

SHORT REPORT

Comparison of API20C with molecular identification of *Candida* spp isolated from bloodstream infections

J Xu, B C Millar, J E Moore, R McClurg, M J Walker, J Evans, S Hedderwick, R McMullan

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A study was carried out to compare the API20C technology with polymerase chain reaction amplification and direct sequencing of the short internal transcribed spacer region 2 (ITS2) for the identification of 58 isolates of invasive candida species obtained from patients with bloodstream infections over the seven year period 1994 to 2000. Overall, there was only one disagreement between the phenotypic and genotypic identification, where the API scheme identified the isolate as *C albicans* but the molecular method identified it as *C dubliniensis*. This study demonstrated that the API20C method is useful in the identification of *Candida* spp isolated from blood culture and that molecular methods do not enhance identifications made using the API20C scheme. However, for correct reporting of *C dubliniensis*, an emerging bloodborne pathogen, it is recommended that all isolates identified as *C albicans* by the API20C scheme are further examined phenotypically and/or genotypically.

Candida species are important fungal pathogens in several patient populations, particularly when causing bloodstream infections. There have been recent reports on the misidentification of several candida species when using phenotypic identification techniques.^{1,2} More recently, molecular identification techniques for fungi have been described, which allow for more accurate and rapid identification of *Candida* spp based on differences within the ribosomal RNA.³ Of these methods, polymerase chain reaction (PCR) amplification of the short internal transcribed spacer 2 (ITS2) region has been described as a rapid and reliable method to characterise yeast infections. At present, most laboratories in the UK use a combination of microscopic tests together with a commercial test system, such as the API20C, API32C, or API candida schema for the identification of clinically important yeasts. Previous work^{4,5} showed that the API20C scheme was more reliable at identifying common (97%) and rare (88%) yeast isolates than the API32C scheme, which could reliably identify 92% of common yeasts and 85% of rare isolates, but there have been few data comparing the use of the API schemes with the recently described ITS sequence based identification methods. Therefore, this study aimed to carry out a comparison of the API20C identification scheme with molecular identification using the ITS rRNA regions. In addition, we wanted to evaluate laboratory parameters associated with these respective phenotypic and genotypic techniques, to help provide guidance on their advantages and disadvantages in diagnostic applications.

"Our study aimed to carry out a comparison of the API20C identification scheme with molecular identification using the internal transcribed spacer region rRNA regions"

MATERIALS AND METHODS

Candida spp isolates (n = 58) were obtained from the blood cultures of 58 patients with bloodborne candidiasis attending Belfast City Hospital during the period 1994 to 2000. All isolates were stored at -80°C in accordance with the method of Moore *et al.*⁶ Isolates were resuscitated from storage on to Sabouraud dextrose agar (Oxoid CM0041; Oxoid Ltd, Basingstoke, UK), and passaged at least twice before phenotypic and genotypic characterisation.

All DNA isolation procedures were carried out in a class II biological safety cabinet in a room geographically separate from that used to set up the reaction mixes and also from the "post-PCR" room to minimise the production of false positive results. Total genomic fungal DNA was extracted from a single colony using the Roche high purity PCR template kit (Roche Diagnostics, East Sussex, UK), in accordance with the manufacturer's instructions, following pretreatment with lyticase (Boehringer Mannheim, East Sussex, UK), as described previously.⁷ All reaction mixes were set up in a PCR hood in a room separate from that used to extract DNA and the amplification and post-PCR rooms to minimise contamination. Reaction mixes (50 µl) were set up as follows: 10mM Tris/HCl, pH 8.3, 50mM KCl, 2.5mM MgCl₂, 200µM (each) dATP, dCTP, dGTP, and dTTP, 1.25 U of Taq DNA polymerase (Amplitaq; Perkin Elmer, Cheshire, UK), 0.2µM (each) of the ITS2 region primers (ITS3/ITS4; namely: ITS3, 5'-GCA TCG ATG AAG AAC GCA GC-3' and ITS4 (reverse) 5'-TCC TCC GCT TAT TGA TAT GC-3', as described previously),³ and 4 µl of DNA template. After a "hot start", the reaction mixtures were subjected to the following thermal cycling parameters in a Perkin Elmer 2400 thermocycler: 96°C for three minutes, followed by 40 cycles of 96°C for one minute, 55°C for one minute, and 72°C for one minute, with a final extension at 72°C for 10 minutes. During each run molecular grade water (LAL Grade; Biowhittaker Inc, Walkersville, Maryland, USA) samples were included randomly as negative controls. Where there was disagreement between the phenotypic and genotypic result using the ITS2 region, a further PCR reaction was set up, amplifying the ITS1-5.8S-ITS2 region, using the primers ITS1 (forward) 5'-TCC GTA GGT GAA CCT GCG G-3' and ITS4 (reverse), as described above. After amplification, aliquots (15 µl) were removed from each reaction mixture and examined by electrophoresis (80 V, 45 minutes) in gels composed of 2% (wt/vol) agarose (Gibco, Paisley, UK) in TAE buffer (40mM Tris, 20mM acetic acid, 1mM EDTA, pH 8.3), stained with ethidium bromide (5 µg/100ml). Gels were visualised under ultraviolet illumination using a gel image analysis system (UVP Products, UK) and all images archived as digital (*.bmp) graphic files.

