Identification of *Candida* species in formalin fixed, paraffin wax embedded oral mucosa by sequencing of ribosomal DNA

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Abstract

Aim—To identify *Candida* species in formalin fixed, paraffin wax embedded tissue by sequencing candidal rDNA.

Methods—Target rDNA sequences were amplified by polymerase chain reaction (PCR) from fresh isolates of *Candida* and from 18 preserved oral mucosal tissue samples (16 cases of chronic hyperplastic candidiasis and two fibroepithelial polyps), shown histologically to contain *Candida*. Identification of *Candida* species within tissue was based on a comparison of the rDNA sequences obtained with those from the fresh isolates of *Candida* and those present in the GenBank database.

Results—The PCR products obtained from 12 of the 18 tissue specimens studied were characteristic of *Candida albicans*. In two of these cases a second, larger PCR product was obtained and these sequences were characteristic of *Candida glabrata*.

Conclusions—Candidal DNA was amplified successfully from formalin fixed, paraffin wax embedded tissue. Sequencing of the PCR product enabled identification of the *Candida* species present.

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Keywords: *Candida*, PCR, rDNA, formalin fixed paraffin wax embedded sections.

The use of modern chemotherapeutic regimens such as immunosuppressive and corticosteroid therapies has been associated with an increase in the incidence of opportunistic infections involving *Candida*.^{1,2} Although deep seated candidal infection in immunocompromised individuals can arise,³ superficial infections of the oral and vaginal mucosa are the most frequently occurring forms of candidiasis.² Confirmation of these infections is made by culture and identification of *Candida* isolates. *Candida albicans* is regarded as the most pathogenic species but other species, such as *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, *C. guilliermondii*, and *C. krusei*, have also been implicated in human disease.⁴

Chronic hyperplastic candidiasis (CHC), a form of candidiasis which affects the oral mucosa, has received particular attention as it has been associated with the development of intra-oral malignancy.⁵ The isolation of *Candida* from the mucosal surface is not sufficient for a diagnosis of CHC because *Candida* are often re-

covered in low numbers and differentiation from the level accepted as normal commensal carriage is difficult.⁶ Diagnosis of CHC, therefore, is based on microscopic examination of lesional tissue for the presence of structures consistent with fungal hyphae. Unfortunately, techniques confirming these structures to be candidal hyphae would not seem to have been reported.

Human soft tissue biopsy material is routinely preserved by fixation in formalin and subsequent embedding in paraffin wax. The advent of versatile gene amplification technologies, including the polymerase chain reaction (PCR),⁷ has enabled the rapid and sensitive amplification of DNA from such archival material. Human gene abnormalities, viral DNA and bacterial DNA have been detected in formalin fixed, paraffin wax embedded tissue using PCR and other molecular techniques.⁸⁻¹¹ However, procedures based on molecular biology have not been reported for the detection of fungi in archival tissue.

Candidal rDNA sequences have been used as a basis for the identification of clinical isolates of medically important *Candida* species either by sequencing or restriction fragment length polymorphism analysis.¹²⁻¹⁴ The rDNA sequences of *Candida* provide suitable targets for PCR as they comprise both conserved and variable regions. Conserved regions are ideal targets for the design of PCR primers whilst the intervening variable regions are often species or strain specific.

The aim of the present study was to develop an approach based on sequence analysis of candidal rDNA regions that would permit identification of *Candida* within formalin fixed, paraffin wax embedded samples of oral mucosa.

Methods

Pure cultures of 11 *Candida* isolates, shown in table 1, were initially examined to establish a reference base of product size and sequence. The identity of all isolates was confirmed by germ tube formation and use of the API-20C carbohydrate assimilation system (bioMérieux; Basingstoke, UK). Isolates were cultured for 24 hours in yeast nitrogen base medium (Difco, East Moseley, UK) at 37°C, and the DNA extracted as described previously.¹⁴ Five microlitres (approximately 100 µg/ml DNA) of the extract was added to 45 µl water and used as template for PCR.

