Candida infections

detection and epidemiology

Cover: 'budding sea-creature' by Ingrid C. Roos
photography: Annemarie Borst

Printed by: Ponsen & Looijen BV, Wageningen
ISBN: 90-393-3084-0

Candida infections

detection and epidemiology

Candida infecties

detectie en epidemiologie

(met een samenvatting in het Nederlands)

Proefschrift
ter verkrijging van de graad van doctor
aan de Universiteit Utrecht
op gezag van de Rector Magnificus
Prof. dr. W.H. Gispen
ingevolge het besluit van het College voor Promoties
in het openbaar te verdedigen
op vrijdag 6 september 2002 des middags te 12.45 uur

door

Annemarie Borst
geboren op 20 mei 1972 te Castricum

promotor: Prof. dr. J. Verhoef
co-promotor: Dr. A.C. Fluit

Het onderzoek beschreven in dit proefschrift werd mogelijk gemaakt door financiële steun van bioMérieux, Boxtel, Nederland (voorheen Organon Teknika).

Het drukken van dit proefschrift werd mede mogelijk gemaakt door Applied Biosystems, Wyeth Pharmaceuticals, Pfizer bv, MSD B.V. en ICN Pharmaceuticals Holland B.V.

here I stand by the mountain
look up to the sky
knowing it's a matter of having to climb
above this place these clouds lie

(Luka Bloom)

Contents

Introduction

OBJECTIVES

Candida species are opportunistic fungal pathogens which cause severe infections in immunocompromised patients. Due to the profound developments in medical care, the number of immunocompromised patients has increased, and so has the number of life-threatening *Candida* infections[1]. At present, *Candida* is the 4th most common bloodstream pathogen in North America and ranks 8th in Europe[13,19]. High-risk groups include: neutropenic cancer patients, bone marrow and organ transplant recipients, patients suffering from AIDS, diabetics, and patients receiving broad-spectrum antibiotics or parenteral nutrition. Attributable mortality of *Candida* infections is as high as 38%, and crude mortality rates exceed 50%[15,29,44,45].

The most commonly used detection method for *Candida* infections, automated blood culture, is inadequate. Many yeast infections remain undetected or are diagnosed only after several days[25]. Furthermore, since different *Candida* species show differences in resistance against antimycotic agents[13,28], identification up to the species level is essential for adequate treatment. In many clinical microbiological laboratories the identification methods are based on phenotypic characteristics. However, some species show a high degree of phenotypic similarity which complicates identification. Commercial tests usually show high sensitivities and specificities for *Candida albicans*, but are less reliable or require further testing for the identification of other species[3,6,12,17].

In addition to the inadequate detection, relatively little is known about the epidemiology of *Candida* infections. Typing of different strains of the same *Candida* species and linking these strain types to data on the presence of virulence factors or resistance to antimycotic agents may improve our understanding of the epidemiology of this yeast, and may help to identify genetic markers for these traits. Several typing methods have been used for *Candida* species, but none of them is considered the golden standard[4,11,30,32,33,38].

The first objective of this thesis was to improve the diagnosis and identification of *Candida* infections. The second objective was to study the epidemiology of *C. albicans*. In particular, we investigated whether a relatively new typing method, Amplified Fragment Length Polymorphism analysis (AFLP), is suitable for typing *C. albicans*. Furthermore, the relationship between the type of infection, geographic origin, and the expression of virulence factors by clinical *C. albicans* isolates was examined.

DETECTION OF *CANDIDA* INFECTIONS

Automated blood culture. Automated blood culture systems are routinely used as diagnostic tool. Since our hospital makes use of the BacT/Alert monitoring system of bioMérieux (formerly Organon Teknika, Boxtel, the Netherlands) we focus here on this device. However, although the contents of the culture media as well as the exact method of detection differs between the different systems, the basic protocol is the same. Blood is inoculated directly into the culture bottles containing proprietary media based on enriched trypticase soy broth. Different media are developed for the growth of aerobic and anaerobic organisms. Furthermore, the development of 'FAN' bottles (fastidious antibiotic neutralization) in some cases increased the detection rate[43]. Special media for detection of fungal growth are

also available[14]. However, because it is labour intensive and expensive to use several systems at the same time, most laboratories only use the standard blood culture bottles. In our hospital, regular anaerobic bottles and FAN aerobic bottles are used.

The bottles are incubated under continuous agitation. A differentially permeable membrane in the bottom of the bottle separates the medium from a pH sensor. This green colored sensor turns yellow when carbon dioxide produced by growing microorganisms diffuses across the membrane and reacts with water generating hydrogen ions. This lowering of the pH is monitored by the instrument every ten minutes during incubation. When the instrument renders a positive signal, further testing is needed to identify the organism grown in the bottle. Usually, a Gram staining is performed, and the blood culture is subcultured on blood agar and, if necessary, other media.

It is known that automated blood culture systems may fail to detect yeasts in up to 65% of the cases[25]. Many blood culture media are not optimal for fungal growth. Also, growth of fungi may be inhibited by the presence of antimycotic agents in the blood of the patient. The question whether terminal subculture of negative blood culture bottles will lead to enhanced detection rates is under debate[23,31,36,39,46]. In the study described in **Chapter 1** of this thesis, we examined whether terminal subculture of negative blood culture bottles improves the detection rate for patients who are at high risk for candidaemia.

NASBA. Since automated blood culture systems often need several days before fungal infections are detected, or even miss these infections entirely, improved detection methods are needed. Nucleic acid amplification technologies provide promising tools for the rapid detection of *Candida* species in clinical materials. Polymerase Chain Reaction (PCR) is the most generally used amplification technique. However, in 1991 Compton described a new amplification method, Nucleic Acid Sequence-Based Amplification (NASBA™), which has several advantages over PCR[7]. A schematic representation of this technique is depicted in Figure 1. The technique is based on the incorporation of a T7 RNA polymerase promotor sequence in one of the primers. This primer anneals to the single stranded RNA target. After primer extension by Avian Myeloblastosis Virus Reverse Transcriptase (AMV-RT), the RNA strand of the resulting RNA/DNA hybrid is degraded by RNase H. The second primer anneals to the single stranded cDNA, and a double stranded cDNA molecule is generated by AMV-RT. This cDNA now contains a double stranded T7 promotor which enables T7 RNA polymerase to generate multiple anti-sense RNA copies. These amplicons serve as templates for the cyclic phase of the amplification, as shown on the right-hand side in Figure 1.

In contrast with PCR, NASBA is an isothermal process which eliminates the need for (expensive) thermal cyclers. By using RNA as target, which is far less stable than DNA, the risk of obtaining false-positive results due to amplification of nucleic acids from dead or degrading cells is reduced. Also, no separate RT step is required for RNA amplification, and RNA can be amplified in a background of DNA molecules. Furthermore, unlike PCR where the initial primer level limits the maximum yield, the T7 RNA polymerase reuses the cDNA, resulting in an exponential increase in RNA amplicons. In addition, these single stranded amplicons are ideal targets for detection with specific probes, without the need for denaturation. The NASBA assay developed in this thesis uses ribosomal RNA as target, which can be present in as many as 10,000 copies per cell. This results in a very sensitive assay. The characteristics and applications of NASBA have recently been reviewed by Deiman et al.[10].

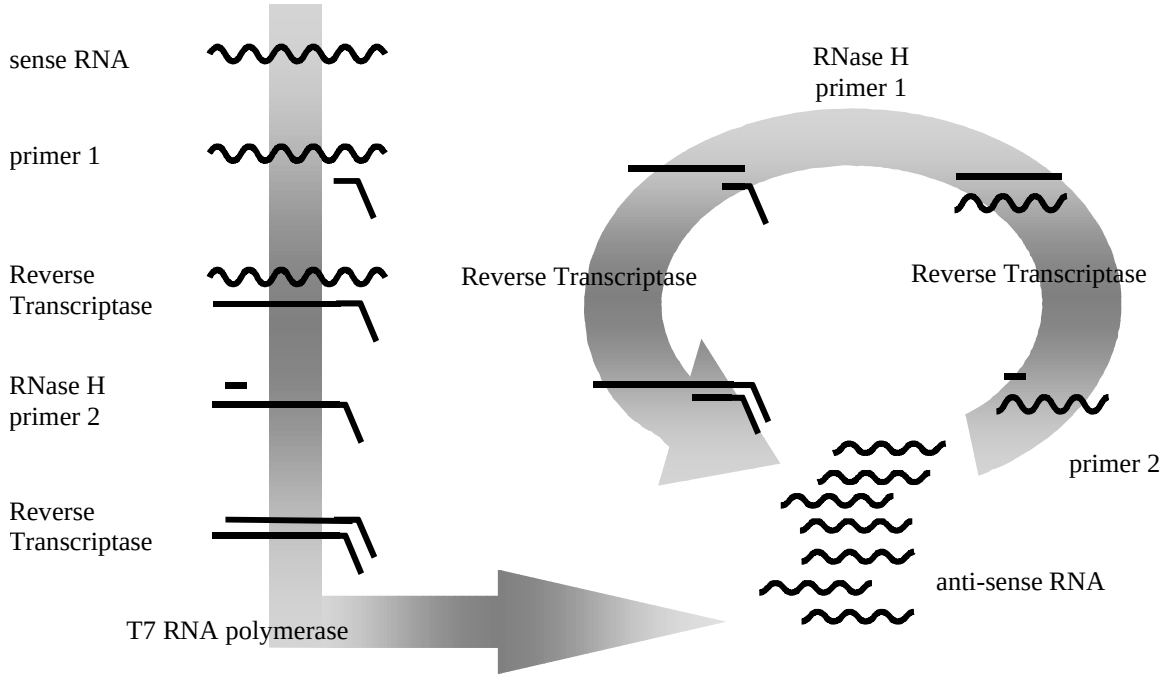


Figure 1

Schematic representation of nucleic acid sequence-based amplification (NASBA)

In **Chapter 2** of this thesis we describe the development of a NASBA assay for the detection of *Candida* species. The primers are based on the conserved regions of the 18S rRNA sequence of medically important fungi. Furthermore, specific probes were designed and tested for the identification of the different medically important species, including *C. glabrata* and *C. krusei*. Sample preparation remains a crucial step in all amplification methods. In case of disseminated infections, it is favorable to use whole blood instead of plasma or serum. The use of whole blood prevents the loss of target due to phagocytosed fungal cells or cells attached to leukocytes or other blood cells. According to Jordan, the use of plasma resulted in a loss of more than 50% of the initial input of *C. albicans*[21]. Rapid whole blood sample preparation methods generally cannot process more than 200 µl blood[21,40]. In **Chapter 2** an improved rapid sample preparation for whole blood samples up to 1 ml is described.

Amplification technologies can also be used to reduce the time needed for species identification after growth is detected in blood culture bottles. Besides detection of *Candida* species directly in blood samples of patients, we wanted to know whether we could use our NASBA assay to quicken and maybe even improve the detection rate of *Candida* species in blood cultures. Therefore, culture-positive as well as negative blood cultures from patients with a proven candidaemia were analyzed, and the results of the NASBA assay were compared with the results of BacT/Alert monitoring. The results of this study are described in **Chapter 3**.

After the encouraging results of the previous studies, a clinical trial was initiated in order to evaluate the use of the NASBA assay for the improved detection of *Candida* species in patients suspected of having candidaemia. Since candidaemia is characterized by a low number of yeast cells in the blood stream, testing of blood culture samples after a short (2 day) culture step was also included. The results of this clinical trial are presented in **Chapter 4**.

Contamination control. Implementation of the NASBA assay in a routine clinical

laboratory requires considerable standardization of the different procedures. In Chapters 3 and 4, our amplicon detection assay was successfully replaced by the NucliSens Basic Kit detection module in combination with the NucliSens reader (bioMérieux (formerly Organon Teknika), Boxtel, the Netherlands). **Chapter 5** describes our efforts to replace our in-house NASBA amplification protocol by the NucliSens Basic Kit amplification module. Unfortunately, impracticable difficulties with false-positive results were encountered due to contaminated components of the kit, and it was decided to continue working with our in-house assay. Over the years, working with sensitive amplification technologies like NASBA has inevitably experienced us in contamination control. The current problems lead us to review the implications of contaminations in diagnosis and research on infectious diseases (**Chapter 6**). In the same review we also discuss the functionality and draw-backs of different methods for prevention and destruction of contaminating DNA and give recommendations on how to improve laboratory practice.

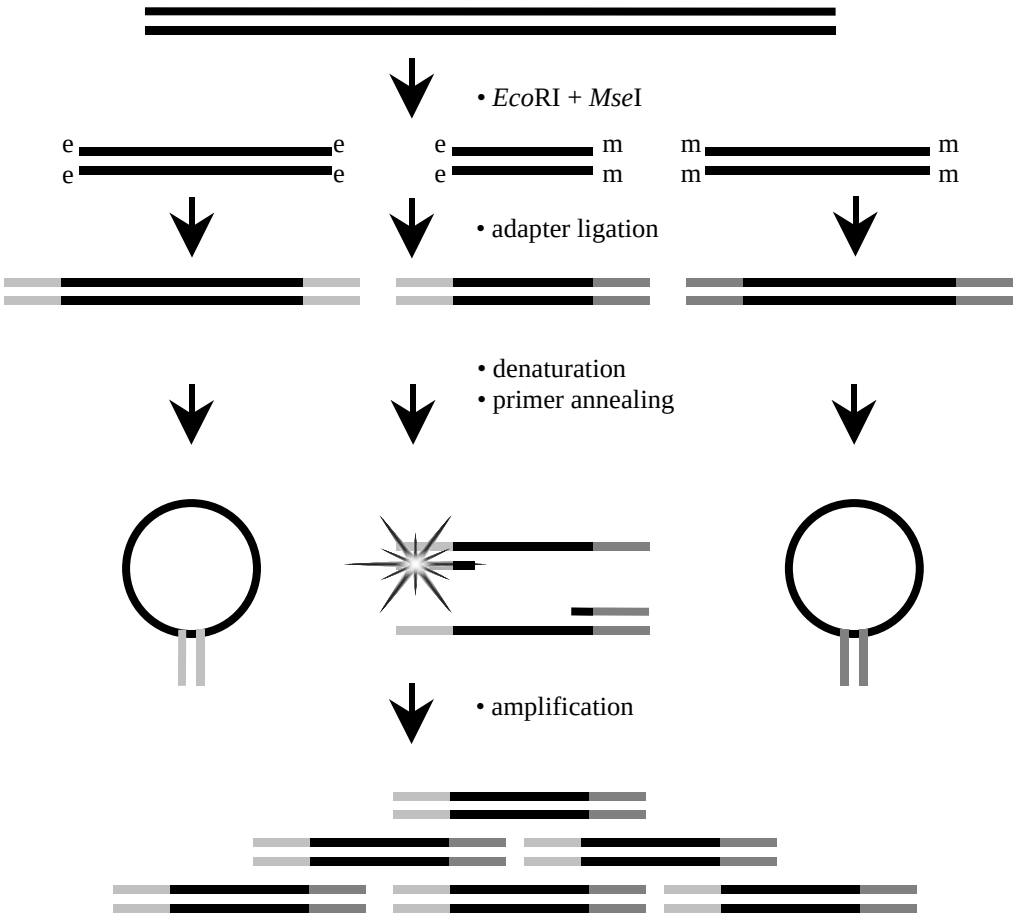


Figure 2

Schematic representation of amplified fragment length polymorphism analysis (AFLP)

AFLP. While *Candida albicans* used to be the main cause of invasive fungal infections, non-*albicans Candida* species like *C. glabrata*, *C. krusei* and *C. parapsilosis* are increasingly isolated. These non-*albicans Candida* species now account for approximately 50% of all *Candida* infections[22]. The different *Candida* species show differences in resistance to antimycotic agents. *C. krusei* is innately resistant to fluconazole, and *C. glabrata* is able to acquire resistance to this drug very rapidly[13,28]. Furthermore, *C. glabrata* infections have been associated with an extremely high mortality[16]. Therefore, to start an adequate treatment as early as possible, it is essential to rapidly detect the causative organism up to the species level. With the NASBA assay developed in this thesis, it is possible to identify most medically important *Candida* species. Other probes can be designed and implemented in the assay when more species need to be identified. However, Amplified Fragment Length Polymorphism analysis (AFLP[41]), may also prove to be an excellent identification tool.