All amplicons were purified using a QIAquick PCR purification kit (Qiagen, Crawley, Sussex, UK) eluted in Tris/HCl

Abbreviations: ITS, internal transcribed spacer region; PCR, polymerase chain reaction

Table 1 Comparison of phenotypic and genotypic identification within a collection of 58 invasive *Candida* spp isolated over the seven year period, 1994–2000

Isolate number	Phenotypic identification (API 20C)	Molecular identification	Small ITS region (ITS3/ITS4)	
			Base pairs	% Homology
1	<i>C albicans</i>	<i>C albicans</i>	295	100
2	<i>C albicans</i>	<i>C albicans</i>	299	100
3	<i>C albicans</i>	<i>C albicans</i>	290	100
4	<i>C albicans</i>	<i>C albicans</i>	303	99
5	<i>C albicans</i>	<i>C albicans</i>	302	99
6	<i>C albicans</i>	<i>C albicans</i>	293	99
7	<i>C parapsilosis</i>	<i>C parapsilosis</i>	276	99
8	<i>C parapsilosis</i>	<i>C parapsilosis</i>	267	99
9	<i>C tropicalis</i>	<i>C tropicalis</i>	295	100
10	<i>C krusei</i>	<i>Issachenkia orientalis*</i>	289	99
11	<i>C krusei</i>	<i>Issachenkia orientalis*</i>	271	99
12	<i>C parapsilosis</i>	<i>C parapsilosis</i>	263	99
13	<i>C krusei</i>	<i>I orientalis*</i>	271	99
14	<i>C albicans</i>	<i>C albicans</i>	294	100
15	<i>C tropicalis</i>	<i>C tropicalis</i>	289	99
16	<i>C parapsilosis</i>	<i>C parapsilosis</i>	277	99
17	<i>C glabrata</i>	<i>C glabrata</i>	320	99
18	<i>C glabrata</i>	<i>C glabrata</i>	332	100
19	<i>C albicans</i>	<i>C albicans</i>	393	100
20	<i>C glabrata</i>	<i>C glabrata</i>	344	100
21	<i>C albicans</i>	<i>C albicans</i>	264	100
22	<i>C albicans</i>	<i>C albicans</i>	289	99
23	<i>C albicans</i>	<i>C dubliniensis</i>	299	100
24	<i>C albicans</i>	<i>C albicans</i>	259	100
25	<i>C albicans</i>	<i>C albicans</i>	269	98
26	<i>C albicans</i>	<i>C albicans</i>	280	100
27	<i>C glabrata</i>	<i>C glabrata</i>	343	99
28	<i>C albicans</i>	<i>C albicans</i>	271	100
29	<i>C albicans</i>	<i>C albicans</i>	294	100
30	<i>C albicans</i>	<i>C albicans</i>	299	100
31	<i>C albicans</i>	<i>C albicans</i>	287	100
32	<i>C glabrata</i>	<i>C glabrata</i>	357	100
33	<i>C glabrata</i>	<i>C glabrata</i>	333	100
34	<i>C glabrata</i>	<i>C glabrata</i>	376	100
35	<i>C parapsilosis</i>	<i>C parapsilosis</i>	272	99
36	<i>C glabrata</i>	<i>C glabrata</i>	300	100
37	<i>C parapsilosis</i>	<i>C parapsilosis</i>	270	99
38	<i>C parapsilosis</i>	<i>C parapsilosis</i>	271	99
39	<i>C tropicalis</i>	<i>C tropicalis</i>	278	100
40	<i>C dubliniensis</i>	<i>C dubliniensis</i>	285	100
41	<i>C albicans</i>	<i>C albicans</i>	289	100
42	<i>C albicans</i>	<i>C albicans</i>	283	100
43	<i>C albicans</i>	<i>C albicans</i>	282	100
44	<i>C albicans</i>	<i>C albicans</i>	300	100
45	<i>C albicans</i>	<i>C albicans</i>	294	99
46	<i>C albicans</i>	<i>C albicans</i>	294	100
47	<i>C glabrata</i>	<i>C glabrata</i>	380	100
48	<i>C albicans</i>	<i>C albicans</i>	294	100
49	<i>C albicans</i>	<i>C albicans</i>	295	100
50	<i>C glabrata</i>	<i>C glabrata</i>	384	100
51	<i>C albicans</i>	<i>C albicans</i>	295	100
52	<i>C albicans</i>	<i>C albicans</i>	283	100
53	<i>C glabrata</i>	<i>C glabrata</i>	380	100
54	<i>C glabrata</i>	<i>C glabrata</i>	375	99
55	<i>C albicans</i>	<i>C albicans</i>	293	99
56	<i>C glabrata</i>	<i>C glabrata</i>	376	100
57	<i>C parapsilosis</i>	<i>C parapsilosis</i>	262	100
58	<i>C albicans</i>	<i>C albicans</i>	295	99