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Table 1 PCR product sizes obtained by amplification of rDNA regions from 11 *Candida* isolates using three primer pairs

Candida isolate	Primer pair and PCR product size (bp)		
	Pcon1 and Pcon2	ITS1 and ITS2	ITS1 and ITS4
<i>C. albicans</i> 121	105*	218	535*
<i>C. albicans</i> 324	105	218	535*
<i>C. albicans</i> 655	105*	218	535* (L47111)
<i>C. tropicalis</i> 340	105*	218	524* (L47112)
<i>C. tropicalis</i> 722	105	218	524*
<i>C. stellatoidea</i> NCPF 3108	105	219	540* (L47114)
<i>C. parapsilosis</i> 937A	105*	229	520* (L47109)
<i>C. krusei</i> 3165	105*	183	510* (L47113)
<i>C. guilliermondii</i> 657b	105*	249*	608* (L47110)
<i>C. kefyr</i> (pseudotropicalis) Y06.07	105*	309	720* (L47107)
<i>C. (Torulopsis) glabrata</i> 955	105*	478* (L47108)	878

bp = base pairs; *products sequenced; GenBank accession number given in brackets.

The archival tissue material had originally been fixed in at least a 10-fold volume of 10% buffered formal saline for 24 hours prior to processing through graded alcohol and xylene and embedding in paraffin wax. Eighteen blocks of tissue shown to contain structures consistent with candidal hyphae on staining with periodic acid-Schiff (PAS) were selected for further study.

Positive control material was prepared by re-suspending *C. albicans* (strain 655) in 0.4% molten agarose which, after solidifying, was preserved in the same manner as test tissues.

Sections (2 × 20 µm) of archival and positive control material were placed in 1.5 ml microtubes and deparaffinised by the addition of xylene (1 ml) for 10 minutes. After centrifugation (12 500 rpm for 10 minutes), the pelleted material was washed once in ethanol and dried. The dried material was resuspended in 200 µl proteinase K buffer (1 mg/ml proteinase K (Sigma, Poole, Dorset, UK), 50 mM Tris/HCl (pH 8.5), 1 mM EDTA, and 0.5% Tween 20) and incubated at 37°C for six days. The solution was then heated to 95°C for 30 minutes to inactivate proteinase K, and 50 µl of the cooled solution used as template for PCR.

Four pairs of PCR primers were used (table 2). The PCR conditions for each set of primers have been described previously.^{11 14 16} Negative controls using sterile water in place of DNA were included alongside each amplification reaction.

The presence and size of PCR products were assessed by agarose gel electrophoresis and ethidium bromide staining.¹⁷ Purification of the PCR products from the tissues and cultures of *Candida* was achieved using the QIAquick Spin PCR Purification Kit (Qiagen, Dorking, UK). In cases where two distinct PCR products of

differing sizes resulted from the same tissue, isolation and purification of each product from the agarose gel was performed using a similar, commercially available system (QIAquick Gel Extraction Kit; Qiagen).

Sequencing of both strands of the purified candidal PCR products (0.5 µg) was performed using the original PCR primers and the Prism Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Perkin Elmer), in accordance with the manufacturer's instructions. The resulting dideoxy products were analysed using an Applied Biosystems 373A automated sequencer. The sequences obtained and those retrieved from GenBank and EMBL databases (SERC, Daresbury Laboratory, Warrington, UK) were aligned using the CLUSTAL v suite of programs, and distance values calculated with the DNA DIST software program (University of Wisconsin Genetics Computer Group).¹⁸