A schematic representation of AFLP is shown in Figure 2. Chromosomal DNA is digested with two restriction enzymes, e.g. *Eco*RI and *Mse*I, and adapters are ligated to the fragments. These adapters are known sequences which are designed in such a way that after ligation the recognition sites for the restriction enzymes are lost. Hence, restriction and ligation can take place in the same reaction tube. Two types of fragments are formed: fragments with the same adapter at both ends and fragments with the two different adapters at each end. The adapters are used as targets for the primers during PCR amplification. However, fragments with the same adapter at both ends will generally form 'stem-loop' structures after denaturation, which makes it impossible for the primers to anneal. Therefore, the amplification products will contain mainly fragments with a different adapter at each end. If necessary, by using one or more selective nucleotides on the 3'-end of the primers, the number of amplified fragments can be reduced to obtain a more distinctive pattern. One of the primers is labeled with a fluorescent dye. The fragments are separated in an automated sequence apparatus, and analyzed using software packages like BioNumerics (Applied Maths, Sint-Martens-Latem, Belgium).

The advantages and disadvantages of AFLP and the NASBA assay are summarized in Table 1. Both methods only need small amounts of starting material and can be obtained in a kit format. The advantage of the NASBA assay over AFLP is that the former can be applied directly on clinical material, which speeds up the whole process. AFLP on the other hand is universally applicable and by storing the patterns, including those of reference strains, in a general accessible database a screening library for identification of species is obtained. Furthermore, for numerous organisms AFLP patterns can also be used for strain typing[35]. In conclusion: the NASBA assay is preferable for rapid diagnosis, whereas AFLP may be helpful to identify unknown isolates from cultures. **Chapter 7** of this thesis describes the use of AFLP as an identification method for medically important *Candida* species.

EPIDEMIOLOGY OF *CANDIDA* INFECTIONS

Virulence. Several putative virulence factors of *C. albicans* have been described[5]. One remarkable virulence factor is the capacity of *C. albicans* to 'switch' between different phenotypes at a relatively high frequency. This switching is characterized by differences in colony morphology, e.g. 'white', 'opaque', 'star', 'stippled', 'hat', 'irregular', 'wrinkle', and 'fuzzy'.

Table 1
Advantages and disadvantages for the NASBA assay and AFLP as identification methods

	Advantages	**Disadvantages**
NASBA assay	- directly on clinical material - small amounts of material are sufficient - rapid - standardization with kit	- several species-specific probes needed - not every species identifiable in current assay
AFLP	- universally applicable - database screening of results - possibilities for strain typing - small amounts of material are sufficient - standardization with kit	- pure sample necessary - less rapid

Phenotypic switching is reversible and is accompanied by changes in antigen expression and tissue affinities. Furthermore, transition between unicellular yeast cells and filamentous growth forms occurs. The formation of (pseudo)hyphae is probably associated with an enhanced capability to invade host tissues. *C. albicans* also produces adhesins, biomolecules that promote the adherence of the yeast to host cells. In addition, the organism secretes hydrolytic enzymes such as phospholipases and secreted aspartyl proteinases. Phospholipases most likely contribute to the pathogenicity of *C. albicans* by damaging host cell membranes, aiding the fungus by invasion of host tissues[24]. Secreted aspartyl proteinases are capable of degrading epithelial and mucosal barrier proteins like collagen, keratin and mucin, as well as antibodies, complement and cytokines[9].

The importance of these enzymes in *C. albicans* virulence has been ascertained by cloning and disrupting the genes involved, and studying the effect of these mutations on the virulence in animal models. Disruption of the phospholipase B gene significantly attenuated virulence in mice and dramatically reduced the ability of the yeast to penetrate host cells[24]. Disruption of the genes involved in secreted aspartyl proteinase production resulted in altered adherence of yeast cells and attenuation of virulence in different animal models[8,18,34,42].

The expression of certain virulence factors may be associated with specific characteristics such as geographic origin of the isolates or the type of infection. Knowledge of such correlations may help to understand the epidemiology of these infections. This may result in improved therapeutic regimens. In **Chapter 8**, the differences in phospholipase and secreted aspartyl proteinase production of a large panel of clinical *C. albicans* isolates obtained from 12 different European countries were studied, and the results were linked to data on the geographic origin of the isolates and the site of infection.

Strain typing. Strain typing is important in various situations[37]. When an outbreak of disease occurs in a hospital (ward), strain typing can be used to investigate whether this outbreak is caused by a single strain, or whether several strains are involved. Furthermore, by testing isolates from patients, health care workers and the hospital environment the origin of the outbreak can be identified and adequate measures to stop the outbreak and prevent future recurrences can be undertaken. Strain typing can also be of use to unravel the relationship between commensal and pathogenic strains. It is generally acknowledged that most infecting

Candida strains originate from the host's commensal flora. However, are all commensals capable of becoming pathogens? Strain typing methods have proven to be valuable tools in research on strain persistence or replacement during different stages of infections. Besides the dynamics of yeast strains within in human being, it is also essential to understand the dynamics of the organism in a human population. Are certain strains or clusters of strains associated with a geographic locale? Are there endemic strains in particular hospital wards?

Besides being a tool for the identification of different species, AFLP may also be suitable for typing *C. albicans*. In fact, AFLP was developed as a typing method, and has been used successfully for many organisms including *Saccharomyces cerevisiae*, a close relative of *C. albicans*[2,35,41]. Several other typing methods have been described for *C. albicans*. However, no method has been accepted as the golden standard. Randomly Amplified Polymorphic DNA analysis (RAPD) has the disadvantage of being less reproducible than AFLP[20,35]. Other fingerprinting techniques which are often used are based on selective parts of the genome. For example, the PCR fingerprinting method described by Meyer et al. uses mini- and microsatellite sequences as targets for the primers[27], reference strand-mediated conformational analysis (RSCA) is based on 18S rRNA sequences[26], and the Ca3 probe used in Southern blot hybridizations of *Eco*RI digested genomic DNA hybridizes with genomic sequences containing repetitive elements[33]. AFLP patterns are a representation of the whole genome. Furthermore, these patterns can easily be stored in (general accessible) databases, which greatly facilitates the exchange of results between laboratories. In **Chapter 9** of this thesis we investigated whether AFLP is suitable for typing *C. albicans*. AFLP was used to type the collection of European clinical *C. albicans* isolates, and the correlation between AFLP type and geographical origin, site of infection and the production of hydrolytic enzymes was studied.

The results of all studies described in this thesis will provide tools for the improvement of diagnosis and identification of *Candida* infections, and contribute to our understanding of the epidemiology of this yeast.

REFERENCES

1. **Abi Said, D., E. Anaissie, O. Uzun, I. Raad, H. Pinzcowski, and S. Vartivarian.** 1997. The epidemiology of hematogenous candidiasis caused by different *Candida* species. Clin. Infect. Dis. 24: 1122-1128
2. **Azumi, M. and N. Goto-Yamamoto.** 2001. AFLP analysis of type strains and laboratory and industrial strains of *Saccharomyces* sensu stricto and its application to phenetic clustering. Yeast 18: 1145-1154
3. **Bernal, S., M.E. Martin, M. Chavez, J. Coronilla, and A. Valverde.** 1998. Evaluation of the new API *Candida* system for identification of the most clinically important yeast species. Diagn. Microbiol. Infect. Dis. 32: 217-221
4. **Boerlin, P., F. Boerlin-Petzold, J. Goudet, C. Durussel, J.L. Pagani, J.P. Chave, and J. Bille.** 1996. Typing *Candida albicans* oral isolates from human immunodeficiency virus-infected patients by multilocus enzyme electrophoresis and DNA fingerprinting. J. Clin. Microbiol. 34: 1235-1248

5. **Calderone, R.A. and W.A. Fonzi.** 2001. Virulence factors of *Candida albicans*. Trends Microbiol. 9: 327-335
6. **Campbell, C.K., K.G. Davey, A.D. Holmes, A. Szekely, and D.W. Warnock.** 1999. Comparison of the API *Candida* system with the AUXACOLOR system for identification of common yeast pathogens. J. Clin. Microbiol. 37: 821-823
7. **Compton, J.** 1991. Nucleic acid sequence-based amplification. Nature 350: 91-92
8. **De Bernardis, F., S. Arancia, L. Morelli, B. Hube, D. Sanglard, W. Schafer, and A. Cassone.** 1999. Evidence that members of the secretory aspartyl proteinase gene family, in particular SAP2, are virulence factors for *Candida* vaginitis. J. Infect. Dis. 179: 201-208
9. **De Bernardis, F., P.A. Sullivan, and A. Cassone.** 2001. Aspartyl proteinases of *Candida albicans* and their role in pathogenicity. Med. Mycol. 39: 303-313
10. **Deiman, B., P. Van Aerle, and P. Sillekens.** 2002. Characteristics and applications of nucleic acid sequence-based amplification (NASBA). Mol. Biotechnol. 20: 163-179
11. **Diaz-Guerra, T.M., J.V. Martinez-Suarez, F. Laguna, and J.L. Rodriguez-Tudela.** 1997. Comparison of four molecular typing methods for evaluating genetic diversity among *Candida albicans* isolates from human immunodeficiency virus-positive patients with oral candidiasis. J. Clin. Microbiol. 35: 856-861
12. **Espinel-Ingroff, A., L. Stockman, G. Roberts, D. Pincus, J. Pollack, and J. Marler.** 1998. Comparison of RapID yeast plus system with API 20C system for identification of common, new, and emerging yeast pathogens. J. Clin. Microbiol. 36: 883-886
13. **Fluit, A.C., M.E. Jones, F.J. Schmitz, J. Acar, R. Gupta, and J. Verhoef.** 2000. Antimicrobial susceptibility and frequency of occurrence of clinical blood isolates in Europe from the SENTRY antimicrobial surveillance program, 1997 and 1998. Clin. Infect. Dis. 30: 454-460
14. **Fricker Hidalgo, H., F. Chazot, B. Lebeau, H. Pelloux, P. Ambroise Thomas, and R. Grillot.** 1998. Use of simulated blood cultures to compare a specific fungal medium with a standard microorganism medium for yeast detection. Eur. J. Clin. Microbiol. Infect. Dis. 17: 113-116
15. **Giamarellou, H. and A. Antoniadou.** 1996. Epidemiology, diagnosis, and therapy of fungal infections in surgery. Infect. Control Hosp. Epidemiol. 17: 558-564
16. **Gumbo, T., C.M. Isada, G. Hall, M.T. Karafa, and S.M. Gordon.** 1999. *Candida glabrata* Fungemia. Clinical features of 139 patients. Medicine 78: 220-227
17. **Hoppe, J.E. and P. Frey.** 1999. Evaluation of six commercial tests and the germ-tube test for presumptive identification of *Candida albicans*. Eur. J. Clin. Microbiol. Infect. Dis. 18: 188-191
18. **Hube, B., D. Sanglard, F.C. Odds, D. Hess, M. Monod, W. Schafer, A.J. Brown, and N.A. Gow.** 1997. Disruption of each of the secreted aspartyl proteinase genes SAP1, SAP2, and SAP3 of *Candida albicans* attenuates virulence. Infect. Immun. 65: 3529-3538
19. **Jarvis, W.R.** 1995. Epidemiology of nosocomial fungal infections, with emphasis on *Candida* species. Clin. Infect. Dis. 20: 1526-1530
20. **Jones, C.J., K.J. Edwards, S. Castaglione, M.O. Winfield, F. Sala, C. VandeWiel, G. Bredemeijer, B. Vosman, M. Matthes, A. Daly, R. Brettschneider, P. Bettini, M. Buiatti, E. Maestri, A. Malcevschi, N. Marmiroli, R. Aert, G. Volckaert, J. Rueda, R. Linacero, A. Vazquez, and A. Karp.** 1997. Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories. Molecular Breeding 3: 381-390
21. **Jordan, J.A.** 1994. PCR identification of four medically important *Candida* species by using a single primer pair. J. Clin. Microbiol. 32: 2962-2967
22. **Kao, A.S., M.E. Brandt, W.R. Pruitt, L.A. Conn, B.A. Perkins, D.S. Stephens, W.S. Baughman, A.L.**

Reingold, G.A. Rothrock, M.A. Pfaller, R.W. Pinner, and R.A. Hajjeh. 1999. The epidemiology of candidemia in two United States cities: results of a population-based active surveillance. Clin. Infect. Dis. 29: 1164-1170

23. **Kennedy, G.T., J.G. Barr, and C. Goldsmith.** 1995. Detection of bacteraemia by the continuously monitoring BacT/Alert system. J. Clin. Pathol. 48: 912-914
24. **Leidich, S.D., A.S. Ibrahim, Y. Fu, A. Koul, C. Jessup, J. Vitullo, W. Fonzi, F. Mirbod, S. Nakashima, Y. Nozawa, and M.A. Ghannoum.** 1998. Cloning and disruption of caPLB1, a phospholipase B gene involved in the pathogenicity of *Candida albicans*. J. Biol. Chem. 273: 26078-26086
25. **Maksymiuk, A.W., S. Thongprasert, R. Hopfer, M. Luna, V. Fainstein, and G.P. Bodey.** 1984. Systemic candidiasis in cancer patients. Am. J. Med. 77: 20-27
26. **McIlhatton, B.P., C. Keating, M.D. Curran, M.F. McMullin, J.G. Barr, J.A. Madrigal, and D. Middleton.** 2002. Identification of medically important pathogenic fungi by reference strand-mediated conformational analysis (RSCA). J. Med. Microbiol. 51: 468-478
27. **Meyer, W., K. Maszewska, and T.C. Sorrell.** 2001. PCR fingerprinting: a convenient molecular tool to distinguish between *Candida dubliniensis* and *Candida albicans*. Med. Mycol. 39: 185-193
28. **Pfaller, M.A., R.N. Jones, G.V. Doern, H.S. Sader, R.J. Hollis, and S.A. Messer.** 1998. International surveillance of bloodstream infections due to *Candida* species: frequency of occurrence and antifungal susceptibilities of isolates collected in 1997 in the United States, Canada, and South America for the SENTRY Program. The SENTRY Participant Group. J. Clin. Microbiol. 36: 1886-1889
29. **Pittet, D., N. Li, and R.P. Wenzel.** 1993. Association of secondary and polymicrobial nosocomial bloodstream infections with higher mortality. Eur. J. Clin. Microbiol. Infect. Dis. 12: 813-819
30. **Pujol, C., S. Joly, S.R. Lockhart, S. Noel, M. Tibayrenc, and D.R. Soll.** 1997. Parity among the randomly amplified polymorphic DNA method, multilocus enzyme electrophoresis, and Southern blot hybridization with the moderately repetitive DNA probe Ca3 for fingerprinting *Candida albicans*. J. Clin. Microbiol. 35: 2348-2358
31. **Reisner, B.S. and G.L. Woods.** 1999. Times to detection of bacteria and yeasts in BACTEC 9240 blood culture bottles. J. Clin. Microbiol. 37: 2024-2026
32. **Robert, F., F. Lebreton, M.E. Bougnoux, A. Paugam, D. Wassermann, M. Schlotterer, C. Tourte-Schaefer, and J. Dupouy-Camet.** 1995. Use of random amplified polymorphic DNA as a typing method for *Candida albicans* in epidemiological surveillance of a burn unit. J. Clin. Microbiol. 33: 2366-2371
33. **Sadhu, C., M.J. McEachern, E.P. Rustchenko-Bulgac, J. Schmid, D.R. Soll, and J.B. Hicks.** 1991. Telomeric and dispersed repeat sequences in *Candida* yeasts and their use in strain identification. J. Bacteriol. 173: 842-850
34. **Sanglard, D., B. Hube, M. Monod, F.C. Odds, and N.A. Gow.** 1997. A triple deletion of the secreted aspartyl proteinase genes SAP4, SAP5, and SAP6 of *Candida albicans* causes attenuated virulence. Infect. Immun. 65: 3539-3546
35. **Savelkoul, P.H., H.J. Aarts, J. de Haas, L. Dijkshoorn, B. Duim, M. Otsen, J.L. Rademaker, L. Schouls, and J.A. Lenstra.** 1999. Amplified-fragment length polymorphism analysis: the state of an art. J. Clin. Microbiol. 37: 3083-3091
36. **Shigei, J.T., J.A. Shimabukuro, M.T. Pezzlo, L.M. de la Maza, and E.M. Peterson.** 1995. Value of terminal subcultures for blood cultures monitored by BACTEC 9240. J. Clin. Microbiol. 33: 1385-1388
37. **Soll, D. R.** 2000. The ins and outs of DNA fingerprinting the infectious fungi. Clin. Microbiol. Rev. 13: 332-370
38. **Thanos, M., G. Schonian, W. Meyer, C. Schweynoch, Y. Graser, T.G. Mitchell, W. Presber, and H.J.**

Tietz. 1996. Rapid identification of *Candida* species by DNA fingerprinting with PCR. J. Clin. Microbiol. 34: 615-621

39. **Tinghitella, T.J. and M.D. Lamagdeleine.** 1995. Assessment of Difco ESP 384 blood culture system by terminal subcultures: failure to detect *Cryptococcus neoformans* in clinical specimens. J. Clin. Microbiol. 33: 3031-3033
40. **Uyttendaele, M., R. Schukkink, B. Van Gemen, and J. Debevere.** 1994. Identification of *Campylobacter jejuni*, *Campylobacter coli* and *Campylobacter lari* by the nucleic acid amplification system NASBA. J. Appl. Bacteriol. 77: 694-701
41. **Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. Van de Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper, and M. Zabeau.** 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res. 23: 4407-4414
42. **Watts, H.J., F.S. Cheah, B. Hube, D. Sanglard, and N.A. Gow.** 1998. Altered adherence in strains of *Candida albicans* harbouring null mutations in secreted aspartic proteinase genes. FEMS Microbiol. Lett. 159: 129-135
43. **Weinstein, M.P., S. Mirrett, L.G. Reimer, M.L. Wilson, S. Smith-Elekes, C.R. Chuard, K.L. Joho, and L.B. Reller.** 1995. Controlled evaluation of BacT/Alert standard aerobic and FAN aerobic blood culture bottles for detection of bacteremia and fungemia. J. Clin. Microbiol. 33: 978-981
44. **Wenzel, R.P.** 1995. Nosocomial candidemia: risk factors and attributable mortality. Clin. Infect. Dis. 20: 1531-1534
45. **Wey, S.B., M. Mori, M.A. Pfaller, R.F. Woolson, and R.P. Wenzel.** 1988. Hospital-acquired candidemia. The attributable mortality and excess length of stay. Arch. Intern. Med. 148: 2642-2645
46. **Ziegler, R., I. Johnscher, P. Martus, D. Lenhardt, and H.M. Just.** 1998. Controlled clinical laboratory comparison of two supplemented aerobic and anaerobic media used in automated blood culture systems to detect bloodstream infections. J. Clin. Microbiol. 36: 657-661

I: Value of terminal subculture of automated blood cultures in patients with candidaemia

Annemarie Borst, Maurine Leverstein-Van Hall, Jan Verhoef, Ad Fluit

Eijkman-Winkler Institute, University Medical Center, Utrecht, the Netherlands

European Journal of Clinical Microbiology and Infectious Diseases (2000), 19: 803-805

BRIEF REPORT

Yeasts have become increasingly important causes of invasive infections in immunocompromised patients. *Candida albicans* is the predominant causative agent in these infections. However, non-*albicans Candida* spp. are increasingly isolated. Automated blood culture systems are routinely used as diagnostic tool. Although the contents of the culture media of these systems as well as the exact method of detection differs between the different systems, the basic protocol is the same: blood is inoculated directly into the culture bottles and the bottles are cultured for 5-7 days, or until a positive growth-signal is obtained.