**I orientalis* is the teleomorphic and sexual form of *C krusei*. All sequences entered in GenBank for *C krusei* have been renamed as *I orientalis*. Resulting sequences for *I orientalis*, *C dubliniensis*, *C tropicalis*, *C glabrata*, *C parapsilosis*, and *C albicans* were subsequently deposited in GenBank with the respective accession numbers, AF417255, AF430249, AF441197, AF441198, AF441199, and AF441200. ITS, internal transcribed spacer region.

(10mM, pH 8.5) before sequencing, particularly to remove dNTPS, polymerases, salts, and primers. For the short ITS2 region, the sequencing primer ITS3 was used in the forward direction and for the large ITS1–5.8S–ITS2 region, the ITS1 primer was used in the forward direction. In both cases, the sequencing primer was labelled with Cy-5' and was used in conjunction with the ALF Express II (Amersham-Pharmacia, Little Chalfont, Buckinghamshire, UK) using the Thermo Sequenase fluorescent labelled primer cycle sequencing kit

with 7-deaza-dGTP (catalogue no: RPN 2438; Amersham Pharmacia Biotech) (96°C for one minute, followed by 25 cycles of 96°C for 10 seconds, 50°C for five seconds, and 60°C for five seconds, followed by a 4°C hold). The resulting sequences obtained were compared with those stored in the GenBank Data system using BLAST alignment software (<http://www.blast.genome.ad.jp/>) and sequence homology identity determined in accordance with the criteria, as described previously by Goldenberger *et al.*⁸

Table 2 Comparison of laboratory parameters of API20C and PCR sequencing techniques

Laboratory parameter	PCR sequence identification	API20C
Time to result		
Culture time	–	72 hours
DNA extraction	1 hour	
PCR amplification	3 hours	
Agarose gel electrophoresis	15 minutes	
Clean up of PCR product	–	
Sequencing protocol	16 hours (overnight)	
Total time	20 hours 15 minutes	72 hours ⁵
Relative cost/isolate	High	Moderate
Ease of use	Complex	Simple
Repeatability	Excellent	Good
Subjectivity	Low	Moderate
Requirements for complex equipment	High	Low
Applicability to routine diagnostic laboratory	Poor	Good

Isolates were identified using the API20C scheme (bioMérieux, Les Halles, France), in accordance with the manufacturer's instructions.

