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# Direct and specific identification of *Cryptococcus neoformans* in biological samples using fluorescently labelled DNA probes

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**Abstract** Fluorescence in situ hybridisation (FISH) is a suitable technique for the rapid, reliable and cultivation-independent identification of microbial pathogens. This study describes the development of fluorescently labelled rRNA-targeted oligonucleotides and a FISH assay to detect and identify *Cryptococcus neoformans* in culture and biological samples. All *C. neoformans* reference and

clinical isolates gave positive signals with the specific oligonucleotide probes, whereas all non-target yeast species gave negative reactions with the same probes. The assay was also successfully applied to the detection of *C. neoformans* cells in cerebrospinal samples from patients with clinical diagnosis of cryptococcosis. The described FISH-based assay revealed to be practical, sensitive and specific for the detection and identification of *C. neoformans* yeasts.

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## Introduction

*Cryptococcus neoformans* is an encapsulated basidiomycetous yeast present in the environment worldwide. Traditionally, *C. neoformans* has been classified into five serotypes, i.e. A (*C. neoformans* var. *grubii*), D (*C. neoformans* var. *neoformans*), AD (hybrids of serotypes A and D which have antigens of the polysaccharide capsule related to those of the former), B and C (previously known as *C. neoformans* var. *gattii*). Currently, the members of serotypes B and C are considered to belong to a phylogenetically related but distinct species, *C. gattii*. The inhalation of *C. neoformans* sensu lato usually results in a self-limited asymptomatic pulmonary infection. However, *C. neoformans* can also cause disseminated disease, particularly in patients with depressed cell-mediated immunity [1], the meninges being the most common site of extrapulmonary infection. The diagnosis of cryptococcosis is based either on direct microscopic examination of clinical samples, such as cerebrospinal fluid (CSF), or on culture and subsequent phenotypic identification of the aetiological agent. In addition to ambiguous results, those conventional assays are time-consuming and lack sensitivity and specificity [1]. Fluorescence in situ hybridisation (FISH) has

**Table 1** List of strains and species used for the optimisation of the fluorescence in situ hybridisation (FISH) assay and respective hybridisation results

Species	Strain	Serotype <sup>a</sup>	Origin	Hybridisation results			
				Cne448	Cne205	EUK516	EUB338
<i>Cryptococcus neoformans</i>	CN137 <sup>T</sup> (= PYCC 3957 <sup>T</sup> ; CBS 132 <sup>T</sup> )	D	Fermenting fruit juice	+	+	+	–
	CN4	A	CSF of an HIV 2-infected patient, Guine Bissau (1992)	+	+	+	–
	CN23	A	CSF of an HIV 1-infected patient, Curry Cabral's Hospital, Lisbon (1995)	+	+	+	–
	CN27	A	CSF of an HIV 1-infected patient, Angola (1995)	+	+	+	–
	CN34	A	CSF of an HIV 1-infected patient, Military's Hospital, Lisbon (1995)	+	+	+	–
	CN38	AD	Blood of an HIV 1-infected patient, Santa Maria's Hospital, Lisbon (1996)	+	+	+	–
	CN40	AD	CSF of an HIV 1-infected patient, Santa Maria's Hospital, Lisbon (1996)	+	+	+	–
	CN43	A	CSF of an HIV 1-infected patient, Institute of Hygiene and Tropical Medicine, Lisbon (1996)	+	+	+	–
	CN48	D	Skin of an HIV 1-infected patient, Curry Cabral's Hospital, Lisbon (1996)	+	+	+	–
	CN52	AD	Blood of an HIV 1-infected patient, Santa Maria's Hospital, Lisbon (1996)	+	+	+	–
	CN54	A	CSF of an HIV 1-infected patient, Cascais's Hospital, Cascais, Portugal (1996)	+	+	+	–
	CN55	A	CSF of an HIV 1-infected patient, St. António's Hospital, Oporto, Portugal (1996)	+	+	+	–
	CN57	A	CSF of an HIV 1-infected patient, Cascais's Hospital, Cascais (1996)	+	+	+	–
	CN59	A	CSF of an HIV 1-infected patient, Santa Maria's Hospital, Lisbon (1996)	+	+	+	–
	CN65	A	CSF of an HIV 1-infected patient, St Antonio's Hospital, Oporto, Portugal (1996)	+	+	+	–
	CN68	D	Skin of an HIV 1-infected patient, Egas Moniz's Hospital, Lisbon (1996)	+	+	+	–
	CN73	AD	Skin of an HIV 1-infected patient, Curry Cabral's Hospital, Lisbon (1996)	+	+	+	–
	CN80	D	CSF of an HIV 1-infected patient, Institute Pasteur, Paris (1997)	+	+	+	–
	CN88	A	CSF of an HIV-infected patient, St. António's Hospital, Oporto (1997)	+	+	+	–
	CN95	A	CSF of an HIV 1-infected patient, Caxias Prisoner Hospital, Lisbon (1997)	+	+	+	–
	CN100	A	CSF of an HIV 1-infected patient, St. José's Hospital, Lisbon (1997)	+	+	+	–
	CN107	AD	CSF of an HIV 1-infected patient, S. Francisco Xavier's Hospital, Lisbon (1997)	+	+	+	–
	CN112	A	Pigeon faeces, Lisbon	+	+	+	–
	CN312	nd	CSF, St. António's Hospital, Oporto (2008)	nd	+	+	–
	CN313	nd	CSF, St. António's Hospital, Oporto (2008)	nd	+	+	–
	CN314	nd	Bronchial aspirate, St. António's Hospital, Oporto (2008)	nd	+	+	–
	CN315	nd	Blood, St. António's Hospital, Oporto (2008)	nd	+	+	–
	CN316	nd	CSF, St. António's Hospital, Oporto (2008)	nd	+	+	–
	CN317	nd	CSF, St. António's Hospital, Oporto (2008)	nd	+	+	–
	CN318	nd	CSF, St. António's Hospital, Oporto (2008)	nd	+	+	–
	CN319	nd	CSF, St. António's Hospital, Oporto (2008)	nd	+	+	–
	CN320	nd	CSF, St. António's Hospital, Oporto (2008)	nd	+	+	–
CN321	nd	CSF, St. António's Hospital, Oporto (2008)	nd	+	+	–	