Results

PCR AMPLIFICATION

Table 1 illustrates the product size (determined by gel electrophoresis and sequencing) following PCR amplification of DNA extracted from 11 pure cultures of *Candida*. PCR products of the same size (105 base pairs) were obtained with all isolates tested with primers Pcon1 and Pcon2. Using the ITS1 and ITS2 primer pair, isolates identified as *C. krusei*, *C. guilliermondii*, *C. kefyr* (pseudotropicalis), and *C. (Torulopsis) glabrata* yielded products of 183, 249, 309, and 478 base pairs, respectively. In the case of the remaining strains, similarly sized products (220–230 base pairs) were obtained (fig 1A). Using primers ITS1 and ITS4, isolates identified as *C. guilliermondii*, *C. kefyr* (pseudotropicalis) and *C. (Torulopsis) glabrata* yielded product sizes of 608, 720 and 878 base pairs, respectively. Products of 510–540 base pairs were obtained from the remaining strains (fig 1B).

PCR AMPLIFICATION OF DNA IN FORMALIN FIXED, PARAFFIN WAX EMBEDDED MATERIAL

Table 3 shows the results of PCR performed on DNA extracted from formalin fixed, paraffin wax embedded material with the different primer pairs. Amplification of human β-actin gene sequences (202 base pairs) with primers AW75 and AW76 occurred in 15 of the 18 tissue samples tested. Products of the expected size were also observed with *C. albicans* embedded in the positive control agarose material using each of the three primer pairs directed

Table 2 Primers used to amplify DNA extracted from *Candida* isolates and archival tissue

Primer	Primer sequence (5'–3')	Target DNA	Expected product size (bp)	Reference
AW75 AW76	CCTTCCTGGGCATGGAGTCCTG GGAGCAATGATCTTGATCTTC	Human β-actin DNA	202	Ghossein <i>et al</i> ¹¹
ITS1 ITS4	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTTGATATGC	ITS1, 5.8S and ITS2 rDNA regions	535 (<i>C. albicans</i>)	White <i>et al</i> ¹⁵
ITS1 ITS2	TCCGTAGGTGAACCTGCGG GCTGCGTTCTTCATCGATGC	ITS1 rDNA region	218 (<i>C. albicans</i>)	White <i>et al</i> ¹⁵
Pcon1 Pcon2	AGTTTCGCGTATGGTCTCCC GTTGCGGCCATATCTAGCAG	Candidal 5S rDNA	105 (<i>C. albicans</i>)	Holmes <i>et al</i> ¹⁶

Figure 1 (A) PCR amplification of candidal rDNA with primers ITS1 and ITS2. (B) PCR amplification of candidal rDNA with primers ITS1 and ITS4. Lanes 1 to 8 contain DNA from *C. glabrata*, *C. guilliermondii*, *C. kefyr*, *C. krusei*, *C. parapsilosis*, *C. stellatoidea*, *C. tropicalis*, and *C. albicans*, respectively. Lane 9 contains a molecular weight marker.

against candidal rDNA. Twelve of the 18 tissue samples yielded PCR products of about 105 base pairs with the Pcon1/Pcon2 primer pair. Products of 218 base pairs were also obtained

Figure 2 PCR amplification of candidal rDNA extracted from formalin fixed, paraffin wax embedded tissue with primers ITS1 and ITS2. Lanes 1 to 6 contain DNA from the following formalin fixed tissue samples: D92.1639, D92.865, D95.227, D92.791, D95.126, and D88.169. Lanes 7 and 8 contain DNA from *C. krusei* and a water control, respectively. Lane 9 contains a molecular weight marker.

from these samples using the ITS1/ITS2 primer pair (fig 2). In two of these 12 tissue samples a second, larger PCR product (478 base pairs) was also observed. Using primers ITS1 and ITS4, 535 base pair PCR products were generated from two of the 18 samples tested.

DIRECT SEQUENCING OF PCR PRODUCTS

The Pcon1/Pcon2 PCR product from five tissue samples and eight *Candida* cultures was sequenced (tables 1 and 3). When Pcon1/Pcon2 product sequences generated from pure cultures of *Candida* and database sequences (*C. parapsilosis*, EMBL accession no. D10535 and *C. albicans*, EMBL accession no. X16634) were compared, it was apparent that there was insufficient variation to enable speciation.