RESULTS AND DISCUSSION

Table 1 shows the phenotypic and genotypic characterisation of the isolates. Overall, there was only one disagreement between the phenotypic and genotypic identifications (isolate 23), where the API scheme identified this isolate as *C albicans* but the molecular method identified this as *C dubliniensis*. In this case, a larger section of the ITS1–5.8S–ITS2 region was sequenced to confirm the initial identification of the smaller ITS2 region.

Over the past few years, molecular techniques have greatly enhanced the diagnosis of causal agents of infectious disease, particularly when the causal agent is difficult to culture and identify.^{9–11} The results from our present study suggest that such a molecular identification scheme may be useful in differentiating *C dubliniensis* from *C albicans*, but is of little benefit in relation to the other non-albicans *Candida* spp. In addition, molecular techniques may be of benefit for the rapid identification of yeasts. Table 2 compares various laboratory parameters of the API20C and PCR schema, as experienced in our study, in the identification of *Candida* spp.

Because previous studies have shown that *C dubliniensis* is often present in mixed cultures with *C albicans*,¹² it is important that routine diagnostic laboratories have the ability to differentiate between these two species. To date, the main problem has been in the selection of representative colonies of *C dubliniensis* from primary plates, before isolates are further characterised to the species level. There have been numerous reports of various phenotypic tests that have been used in an attempt to differentiate between these two species. These tests, including characteristic appearance on indicator media, chlamydospore formation, growth/restricted growth at raised temperature, carbohydrate assimilation, and intracellular β -D-glucosidase activity, have had varying degrees of success.¹² Initially, it was suggested that the use of CHRO-Magar was a suitable means of discrimination; however, subsequent studies have shown this phenotypic characteristic to be an unreliable marker.^{12–13} Tintelnot and colleagues¹² found that a more reliable phenotypic test was growth at increased temperature (42°C), where 117 of 117 *C albicans* and 53 of 53 *C dubliniensis* isolates were able to grow and not grow, respectively. However, Tintelnot and colleagues¹² and other workers¹³ have shown that increased growth temperature cannot be used as the sole marker on all occasions.

Take home messages

- The API20C technology was compared with polymerase chain reaction amplification and direct sequencing of the short internal transcribed spacer region (ITS2) for the identification of 58 isolates of invasive candida species
- Overall, there was only one disagreement: the API scheme identified the isolate as *C albicans* but the molecular method identified it as *C dubliniensis*.
- Thus, the API20C method is useful in the identification of *Candida* spp isolated from blood culture and molecular methods do not enhance its use
- However, for correct reporting of *C dubliniensis*, an emerging bloodborne pathogen, we recommend that all isolates identified as *C albicans* by the API20C scheme are further examined phenotypically and/or genotypically

“Molecular differentiation may be of increasing importance, given the emerging role of *C dubliniensis* in the current literature”

Consequently, because of the variable phenotypic results and evidence of the presence of atypical *C albicans* organisms, various workers have examined the usefulness of molecular assays to aid in the discrimination of these two species.^{14–17} Of these, the method of Elie and colleagues¹⁴ is particularly useful because it does not rely on colony picking from primary plates, and DNA can be extracted from the entire candida flora represented on the plate and differentiated by the use of specific probes. Such molecular differentiation may be of increasing importance, given the emerging role of *C dubliniensis* in the current literature.¹⁸

In conclusion, our study demonstrated that the API20C is a useful method in the identification of *Candida* spp isolated from blood culture and that molecular methods do not enhance API20C identification. However, for correct reporting of *C dubliniensis*, we recommend that all isolates identified as *C albicans* by the API20C scheme are further examined phenotypically and/or genotypically.

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Authors' affiliations

J Xu, B C Millar, J E Moore, R McClurg, R McMullan, Northern Ireland Public Health Laboratory, Department of Bacteriology, Belfast City Hospital, Belfast BT9 7AD, UK

M J Walker, J Evans, Mycology Reference Laboratory, Department of Microbiology, The Royal Group of Hospitals, Grosvenor Road, Belfast BT12 6BA, UK

S Hedderwick, Department of Infectious Diseases, The Royal Group of Hospitals

Correspondence to: Dr John E Moore, Northern Ireland Public Health Laboratory, Department of Bacteriology, Belfast City Hospital, Belfast BT9 7AD, UK; jemoore@niph.dnet.co.uk

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