**Table 1** (continued)

Species	Strain	Serotype <sup>a</sup>	Origin	Hybridisation results			
				Cne448	Cne205	EUK516	EUB338
	CN322	nd	CSF, St. António's Hospital, Oporto (2008)	nd	+	+	–
	CN323	nd	CSF, St. António's Hospital, Oporto (2008)	nd	+	+	–
	CN324	nd	CSF, St. António's Hospital, Oporto (2008)	nd	+	+	–
	CN325	nd	CSF, St. António's Hospital, Oporto (2008)	nd	+	+	–
	CN326	nd	CSF, St. António's Hospital, Oporto (2008)	nd	+	+	–
	CN327	nd	CSF, St. António's Hospital, Oporto (2008)	nd	+	+	–
	CN328	nd	CSF, St. António's Hospital, Oporto (2008)	nd	+	+	–
	CN329	nd	CSF, St. António's Hospital, Oporto (2008)	nd	+	+	–
	CN330	nd	CSF, St. António's Hospital, Oporto (2008)	nd	+	+	–
	CN331	nd	CSF, St. António's Hospital, Oporto (2008)	nd	+	+	–
	CN332	nd	CSF, St. António's Hospital, Oporto (2008)	nd	+	+	–
	CN333	nd	CSF, St. António's Hospital, Oporto (2008)	nd	+	+	–
	CN334	nd	CSF, St. António's Hospital, Oporto (2008)	nd	+	+	–
	CN335	nd	Blood, St. António's Hospital, Oporto (2008)	nd	+	+	–
	CN336	nd	CSF, St. António's Hospital, Oporto (2008)	nd	+	+	–
	CN337	nd	Bronchoalveolar lavage, St. António's Hospital, Oporto (2008)	nd	+	+	–
	CN338	nd	Urine, St. António's Hospital, Oporto (2008)	nd	+	+	–
	CN339	nd	CSF, St. António's Hospital, Oporto (2008)	nd	+	+	–
	CN340	nd	Blood, St. António's Hospital, Oporto (2008)	nd	+	+	–
	CN341	nd	CSF, St. António's Hospital, Oporto (2008)	nd	+	+	–
	CN342	nd	CSF, St. António's Hospital, Oporto (2008)	nd	+	+	–
	CN343	nd	CSF, St. António's Hospital, Oporto (2008)	nd	+	+	–
	CN344	nd	CSF, St. António's Hospital, Oporto (2008)	nd	+	+	–
	CN345	nd	CSF, St. António's Hospital, Oporto (2008)	nd	+	+	–
	CN346	nd	CSF, St. António's Hospital, Oporto (2008)	nd	+	+	–
	CN347	nd	CSF, St. António's Hospital, Oporto (2008)	nd	+	+	–
	CN348	nd	CSF, St. António's Hospital, Oporto (2008)	nd	+	+	–
	CN349	nd	CSF, St. António's Hospital, Oporto (2008)	nd	+	+	–
<i>Cryptococcus gattii</i>	CN139 (= PYCC 5025)	B	Eucalyptus, Australia	nd	+	+	–
	CBS 10101	C	Cheata, South Africa	nd	+	+	–
<i>Candida albicans</i>	IHMT 2588/96	na	Human oesophagus biopsy (1996)	–	–	+	–
<i>Candida curvata</i>	IHMT 658/97	na	Human vaginal exudate (1997)	–	–	+	–
<i>Candida famata</i>	IHMT 415/97	na	Human faeces (1998)	–	–	+	–
<i>Candida glabrata</i>	PYCC 2418 <sup>T</sup> (= CBS 138 <sup>T</sup> )	na	Human faeces	–	–	+	–
	IHMT 128/98	na	Human vaginal exudate (1998)	–	–	+	–
<i>Candida guilliermondii</i>	IHMT 45/99	na	Blood (1999)	–	–	+	–
<i>Candida kefyr</i>	IHMT 422/98	na	Human vaginal exudate (1998)	–	–	+	–
<i>Candida krusei</i>	IHMT 527/97	na	Human vaginal exudate (1997)	–	–	+	–
<i>Candida tropicalis</i>	IHMT 388/98	na	Human vaginal exudate (1998)	–	–	+	–