There has been an ongoing debate about the need of subculturing automated blood culture bottles that have remained negative. Some authors reported that yeasts often are detected only after terminal subculturing[3,4]. However, in recent years several authors claimed that all important pathogens (including yeasts) are detected by the automated blood culture system within the standard incubation time. For example, Reisner and Woods[2], stated that when using the Bactec 9240 system, 4 days of culturing is sufficient to detect all bacteria, and no more than 6 days are required to detect all important yeasts. Longer incubation times would only result in detection of contaminants. Ziegler et al.[5] compared different culture media in the Bactec 9240 and the BacT/Alert blood culture systems, and concluded that for both systems a 5-day incubation period is sufficient, and no terminal subculture is required. The same incubation time was recommended by Kennedy et al.[1] These authors declare that clinically relevant pathogens show a quick growth, and slow growing organisms are mainly contaminants.

The objective of our study was to evaluate whether these recent findings also hold true for patients who are at high risk for candidaemia. Therefore, positive as well as negative blood cultures obtained from patients with a culture-proven candidaemia were investigated. The results of BacT/Alert monitoring alone were compared with BacT/Alert monitoring in combination with blind subculturing of the negative blood cultures.

Ten patients were included. Nine patients were hospitalized in a university hospital, one patient was hospitalized in a children's hospital. Only the child was treated with antimycotic agents at the moment of inclusion. All blood cultures were taken on clinical indication and handled in the same microbiology lab. Blood samples were divided over two blood culture bottles: one FAN aerobic and one regular anaerobic BacT/Alert bottle. For the patient from the children's hospital, Pedi-BacT bottles were used (all bottles: Organon Teknika, the Netherlands). The blood culture bottles were incubated in the BacT/Alert monitoring system for 7 days, or until a positive signal was obtained. All bottles were subcultured on blood-agar and mold-agar.

For three patients, subculturing of the negative blood culture bottles resulted in extra information. Table 1 depicts the patient characteristics and the risk factors for invasive candidiasis present at the time of detection for these patients. For patients 2 and 3 (Table 1), the extra blood cultures found positive confirmed the diagnosis but did not change the treatment regimens. From the third patient however, positive subcultures were obtained when BacT/Alert monitoring alone would suggest that the infection was adequately treated (patient no. 1; Table 1). Nineteen blood cultures were drawn over a period of 19 days. Three of the six blood cultures found positive only after subculturing were drawn from the patient when

treatment with antimycotics (Amphotericin B) had already been started. Yeast was detected up to 7 days after the last BacT/Alert-positive blood culture. Treatment with Amphotericin B was continued until the patient died.

Our findings show, that subculturing of negative blood culture bottles can lead to additional information which may be clinically relevant. Therefore, we would like to comment that further evaluation of routine terminal subculturing of negative blood cultures from patients with suspected candidaemia and patients under treatment for candidaemia might be valuable.

Table 1
Clinical data and results of BacT/Alert monitoring and subculturing of the blood cultures

Patient no.	Underlying condition	Risk factors[a]	BCB (total)	BacT/Alert +		Subculture (add. +)		*Candida* spp. isolated
				AE	AN	AE	AN	
1	infected echinococcal cyst	a, c, d, e, f, g	19[b]	0	2	5	1	*C. glabrata*
2	colostomy	a, b, c, d, e, g	12	3	1	0	2	*C. albicans*
3	*Candida* endocarditis around prosthetic valve	a, b, g	8	1	0	0	1	*C. albicans*

BCB: blood culture bottles; AE: aerobic blood cultures; AN: anaerobic blood cultures

[a] a: broad-spectrum antibiotics; b: dialysis; c: intratracheal tube; d: laparotomy; e: septic shock; f: colonization with *Candida* spp.; g: arterial or central venous catheter

[b] data of one bottle not available

REFERENCES

1. **Kennedy, G.T., J.G. Barr, and C. Goldsmith.** 1995. Detection of bacteraemia by the continuously monitoring BacT/Alert system. J. Clin. Pathol. 48: 912-914
2. **Reisner, B.S. and G.L. Woods.** 1999. Times to detection of bacteria and yeasts in Bactec 9240 blood culture bottles. J. Clin. Microbiol. 37: 2024-2026
3. **Shigei, J.T., J.A. Shimabukuro, M.T. Pezzlo, L.M. de la Maza, and E.M. Peterson. 1995.** Value of terminal subcultures for blood cultures monitored by Bactec 9240. J. Clin. Microbiol. 33: 1385-1388
4. **Tinghitella, T.J. and M.D. Lamagdeleine.** 1995. Assessment of Difco ESP 384 blood culture system by terminal subcultures: failure to detect *Cryptococcus neoformans* in clinical specimens. J. Clin. Microbiol. 33: 3031-3033
5. **Ziegler, R., I. Johnscher, P. Martus, D. Lenhardt, and H.M. Just.** 1998. Controlled clinical laboratory comparison of two supplemented aerobic and anaerobic media used in automated blood culture systems to detect bloodstream infections. J. Clin. Microbiol. 36: 657-661

II: Nucleic acid sequence-based amplification (NASBA) detection of medically important *Candida* species

Myra Widjojoatmodjo[1], Annemarie Borst[1], Rianne Schukkink[2], Adrienne Box[1], Nicole Tacken[2], Bob van Gemen[2], Jan Verhoef[1], Bert Top[2], Ad Fluit[1]

[1] Eijkman-Winkler Institute, University Medical Center, Utrecht, the Netherlands
[2] Organon Teknika, Boxtel, the Netherlands

Journal of Microbiological Methods (1999), 38: 81-90

ABSTRACT

Nucleic Acid Sequence Based Amplification (NASBA), an isothermal amplification technique for nucleic acids, was evaluated for the identification of medically important *Candida* species using primers selected from 18S rRNA sequences conserved in fungi. An RNA fragment of 257 nucleotides was amplified for *Candida albicans*. Nineteen different fungi were tested for rRNA amplification with the NASBA. All were positive when analyzed on agarose gel, whereas human RNA was negative. For the identification of *Candida* species, NASBA amplification products were analyzed in an enzyme bead-based detection format, using species specific biotinylated probes and a generic *Candida* HRPO probe or a membrane-based system using biotinylated probes and avidin-HPRO. Discrimination of the major human pathogenic *Candida* spp. was based on a panel of biotinylated probes for *Candida krusei*, *Candida tropicalis*, *Candida albicans*, *Candida glabrata*, and *Candida lusitaniae*. Using rRNA dilutions obtained from pure cultures of *C. albicans*, the combination of NASBA and the enzymatic bead-based detection yielded a sensitivity equivalent to 0.01 cfu. In a model system using 1 ml of artificially contaminated blood as few as 1-10 cfu of *C. albicans* could be detected. Testing of 68 clinical blood samples from patients suspected of candidemia showed that eight samples were positive for *C. albicans* and one for *C. glabrata*. Testing of 13 clinical plasma samples from patients suspected of fungemia identified the presence of *C. albicans* in two specimens. The whole procedure of sample preparation, amplification and identification by hybridization can be performed in one day. This speed and the observed sensitivity of the assay make the NASBA a good alternative to PCR for the detection of candidemia.

INTRODUCTION

Opportunistic fungal infections are most often seen in immunocompromised patients. Candidiasis accounts for the majority of fungal infections in these patients. The diagnosis of systemic candidiasis has proved to be difficult. Blood cultures remain the major tool for the diagnosis of candidiasis, but fail to detect 50-70% of the cases. Detection of antibodies, antigens and metabolites have been investigated extensively, but none is satisfactory[1,3,13,18]. It is essential to have a rapid, reliable detection method which enables therapy to start as early as possible.

Fungemia is characterized by low numbers of yeast cells in the bloodstream. As few as 1-10 colony forming units (cfu) per ml of blood may be present. Therefore, amplification technologies provide promising methods for the rapid detection of fungemia. Several groups have shown the feasibility of PCR for the detection of candidemia[6,7,10-12,14,17,20,22,23,25,26,28,32]. However, sample volumes were either to small or the PCR amplification had good sensitivity but required a cumbersome sample preparation. More rapid sample preparation methods have been described, but these methods cannot process more than 200 µl blood[19,31].

An alternative approach to PCR is RNA amplification by NASBA (Nucleic Acid Sequence Based Amplification)[9,21]. NASBA is an isothermal nucleic acid amplification system of RNA that utilizes the simultaneous action of three enzymes: avian myeloblastosis virus reverse transcriptase (AMV-RT), RNase H and T7 RNA polymerase. Initially, a primer containing a

T7 RNA promotor sequence anneals to the single stranded RNA. After primer extension by AMV-RT, the RNA strand of the resulting RNA/DNA hybrid is degraded by RNase H. Then, the second primer anneals to the single stranded cDNA and AMV-RT generates a double stranded cDNA molecule containing a double stranded T7 promotor. Subsequently, T7 RNA polymerase initiates transcription and generates multiple anti-sense RNA copies, which now serve as template for the cyclic phase of the amplification. A major advantage of NASBA over PCR is that it is performed isothermally at 41°C and no separate RT step is required for RNA amplification. An additional advantage when using rRNA as target is that as many as 10,000 copies of rRNA can be present per cell. When HIV-1 RNA is used as input, the detection level with NASBA was as low as 10 molecules[21].

This is the first study using NASBA for the detection of fungi in clinical samples. This study investigated the use of the NASBA technique in combination with an improved rapid sample preparation method for the detection of *Candida* species spiked in blood specimens and specimen from patients suspected of candidemia. Whereas in most studies either plasma or serum was used for sample preparation, we studied the recovery from whole blood, which had the advantage that phagocytosed fungal cells or cells attached to leukocytes or other blood cells were also detected. Primers for NASBA were based on the conserved regions of the 18S rRNA sequence of medically important fungi[15]. For the identification of medically important *Candida* species, specific biotinylated probes were developed for *C. albicans, C. glabrata, C. krusei, C. tropicalis*, and *C. lusitaniae*. These probes were used in a microtiter format hybridization assay to identify amplified RNA.

MATERIALS AND METHODS

Fungal species. *C. albicans* (CBS 562), *C. tropicalis* (CBS 2310), *C. krusei* (*Issatchenkia orientalis*) (CBS 573), *C.* (*Torulopsis*) *glabrata* (CBS 138), *C. guilliermondii* (CBS 2024), *C. viswanathii* (CBS 4024), *C. lusitaniae* (CBS 4413), *C. parapsilosis* (CBS 2195), *Kluyveromyces marxianus* (CBS 607), *Kluyveromyces lactis* (CBS 739), *Torulasporula delbrueckii* (CBS 133), *Saccharomyces cerevisiae* (CBS 375), *Aspergillus niger* (CBS102.12), *A. nidulans* (CBS 100.20), *A. terreus* (CBS 106.25), *Penicillium chrysogenum* (CBS195.46), *P. marneffei* (CBS 549.77), *Paecilomyces variotii* (CBS 339.51) were obtained from the Centraal Bureau voor Schimmelcultures (CBS, Baarn, The Netherlands). *Cryptococcus neoformans* NIH3413. Clinical isolates were collected from the University Hospital Utrecht or as part of the SENTRY Antimicrobial Surveillance Program, in which 24 European university hospitals participate. Clinical isolates were identified either by VITEK or API (bioMérieux, France). Yeasts were grown on Sabouraud Dextrose Agar at 25°C for 1-2 days. Patient blood samples were obtained from the Antoni van Leeuwenhoek Ziekenhuis, Amsterdam and the University Hospital Utrecht. Plasma samples were obtained from the Daniel den Hoed Kliniek, Rotterdam.

Purification of target RNA. Blood was collected in EDTA tubes. To determine the sensitivity of the assay, blood samples were spiked with *C. albicans*. The collected blood samples were stored at -70°C until further use. Blood cells were lysed according to a modified protocol from Van Deventer[32]. Briefly, 0.9 ml of lysis buffer (0.32 M sucrose, 10 mM Tris-

HCl (pH 7.5), 5 mM $MgCl_2$, 1% Triton X-100) was added to 1.0 ml of thawed blood. After lysis, cell debris and *Candida* cells were pelleted by centrifugation at 16,000 x g for 5 min. The pellet was resuspended in 1.8 ml of lysis buffer with 15-75 U/ml DNase I (RNase-free, Boehringer Mannheim, Mannheim, Germany) and incubated for 30 min. at 37°C. After centrifugation at 16,000 x g for 5 min., the pellet was resuspended in 350 µl Qiagen RNeasy RLT solution (Qiagen, Hilden, Germany). Before loading the mixture onto a Qiagen RNeasy total RNA isolation column the suspension was sonicated for 5 min. in a Branson 221 sonication bath (Lameris, Breukelen, the Netherlands) and 250 µl ethanol (96%) was added. The isolation was performed according to the instructions of the manufacturer with a minor modification: RNA was eluted from the column with 75 µl water for 10 min. at 56°C. Columns were heated in a multiblock heater (Lab-line, Beun de Ronde, Abcoude, the Netherlands).

Primers and probes. The oligonucleotides used in this study are summarized in Table 1. Conserved primers were chosen from the 18S rRNA sequence alignment to amplify yeast and fungal specific sequences. Biotinylated primers were used for the identification of *Candida* species.

NASBA. Five µl target RNA in water was added to a pre-reaction mixture. The final volume of 15 µl contained 53 mM Tris-HCl (pH 8.5), 16 mM $MgCl_2$, 93 mM KCl, 6.7 mM DTT, 1.3 mM of each dNTP, 2.7 mM of each rNTP, 20% (v/v) dimethyl sulfoxide, and 0.27 µM of each primer. This mixture was first incubated at 65°C for 5 min. followed by 5 min. at 41°C. Then 5 µl of an enzyme mixture containing 2.1 µg BSA (Boehringer Mannheim), 6.4 U AMV-RT (Seigaku, Rockville, MD), 0.08 U RNase H (Pharmacia, Uppsala, Sweden), and 32 U T7 RNA polymerase (Pharmacia) in 1.5 M sorbitol was added. The reaction mixture, a 20 µl total volume, was incubated for 90 min. at 41°C. After amplification 2.5-5 µl was analyzed on a 2% agarose gel (Pronarose, Hispanagar, Burgos, Spain).

Table 1

Oligonucleotides used for NASBA and hybridization analysis.

