Rapid and accurate identification of species belonging to the *Candida parapsilosis* complex by real-time PCR and melting curve analysis

Constantin Hays, Chantal Duhamel, Vincent Cattoir and Julie Bonhomme

Correspondence

Julie Bonhomme

bonhomme-j@chu-caen.fr

Microbiology Laboratory, Côte de Nacre University Hospital, Caen, France

Candida parapsilosis is the second most frequent *Candida* species isolated from blood cultures. Since 2005, *C. parapsilosis* has been divided into three distinct species based on genetic traits: *Candida parapsilosis*, *Candida metapsilosis* and *Candida orthopsilosis*. The aim of this study was to develop a rapid real-time PCR assay able to distinguish these closely related species via a melting curve analysis. This identification method was optimized by using reference strains and well-characterized clinical isolates of *Candida* species. A single set of consensus primers was designed to amplify a 184 bp portion of the *SADH* gene in order to identify species based on the unique melt profile resulting from DNA sequence variations from each species of the complex. PCR products were detected with SYBR Green fluorescent dye and identification was established by melting curve analysis. For validation of the technique, a total of 116 clinical isolates, phenotypically identified as *C. parapsilosis*, were tested by real-time PCR and results were further compared with PCR-RFLP patterns of the *SADH* gene, used as the reference method. The melting curve analysis of amplified DNA could differentiate between *C. parapsilosis* (83.5 °C), *C. metapsilosis* (82.9 °C) and *C. orthopsilosis* (82.1 °C), with a sensitivity and specificity comparable to those of the reference method. One hundred and fourteen *C. parapsilosis* and two *C. orthopsilosis* isolates were identified among the clinical isolates. This method provides a simple, rapid and reliable identification of species belonging to the *C. parapsilosis* complex. This novel approach could be helpful for clinical and epidemiological investigations.

Received 28 September 2010

Accepted 22 December 2010

INTRODUCTION

Among *Candida* species, *Candida parapsilosis* is the second most frequently isolated from bloodstream infections in many regions of the world, especially in Latin America and Asia, while it is also common in Europe (Almirante *et al.*, 2006). *C. parapsilosis* emerged in the 1990s and its incidence is still increasing (Playford *et al.*, 2010). This is partly due to the massive usage of medical devices, notably among immunocompromised patients, and to the selective pressure exerted by the intensive use of azoles and more recently of echinocandins (Bassetti *et al.*, 2009; Forrest *et al.*, 2008; Trofa *et al.*, 2008). *C. parapsilosis* is also known to be a nosocomial yeast responsible for bloodstream infections, mainly in intensive care units, transmitted by the hands of healthcare workers (van Asbeck *et al.*, 2007). Moreover, its ability to form biofilms on medical implants, like *Candida albicans*, confers increased resistance to antifungal agents, leading to a real therapeutic problem (Kuhn *et al.*, 2002; Růžička *et al.*, 2007).

Since 2005, *C. parapsilosis* has been considered to be a species complex, including three distinct species based on genetic criteria: *C. parapsilosis*, *Candida metapsilosis* and

Candida orthopsilosis (Tavanti *et al.*, 2005). Epidemiological and clinical features corresponding to each species have been poorly documented since their phenotypic identification remains unreliable. Therefore, the rapid identification of *Candida* species in clinical laboratories appears essential for the management of patients with candidaemia.

The aim of this study was to develop a rapid real-time PCR assay able to distinguish these three closely related species via a melting curve analysis. After optimization of this technique, we tested 116 clinical isolates, previously identified by conventional methods as *C. parapsilosis*, and compared with PCR-RFLP patterns of the *SADH* gene, used as the reference method.

METHODS

Clinical isolates and reference strains. A total of 116 clinical isolates identified as *C. parapsilosis* using the Vitek 2 YST card or API ID 32 C (bioMérieux) were collected from 79 patients hospitalized in the Caen University Hospital from October 2006 to September 2010. They were recovered from blood (36), central venous catheter (22), urine (15), respiratory tract (10), faeces (13) or others (20). *C. parapsilosis*

ATCC 22019, *C. metapsilosis* CBS 10907 and *C. orthopsilosis* CBS 10906 were included in the study as reference strains. Three clinical strains, identified as *C. orthopsilosis* by sequencing, were also studied (gift from M.-E. Bougnoux, Hôpital Necker-Enfants Malades, Paris, France). Other *Candida* species of clinical interest were used to ensure the technique specificity: *C. albicans*, *Candida glabrata*, *Candida tropicalis*, *Candida krusei*, *Candida kefyr*, *Candida lusitanae*, *Candida dubliniensis*, *Candida famata*, *Candida colliculosa* and *Candida sphaerica*. A clinical isolate identified as *Saccharomyces cerevisiae* and the *Lodderomyces elongiporus* reference strain CBS 2605 were also tested. Each strain was cultured in YPD liquid medium (2 % glucose, 2 % peptone, 1 % yeast extract) overnight at 30 °C with agitation and stored at –80 °C.