**Table 1** (continued)

Species	Strain	Serotype <sup>a</sup>	Origin	Hybridisation results			
				Cne448	Cne205	EUK516	EUB338
<i>Candida parapsilosis</i>	IHMT 195/98	na	Human vaginal exudate (1998)	–	–	+	–
<i>Cryptococcus albidus</i>	PYCC 2409 <sup>T</sup> (= CBS 142 <sup>T</sup> )	na	Atmosphere (Japan)	–	–	+	–
	CN71	na	Blood	–	–	+	–
<i>Cryptococcus laurentii</i>	PYCC 3966 <sup>T</sup> (= CBS 139 <sup>T</sup> )	na	Palmwine (Congo)	–	–	+	–
<i>Debaryomyces hansenii</i>	CN15	na	Pigeon excreta	–	–	+	–
<i>Rhodotorula glutinis</i>	PYCC 4177 <sup>T</sup> (= CBS 20 <sup>T</sup> )	na	Atmosphere	–	–	+	–
<i>Saccharomyces cerevisiae</i>	PYCC 4455 <sup>T</sup> (= CBS 1171 <sup>T</sup> )	na	Brewer's top yeast (Netherlands)	–	–	+	–
<i>Sirobasidium magnum</i>	PYCC 5289	na	Unknown	–	–	+	–
<i>Trichosporon cutaneum</i>	IHMT 256/97	na	Human vaginal exudate (1997)	–	–	+	–

<sup>a</sup> According to Martins [6]

nd = not determined; na = not applicable; T = type strain; CSF = cerebrospinal fluid

All strains are maintained at the Mycology Lab ('CN' and 'IHMT' strains) of the Institute of Hygiene and Tropical Medicine (IHMT/UNL), Lisbon, Portugal, at the Portuguese Yeast Culture Collection ('PYCC' strains), Caparica, Portugal, and at the Centraalbureau voor Schimmelcultures ('CBS' strains), Utrecht, The Netherlands

been widely used for the detection and identification of microorganisms, with emphasis on prokaryotic organisms, allowing the direct microscopic visualisation and quantification of individual cells in their natural environments [2, 3]. FISH has also been used to detect and identify fungal organisms, including clinically relevant yeasts such as *Candida* species [4, 5]. The aim of this study was to optimise and evaluate the practicability, sensitivity and specificity of a FISH-based assay for the identification of *C. neoformans* directly in clinical samples.