Position[a]	5'-label	sequence (5' to 3')	name	description
55-76	-	ATGTCTAAGTATAAGCAATTTA	p2.1	forward NASBA primer
271-253	-	*AATTCTAATACGACTCACTATAGGGAG-AGA*CATGCGATTCGAAAAGTTA[b]	p1.1	reverse NASBA primer with T7 promotor site
157-174	HRPO	TCTAGAGCTAATACATGC	1727	yeast/fungi probe
180-203	biotin	ATCCCGACTGTTTGGAAGGGATGT	1913	*C. albicans, C. tropicalis, C. parapsilosis* probe
227-244	biotin	CCCTCGGGCCTTTTGATG	95.66	*C. krusei* probe
180-203	biotin	ATCTCGACCTCTTGGAAGAGATGT	1912	*C. glabrata* probe
180-203	biotin	AGCCCGACCTCTGGAAGGGCTGTA	1914	*C. lusitaniae* probe
222-238	biotin	CAATGTCTTCGGACTCTT	2176	*C. tropicalis* probe
87-110	biotin	CTGCGAATGGCTCATTAAATCAGT	UNI2	universal yeast/fungi probe

[a] position numbering of *C. albicans*

[b] italics: T7 promotor sequence

Enzymatic bead-based detection of NASBA products. Streptavidin-coated magnetic beads (Dynabeads M280-Streptavidin, Dynal, Oslo, Norway), washed once with 1% BSA in phosphate buffered saline were coated with 1 nmol biotinylated capture probe (Table 1) per 300 µl magnetic beads and incubated for 1 h at room temperature with continuous shaking. After incubation, the beads were recovered by magnetic force and washed once in 5 x SSPE (0.75 M NaCl, 50 mM sodium phosphate buffer (pH 7.4), 5 mM EDTA) plus 0.1% SDS, and once in phosphate buffered saline (PBS) containing 0.1% BSA. The beads were resuspended in the original volume of PBS with 0.1% BSA and stored at 4°C until used. One and a half µl of the NASBA product was incubated with 5 µl of the capture probe-coated magnetic beads in 50 µl hybridization mixture (5 x SSPE, 0.1% SDS, 0.1% blocking reagent (Boehringer Mannheim), and 10 µg/ml salmon sperm DNA) for 30 min. at 45°C in a flexible microtiter plate with shaking (Titertek, Flow Laboratories, Irvine, Ayrshire, Scotland). After recovery of the magnetic beads, they were incubated with 50 µl hybridization mix with the HRPO detection probe (1727) for 30 min. at 45°C with shaking. The beads were washed with 100 µl 2 x SSC (2 x SSC equals 0.3 M NaCl, 30 mM sodium citrate (pH 7.2), 0.1% BSA), TBS (0.1 M Tris-HCl (pH 8.0), 0.15 M NaCl) plus 0.2% Tween 20, and TBS, respectively. Then, 50 µl substrate was added (0.1 mg/ml 3,3',5',5-tetramethylbenzidine, 0.15 mg/ml urea peroxide in 0.1 M sodium acetate (pH 6.0)). After 10 min. incubation at room temperature the reaction was stopped with 50 µl 2 M H_2SO_4. The A_{450} was measured in a Bio-Rad model 3550 microplate reader (Bio Rad, Hercules, CA). Samples were regarded as positive when the optical density was 3 times higher than the average of the negative controls.

Membrane-based identification. Two µl NASBA product was spotted onto Z-probe membrane strips (Bio-Rad) which were rinsed with MilliQ water and dried. The filter with the spots was dried and baked for 30 min. at 80°C. The strips were covered with approximately 5 ml preheated hybridization mix containing 7% SDS, 5 x SSC, 20 mM sodium phosphate and 10 x Denhardt's solution. Twelve and a half µl biotin-probe (5 µM) was added and hybridization was allowed for 2 h at 50°C. The strips were washed twice for 5 min. with preheated 1% SDS in 3 x SSC at 50°C. Then the strips were washed once with 0.1% SDS in 2 x SSPE (1 x SSPE equals 100 mM di-sodium phosphate (pH 7.4), 175 mM NaCl, 1 mM EDTA) for 10 min. at room temperature. The strips were blocked with 0.5% SDS and 5% skimmed milk in 5 x SSPE for 15 min. at room temperature. The strips were washed once for 2 min. with 0.1% SDS in 2 x SSPE at room temperature. Two µl streptavidin-HRPO conjugate in 5 ml 0.1% SDS, 5 x SSPE was added to each strip and incubated for 30 min. at room temperature. Strips were washed twice for 1 min. and once for 10 min. with 0.1% SDS in 2 x SSPE and twice for 2 min. with 2 x SSPE at room temperature. Excess fluid was removed on filter paper and 5 ml substrate (2.5 mg diamino-benzidine, 1.25 mg $CoCl_2$, 0.02% nickel sulphate, 0.011% H_2O_2 in phosphate buffered saline) was added. Color development was stopped by rinsing with tap water.

Controls. To monitor the testing of samples several positive and negative controls were developed. A positive control for the whole procedure including RNA isolation was generated by adding 100 µl containing 1 x 10^8 cfu *C. albicans* to 0.9 ml lysis buffer, as a negative control 1 ml lysis buffer was used. The controls were centrifuged for 5 min. at 16,000 x g and the pellet was resuspended in 1.8 ml lysis buffer with 15-75 U/ml DNase I. Controls were treated further as described above. In addition to the controls described above, which also include

sample preparation, separate controls for the amplification were used. One positive control and two negative controls were used for each NASBA run. In the negative controls no template was added. The positive control consisted of the addition of 70 pg *C. albicans* RNA to the NASBA pre-reaction mixture. *C. albicans* control RNA was obtained by the transcription of the PCR product with primer p1.1 and p2.1 (Table 1) from the 18S rRNA genes, which was cloned into pGEM-T (Promega, Madison, WI). The negative controls were positioned at the beginning and the end of the series of samples which were tested. Finally for each species a bead-assay control was added. The target for hybridization was generated by amplifying the rRNA isolated from 1 x 10^7 cfu of the respective species with primers p1.1 and p2.1 as described above.

RESULTS

NASBA. Positive NASBA results were obtained for all fungi examined, including *C. albicans, C. tropicalis, C. krusei, C. glabrata, C. guilliermondii, C. viswanathii, C. lusitaniae, C. parapsilosis, K. marxianus, K. lactis, T. delbrueckii, S. cerevisiae, A. fumigatus, A. flavus, A. niger, A. nidulans, A. terreus, P. chrysogenum, P. marneffei, Paecilomyces variotii,* and *C. neoformans*. No amplification products were obtained with RNA isolated from *Escherichia coli, Staphylococcus aureus* and RNA isolated from human blood (data not shown).

Identification of *Candida* spp. Biotinylated probes developed for the discrimination of the medically important *Candida* species were tested with amplification products obtained from yeasts, fungi, and human RNA (Table 2). The probes for *C. glabrata*, *C. krusei*, and *C. lusitaniae* were specific for these species. The *C. albicans* probe 1913 reacted not only with *C. albicans* but also with *C. tropicalis, C. viswanathii, C. parapsilosis,* and *C. guilliermondii*. The *C. tropicalis* probe 2176 could be used to discriminate *C. tropicalis* from *C. albicans*, although it was not specific for *C. tropicalis* since it also hybridizes with *K. marxianus*, *K. lactis*, *S. cerevisiae,* and *T. delbrueckii*. All other fungi tested reacted only with the universal fungal probe UNI2, and were negative with the other *Candida* probes.

Thirty-two clinical isolates of *Candida* spp. were tested by NASBA amplification and enzyme bead-based identification. Twenty-five strains of *C. albicans*, 4 strains of *C. glabrata*, and 2 strains of *C. tropicalis* were identified correctly. One strain was identified as *C. tropicalis*, whereas conventional methods identified it as *C. albicans*.

Another 31 clinical isolates including 9 *C. glabrata*, 10 *C. parapsilosis*, 9 *C. tropicalis*, and 3 *C. guilliermondii* isolates yielded the expected results in the membrane-based identification system, except for 1 *C. glabrata* isolate which was identified as *C. parapsilosis* with conventional methods.

Sensitivity of detection of *C. albicans* by NASBA. Nucleic acids isolated from 2 x 10^4 cfu *C. albicans* were eluted in 100 μl water. A 10-fold dilution series was prepared from this stock solution and 5 μl of the dilution series was tested by NASBA. A 10^4-fold dilution, equivalent to 1 cfu was still positive on agarose gel (Fig. 1), whereas a 10^6-fold dilution, equivalent to 0.01 cfu was still positive when analyzed with enzymatic bead-based detection. Assuming that a *Candida* cell contains approximately 10^4 rRNA molecules[34], a sensitivity of 0.01 cfu is equivalent to 10 molecules of rRNA. When a tenfold dilution range was prepared from whole

C. albicans cells, followed by RNA isolation, NASBA amplification and enzymatic bead-based detection, reproducibly a sensitivity of 1-10 cfu was obtained.

Preparation of clinical samples. Blood samples were stored at -70°C until further processing. Short term storage of the blood samples had no negative effect on the sensitivity of the NASBA amplification. On the contrary, lysis of blood cells was more complete, and less dependent on blood composition than without storage at -70°C.

Several rapid methods have been described for blood sample preparation for PCR of *Candida* spp. Removal of erythrocytes by centrifugation and the use of the supernatant (plasma)[19] resulted in a loss of more than 50% of the initial input of *C. albicans*. A rapid method described by Fujita[12] which included lysis of erythrocytes and leukocytes in TE buffer with proteinase K and Tween 20 detergent followed by pelleting the yeast cells was not suitable for the reproducible processing of 1 ml blood, because of the low numbers of yeast cells present in our samples. The first steps of the method described by Buchmann[6] included lysis of blood cells by detergents, breakdown of human DNA by DNase, pelleting of the yeast cells, followed by a labour intensive DNA extraction from spheroplasts. The method described by Van Deventer[32], which was based on the Buchmann method, but included glass beads for the isolation of *Candida* DNA, was not directly suitable for processing more than 200 µl blood. We optimized this method by preceding the sample preparation with freeze/thawing the sample, optimizing the lysis of blood and yeast cells, and the use of a RNeasy column to bind fungal nucleic acids in the presence of guanidine thiocyanate[4]. This resulted in a rapid sample preparation method. Using this method, we were able to process 1 ml of blood with a sensitivity of 1 cfu per ml of whole blood in a reproducible manner (Fig. 2). The data obtained by agarose gel electrophoresis were confirmed by an enzymatic bead assay with *C. albicans* and universal probe (Table 3). The whole procedure of blood lysis, RNA isolation, NASBA and enzymatic bead-based identification could be performed in one day.

Table 2
Specificities of the species specific biotinylated probes in the enzyme bead-based detection assay with NASBA products obtained from the amplification of rRNA (derived from 10^3 cfu) from different yeasts.

RNA isolated from	UNI2 fungi	1912 *glabrata*	95.66 *krusei*	1914 *lusitaniae*	1913 *albicans*	2176 *tropicalis*
C. glabrata	+	+	-	-	-	-
C. krusei	+	-	+	-	-	-
C. lusitaniae	+	-	-	+	-	-
C. albicans	+	-	-	-	+	-
C. tropicalis	+	-	-	-	+	+
C. viswanathii	+	-	-	-	+	-
C. guilliermondii	+	-	-	-	+	-
C. parapsilosis	+	-	-	-	+	-
K. marxianus	+	-	-	-	-	+
K. lactis	+	-	-	-	-	+
S. cerevisiae	+	-	-	-	-	+

Contamination. Contamination is a major problem for amplification techniques, especially when 'universal' primers are used. Amplicon contamination has to be controlled by careful separation of amplification products from target or buffer, enzyme or nucleotide mixtures. Contamination of reagents and samples by environmental fungal RNA is more difficult to avoid, because of the ubiquitous nature of yeasts and fungi. A number of our negative controls showed amplification products on agarose gel. However, analysis in a bead assay showed that the amplification products were not obtained from *Candida* spp. present in the same assay, but appeared to be from other genera, e.g. *Saccharomyces*, *Kluyveromyces*, or other related yeasts. This problem infrequently occurred when no target rRNA from a sample was present. In addition we observed differences in the frequency when using different batches of reagents or water.

Clinical samples. Sixty-eight blood samples from patients suspected of candidemia were evaluated in the NASBA and bead assay in series of ten samples. In addition to the samples a number of controls were included. A negative control and positive control for the whole procedure including RNA isolation were included. A positive control for the NASBA only and two negative controls for the amplification, one at the beginning and one at the end of the amplification series, were added. Finally, for each probe an enzymatic bead-based detection control was added. Blood samples were considered positive when the A_{450} reading in the enzymatic bead-based detection assay was three times the average of the negative control of the NASBA amplification. Using these criteria, nine of the blood samples were positive. Eight samples reacted with the *C. albicans* probe and one sample with the *C. glabrata* probe. In addition, thirteen plasma samples were tested. Two of the samples were positive and contained *C. albicans*.

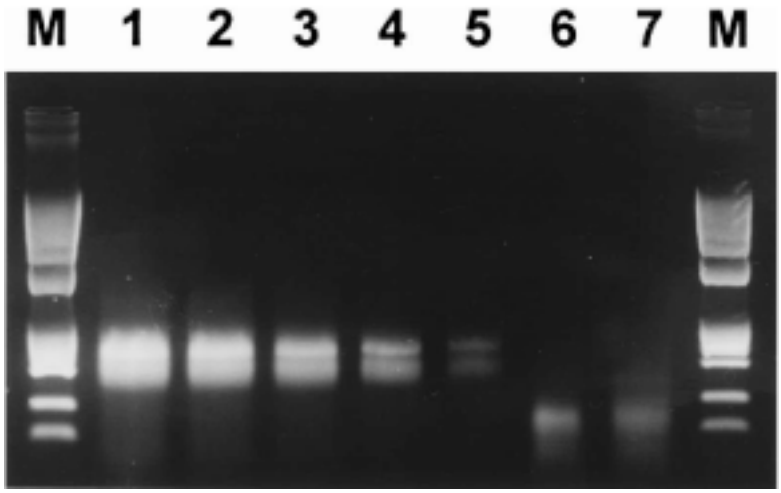


Figure 1
Detection levels of NASBA using agarose gel electrophoresis analysis on a 10-fold dilution range of rRNA isolated from 2 x 10^4 cfu *C. albicans*. Lane 1: rRNA dilution equivalent to 1000 cfu; lane 2: rRNA dilution equivalent to 100 cfu; lane 3: rRNA dilution equivalent to 10 cfu; lane 4: rRNA dilution equivalent to 1 cfu; lane 5: rRNA dilution equivalent to 0.1 cfu; lane 6: rRNA dilution equivalent to 0.01 cfu; lane 7: rRNA dilution equivalent to 0.001 cfu and lanes M: lambda *Pst*I digest as marker. Lane 1-5: The amplification product consists of a DNA/RNA hybrid (upper band) and RNA (lower band).

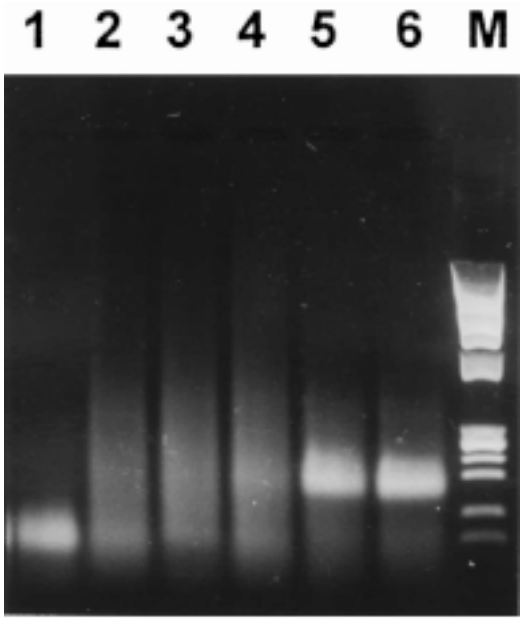


Figure 2
Agarose gel electrophoretic analysis of NASBA products after amplification of rRNA isolated from 1 ml blood samples spiked with increasing numbers of *C. albicans*. Lane 1: negative control for the NASBA; lane 2: blood sample without *C. albicans*; lane 3: 1 cfu/ml blood; lane 4: 10 cfu/ml blood; lane 5: 100 cfu/ml blood; lane 6: 1000 cfu/ml blood; lane M: lambda *PstI* digest as marker. The samples were also analyzed in the enzymatic bead-based detection system (Table 3).

DISCUSSION

We have developed a nucleic acid sequence-based amplification assay for the detection of *Candida* species. Amplification of nucleic acids by NASBA has a major advantage over PCR: the use of multicopy rRNA targets results in high sensitivities[21,33]. The primers chosen in the 18S rRNA can be used for NASBA of rRNA from all yeast and fungal species tested, including *Candida* spp., *Aspergillus* spp., and *C. neoformans*. The probes which were chosen in the amplified region could be used for the identification of medically important *Candida* spp. The detection limit obtained in terms of rRNA molecules (analytical sensitivity) was approximately 10-100 copies, equivalent to less than 1 cfu *C. albicans*. PCR is known to be sensitive to inhibitory compounds from blood, such as hemoglobin[16]. NASBA is relatively insensitive to inhibitors from blood. Due to the presence of thousands of copies of 18S rRNA the sample can be diluted.