Sequencing of PCR products obtained from pure cultures of *Candida* (table 1) with the ITS1 and ITS2 primers showed that each *Candida* species yielded an identifiable product se-

Table 3 PCR product sizes obtained by amplification of DNA from formalin fixed, paraffin wax embedded tissues using four different primer pairs

Tissue reference	Origin of tissue†	Primer pair and product size (bp)			
		AW75 and AW76	Pcon1 and Pcon2	ITS1 and ITS2	ITS1 and ITS4
Positive control					
D88.169	CHC	202	105	218	535
D88.389	CHC	No product	105*	218*	No product
D88.404	CHC	No product	No product	No product	No product
D89.912	CHC	202	No product	No product	No product
D90.665	CHC	202	105	218 and 479	No product
D91.697	CHC	202	105	218	No product
D91.98	CHC	202	No product	No product	No product
D92.641	CHC	202	105	218*	No product
D92.791	CHC	202	No product	No product	No product
D92.865	CHC	202	105*	218 and 479*	No product
D92.1000	CHC	No product	105*	218*	No product
D92.1639	CHC	202	No product	No product	No product
D93.1497	CHC	202	105*	218*	No product
D93.1498	CHC	202	105	218*	No product
D93.1521	CHC	202	105*	218	535
D95.126	Fibroepithelial polyp	202	105*	218	535
D95.227	Fibroepithelial polyp	202	105	218	No product
D95.400	CHC	202	105	218*	No product
			No product	No product	No product

† Tissues previously shown to contain structures consistent with *Candida* hyphae by microscopic examination; CHC = chronic hyperplastic candidiasis; * products sequenced.


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C albicans X71088† TCCGTAGGTGAACCTGCGGAAGGATCATTACTGATTTGCTTAATTGCACC
C albicans 655 TCCGTAGGTGAACCTGCGGAAGGATCATTACTGATTTGGTGAATTGCACC
C tropicalis 340 TCCGTAGGTGAACCTGCGGAAGGATCATTACTGATTTGCTTAATTGCACC
C krusei 3165 TCCGTAGGTGAACCTGCGGAAGGATCATTACTG- -TGATTTA- - -GTACT
D88.169 TCCGTAGGTGAACCTGCGGAAGGATCATTACTGATTTGCTTAATTGCACC
D91.98 TCCGTAGGTGAACCTGCGGAAGGATCATTTXCTGATTTGCXTAATTGCACC
D92.865 TCCGTAGGTGAACCTGCGGAAGGATCATTACTGATTTGCTTAATTGCACC
D92.1639 TCCGTAGGTGAACCTGCGGAAGGATCATTACTGATTTGCTTAATTGCACC
D93.1497 TCCGTAGGTGAACCTGCGGAAGGATCATTACTGATTTGCTTAATTGCACC
D95.227 TCCGTAGGTGAACCTGCGGAAGGATCATTACTGATTTGCTTAATTGCACC
***** ** * * *

C albicans X71088† ACATGTGTTTTCTTTGAAACAACTTGCTTTGGCGGTGGGCC- CAGCCT
C albicans 655 ACATGTGTTTTCTTTGAAACAACTTGCTTTGGCGGTGGGCC- CAGCCT
C tropicalis 340 ACATGTGTTTTTATTG- AACAAATTT- CTTTGGTGGCGGGAGCAATCCT
C krusei 3165 ACAC- TGCGTGAGC- -GGAACGAAX- - - - -ACAACAA- - - -CACCT
D88.169 ACATGTGTTTTGTTTTGGA- CAAACTTGCTTTGGCGGTGGGCCTCTACCT
D91.98 ACATGTGTTTTGTTTTGGA- CCAACTTGCTTTGGCGGTGGGCCTCTACCT
D92.865 ACATGTGTTTTGTTTTGGA- CAAACTTGCTTTGGCGGTGGGCCTCTACCT
D92.1639 ACATGTGTTTTGTTTTGGA- CAAACTTGCTTTGGCGGTGGGCCTCTACCT
D93.1497 ACATGTGTTTTCTTTGAAACAACTTGCTTTGGCGGTGGGCC- CAGCCT
D95.227 ACATGTGTTTTCTTTGAAACAACTTGCTTTGGCGGTGGGCC- CAGCCT
*** ** * * * * *