## Materials and methods

Sixty-one *C. neoformans* and two *C. gattii* isolates of several origins, most of them obtained from human immunodeficiency virus (HIV)-positive patients, were used in this work (Table 1). Eighteen strains belonging to other yeasts species, including clinical isolates, were used as negative controls (Table 1). All yeasts were grown aerobically under continuous shaking in YM broth (0.3% w/v malt extract, 0.2% yeast extract, 0.5% peptone, 1% glucose) at 25°C. Cells were harvested in the exponential growth phase (optical density of 2.5 at 600 nm) by centrifugation for 5 min at 13,000 rpm. Cells were washed once with 1× phosphate-buffered saline (PBS) (130 mM sodium chloride, 10 mM sodium phosphate buffer, pH 7.2) and fixed for 4 h with 4% w/v paraformaldehyde in PBS at 4°C. After fixation, cells were washed

twice with PBS, resuspended in one volume of PBS and one volume of cold absolute ethanol and stored at –20°C until use. A total of 18 CSF and 10 blood samples from HIV-positive patients with clinical diagnosis of cryptococcosis received for routine conventional testing at the Institute of Hygiene and Tropical Medicine and at the Hospital Santa Maria, both in Lisbon, were also included in this study. Additionally, negative CSF samples were artificially infected with *C. neoformans* PYCC 3957<sup>T</sup> type strain (seven samples) or other clinically relevant yeasts species (seven samples) and used as positive and negative controls, respectively. Each biological sample was also examined for the presence of encapsulated budding yeast by India ink preparation using microscopy (CSF samples), for the presence of cryptococcal polysaccharide antigen by latex agglutination test (Pastorex Crypto Plus, Bio-Rad) (CSF and blood samples) and by culture on Sabouraud glucose agar (CSF samples), according to standard procedures [1, 6]. Additionally, 1 ml of each CSF sample was centrifuged at 13,000 rpm for 5 min to pellet yeast cells, which was then washed once with 500 µl of 1× PBS and fixed with 4% paraformaldehyde as described above.

Blood samples were treated as previously described [7] and the resulting lysates were treated as for CSF samples. Two 26S rRNA-targeted species-specific oligonucleotide probes were designed for *C. neoformans*, which are also complementary to the closely related *C. gattii*: Cne448 (5'-CCAGCCCTTATCCACCGA) and Cne205 (5'-

GTCCGTTACTTGGGAGT). Probes EUK516 (5'-ACCA GACTTGCCCTCC), universal for eukaryotic organisms, and EUB338 (5'-GCTGCCTCCCGTAGGAGT), targeting all bacteria, were used as positive and negative hybridisation controls, respectively [8]. All probes were labelled at their 5'-end with the fluorochrome Cy3 (MWG Biotech, Ebersberg, Germany). Approximately  $10^6$  fixed yeast cells obtained from cultures, and whole fixed pellets obtained from clinical samples, were hybridised in 20  $\mu$ l of hybridisation buffer (0.9 M NaCl, 0.01% w/v SDS, 20 mM Tris-HCl, pH 7.2 and 45% formamide for Cne448, 10% for Cne205 and 20% for EUK516 and EUB338), with 1.5 ng of Cy3-labelled probe and incubated at 46°C for 2 h. After incubation, cells were pelleted by centrifugation and resuspended in 1 ml of prewarmed washing buffer (20 mM Tris-HCl, pH 8.0, 0.01% w/v SDS, 5 mM EDTA and 400 mM NaCl for Cne448, 125 mM for Cne205 and 225 mM for EUK516 and EUB338 probes) for 30 min at 46°C. The suspension was

then centrifuged and the pellet resuspended in 50  $\mu$ l of 1× PBS, placed on ice and analysed within 3 h. For each sample, 10  $\mu$ l of hybridised suspension was spotted onto precleaned microscopic slides and dried at 37°C for 20 min. The slides were then mounted with Vectashield solution (Vector, Burlingame, CA, USA) and examined with an Olympus BX50 microscope, fitted for epifluorescence microscopy with a U-ULH 100W mercury high-pressure bulb and a U-MA1007 filter set for the fluorochrome Cy3 (Olympus). Microphotographs were taken using a digital camera (Olympus C3030-Z00M) and edited using standard software (Adobe Photoshop 6.0, Adobe).

