quantified in this assay, *in situ* hybridization appears to be a suitable method to study such phenomena in *in vitro* models with mixtures of different *Candida* species.

As a consequence of this study which has demonstrated rRNA-targeted oligonucleotide probes to be applicable for the specific detection of *Candida* species, our present investigations aim at the design of probes specific for single species and also of a probe that recognizes all clinically important members of the genus. Probe O20 is currently used to evaluate the described method for the detection of *Candida* in clinical material. Using a biotinylated, 5S rRNA-targeted oligonucleotide, Montone & Litzky (1995) were able to detect *Aspergillus* hyphae

and conidiospores in tissue specimens from patients with culture-proven aspergillosis. The direct detection by *in situ* hybridization would bring about a fast identification of the infecting organism, as the procedure takes less than a day, in contrast to methods requiring cultivation and phenotypic characterization. Even PCR protocols that rely on hybridization of the amplified product with a species-specific probe for species identification have been described to take 2 d (Jordan, 1994; van Deventer *et al.*, 1995). *Candida* species other than *C. albicans* have been isolated with increasing frequency during the last few years and they differ in their susceptibility to antifungal agents (Wingard *et al.*, 1991, 1993). *C. krusei*, for example, is reported to be intrinsically resistant to the widely used drug fluconazole (Vanden Bossche *et al.*, 1994), and *C. lusitanae* has been associated with amphotericin B resistance (Pfaller *et al.*, 1994). A rapid identification of the infecting species would therefore facilitate the decision about appropriate treatment.

Many epidemiological studies have shown that in cases in which resistance to azoles occurs during therapy, *C. albicans* is often replaced by *C. krusei* or *C. glabrata*, species with higher resistance against these antifungals (for a recent review, see Rex *et al.*, 1995). Most of the studies, however, described only single isolates from each episode, and it is not possible to decide if the newly detected resistant species came from an outside source or if it was already present during previous episodes. Examination of the clinical samples from patients infected with different *Candida* species by fluorescent *in situ* hybridization with probes of various specificities would yield information about the population structure. In addition, mixed infections by fungi and bacteria, e.g. *C. albicans* and *Staphylococcus aureus* or *Enterococcus* spp. (Rüchel *et al.*, 1991) should be detectable by using differently labelled probes which are specific for the respective species.

In conclusion, the study demonstrated that *in situ* hybridization with fluorescently labelled, rRNA-targeted oligonucleotide probes is capable of specifically detecting and identifying *Candida* species. In future, the method may therefore be applied as a new diagnostic tool.

ACKNOWLEDGEMENTS

This study was supported by the Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie (BMBF grant: O1 K1 8906-0) and the Fonds der Chemischen Industrie. We thank Prof. Hannelore Bernhard, Cornelia Heeg and Birgit Hoffmann for the identification and sending of *Candida* strains and Dr Gerwald Köhler and Dr Ingeborg Mühlendorfer for critical reading of the manuscript. Sibylle Schadhauer is gratefully acknowledged for the preparation of the FLUOS-labelled probe O20.

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Received 22 March 1996; accepted 5 July 1996.