C albicans X71088† GCCGCCAGAGGTC- TAAACTTACAACCA- ATTTTTTATCAACTTGTACAC
C albicans 655 GCCGCCAGAGGTC- TAAACTTACAACCA- ATTTTTTATCAACTTGTACAC
C tropicalis 340 ACCGCCAGAGGTTATAA- -CTA- AACCAAACTTTTATTTACA- GTCAAA
C krusei 3165 - - - - -AAAATGTGGAAT- ATAGC- - - - -ATATAGTCGACAAG
D88.169 GCCGCCAGAGGACATAAACTTACAACCAATTTTTTATAAACTTGTACAG
D91.98 GCCGCCAGAGGACATAAACTTACAACCAATTTTTTATAAACTTGTACAG
D92.865 CCCGCCAGAGGACATAAACTTCCAACCAATTTTTTATACACTTGTACAG
D92.1639 CCCGCCAGAGGACATACTCACAACCATATTTTTTATAAACTTGTACAG
D93.1497 GCCGCCAGAGGTC- TAAACTTACAACCA- ATTTTTTATCAACTTGTACAC
D95.227 GCCGCCAGAGGTC- TAAACTTACAACCA- ATTTTTTATCAACTTGTACAC
* * * * *

C albicans X71088† CCAGATT- ATTACT- TAATAGTC- AAAACTTTCAACAACGGATCTCTTGG
C albicans 655 CCAGATT- ATTACT- -AATAGTC- AAAACTTTCAACAACGGATCTCTTGG
C tropical 340 CTTGATTTATTATTACAATAGTC- AAAACTTTCAACAACGGATCTCTTGG
C krusei 3165 AGAAATCTACGAXA- -AACAXACAAAACCTTACTAACAACGGATCTCTTGG
D88.169 - -AGATT- ATT- TT- TAATAGTC- AAAACTTTCAACAACGGATCTCTTGG
D91.98 - -AGATT- ATT- TT- TAATAGTC- AAAACTTTCAAXAACGGATCTCTTGG
D92.865 - -AGATT- ATT- TT- TAATAGAC- AAAACTTTCAACAACGGATCTCTXGG
D92.1639 - -AGACT- ATT- TT- TAATAGTCCAAAACCTTCAACAACGGATCTCTTGG
D93.1497 CCAGATT- ATTACT- -AATAGTC- AAAACTTCCAACAACGGATCTCTTGG
D95.227 CCAGATT- ATTACT- TAATAGTC- AAAACTTTCAACAACGGATCTCTTGG
* * * * *

C albicans X71088† TTCTCGCATCGATGAAGAACGCAGC
C albicans 655 TTCTCGCATCGATGAAGAACGCAGC
C tropicalis 340 TTCTCGCATCGATGAAGAACGCAGC
C krusei 3165 TTCTCGCATCGATGAAGAACGCAGC
D88.169 TTCTCGCATCGATGAAGAACGCAGC
D91.98 TXCTCGCATCGATGAAGAACGCAGC
D92.865 TXCTCGCATCGATGAAGAACGCAGC
D92.1639 GTCTCGCATCGATGAAGAACGCAGC
D93.1497 TTCTCGCATCGATGAAGAACGCAGC
D95.227 TXCTCGCATCGATGAAGAACGCAGC
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† = EMBL accession number; * indicates identical bases.

Figure 3 CLUSTAL V multiple sequence alignment of ITS1 and ITS2 PCR products amplified from pure cultures of *Candida* and archival tissue.