## Results and discussion

Positive hybridisation signals were determined as bright orange fluorescent yeast cells. All 61 *C. neoformans* and

**Table 2** List of cerebrospinal fluid (CSF) and blood samples analysed by conventional diagnostic methods and by FISH with the probe Cne205

IHMT no.	Sample	Microscopy with India ink <sup>a</sup>	Sabouraud culture <sup>b</sup>	Title of cryptococcal polysaccharide antigen <sup>c</sup>	FISH <sup>d</sup>
383/97	CSF	Negative	Negative	1:10,000	++
393/97	CSF	Negative	Negative	1:1,000	++
397/97	CSF	Negative	Positive	1:10,000	++
421/97	CSF	Positive	Negative	1:10,000	++
580/97	CSF	Positive	Positive	1:1,000	++
594/97	CSF	Positive	Negative	1:10,000	++
600/97	CSF	Positive	Positive	1:10,000	++
37/99	CSF	Positive	Positive	1:10,000	++
59/99	CSF	Negative	Negative	Negative	Negative
277/99	CSF	Positive	Positive	1:10,000	++
99/01	CSF	Positive	Positive	1:2,048	++
167/01	CSF	Positive	Positive	1:1,000	++
186/01	CSF	Positive	Positive	1:10,000	++
190/01	CSF	Negative	Negative	Negative	Negative
197/01	CSF	Negative	Negative	Negative	Negative
202/01	CSF	Negative	Negative	Negative	Negative
54/02	CSF	Positive	Positive	1:1,000	++
274/02	CSF	Negative	Positive	1:1,500	++
394/97	Blood	nd	nd	1:10,000	+
523/97	Blood	nd	nd	1:10,000	+
525/97	Blood	nd	nd	1:10	Negative
595/97	Blood	nd	nd	1:10,000	+
597/97	Blood	nd	nd	1:10,000	+
621/97	Blood	nd	nd	1:1,000	+
625/97	Blood	nd	nd	1:1,000	+
187/01	Blood	nd	nd	1:10,000	+
110/02	Blood	nd	nd	1:100	+
275/02	Blood	nd	nd	1:1,000	Negative

<sup>a</sup> Positive: detection of encapsulated yeast cells on samples

<sup>b</sup> Positive: *Cryptococcus neoformans* colonies detected

<sup>c</sup> Positive: detection of *C. neoformans* antigen presence in the clinical sample

<sup>d</sup> Detection of fluorescent yeast cells with FISH probe Cne205: ++, bright and intense fluorescently labelled yeast cells; +, yeast cells with a faint fluorescent signal; Negative, no fluorescent yeast cells detected

CSF = cerebrospinal fluid; nd = not determined

two *C. gattii* isolates gave positive signals with Cne448 and/or Cne205 probes, whereas all non-target species gave negative reactions with the same probes (Table 1). It was also possible to identify *C. neoformans* cells with the specific probes in mixed samples with other yeast species. All yeast species tested yielded strong fluorescent signals with the universal probe EUK516. None of the yeast cells showed any fluorescent signal with the EUB338 probe (Table 1). FISH with the Cne205 probe always showed stronger fluorescent signals than with the Cne448 probe. This can be explained since the complementary target of the later probe is located in a low-accessibility rRNA region for FISH [9]. Probe Cne205, which presented the brightest fluorescent signals, was evaluated for the detection of *C. neoformans* in clinical samples. A group of cerebrospinal and serum fluid samples was firstly examined and the direct identification of *C. neoformans* cells in all artificially infected samples was achieved. The assay was then applied in cerebrospinal and serum samples from patients with clinical diagnosis of cryptococcosis, in parallel with other conventional diagnostic methods (Table 2). Ten culture-positive CSF samples revealed to be also positive by FISH. Additionally, four culture-negative CSF samples revealed to be FISH-positive (two of these samples were also positive by direct microscopy with India ink; cryptococcal antigens were detected in all four). The detection limit of our Cne205 probe-based FISH assay was estimated to be  $10^2$  cells/ml when using serially diluted artificially spiked CSF samples (data not shown). Four CSF samples were negative by all tests performed. The detection of *C. neoformans* from blood samples was more difficult, with cells presenting less intense fluorescence signals (Table 2).

Other groups have recently reported positive outcomes on the introduction of FISH-based assays in the routine of their clinical laboratories [4, 10]. The incorporation of these assays as part of the initial yeast laboratorial identification algorithm can result in substantial savings for hospitals [4]. FISH associates the fastness, sensitivity and specificity of molecular diagnostic methods with the observation of morphologic features of the aetiological agents of disease, contributing to a reliable identification of the pathogens directly in their substrates and leading to the consequent definition of the most appropriate treatment. To the best of our knowledge, we describe in this study the first FISH-based assay for the detection and identification of *C. neoformans* yeasts, which revealed to be practical, sensitive

and specific when compared to the conventional methods. FISH may constitute a valuable complementary tool to conventional *C. neoformans* laboratory analysis, especially when using CSF samples. It can be automated and implemented in hospital laboratories with a great number of analyses per day.

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