A side effect of this extremely high sensitivity is the increased danger of contamination[2]. In fact a low level contamination with fungal rRNA was present in some batches of enzyme preparations and/or reagents (data not shown). A similar phenomenon has been observed for bacterial and yeast DNA in the PCR when either universal 16S or 18S rRNA primers were used[5,28]. The problems caused by low levels of RNA could be alleviated by the use of specific probes.

Identification of *Candida* spp. was based on its usefulness for therapy in clinical specimens. *C. albicans* is medically the most frequently encountered pathogenic yeast, but *C. glabrata*, *C. krusei*, *C. parapsilosis*, *C. lusitaniae*, and *C. tropicalis* are important emerging pathogens[24,30]. Especially, it is mandatory to identify *C. glabrata*, *C. krusei*, and *C. tropicalis*, because these species show a high prevalence of resistance against fluconazole, whereas *C. lusitaniae* isolates increasingly are reported to be Amphotericin resistant[8,27,29]. Consequently, specific biotinylated probes were developed for *C. albicans*, *C. glabrata*, *C. krusei*, *C. tropicalis*, and

Table 3
A_{450}-values obtained in the enzymatic bead-based detection assay with the *C. albicans* probe 1913 and the universal *Candida* probe UNI2 after NASBA of rRNA isolated from blood samples spiked with increasing numbers of *C. albicans*.

Sample	Lane no.[a]	*C. albicans* probe	UNI2 probe
negative control NASBA	1	0.071	0.124
negative blood	2	0.096	0.104
1 cfu/ml	3	0.091	0.099
10 cfu/ml	4	0.321	0.461
100 cfu/ml	5	0.931	0.790
1000 cfu/ml	6	0.751	0.806

[a] The lane no. corresponds to the lane number in Fig. 2.

C. lusitaniae. Identification of the NASBA products used a microtiter format, bead-based detection assay using biotin-labeled specific probes coupled to streptavidin-coated magnetic beads and detection with a generic probe which was labeled with HRPO. The use of streptavidin-coated magnetic beads as solid support for the biotinylated probes results in shorter diffusion distances and greater surface areas compared to standard hybridization assays. In turn, this led to shorter incubation times. An additional advantage of NASBA is that the resulting ssRNA product directly can be used in a hybridization assay, whereas PCR products have to be denatured[19] with the inevitable chance of renaturing. The hybridization assay was approximately 10 times more sensitive than agarose gel electrophoresis.

A number of papers described the use of PCR for the diagnosis of candidemia[17,20,22,23,25,26,28,32], but sample preparation was either laborious or could not cope with larger volumes of blood, which are required to detect the low number of microorganisms encountered in blood. Therefore, we focused on a rapid sample preparation method which could accommodate volumes up to 1 ml. Most studies used the method described by Buchmann[6]. Although this method was highly efficient[6,28], it was labour intensive. Our method is a simplified version of the method described by Buchmann. Blood cells were selectively lysed, fungal cells pelleted and RNA was isolated using guanidine thiocyanate and silica spin columns. This method resulted in a clinical sensitivity of 1-10 cfu per ml of whole blood. The procedure of blood lysis and RNA isolation took less than 3 hours and NASBA with enzymatic bead-based identification takes approximately 5 hours. Taken together, the complete procedure could be performed in one day. The lysis method developed was not only suitable for obtaining RNA from *Candida*, but also *Aspergillus* spp. The lysis of cryptococci requires a minor modification because the sample had to be incubated with lyticase from *Trichoderma harzianum* before the addition of guanidine thiocyanate (data not shown).

Evaluation of this NASBA protocol on 68 blood specimens obtained from patients suspected of candidemia suggested the presence of *C. albicans* in 8 specimens and in one specimen *C. glabrata*. Evaluation of 13 serum samples from patients suspected of candidemia identified two specimens with *C. albicans*. These data clearly show that NASBA in combination with the enzymatic bead-based identification can be used for the detection of *Candida* spp. in blood without prior need for culture. In fact the number of positive samples may increase when long term storage of the samples (which was more than 3 to 6 months for

many of our samples) can be avoided, because RNA appears to degrade during long term storage at -70°C.

More research has to be performed on a clinical specimen, but the prospect for NASBA detection and identification of yeasts in blood seems good. The speed and accuracy of this nucleic acid amplification technology can result in a more rapid diagnosis of candidemia and thereby reducing morbidity and mortality.

ACKNOWLEDGEMENTS

The authors wish to thank Drs. Rodenhuis and M.J. Holtkamp from the Antoni van Leeuwenhoekhuis in Amsterdam, the Netherlands, Dr. J.J. Cornelissen from the Daniel den Hoed Kliniek in Rotterdam, the Netherlands, and Drs. M. Rozenberg-Arska and A.W. Dekker form the University Hospital Utrecht, Utrecht, the Netherlands, for supplying blood and plasma specimens.

REFERENCES

1. **Baumgartner, C., A. Freydire, and Y. Gille.** 1996. Direct identification and recognition of yeast species from clinical material using Albicans ID and CHROMagar *Candida* plates. J. Clin. Microbiol. 34: 454-456
2. **Blais, B.W., G. Turner, R. Sooknanan, and L.T. Malek.** 1997. A nucleic acid sequence-based amplification system for detection of *Listeria monocytogenes hlyA* sequences. Appl. Environm. Microbiol. 63: 310-313
3. **Bodey, G.P. and E.J. Anaissie.** 1989. Chronic systemic candidiasis. Eur. J. Clin. Microbiol. Infect. Dis. 8: 98-101
4. **Boom, R., C.J.A. Sol, M.M.M. Salimans, C.L. Jansen, and P.M.E. Wertheim-van Dillen.** 1990. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. 28: 495-503
5. **Böttger, E.C.** 1990. Frequent contamination of *Taq* polymerase with DNA. FEMS Microbiol. Lett. 65: 171-176
6. **Buchmann, T.G., M. Rossier, W.G. Merz, and P. Charache.** 1990. Detection of surgical pathogens by in vitro amplification. Part I. Rapid identification of *C. albicans* by in vitro amplification of a fungus-specific gene. Surgery 108: 338-347
7. **Burgener-Kairuz, P., J.-P. Zuber, P. Jaunin, T.G. Buchmann, J. Bille, and M. Rossier.** 1994. Rapid detection and identification of *Candida albicans* and *Torulopsis (Candida) glabrata* in clinical specimens by species-specific nested PCR amplification of a cytochrome P-450 lanosterol-α-demethylase (l1A1) gene fragment. J. Clin. Microbiol. 32: 1902-1907
8. **Chavanet, P., J. Lopez, M. Grappin, A. Bonnin, M. Duong, A. Waldner, M. Buisson, P. Camerlynck, and H. Portier.** 1994. Cross-sectional study of the susceptibility of *Candida* isolates to antifungal drug *in vitro-in vivo* correlation in HIV patients. AIDS 8: 945-950
9. **Compton, J.** 1991. Nucleic acid sequence-based amplification. Nature 350: 91-92
10. **Crampin, A.C. and R.C. Matthews.** 1993. Application of the polymerase chain reaction to the diagnosis of

candidosis by amplification of an HSP90 gene fragment. J. Med. Microbiol. 39: 233-238

11. **Einsele, H., H. Hebart, G. Roller, J. Löffler, I. Rithenhöfer, C. Müller, R. Bowden, J. van Burik, D. Engelhard, L. Kanz, and U. Schumacher.** 1997. Detection and identification of fungal pathogens in blood by using molecular probes. J. Clin. Microbiol. 35: 1353-1360
12. **Fujita, S., B.A. Lasker, T.J. Lott, E. Reiss, and C. Morrison.** 1995. Microtitration plate enzyme immunoassay to detect PCR-amplified DNA from *Candida* species in blood. J. Clin. Microbiol. 33: 962-967
13. **Gutiéterrez, J., C. Maroto, G. Piédrola, E. Martin, and J.A. Perez.** 1993. Circulating *Candida* antigens and antibodies: useful markers of candidemia. J. Clin. Microbiol. 31: 2550-2552
14. **HeeShin, J., F.S. Nolte, and C.J. Morrison.** 1997. Rapid identification of *Candida* species in blood cultures by a clinically useful PCR method. J. Clin. Microbiol. 35: 1454-1459
15. **Hendriks, L., A. Goris, Y. Van de Peer, J. Neefs, M. Vancanneyt, K. Kersters, G.L. Hennebert, and R. De Wachter.** 1991. Phylogenetic analysis of five medically important *Candida* species as deduced on the basis of small ribosomal subunit RNA sequences. J. Gen. Microbiol. 137: 1223-1230
16. **Higushi, R.** 1989. Simple and rapid sample preparation of samples for PCR. In: PCR technology: principles and applications for DNA amplification (Ed. G.A. Ehrlich), Stockton Press, New York, p31-38
17. **Holmes, A.R., R.D. Cannon, M.G. Shepard, and H.F. Jenkinson.** 1994. Detection of *Candida albicans* and other yeasts in blood by PCR. J. Clin. Microbiol. 32: 228-231
18. **Jones, J.M.** 1990. Laboratory diagnosis of invasive candidiasis. Clin. Microbiol. Rev. 3: 32-45
19. **Jordan, J.A.** 1995. PCR identification of four medically important *Candida* species by using a single primer pair. J. Clin. Microbiol. 32: 2962-2967
20. **Kan, V.L.** 1993. Polymerase chain reaction for the diagnosis of candidemia. J. Infect. Dis. 168: 779-783
21. **Kievits, T., B. Van Gemen, D. Van Strijp, R. Schukkink, M. Dircks, H. Adriaanse, L. Malek, R. Sooknanan, and P. Lens.** 1991. NASBA™ isothermal enzymatic in vitro nucleic acid amplification optimized for the diagnosis of HIV-1 infection. J. Virol. Methods 35: 273-286
22. **Miyakawa, Y., T. Mabuchi, and Y. Fukazawa.** 1993. New method for detection of *Candida albicans* in human blood by polymerase chain reaction. J. Clin. Microbiol. 31: 3344-3347
23. **Morace, G., M. Sanguinetti, B. Posteraro, G.L. Cascio, and G. Fadda.** 1997. Identification of various medically important *Candida* species in clinical specimen by PCR-restriction enzyme analysis. J. Clin. Microbiol. 35: 667-672
24. **Nguyen, M.H., J.E. Peacock, A.J. Morris, D.C. Tanner, M.L. Nguyen, D.R. Snydman, M.M. Wagener, M.G. Rinaldi, and V.L. Yu.** 1996. The changing face of candidemia: emergence of non-*Candida albicans* species and fungal resistance. Am. J. Med. 100: 617-623
25. **Niesters, H.G.M., W.H.F. Goessens, J.F.M.G. Meis, and W.G.V. Quint.** 1993. Rapid, polymerase chain reaction-based identification assays for *Candida* species. J. Clin. Microbiol. 31: 904-910
26. **Polanco, A.M., J.L. Rodríguez-Tudela, and J.V. Martínez-Suárez.** 1995. Detection of pathogenic fungi in human blood by the polymerase chain reaction. Eur. J. Clin. Microbiol. Infect. Dis. 14: 618-621
27. **Price, M.F., M.T. LaRocco, and L.O. Gentry.** 1994. Fluconazole susceptibilities of *Candida* species and distribution of species recovered from blood cultures over a 5-year period. Antimicrob. Agents Chemother. 38: 1422-1424
28. **Rand, K.H., H. Houck, and M. Wolff.** 1994. Detection of candidemia by polymerase chain reaction. Mol. Cell. Probes 8: 215-221
29. **Rex, J.H., M.G. Rinaldi, and M.A. Pfaller.** 1995. Resistance of *Candida* species to fluconazole. Antimicrob. Agents Chemother. 39: 1-8
30. **Samaranayake, Y.H. and L.P. Samaranayake.** 1994. *Candida krusei*: biology, epidemiology,

pathogenicity and clinical manifestations of an emerging pathogen. J. Med. Microbiol. 41: 295-310

31. **Uyttendaele, M., R. Schukkink, B. Van Gemen, and J. Debevere.** 1994. Identification of *Campylobacter jejuni*, *Campylobacter coli* and *Campylobacter lari* by nucleic acid amplifcation system NASBA^{R}. J. Appl. Bacteriol. 77: 694-701
32. **Van Deventer, A.J.M., W.H.F. Goessens, A. Van Belkum, H.J.A. VanVliet, E.W.M. Van Etten, and H.A. Verbrugh.** 1995. Improved detection of *Candida albicans* by PCR in blood of neutropenic mice with systemic candidiasis. J. Clin. Microbiol. 33: 625-628
33. **Van Gemen, B., T. Kievits, R. Schukkink, D. Van Strijp, L.T. Malek, R. Sooknanan, H.G. Huisman, and P. Lens.** 1993. Quantification of HIV-1 RNA in plasma using NASBA^{TM} during HIV-1 primary infection. J. Virol. Methods 43: 177-188
34. **Wilson, K.H., R.B. Blitchington, and R.C. Greene.** 1990. Amplification of bacterial 16S RNA with PCR. J. Clin. Microbiol. 28: 1942-1946

III: Detection of *Candida* spp. in blood cultures using nucleic acid sequence-based amplification (NASBA)

Annemarie Borst, Maurine Leverstein-Van Hall, Jan Verhoef, Ad Fluit

Eijkman-Winkler Institute, University Medical Center, Utrecht, the Netherlands

Diagnostic Microbiology and Infectious Disease (2001), 39: 155-160

ABSTRACT

Candida spp. are the main causes of fungal infections in immunocompromised patients. It is known, that the routinely used automated blood culture systems may fail to detect yeasts. We therefore investigated, whether Nucleic Acid Sequence-Based Amplification (NASBA) can be used to improve the detection rate of *Candida* spp. in blood cultures. Culture-positive as well as negative blood cultures from patients with a proven candidaemia were analyzed, and the results of BacT/Alert monitoring were compared with the results of NASBA-based detection of yeast RNA. With the NASBA-assay, the number of positive blood cultures increased from 21% to 34%. The NASBA-assay may confirm the diagnosis and demonstrate the need for prolonged treatment. In addition it may shorten the time to detection. In summary, using NASBA for the detection of yeast RNA in blood cultures, we have shown for the first time that it is possible to improve the detection rate of yeasts in blood cultures by using amplification technology.

INTRODUCTION

Most fungal species involved in human disease are opportunistic pathogens and only cause problems in debilitated individuals. However, due to the profound developments in medical care, the number of immunocompromised patients has increased, and yeasts and fungi are found more often as causes of systemic infections. High-risk groups include: neutropenic cancer patients, transplant recipients and patients receiving broad-spectrum antibiotics or parenteral nutrition[20]. Candidiasis accounts for the majority of fungal infections in these patients. In North America, *Candida* is the 4th most common bloodstream pathogen, and in Europe *Candida* spp. rank 8th[5,8]. The main species is *Candida albicans*, but in recent years other species such as *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, *C. lusitaniae* and *C. krusei* were isolated more often[1,15,16,21,24]. These different *Candida* spp. show differences in resistance against antimycotic drugs. For example, *C. krusei* and many strains of *C. glabrata* show resistance against fluconazole[16]. Therefore, a rapid detection and identification to the species-level is essential to start an adequate treatment as soon as possible.

Automated blood culture systems are routinely used as diagnostic tool. However, in many cases these blood cultures fail to detect yeasts (up to 65%) or need prolonged incubation times or terminal subculturing of negative blood culture bottles before yeast growth can be detected[11,18,19]. This may be caused by the fact that many blood culture media are not optimal for fungal growth, by the presence of antimycotics in the blood and/or by a low number of organisms present in the blood. Special media for detection of fungal growth are available[6]. However, because it is labour intensive and expensive to use several systems at the same time, most laboratories only use the standard blood culture bottles.

Because in fungemia only 1-10 colony forming units (cfu) per ml blood may be present, amplification technologies provide promising methods for the rapid detection of fungemia. Several groups have published good results when using PCR on DNA isolated from whole blood for the detection of candidaemia. However, one of the problems when using PCR is the possibility of detecting naked DNA derived from dead and degrading yeast cells instead of live

yeasts, which results in false-positives[4,13]. Also, in many cases only small sample volumes could be used, or a long and cumbersome sample preparation was needed[2,7,9,12-14,17].