DNA extraction. Yeast genomic DNA was extracted with the MasterPure Yeast DNA Purification kit (Epicentre Biotechnologies) according to the manufacturer's instructions.

Real-time PCR and melting curve analysis. After aligning the *SADH* sequences of the three species, a single set of consensus primers was carefully designed to amplify a 184 bp portion of the gene, in order to identify species based on the unique melt profile resulting from DNA sequence variations from each *C. parapsilosis* complex species. The 25 µl reaction mixture consisted of 12.5 µl SYBR Green PCR Master Mix 2× (Qiagen), 2.5 µl forward and reverse primers (10 µmol l⁻¹) (SADH-F 5'-GCTGCGGCTTCAACTGATGC-3' and SADH-R 5'-CTTGGTCACGAGCCTCC-3'), 5.5 µl distilled water and 2.0 µl DNA sample (approx. 150 ng). Real-time PCR and melting curve analysis were carried out using a Rotor-Gene Q thermocycler (Qiagen). The experimental conditions used were as follows: 5 min at 95 °C, followed by 40 cycles of 10 s at 95 °C, 30 s at 64 °C and 20 s at 72 °C. Melting curves were generated after the last extension step; the temperature was ramped from 55 °C to 95 °C, 0.5 °C every 5 s. Melting curves were analysed by the Rotor-Gene 1.7 software (Qiagen).

PCR-RFLP analysis. Results for each strain identified by real-time PCR and melting curve analysis were compared to those obtained with PCR-RFLP used as the reference method, as previously described (Asadzadeh *et al.*, 2009). Briefly, primers SADHF2 (5'-TGGATTG-TGGGGAYAAATTATGT-3') and SADHR2 (5'-GTCAATACRGCAT-CAGTTGAAGC-3') were used to amplify a 310 bp fragment of the *SADH* gene. Subsequently, PCR products were digested with the restriction enzyme *BanI*.

Statistical analysis. Student's *t* test was used to establish differences between test groups. A *P*-value of <0.05 was considered significant.

RESULTS AND DISCUSSION

The primers SADH-F and SADH-R were designed to amplify a portion of the *SADH* gene, with a DNA G+C content difference between the three species (*C. parapsilosis*, 45.1 mol%; *C. metapsilosis*, 43.5 mol%; and *C. orthopsilosis*, 41.3 mol%). This difference allowed species identification with three significantly different melting temperatures: 83.5 ± 0.19 °C, 82.9 ± 0.15 °C and 82.1 ± 0.25 °C for *C. parapsilosis*, *C. metapsilosis* and *C. orthopsilosis*, respectively ($P < 0.05$) (Fig. 1). A difference of 0.7 °C maximum in the melting peak temperatures could be observed between different runs, but not within the same run. This disparity probably resulted from variations in the temperature profile created by the thermocycler, requiring the use of reference strains DNA in each set as positive controls. No amplification was observed with *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. krusei*, *C. kefyr*, *C. lusitanae*, *C. dubliniensis*, *C. famata*, *C. colliculosa*, *C. sphaerica*, *S. cerevisiae* or *L. elongiporus* (specificity, 100 %). The latter species is very close phenotypically and genetically to *C. parapsilosis*, formerly considered as its teleomorph, resulting in misidentification by conventional methods (Lockhart *et al.*, 2008a). Concerning the PCR-RFLP reference method, the *BanI*-digested PCR products generated two fragments of 166 and 144 bp, two fragments of 268 and 42 bp, and one undigested fragment of 310 bp for *C. parapsilosis*, *C. metapsilosis* and *C. orthopsilosis*, respectively, as expected.