Table 4 DNA distance values for ITS1 and ITS2 primer generated PCR products amplified from pure cultures of *Candida* and archival tissue

	C albicans EMBL X71088	C albicans 655	C tropicalis 340	C krusei 3165	D88.169	D91.98	D92.865	D92.1639	D93.1497	D95.227
<i>C. albicans</i>										
EMBL X71088	0-0000									
<i>C. albicans</i> 655	0-0140	0-0000								
<i>C. tropicalis</i> 340	0-1078	0-1206	0-0000							
<i>C. krusei</i> 3165	0-3856	0-4103	0-3625	0-0000						
D88.169	0-0436	0-0594	0-1216	0-3324	0-0000					
D91.98	0-0497	0-0660	0-1305	0-3441	0-0047	0-0000				
D92.865	0-0650	0-0819	0-1354	0-3356	0-0190	0-0243	0-0000			
D92.1639	0-0693	0-0861	0-1524	0-3612	0-0284	0-0340	0-0387	0-0000		
D93.1497	0-0046	0-0188	0-1089	0-3794	0-0488	0-0550	0-0706	0-0748	0-0000	
D95.227	0-0000	0-0141	0-1089	0-3914	0-0438	0-0497	0-0650	0-0697	0-0046	0-0000

base pairs) generated from the test tissue samples revealed greatest homology with the product obtained from *C. albicans*, and also concurred with previously reported sequences (EMBL accession no. X71088) for the targeted region of *C. albicans* rDNA (fig 3, table 4). The sequence of the larger (478 base pairs) PCR product obtained from tissue D92.791 had greatest homology with sequences obtained from the pure culture of *C. glabrata*.

Discussion

The presence of bacteria, protozoa and viruses in formalin fixed, paraffin wax embedded material has been demonstrated by PCR.^{9-11 19 20} However, the detection of fungi in such material using gene amplification techniques has not been reported. The results of the present study have demonstrated that it is possible to detect and identify *Candida* species present in formalin fixed, paraffin wax embedded tissue by analysis of the primary structure of PCR amplified rDNA.

C. albicans was detected in 12 of the 18 specimens examined. *C. glabrata* was also present in two of these 12 samples. PCR products were not obtained with any of the four primer pairs used in three samples, whilst candidal DNA was not amplified in six. There are several possible explanations for the failure to produce a PCR product. Previous studies using PCR on formalin fixed, paraffin wax embedded tissues have noted the presence of endogenous inhibitors originating from the tissue.²¹ Furthermore, the size of extracted DNA may be relevant as degradation of target DNA during fixation in formalin has been documented.²² In this study three different fungal specific primer pairs were assessed in separate PCR protocols. Amplification was less successful as the length of target increased. This observation could be related to the decreased efficiency of the PCR on larger targets in formalin fixed material; the intermittent failure of PCR to amplify target DNA greater than 300 base pairs has been reported.²¹ The relatively high number of *Candida* within the agarose control preparation compared with the levels detected in the tissue samples may have partially compensated for this problem and permitted amplification of 535 base pair targets in this material.

Although PCR products were generated by both the Pcon1/Pcon2 and ITS1/ITS2 primer pairs, only the latter products were found to

be suitable for the identification of *Candida* species. These primers amplified targets in 12 of the 18 specimens tested and sequencing of these products enabled the identification of both *C. albicans* and *C. glabrata*. Products identified as originating from *C. glabrata* were obtained from two samples, along with products characteristic of *C. albicans*. This finding may indicate infection involving two species of *Candida*.

The ability to identify *Candida* within human tissue provides an opportunity to increase our knowledge of the role of candidal species in disease. This is particularly important for patients with CHC because of the association between this form of candidiasis and the development of oral cancer.

In conclusion, this study has demonstrated that *Candida* species can be detected and identified in formalin fixed, paraffin wax embedded tissues by sequencing PCR amplified candidal rDNA sequences.

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