We have shown previously, that using Nucleic Acid Sequence-Based Amplification (NASBA[3]) we were able to detect 10 molecules of rRNA when using RNA isolated from cultures of *C. albicans*, and 1-10 cfu *C. albicans* in one ml of whole blood. For this RNA-amplification method, we made use of primers directed against conserved regions of the 18S rRNA of medically important fungi. For the identification of different *Candida* spp., specific biotinylated probes were used[23]. The advantage of using rRNA as target is, that as many as 10,000 copies of rRNA can be present per cell. This accounts for the high sensitivity of our assay. Also, because RNA degrades relatively quickly after cell-death, amplification-products found are mainly derived from living yeast cells.

The aim of our present study was to investigate whether NASBA can be used to improve the detection rate of *Candida* spp. in blood cultures. For this, we analyzed culture-positive as well as negative blood cultures from patients with a proven candidaemia, and compared the results of BacT/Alert monitoring with the results of NASBA-based detection of yeast RNA.

MATERIALS AND METHODS

Fungal species. *Candida albicans* (CBS 562) was obtained from the Centraal Bureau voor Schimmelcultures (CBS, Baarn, the Netherlands). Yeasts were grown on Sabouraud Dextrose Agar (SDA) at 35-37°C for 2 days.

Clinical blood cultures. Clinical blood culture samples were obtained from the University Medical Center Utrecht between September 1998 and November 1999. During this period, all patients with at least one blood culture positive for *Candida* were included in the study. Furthermore, 5 uninfected patients and 5 patients who were colonized with *Candida* were randomly selected. All blood cultures were taken on clinical indication, and handled in the same microbiology lab. Blood from the patients was cultured in sets of blood culture bottles: one FAN aerobic and one regular anaerobic BacT/Alert bottle. In case of children, Pedi-BacT bottles were used (Organon Teknika, Boxtel, the Netherlands). Culture of the blood culture bottles was performed under routine laboratory conditions. Blood cultures were cultured in the BacT/Alert monitoring system for 7 days, or until a positive signal was obtained. For the NASBA-assay, one ml samples were taken from the bottles and stored at -70°C until further use.

Spiking experiments. For the spiking experiments, whole blood of healthy volunteers was added to BacT/Alert blood culture medium (1:4 v/v), the mixture was incubated for 24 hours at 37°C, and 1 ml samples were taken. *C. albicans* was grown on SDA plates for 2 days, and suspended in physiological salt solution. Ten-fold dilutions containing 10^6 down to 1 cfu/50 µl were made and spiked to 1 ml blood culture samples. These spiked samples were frozen at -70°C for at least 20 minutes and further treated as described below. To verify the number of yeast cells in the solutions, samples of 100 µl physiological salt solution containing a calculated amount of 10 or 100 cfu *C. albicans* were plated on SDA. Colonies were counted after 2 days of culturing at 35-37°C.

Extraction of RNA. Blood culture samples were frozen at -70°C for at least 20 minutes.

After thawing, 0.9 ml Lysis Buffer (0.32 M sucrose; 10 mM Tris-HCl (pH 7.5); 5 mM $MgCl_2$; 1% Triton X100) was added and the samples were centrifuged for 5 minutes at 13,000 g. The pellet was resuspended in 100 µl Enzyme Buffer containing 2 mg/ml lyticase (Sigma-Aldrich, Steinheim, Germany), 2 mg/ml lysing enzymes (Sigma-Aldrich) and 0.17% ß-mercaptoethanol in 50 mM Tris-HCl (pH 7.5)/10 mM EDTA, and incubated at 37°C for 10 minutes. One ml RNAzol (Campro Scientific, Veenendaal, the Netherlands) was added and RNA was extracted according to the manufacturer, with minor modifications: 600 µl isopropanol was added to the aqueous phase instead of 500 µl; after washing with ethanol the pellet was dried for 10 minutes at 56°C, and RNA was dissolved for 10 minutes at 56°C in 20-40 µl water that was treated with UV-light for 2 hours. Samples were stored at -70°C until further use.

Primers and probes. The oligonucleotides used in this study are depicted in Table 1. When ECL-detection (electrochemiluminescence) was used, primer 2 was extended at the 5'-end with the following sequence: 5'- GAT GCA AGG TCG CAT ATG AG-3'. The specificity of the probes and the sensitivity of the NASBA-assay were described previously by Widjojoatmodjo et al.[23]

NASBA. Five µl RNA sample was taken up in a pre-reaction mixture with a final volume of 15 µl, containing 53 mM Tris-HCl (pH 8.5), 16 mM $MgCl_2$, 93 mM KCl, 6.7 mM DTT, 1.3 mM of each dNTP, 2.7 mM of each rNTP, 20% (v/v) dimethyl sulfoxide and 0.27 µM of each primer. The whole mixture was first incubated at 65°C for 5 minutes followed by 5 minutes at 41°C. Then 5 µl of an enzyme mixture containing 2.1 µg BSA, 6.4 U AMV-RT (Seigaku, Rockville, MD), 0.08 U RNase H and 32 U T7 RNA polymerase in 1.5 M sorbitol was added. The reaction mixture was incubated for 90 minutes at 41°C.

Controls. One positive and at least two negative controls for the amplification were used for each NASBA reaction. As a positive control for the amplification, 0.70 fg *C. albicans* RNA was used. In the negative controls, no template was added. Negative controls were positioned at the beginning and end of the series of samples that was tested. As a negative control for the whole procedure of isolation and amplification, 50 µl of physiological salt solution was added to samples containing blood and blood culture medium. These samples were treated as described for the spiking experiments.

Dot-blot analysis of NASBA products. Two µl NASBA product was spotted onto Z-probe membrane strips (BioRad, Hercules, CA) which were rinsed with water and dried. The filter with the spots was dried and baked for 30 min. at 80°C. The strips were covered with approximately 5 ml preheated hybridization mix containing 7% SDS, 5 x SSC, 20 mM NaH_2PO_4 and 10 x Denhardt's solution. Twelve and a half µl biotinylated probe (5 µM; Table 1) was added and hybridization was allowed for at least 2 h at 50°C. The strips were washed twice for 5 min. with preheated 1% SDS in 3 x SSC at 50°C. Then the strips were washed once with 0.1% SDS in 2 x SSPE (1 x SSPE equals 100 mM Na_2HPO_4 (pH 7.4), 175 mM NaCl, 1 mM EDTA) for 10 min. at room temperature. The strips were blocked with 0.5% SDS and 5% skimmed milk in 5 x SSPE for 15 min. at room temperature. The strips were washed once for 2 min. with 0.1% SDS in 2 x SSPE at room temperature. Two µl streptavidin-HRPO conjugate in 5 ml 0.1% SDS, 5 x SSPE was added to each strip and incubated for 30 min. at room temperature. Strips were washed twice for 1 min. and once for 10 min. with 0.1% SDS in 2 x SSPE and twice for 2 min. with 2 x SSPE at room temperature. Excess fluid was removed on filter paper and 5 ml substrate (2.5 mg diaminobenzidine, 1.25 mg $CoCl_2$, 0.02% nickel

sulphate, 0.011% H_2O_2 in phosphate buffered saline) was added. Color development was stopped by rinsing with tap water.

ECL-detection. For ECL-detection, the NucliSens Basic Kit Detection Reagents in combination with the NucliSens Reader (Organon Teknika, Boxtel, the Netherlands) were used according to the manufacturer. Amplification products were simultaneously hybridized to the specific capture probes (Table 1) as well as to the generic ECL probe provided in the Basic Kit. This probe hybridizes to the generic sequence, incorporated by primer 2 during amplification. Amplification products were diluted 1:20 before hybridization with capture probes 1912, 1913, 1914 or 9566, and a 1:200 dilution was used when amplicons were hybridized with probes 2176 or UNI2. Hybridization took place at 41°C for 30 minutes. ECL-signals were considered positive when ≥ 17% of the Instrument Reference Solution signal.

Table 1

The primers and probes used in this study.

Primer	Sequence (5' to 3')	
primer 1	*AATTCTAATACGACTCACTATAGGGAGAGA*-CATGCGATTCGAAAAGTTA[a]	
primer 2	*GATGCAAGGTCGCATATGAG*-ATGTCTAAGTATAAGCAATTTA[b]	
Probe	Sequence (5' to 3')	Target
1912	ATCTCGACCTCTTGGAAGAGATGT	*C. glabrata*
1913	ATCCCGACTGTTTGGAAGGGATGT	*C. albicans; C. tropicalis; C. parapsilosis; C. viswanathii; C. guilliermondii*
1914	AGCCCGACCTCTGGAAGGGCTGTA	*C. lusitaniae*
2176	CAATGTCTTCGGACTCTT	*C. tropicalis*
9566	CCCTCGGGCCTTTTGATG	*C. krusei*
UNI2	CTGCGAATGGCTCATTAAATCAGT	universal yeast/fungi probe

[a] italics: T7 promotor sequence
[b] italics: generic sequence used for ECL-detection

RESULTS

Spiking experiments. NASBA-products of RNA isolated from blood cultures spiked with *C. albicans* were analyzed by dot-blot as well as used for ECL-detection. When aerobic medium was used, dot-blot analysis resulted in a detection limit of 10^4 cfu/ml, whereas with ECL-detection a detection limit of 10^3 cfu/ml was repeatedly found. When anaerobic medium was used, 10-100 cfu/ml could still be detected using dot-blot analysis or ECL-detection.

Negative controls. For the negative controls, the isolation and amplification procedures were performed on 1 ml samples of aerobic and anaerobic BacT/Alert blood-culture medium. For both types of medium, 10 different Lot-numbers were used. None of the probes used showed any hybridization when the NASBA-products were analyzed by dot-blot.

In addition, blood from 10 different healthy volunteers was mixed with blood culture medium (aerobic and anaerobic) and processed as described. NASBA-products were dot-blotted and hybridized with the biotinylated probes. No hybridization was observed with any of the *Candida*-probes.

Table 2

Patient information and comparison between BacT/Alert monitoring of clinical blood cultures and NASBA detection of yeast RNA

Patient no.	Sex	Age (yrs)	Underlying conditions	Risk factors[a]	no. BCB (total)	BacT/Alert +	NASBA +[d]	spp.
1	M	46	infected echinococcal cyst	a, c, d, e, g, h	19[b]	2	8	*C. glabrata*
2	F	47	colostomy	a, b, c, d, e, h	12	4	6	*C. albicans*
3	M	57	*Candida* endocarditis around prosthetic valve	a, b, h	8	1	5	*C. albicans*
4	M	56	aorto-enteral fistula, aorto-bifemural prosthesis	a, b, c, d, e, h, i	10	1	1	*C. albicans*
5	F	83	pancreatitis	a, d, h	4	1	1	*C. albicans*
6	F	71	cholecystectomy	a, c, e, g, h	5[b]	1	1	*C. glabrata*
7	F	68	ALS, pneumonia	a, c, e, g, h, i	12	1	1	*C. albicans*
8	F	51	RA, intestinal bleeding	a, c, e, f, g, h	6	1	1	*C. albicans*
9	M	46	pneumonia, respiratory insufficiency	a, b, c, g, h, i	6	1	1	*C. albicans*
10	M	6	CF, DM, port-a-cath infected with *Candida*	a, c, g, h, i	9[c]	6	6	*C. albicans*
				total:	91	19 (21%)	31 (34%)	

BCB: blood-culture bottles; ALS: amyotrophic lateral sclerosis; RA: rheumatoid arthritis; CF: cystic fibrosis; DM: diabetes mellitus

[a] a: broad-spectrum antibiotics; b: dialysis; c: intratracheal tube; d: laparotomy; e: septic shock; f: steroids; g: colonization with *Candida* spp.; h: arterial or central venous catheter; i: parenteral nutrition

[b] data of one bottle not available

[c] Pedi-BacT blood-culture bottles

[d] all samples that were BacT/Alert-positive, were also positive in the NASBA-assay

Uninfected patients. Patients were considered not infected when no *Candida* spp. were found in any culture (blood- as well as other cultures) and no risk-factors for candidaemia were present. One ml samples were taken from blood cultures of these patients and processed as described. Ten aerobic and ten anaerobic blood cultures derived from five different patients were examined. None of the NASBA-products hybridized with any of the *Candida*-probes.

Patients colonized with *Candida* spp. Patients were considered colonized when blood cultures were negative for yeast, but *Candida* spp. were cultured from another site. Ten aerobic and ten anaerobic blood cultures derived from five different patients were examined. *Candida* spp. were cultured from sputum (patients 1, 2, 3 and 5), catheter tip (patients 1, 2 and 5), urine (patients 1 and 4), throat swab, anal swab and wound fluid (patient 1) and bronchial lavage and aspirate of the sinus cavity (patient 5). One NASBA-product derived from an anaerobic blood culture bottle from patient 4 hybridized with probe 2176.

Patients with a culture-proven candidaemia. Ten different patients with at least one blood culture positive for *Candida* spp. were included. Nine patients were hospitalized in a university hospital, one patient was hospitalized in a children's hospital. One ml samples were taken from positive as well as negative blood cultures and processed as described.

Patient information and the results of BactT/Alert monitoring and the NASBA-assay are depicted in Table 2. All samples that were BacT/Alert-positive, were also positive in the NASBA-assay. In patients 1, 2 and 3, yeast RNA was detected in cultures that remained negative in the BacT/Alert monitoring system. In 9 of these 12 BacT/Alert-negative/NASBA-positives, this result was confirmed when the same *Candida* spp. was isolated after subculturing the blood culture bottles (data not shown). Table 3 shows the time-course over which the blood cultures of patients 1, 2 and 3 were drawn. Patient 1 was treated with amphotericin B at time of blood sampling for 14 out of 19 blood cultures.

Table 3
Time course of blood sampling and the results of BacT/Alert monitoring and the NASBA-assay for patients 1, 2 and 3

Patient	Day no.[a]	no. BCB (total)	BacT/Alert +	NASBA +	Antimycotic treatment[c]
1	1	1[b]	1	1	no
	7	4	1	4	no
	8	2	-	-	yes
	9	4	-	-	yes
	14	6	-	3	yes
	19	2	-	-	yes
2	1	6	-	-	no
	5	6	4	6	no
3	1	2	-	2	no
	2	2	-	1	no
	3	2	1	1	no
	4	2	-	1	no

BCB: blood culture bottles
[a] day of blood sampling
[b] data of one bottle not available
[c] at time of blood sampling

DISCUSSION

In our experiments, we have obtained a detection limit for *C. albicans* in blood cultures of 10^3 cfu/ml for aerobic and 10-100 cfu/ml for anaerobic medium. The lower sensitivity for aerobic medium compared to anaerobic medium might be caused by the presence of 'Ecosorb', a substance containing absorbent charcoal material and Fuller's earth[22]. Ecosorb components may interfere with the RNA isolation or residual particles may inhibit the amplification. However, the sensitivity may not be a problem, since Shigei et al.[18] and Tinghitella and Lamagdeleine[19] have shown, that some automated blood culture instruments may fail to detect yeasts in spite of good growth of the organisms in the culture bottles, as was demonstrated by confluent growth after subculturing. Flahaut et al.[4] have used PCR for the detection of *C. albicans* in clinical samples, and report a detection limit of 20 cfu/ml for blood cultures. However, since they do not mention the type of blood culture medium used, it is not possible to compare their results with ours.

In the experiments where blood cultures from patients colonized with *Candida* spp. were used, we observed that one of the NASBA-products hybridized with probe 2176, which is used for the identification of *Candida tropicalis*. However, if the amplification product was obtained from *Candida tropicalis*, it should also hybridize with probe 1913, specific for *Candida albicans, C. tropicalis, C. viswanathii, C. parapsilosis* and *C. guilliermondii*, which it did not[23]. Probe 2176 cross-hybridizes with *Saccharomyces cerevisiae*, an organism that is one of the main causes of contaminations in fungal amplification assays[10]. Therefore, it is likely that our NASBA-product was derived from a *S. cerevisiae* contamination. All other samples from colonized patients, and also all samples from uninfected patients, healthy volunteers and culture medium alone were negative, which indicates that our assay has a low risk of generating false-positives.

When looking at clinical blood cultures from patients with a culture-proven candidaemia, yeast RNA was detected in cultures that had remained negative in the BacT/Alert monitoring system in three out of ten patients. With the NASBA-assay, the number of positive blood cultures increased from 21% to 34%. In patient 1, six blood culture bottles were positive only in the NASBA-assay. *Candida glabrata* was recovered after subculturing these six blood culture bottles, proving that these findings were not false-positives. Three blood culture bottles were positive in the NASBA-assay after the patient had been treated with amphotericin B for 7 days. In this case, BacT/Alert monitoring alone would have suggested that the infection was adequately treated.

For patient 2, the NASBA-assay led to detection of the yeast in the anaerobic bottles of two blood-culture sets, of which the aerobic bottles had already been positive in the BacT/Alert monitoring system. In this case, the additional positive bottles supported the diagnosis.