Since *C. parapsilosis* was considered a species complex and divided into three species, several identification methods have been developed, based on PCR-RFLP (Tavanti *et al.*, 2005; Asadzadeh *et al.*, 2009) and internal transcribed spacer sequencing (Gomez-Lopez *et al.*, 2008; Borman *et al.*, 2009). An amplification fragment length polymorphism method has also been used not only to confirm species identification, but also to evaluate genetic relatedness of *C. parapsilosis* (Tavanti *et al.*, 2010), *C. metapsilosis* (Hensgens *et al.*, 2009) and *C. orthopsilosis* (Tavanti *et al.*, 2007). All these techniques are time-consuming and not adapted for routine diagnosis. In 2009, a real-time PCR with melting temperature

Fig. 1. Characteristic melting peaks with genus-specific forward and reverse primers for identification of *C. parapsilosis* (peak numbered 3), *C. metapsilosis* (peak numbered 2) and *C. orthopsilosis* (peak numbered 1).

analysis was developed to identify six *Candida* species of clinical interest, including *C. parapsilosis* complex species (Khan *et al.*, 2009). However, this technique has not yet been optimized to enable species-level identification of the *C. parapsilosis* complex. In our study, the melting curve analysis of amplified DNA could differentiate between the three species of the *C. parapsilosis* complex with a sensitivity and specificity comparable to those of the PCR-RFLP reference method.

The 116 clinical isolates, previously identified by conventional methods as *C. parapsilosis*, were correctly amplified and detected with SYBR Green fluorescent dye, as were the three *C. orthopsilosis* clinical strains. Based on the melting curve analysis, 114 (98.3 %) and two (1.7 %) were identified as *C. parapsilosis* and *C. orthopsilosis*, respectively, with 100 % correlation with the PCR-RFLP reference method. The three *C. orthopsilosis* clinical strains were correctly identified with the two methods. No *C. metapsilosis* was identified among these clinical isolates. Although epidemiological data are still rare, this species distribution seemed unusual. Indeed, among *C. parapsilosis* complex species, the percentage of *C. orthopsilosis* and *C. metapsilosis* was low compared to that in Asia (11 % of *C. orthopsilosis* and 2.6 % of *C. metapsilosis*), Europe and the Middle East (3.5 % of *C. orthopsilosis* and 2.9 % of *C. metapsilosis*), South America (10.9 % of *C. orthopsilosis* and 1.3 % of *C. metapsilosis*) and North America (5 % of *C. orthopsilosis* and 0.6 % of *C. metapsilosis*) (Lockhart *et al.*, 2008b). Europe is generally considered to be the region with the higher rate of *C. metapsilosis* (Lockhart *et al.*, 2008b).

In this study, 56 % of the *C. parapsilosis* isolates were from patients over 60 years of age, with a median age of 55 years. *C. parapsilosis* is usually described as an important pathogen in children and neonates. We found that 8.9 % of the *C. parapsilosis* isolates were from children under 1 year, consistent with the majority of publications (Filioti *et al.*, 2007).

This method provides a simple, rapid (less than 3 h, including DNA extraction) and reliable identification of species belonging to the *C. parapsilosis* complex. In addition, it seems to be cost-effective since no specific probes are needed in this assay. This novel approach may be helpful for clinical and epidemiological investigations. To our knowledge, we have developed the first rapid and accurate real-time PCR for species differentiation within the *C. parapsilosis* complex.

ACKNOWLEDGEMENTS

We are sincerely grateful to Marie-Elisabeth Bougnoux for her pertinent advice and for providing us with reference strains and *C. orthopsilosis* clinical strains.

REFERENCES

- Almirante, B., Rodríguez, D., Cuenca-Estrella, M., Almela, M., Sanchez, F., Ayats, J., Alonso-Tarres, C., Rodríguez-Tudela, J. L., Pahissa, A. & the Barcelona Candidemia Project Study Group (2006). Epidemiology, risk factors, and prognosis of *Candida parapsilosis* bloodstream infections: case-control population-based surveillance study of patients in Barcelona, Spain, from 2002 to 2003. *J Clin Microbiol* **44**, 1681–1685.
- Asadzadeh, M., Ahmad, S., Al-Sweih, N. & Khan, Z. U. (2009). Rapid molecular differentiation and genotypic heterogeneity among *Candida parapsilosis* and *Candida orthopsilosis* strains isolated from clinical specimens in Kuwait. *J Med Microbiol* **58**, 745–752.
- Bassetti, M., Ansaldi, F., Nicolini, L., Malfatto, E., Molinari, M. P., Mussap, M., Rebesco, B., Bobbio Pallavicini, F., Icardi, G. & Viscoli, C. (2009). Incidence of candidaemia and relationship with fluconazole use in an intensive care unit. *J Antimicrob Chemother* **64**, 625–629.
- Borman, A. M., Linton, C. J., Oliver, D., Palmer, M. D., Szekely, A., Odds, F. C. & Johnson, E. M. (2009). Pyrosequencing analysis of 20 nucleotides of internal transcribed spacer 2 discriminates *Candida parapsilosis*, *Candida metapsilosis*, and *Candida orthopsilosis*. *J Clin Microbiol* **47**, 2307–2310.
- Filioti, J., Spiroglou, K., Panteliadis, C. P. & Roilides, E. (2007). Invasive candidiasis in pediatric intensive care patients: epidemiology, risk factors, management, and outcome. *Intensive Care Med* **33**, 1272–1283.
- Forrest, G. N., Weekes, E. & Johnson, J. K. (2008). Increasing incidence of *Candida parapsilosis* candidemia with caspofungin usage. *J Infect* **56**, 126–129.
- Gomez-Lopez, A., Alastruey-Izquierdo, A., Rodríguez, D., Almirante, B., Pahissa, A., Rodríguez-Tudela, J. L., Cuenca-Estrella, M. & the Barcelona Candidemia Project Study Group (2008). Prevalence and susceptibility profile of *Candida metapsilosis* and *Candida orthopsilosis*: results from population-based surveillance of candidemia in Spain. *Antimicrob Agents Chemother* **52**, 1506–1509.
- Hensgens, L. A., Tavanti, A., Mogavero, S., Ghelardi, E. & Senesi, S. (2009). AFLP genotyping of *Candida metapsilosis* clinical isolates: evidence for recombination. *Fungal Genet Biol* **46**, 750–758.
- Khan, Z., Mustafa, A. S. & Alam, F. F. (2009). Real-time LightCycler polymerase chain reaction and melting temperature analysis for identification of clinically important *Candida* spp. *J Microbiol Immunol Infect* **42**, 290–295.
- Kuhn, D. M., Chandra, J., Mukherjee, P. K. & Ghannoum, M. A. (2002). Comparison of biofilms formed by *Candida albicans* and *Candida parapsilosis* on bioprosthetic surfaces. *Infect Immun* **70**, 878–888.
- Lockhart, S. R., Messer, S. A., Pfaller, M. A. & Diekema, D. J. (2008a). *Lodderomyces elongisporus* masquerading as *Candida parapsilosis* as a cause of bloodstream infections. *J Clin Microbiol* **46**, 374–376.
- Lockhart, S. R., Messer, S. A., Pfaller, M. A. & Diekema, D. J. (2008b). Geographic distribution and antifungal susceptibility of the newly described species *Candida orthopsilosis* and *Candida metapsilosis* in comparison to the closely related species *Candida parapsilosis*. *J Clin Microbiol* **46**, 2659–2664.
- Playford, E. G., Nimmo, G. R., Tilse, M. & Sorrell, T. C. (2010). Increasing incidence of candidaemia: long-term epidemiological trends, Queensland, Australia, 1999–2008. *J Hosp Infect* **76**, 46–51.
- Růžicka, F., Holá, V., Votava, M. & Tejkalová, R. (2007). Importance of biofilm in *Candida parapsilosis* and evaluation of its susceptibility to antifungal agents by colorimetric method. *Folia Microbiol (Praha)* **52**, 209–214.
- Tavanti, A., Davidson, A. D., Gow, N. A., Maiden, M. C. & Odds, F. C. (2005). *Candida orthopsilosis* and *Candida metapsilosis* spp. nov. to replace *Candida parapsilosis* groups II and III. *J Clin Microbiol* **43**, 284–292.
- Tavanti, A., Hensgens, L. A., Ghelardi, E., Campa, M. & Senesi, S. (2007). Genotyping of *Candida orthopsilosis* clinical isolates by

amplification fragment length polymorphism reveals genetic diversity among independent isolates and strain maintenance within patients. *J Clin Microbiol* **45**, 1455–1462.

Tavanti, A., Hensgens, L. A., Mogavero, S., Majoros, L., Senesi, S. & Campa, M. (2010). Genotypic and phenotypic properties of *Candida parapsilosis sensu strictu* strains isolated from different geographic regions and body sites. *BMC Microbiol* **10**, 203.

Trofa, D., Gácser, A. & Nosanchuk, J. D. (2008). *Candida parapsilosis*, an emerging fungal pathogen. *Clin Microbiol Rev* **21**, 606–625.

van Asbeck, E. C., Huang, Y.-C., Markham, A. N., Clemons, K. V. & Stevens, D. A. (2007). *Candida parapsilosis* fungemia in neonates: genotyping results suggest healthcare workers hands as source, and review of published studies. *Mycopathologia* **164**, 287–293.