In the third patient, yeast was detected in four blood-culture bottles that had remained negative in the BacT/Alert monitoring system. We were not able to recover the yeast by subculturing in three of these four BacT/Alert-negative/NASBA-positives. However, since the patient was not treated with antimycotic agents at any time, it is not likely that the amplification product was derived from 'naked RNA' from degrading yeast cells. Yeast RNA was detected in blood culture bottles that were taken two days earlier than the first blood-culture that became positive in the BacT/Alert monitoring system. Even though this finding

supports our hypothesis that by using NASBA it may be possible to improve the detection rate as well as shorten the time to detection, in this case it was not significant since the patient had died before candidaemia was diagnosed.

Our study is the first to describe the use of RNA-amplification for detection of yeasts in blood cultures. Flahaut et al.[4] have used PCR for the detection of *C. albicans* in clinical samples. In their study, the results of the PCR were in complete accordance with the results of blood culturing. To our knowledge, we have shown for the first time that it is possible to improve the detection rate of yeasts in blood cultures by using an amplification technology.

ACKNOWLEDGEMENTS

This work was supported by a grant from Organon Teknika.

We would like to thank Bob van Gemen, Peter Haima and Peter Sillekens for the useful discussions.

REFERENCES

1. **Abi Said, D., E. Anaissie, O. Uzun, I. Raad, H. Pinzcowski, and S. Vartivarian.** 1997. The epidemiology of hematogenous candidiasis caused by different *Candida* species. Clin. Infect. Dis. 24: 1122-1128
2. **Burgener Kairuz, P., J.P. Zuber, P. Jaunin, T.G. Buchman, J. Bille, and M. Rossier.** 1994. Rapid detection and identification of *Candida albicans* and *Torulopsis* (*Candida*) *glabrata* in clinical specimens by species-specific nested PCR amplification of a cytochrome P-450 lanosterol-alpha-demethylase (L1A1) gene fragment. J. Clin. Microbiol. 32: 1902-1907
3. **Compton, J.** 1991. Nucleic acid sequence-based amplification. Nature 350: 91-92
4. **Flahaut, M., D. Sanglard, M. Monod, J. Bille, and M. Rossier.** 1998. Rapid detection of *Candida albicans* in clinical samples by DNA amplification of common regions from *C. albicans*-secreted aspartic proteinase genes. J. Clin. Microbiol. 36: 395-401
5. **Fluit, A. C., M.E. Jones, F.J. Schmitz, J. Acar, R. Gupta, and J. Verhoef.** 2000. Antimicrobial susceptibility and frequency of occurrence of clinical blood isolates in Europe from the SENTRY Antimicrobial Surveillance Program, 1997 and 1998. Clin. Infect. Dis. 30: 454-460
6. **Fricker Hidalgo, H., F. Chazot, B. Lebeau, H. Pelloux, P. Ambroise Thomas, and R. Grillot.** 1998. Use of simulated blood cultures to compare a specific fungal medium with a standard microorganism medium for yeast detection. Eur. J. Clin. Microbiol. Infect. Dis. 17: 113-116
7. **Fujita, S., B.A. Lasker, T.J. Lott, E. Reiss, and C.J. Morrison.** 1995. Microtitration plate enzyme immunoassay to detect PCR-amplified DNA from *Candida* species in blood. J. Clin. Microbiol. 33: 962-967
8. **Jarvis, W. R.** 1995. Epidemiology of nosocomial fungal infections, with emphasis on *Candida* species. Clin. Infect. Dis. 20: 1526-1530
9. **Jordan, J. A.** 1994. PCR identification of four medically important *Candida* species by using a single primer pair. J. Clin. Microbiol. 32: 2962-2967
10. **Loeffler, J., H. Hebart, R. Bialek, L. Hagmeyer, D. Schmidt, F.P. Serey, M. Hartmann, J. Eucker, and**

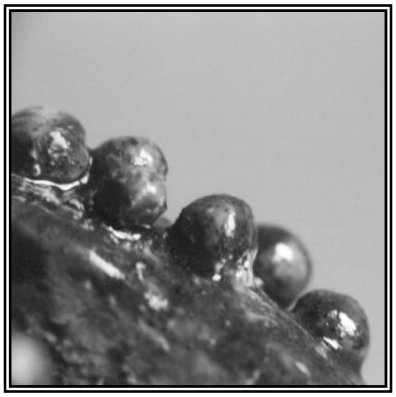
H. Einsele. 1999. Contaminations occurring in fungal PCR assays. J. Clin. Microbiol. 37: 1200-1202

11. **Maksymiuk, A.W., S. Thongprasert, R. Hopfer, M. Luna, V. Fainstein, and G.P. Bodey.** 1984. Systemic candidiasis in cancer patients. Am. J. Med. 77: 20-27
12. **Miyakawa, Y., T. Mabuchi, and Y. Fukazawa.** 1993. New method for detection of *Candida albicans* in human blood by polymerase chain reaction. J. Clin. Microbiol. 31: 3344-3347
13. **Morace, G., L. Pagano, M. Sanguinetti, B. Posteraro, L. Mele, F. Equitani, G. D'Amore, G. Leone, and G. Fadda.** 1999. PCR-restriction enzyme analysis for detection of *Candida* DNA in blood from febrile patients with hematological malignancies. J. Clin. Microbiol. 37: 1871-1875
14. **Morace, G., M. Sanguinetti, B. Posteraro, G. Lo Cascio, and G. Fadda.** 1997. Identification of various medically important *Candida* species in clinical specimens by PCR-restriction enzyme analysis. J. Clin. Microbiol. 35: 667-672
15. **Nguyen, M. H., J.E. Peacock Jr., A.J. Morris, D.C. Tanner, M.L. Nguyen, D.R. Snydman, M.M. Wagener, M.G. Rinaldi, and V.L. Yu.** 1996. The changing face of candidemia: emergence of non-*Candida albicans* species and antifungal resistance. Am. J. Med. 100: 617-623
16. **Pfaller, M. A., R.N. Jones, G.V. Doern, H.S. Sader, R.J. Hollis, and S.A. Messer.** 1998. International surveillance of bloodstream infections due to *Candida* species: frequency of occurrence and antifungal susceptibilities of isolates collected in 1997 in the United States, Canada, and South America for the SENTRY Program. The SENTRY Participant Group. J. Clin. Microbiol. 36: 1886-1889
17. **Rand, K. H., H. Houck, and M. Wolff.** 1994. Detection of candidemia by polymerase chain reaction. Mol. Cell. Probes 8: 215-221
18. **Shigei, J. T., J.A. Shimabukuro, M.T. Pezzlo, L.M. De la Maza, and E.M. Peterson.** 1995. Value of terminal subcultures for blood cultures monitored by BACTEC 9240. J. Clin. Microbiol. 33: 1385-1388
19. **Tinghitella, T. J. and M.D. Lamagdeleine.** 1995. Assessment of Difco ESP 384 blood culture system by terminal subcultures: failure to detect *Cryptococcus neoformans* in clinical specimens. J. Clin. Microbiol. 33: 3031-3033
20. **Vincent, J. L., E. Anaissie, H. Bruining, W. Demajo, M. El Ebiary, J. Haber, Y. Hiramatsu, G. Nitenberg, P.O. Nystrom, D. Pittet, T. Rogers, P. Sandven, G. Sganga, M.D. Schaller, and J. Solomkin.** 1998. Epidemiology, diagnosis and treatment of systemic *Candida* infection in surgical patients under intensive care. Intensive Care Med. 24: 206-216
21. **Voss, A., J.A. Kluytmans, J.G. Koeleman, L. Spanjaard, C.M. Vandenbroucke Grauls, H.A. Verbrugh, M.C. Vos, A.Y. Weersink, J.A. Hoogkamp Korstanje, and J.F. Meis.** 1996. Occurrence of yeast bloodstream infections between 1987 and 1995 in five Dutch university hospitals. Eur. J. Clin. Microbiol. Infect. Dis. 15: 909-912
22. **Weinstein, M. P., S. Mirrett, L.G. Reimer, M.L. Wilson, S. Smith Elekes, C.R. Chuard, K.L. Joho, and L.B. Reller.** 1995. Controlled evaluation of BacT/Alert standard aerobic and FAN aerobic blood culture bottles for detection of bacteremia and fungemia. J. Clin. Microbiol. 33: 978-981
23. **Widjojoatmodjo, M. N., A. Borst, R.A.F. Schukkink, A.T.A. Box, N.M.M. Tacken, B. van Gemen, J. Verhoef, B. Top, and A.C. Fluit.** 1999. Nucleic acid sequence-based amplification (NASBA) detection of medically important *Candida* species. J. Microbiol. Meth. 38: 81-90
24. **Wingard, J. R.** 1995. Importance of *Candida* species other than *C. albicans* as pathogens in oncology patients. Clin. Infect. Dis. 20: 115-125

IV: Clinical evaluation of a NASBA-based assay for detection of *Candida* spp. in blood and blood cultures

Annemarie Borst[1], Jan Verhoef[1], Edwin Boel[2], Ad Fluit[1]

[1] Eijkman-Winkler Institute, University Medical Center, Utrecht, the Netherlands
[2] Laboratory for Medical Microbiology, PAMM, Veldhoven, the Netherlands



Clinical Laboratory (2002). In Press.

SUMMARY

The number of life-threatening opportunistic fungal infections has shown a dramatic increase. However, the diagnosis of candidaemia remains difficult. Nucleic acid amplification assays may improve the detection rate and decrease the time needed for detection and identification of *Candida* spp. Whole blood samples of patients suspected of having candidaemia were analyzed using Nucleic Acid Sequence-Based Amplification (NASBA). Furthermore, aliquots of blood cultures of the patients after 2 days of culturing were tested. Eleven data sets from ten patients in two hospitals were generated. None of the whole blood samples was positive in the NASBA assay. Eight samples were positive in the NASBA assay after two days of culturing, whereas only two additional positive samples were found after longer incubation periods. Thus, a two-day culture step is sufficient to greatly improve the sensitivity of the NASBA assay. The NASBA assay detected *Candida* RNA in three patients. In one patient, the yeast was not detected by automated blood culturing, in another patient the NASBA assay detected the infection two days earlier than the blood culture system.

KEY WORDS

Candida spp., Nucleic Acid Sequence-Based Amplification (NASBA), blood culture

INTRODUCTION

The number of life-threatening opportunistic fungal infections in immunocompromised patients has shown a dramatic increase[1]. *Candida* spp. account for the majority of these infections. The attributable mortality of *Candida* infections is approximately 38%[19], and crude mortality rates exceed 50%[7,12,18]. The diagnosis of candidemia remains difficult. Automated blood culture systems are routinely used, but fail to detect yeasts in many cases[2,11]. Furthermore, prolonged incubation times or terminal subculturing of negative blood culture bottles may be necessary before yeast growth can be detected[3,14,17].

Because of their sensitivity and speed, nucleic acid amplification assays may play an important role in improving the detection rate and decreasing the time needed for detection and identification of *Candida* spp. As little as 10 molecules of rRNA can be detected with a Nucleic Acid Sequence-Based Amplification (NASBA-) assay[20]. This assay makes use of primers directed against conserved regions of the 18S rRNA of medically important fungi and specific probes for the identification of different *Candida* spp. When using whole blood samples, 1-10 cfu of *C. albicans* could still be detected.

In a previous study, we showed that the NASBA assay could detect candidemia, whereas no *Candida* RNA was detected in blood culture medium alone, or in blood cultures from healthy volunteers, uninfected patients or patients colonized with *Candida* spp. When we used the NASBA assay to analyze culture-positive as well as negative blood cultures from patients with a proven candidemia after 7 days of culturing, we showed that compared with automated blood culturing, the number of positive blood cultures increased from 21% to 34%[4].

Here, we present the results of a clinical evaluation of the NASBA assay. Whole blood samples of patients suspected of having candidemia were analyzed. However, since candidemia is characterized by a low number of yeast cells in the bloodstream, a short culture step may further increase the detection rate. Therefore, aliquots of blood cultures from the patients after 2 days of culturing were also tested. The results were compared with automated blood culturing as well as results from the NASBA assay on aliquots of blood cultures after 7 days of culturing.

MATERIALS AND METHODS

Clinical samples. Blood and blood culture samples were obtained from the University Medical Center (UMC), Utrecht, the Netherlands and the Catharina Hospital, Eindhoven, the Netherlands, between March 2000 and June 2001. The study design was approved by the Medical Ethics Committee of the UMC Utrecht (protocol no. 99/104). Patients in the intensive care unit (ICU) were included when there was a clinical suspicion of candidemia and a presence of two or more systemic inflammatory response syndrome (SIRS)-criteria: temperature < 36°C or > 38°C; tachycardia > 90 beats/min.; CO_2 < 32 mm Hg; respiratory rate > 24 breaths/min.; leukocytes < 4 x 10^9/l or > 12 x 10^9/l; > 10% immature (band) forms. Patients in the hematology ward were included when they had neutropenia (# 100 granulocytes/µl) and did not respond to broad spectrum antibiotic treatment for 48-72 hours. In the Catharina Hospital Eindhoven, the inclusion criteria also included culturing of yeasts from 2 or more foci. Patients or their relatives were required to comprehend and sign an informed consent form. EDTA-blood was drawn from the patients on day 1 (before treatment with antimycotic agents was started), divided into 1 ml aliquots, and stored at -70°C. Two blood culture sets were drawn on day 1: one from a central venous catheter (if present) and one from a peripheral vein (if possible). Each blood culture set consisted of one aerobic and one anaerobic blood culture bottle (BacT/Alert, Organon Teknika, the Netherlands). In the UMC Utrecht, FAN aerobic bottles were used. On days 3 and 5 another blood culture set was drawn from a peripheral vein (if possible, otherwise from the central venous catheter). Blood cultures were cultured in the BacT/Alert monitoring system for 7 days. After 2 days of culturing, three 1 ml samples were taken from each bottle and stored at -70°C until use in the NASBA assay. This was repeated after 7 days of culturing, or when blood cultures were positive in the BacT/Alert monitoring system. The species was identified using CHROMagar plates (bioMérieux, Den Bosch, the Netherlands) and VITEK analysis (bioMérieux).

Extraction of RNA. Blood and blood culture samples were frozen at -70°C for at least 20 minutes. After thawing, 0.9 ml lysis buffer (0.32 M sucrose; 10 mM Tris-HCl (pH 7.5); 5 mM $MgCl_2$; 1% Triton X100) was added and the samples were centrifuged for 5 minutes at 13,000 g. The supernatant was removed, and this step was repeated once. The pellet was then resuspended in 100 µl enzyme buffer containing 2 mg/ml lyticase (Sigma-Aldrich, Steinheim, Germany), 4 mg/ml lysing enzymes (Sigma-Aldrich) and 0.17% ß-mercaptoethanol in 50 mM Tris-HCl (pH 7.5)/10 mM EDTA, and incubated at 37°C for 10 minutes. When FAN aerobic blood cultures were used, the samples were centrifuged for 1 minute at 13,000 g , and the supernatant was transferred to a fresh tube. One ml RNAzol (Campro Scientific, Veenendaal,

the Netherlands) was added and RNA was extracted according to the manufacturer's instructions, with minor modifications: 600 µl isopropanol was added to the aqueous phase instead of 500 µl; after washing with ethanol the pellet was dried for 10 minutes at 56°C, and RNA was dissolved for 10 minutes at 56°C in 40 µl water that was treated with UV-light for 2 hours. Samples were stored at -70°C until further use.

Primers and probes. The oligonucleotides used in this study are shown in Table 1. The specificity of the probes and the sensitivity of the NASBA assay have been described previously[4,20].

Table 1

The primers and probes used in this study

Primer	Sequence (5' to 3')	
primer 1	*AATTCTAATACGACTCACTATAGGGAGAGA*-CATGCGATTCGAAAAGTTA[a]	
primer 2	*GATGCAAGGTCGCATATGAG*-ATGTCTAAGTATAAGCAATTTA[b]	
Probe	Sequence (5' to 3')	Target
1912	ATCTCGACCTCTTGGAAGAGATGT	*C. glabrata*
1913	ATCCCGACTGTTTGGAAGGGATGT	*C. albicans; C. tropicalis; C. parapsilosis; C. viswanathii; C. guilliermondii*
1914	AGCCCGACCTCTGGAAGGGCTGTA	*C. lusitaniae*
2176	CAATGTCTTCGGACTCTT	*C. tropicalis*
9566	CCCTCGGGCCTTTTGATG	*C. krusei*

[a] italics: T7 promotor sequence
[b] italics: generic sequence used for ECL-detection

NASBA. Five µl RNA samples were taken up in a pre-reaction mixture with a final volume of 15 µl, containing 53 mM Tris-HCl (pH 8.5), 16 mM $MgCl_2$, 93 mM KCl, 6.7 mM DTT, 1.3 mM of each dNTP, 2.7 mM of each rNTP, 20% (v/v) dimethyl sulfoxide and 0.27 µM of each primer. The whole mixture was first incubated at 65°C for 5 minutes followed by 5 minutes at 41°C. Then 5 µl of an enzyme mixture containing 2.1 µg BSA, 6.4 U AMV-RT (Seigaku, Rockville, MD), 0.08 U RNase H and 32 U T7 RNA polymerase in 1.5 M sorbitol was added. The reaction mixture was incubated for 90 minutes at 41°C.

Controls. One positive and at least two negative controls for the amplification were used for each NASBA reaction. As a positive control for the amplification, 0.70 fg *C. albicans* RNA was used. In the negative controls, no template was added. Negative controls were positioned at the beginning and end of the series of samples that was tested. As a positive control for the whole procedure of isolation and amplification, two blood samples of each patient were spiked with 50 µl of physiological salt solution containing 10^2 and 10^4 colony forming units (cfu) of *C. albicans* (CBS 562, Centraal Bureau voor Schimmelcultures, Utrecht, the Netherlands).

ECL-detection. For electrochemiluminescence (ECL)-detection, the NucliSens Basic Kit Detection Reagents in combination with the NucliSens Reader (Organon Teknika, Boxtel, the Netherlands) were used according to the manufacturer's instructions. Amplification products were simultaneously hybridized to the specific capture probes (Table 1) as well as to the

generic ECL probe provided in the Basic Kit. This probe hybridizes to the generic sequence, incorporated by primer 2 during amplification. Amplification products were diluted 1:20 before hybridization with capture probes 1912, 1913, 1914 or 9566, and a 1:200 dilution was used when amplicons were hybridized with probe 2176. The ECL procedure involves standard use of 5 ml tubes (Falcon, Becton Dickinson, le Pont de Claix, France) for the relatively small sample volumes, which greatly reduces the risk of carry-over contaminations. Furthermore, fresh filtertips were used for each pipeting step and all contaminated waste was disposed in closed plastic bags. Hybridization took place at 41°C for 30 minutes. ECL-signals were considered positive when ≥ 50% of the positive control (0.70 fg *C. albicans* RNA).

RESULTS

Ten patients were included in the trial: 4 patients from the UMC Utrecht and 6 patients from the Catharina Hospital Eindhoven. One patient was suspected of candidemia on two different occasions. From this patient, two data sets were obtained.

None of the whole blood samples was positive in the NASBA assay. Blood cultures as well as the NASBA assay were negative for patients 1, 2, 3, 4 (two data sets), and 9. Bacteria, but not yeasts, were cultured in blood cultures of patients 6, 8 and 10 (patient 6: *Enterococcus faecalis* and *Escherichia coli*; patient 8: *Staphylococcus epidermidis*; patient 10: *Staphylococcus epidermidis* and *Staphylococcus haemolyticus*). NASBA assays performed on samples from patients 6 and 8 were all negative for yeast. However, several blood culture samples of patient 10 hybridized with probe 1913 or 2176 in the NASBA assay (Table 2).

Blood cultures from two patients were positive for *C. albicans* (patients 5 and 7, Table 2). All *C. albicans* positive cultures were taken on day 1: three out of four blood cultures (two anaerobic bottles and one aerobic bottle) of patient 5, and one (aerobic) blood culture of patient 7. Blood cultures taken on days 3 and 5 of patient 5 showed bacterial growth (*Enterococcus faecalis* and *Staphylococcus aureus*, Table 2). Several samples of patients 5 and 7 hybridized with probes 1913 and/or 2176 in the NASBA assay (Table 2).

DISCUSSION

Eleven data sets from ten patients in two hospitals were obtained over a period of 16 months. This low number is due in part to the fact that during the study the therapeutic regimen in the University Medical Center Utrecht was changed: all patients at the surgical ICU received fluconazole-prophylaxis during the second half of the study period, and therefore did not meet the inclusion criteria (according to which the first samples should be obtained before antimycotic treatment was commenced). However, the number of patients that were included during the first half of the study period and from the second hospital was still low.

Although some patients who fitted the inclusion criteria may have been missed, these numbers are indicative of the low number of patients suspected of candidemia in the Netherlands. It is possible that this truly reflects a minimal number of systemic *Candida* infections. It is known that in Europe, *Candida* spp. account for less bloodstream infections

Table 2

Comparison between BacT/Alert monitoring of clinical blood cultures and NASBA detection of yeast RNA

Patient no.	**Sample**[1]	**BacT/Alert**[2]	**NASBA**[2] **day 2**	**NASBA**[2] **day 7**
5	1a	-	1913	n.d.
	1b	*Candida albicans*	1913	n.d.
	1c	*Candida albicans*	-	n.d.
	1d	*Candida albicans*	-	n.d.
	3a	-	-	-
	3b	*(Enterococcus faecalis)*	-	-
	5a	*(Enterococcus faecalis)*	-	-
	5b	*(Staphylococcus aureus)*	1913	-
7	1a	*Candida albicans*	-	1913[3]
	1b	-	-	-[3]
	1c	-	2176	-
	1d	-	-	-
	3a	-	-	-
	3b	-	-	-
	5a	-	-	-
	5b	-	1913 + 2176	-
10	1a	-	2176	2176
	1b	-	2176	1913
	1c	-	-	1913
	1d	-	-	-
	3a	*(Staphylococcus epidermidis)*	1913	-
	3b	*(Staphylococcus epidermidis)*	-	-
	5a	*(Staphylococcus epidermidis; S. haemolyticus)*	-	-
	5b	*(Staphylococcus epidermidis; S. haemolyticus)*	-	-

[1] 1a = aerobic blood culture, day 1, central venous catheter
1b = anaerobic blood culture, day 1, central venous catheter
1c = aerobic blood culture, day 1, peripheral blood
1d = anaerobic blood culture, day 1, peripheral blood
3a = aerobic blood culture, day 3
3b = anaerobic blood culture, day 3
5a = aerobic blood culture, day 5
5b = anaerobic blood culture, day 5

[2] - = negative
n.d. = no data (cultures were positive for yeast after 2-4 days of culturing)
day 2 = NASBA on blood culture samples after 2 days of culturing
day 7 = NASBA on blood culture samples after 7 days of culturing
1913 = NASBA product hybridized with probe 1913
2176 = NASBA product hybridized with probe 2176

[3] samples were taken from blood cultures on day 3 instead of day 7

than, e.g., in North America, where *Candida* spp. are the fourth most common bloodstream pathogens[8]. In Europe, *Candida* spp. rank 8th[6]. However, it is also believed that the number of systemic *Candida* infections is underestimated. Tan et al. reported in 1992 that the number of systemic mycoses in the Netherlands may be as high as hundreds per year, even though only a few cases were reported in their survey[15]. Although these data are quite old, the fact that only four patients from the University Medical Center Utrecht were included in our study (and therefore suspected of having candidemia) over a period of 16 months, whereas around 40 blood cultures positive for *Candida* spp. are seen annually in this hospital, supports this latter hypothesis. Furthermore, taking into account that in up to 65% of the cases automated blood culture systems fail to detect yeasts[11], the true number of systemic *Candida* infections may be much higher than expected.

We have previously established a detection limit for *C. albicans* of 1-10 cfu/ml blood[20]. In the present study, none of the whole blood samples was positive in the NASBA assay. *Candida* spp. were cultured from the blood of two of the patients, and *Candida* RNA was detected in blood cultures of three. It seems likely that the other patients were not infected. The number of cells in the blood of the three patients with *Candida* RNA-positive blood culture samples may have been too low to detect without a culture step. The number of *Candida* cells in candidemia has been quantitated by using colony counts obtained directly on blood agar plates by the lysis centrifugation technique. It was shown that 26.5% of all samples had < 1 cfu/ml blood, and that another 27.9% had between 1 and 10 cfu/ml[13]. Eight samples were positive in the NASBA assay after two days of culturing. Only two additional positive samples were found: one after three days, and one after seven days of culturing. This indicates that a two-day culture step is sufficient to greatly improve the sensitivity of the NASBA assay.

One sample each of patients 3 and 9 hybridized with probe 2176 in the NASBA assay (results not shown). When NASBA products hybridize with probe 2176 as well as probe 1913, the species is identified as *C. tropicalis*[20]. However, in this case the products hybridized with probe 2176 only. Probe 2176 cross-hybridizes with *Saccharomyces cerevisiae*, which is one of the main causes of contaminations in fungal amplification assays[9]. Therefore, it is likely that these NASBA-products were derived from a *S. cerevisiae* contamination.

The anaerobic blood culture taken from the central venous catheter of patient 5 on day 1 was positive for *C. albicans* after 4 days of growth (Table 2). Samples from both bottles of this blood culture set hybridized with probe 1913 in the NASBA assay. This probe hybridizes with *C. albicans*, *C. tropicalis*, *C. viswanathii*, *C. parapsilosis* and *C. guilliermondii*. Because *C. albicans* was grown from this blood culture, it is most likely RNA of this species which was detected. Since the samples for the NASBA assay were taken after two days of growth, the NASBA assay clearly led to earlier detection. Both the aerobic as well as the anaerobic blood culture taken from a peripheral vein instead of the central venous catheter of the same patient (patient 5) on the same day (day 1) showed growth of *C. albicans*. However, samples taken from these bottles did not hybridize with any of the *Candida* probes used. It is known that nucleic acid amplification assays can be inhibited when large amounts of target DNA are present. Therefore, the extracted RNA from these samples was diluted 5 and 10x, and another NASBA was performed (results not shown). Again, the samples did not hybridize with any of the probes. Since yeast growth was detected in these blood cultures very rapidly (in the aerobic bottle after 2 days, and in the anaerobic bottle after 3 days of culturing), it is possible that the

number of yeast cells in the samples was extremely high. In that case, a greater than 10x dilution may have been necessary in order to detect target RNA. This was not further examined, due to lack of material.

Blood cultures from the same patient (patient 5) taken on days 3 and 5 showed bacterial, but not yeast growth. However, a sample taken after two days of culturing from a blood culture of day 5 hybridized with probe 1913 (Table 2). A sample taken from the same blood culture bottle after seven days of culturing did not show any results in the NASBA assay. It is possible that the yeast cells were degraded by antifungal agents present in the blood of the patient. Patient 5 had been treated with fluconazole for 5 days at the time of blood sampling for this culture. The fact that yeast RNA was still detectable in a sample that was drawn when the patient was receiving antifungal treatment may indicate treatment failure. This may have remained undetected by the automated blood culture system because of bacterial overgrowth.

One blood culture from patient 7 showed growth of *C. albicans* after three days. A sample from this bottle taken after three days of culturing was also positive in the NASBA assay (1a, Table 2). A sample from another blood culture bottle from day 1 hybridized with probe 2176 (1c, Table 2). Another sample, taken after two days of culturing of a blood culture from day 5, hybridized with probe 2176 as well as 1913 (indicative for *C. tropicalis*; 5b, Table 2). Since only *C. albicans* was cultured and no *C. tropicalis* was ever found in any culture from this patient, the most likely explanation for these results is that sample 1c is a *S. cerevisiae* contaminant, as described above, and sample 5b contains a mixture of *C. albicans* and *S. cerevisiae* RNA.

Several samples from patient 10 hybridized with either probe 2176 or 1913 (Table 2). Since all negative controls were free of contaminants and because of the stringent measures for prevention of contaminations that are taken in our laboratory, it is not likely that all samples which hybridized only with probe 2176 are the result of *S. cerevisiae* contaminations of the NASBA assay. In rare cases, *S. cerevisiae* causes bloodstream infections (Taylor et al. reported one case in their hospital over a seven year period[16]). A combined infection with *S. cerevisiae* and *C. albicans* is therefore possible. It is, however, striking that all three samples which hybridized with probe 2176 were taken from one blood culture set. *S. cerevisiae*, also known as 'baker's yeast', is used widely in industrial baking and brewing, and is commonly present in the environment. Since the three samples were taken from blood cultures which were drawn at the same time from a central venous catheter, it is very well possible that the blood cultures were contaminated with yeast cells from the skin of either the patient or the health care worker, for instance. The fact that blood cultures in many cases fail to detect yeasts might explain why they were not detected by the automated blood culture system.

Three samples from patient 10 hybridized with probe 1913, indicating that this patient possibly suffered from candidemia. No *Candida* spp. were cultured from the blood of this patient. However, we have previously shown, that the NASBA assay performed on aliquots of blood cultures is more sensitive than the BacT/Alert monitoring system[4].

Our NASBA assay uses separate amplification- and detection procedures. Real-time detection of *C. albicans* with the Light Cycler system has been used successfully[10]. It is possible to combine NASBA amplification with the use of molecular beacons. This results in an isothermal real-time detection assay, without the need of expensive thermal cyclers. Quantification of the NASBA products is possible with ECL- as well as molecular beacon

detection[5].

In conclusion: in three out of ten patients the NASBA assay detected *Candida* RNA. In one patient the yeast was not detected by automated blood culturing, in another patient the NASBA assay detected the infection two days earlier than the blood culture system. This is encouraging for the use of NASBA for the detection of systemic *Candida* infections. However, this study should be repeated with a larger number of patients before sound conclusions can be made.

ACKNOWLEDGEMENTS

We would like to thank M.A. Leverstein-Van Hall, H.J. van Leeuwen, Dr. A.W. Dekker, A. Vos, Dr. A.J.G.H. Bindels, and the technicians from the clinical microbiology laboratories for their contribution to this work.

Annemarie Borst is supported by a grant from Organon Teknika.

REFERENCES

1. **Abi Said, D, E. Anaissie, O. Uzun, I. Raad, H. Pinzcowski, and S. Vartivarian.** 1997. The epidemiology of hematogenous candidiasis caused by different *Candida* species. Clin. Infect. Dis. 24: 1122-1128
2. **Berenguer, J., M. Buck, F. Witebsky, F. Stock, P.A. Pizzo, and T.J. Walsh.** 1993. Lysis-centrifugation blood cultures in the detection of tissue-proven invasive candidiasis. Disseminated versus single-organ infection. Diagn. Microbiol. Infect. Dis. 17: 103-109
3. **Borst, A., M. Leverstein-Van Hall, J. Verhoef, and A. Fluit.** 2000. Value of terminal subculture of automated blood cultures in patients with candidaemia. Eur. J. Clin. Microbiol. Infect. Dis. 19: 803-805
4. **Borst, A., M.A. Leverstein-Van Hall, J. Verhoef, and A.C. Fluit.** 2001. Detection of *Candida* spp. in blood cultures using nucleic acid sequence-based amplification (NASBA). Diagn. Microbiol. Infect. Dis. 39: 155-160
5. **Deiman, B., P. Van Aerle, and P. Sillekens.** 2002. Characteristics and applications of nucleic acid sequence-based amplification (NASBA). Mol. Biotechnol. 20: 163-179
6. **Fluit, A.C., M.E. Jones, F.J. Schmitz, J. Acar, R. Gupta, and J. Verhoef.** 2000. Antimicrobial susceptibility and frequency of occurrence of clinical blood isolates in Europe from the SENTRY antimicrobial surveillance program, 1997 and 1998. Clin. Infect. Dis. 30: 454-460
7. **Giamarellou, H. and A. Antoniadou.** 1996. Epidemiology, diagnosis, and therapy of fungal infections in surgery. Infect. Control Hosp. Epidemiol. 17: 558-564
8. **Jarvis, W.R.** 1995. Epidemiology of nosocomial fungal infections, with emphasis on *Candida* species. Clin. Infect. Dis. 20: 1526-1530
9. **Loeffler, J., H. Hebart, R. Bialek, L. Hagmeyer, D. Schmidt, F.P. Serey, M. Hartmann, J. Eucker, and H. Einsele.** 1999. Contaminations occurring in fungal PCR assays. J. Clin. Microbiol. 37: 1200-1202
10. **Loeffler, J., N. Henke, H. Hebart, D. Schmidt, L. Hagmeyer, U. Schumacher, and H. Einsele.** 2000. Quantification of fungal DNA by using fluorescence resonance energy transfer and the light cycler system. J. Clin. Microbiol. 38: 586-590

11. **Maksymiuk, A.W., S. Thongprasert, R. Hopfer, M. Luna, V. Fainstein, and G.P. Bodey.** 1984. Systemic candidiasis in cancer patients. Am. J. Med. 77: 20-27
12. **Pittet, D., N. Li, and R.P. Wenzel.** 1993. Association of secondary and polymicrobial nosocomial bloodstream infections with higher mortality. Eur. J. Clin. Microbiol. Infect. Dis. 12: 813-819
13. **Rand, K.H., H. Houck, and M. Wolff.** 1994. Detection of candidemia by polymerase chain reaction. Mol. Cell. Probes 8: 215-221
14. **Shigei, J.T., J.A. Shimabukuro, M.T. Pezzlo, L.M. de la Maza, and E.M. Peterson.** 1995. Value of terminal subcultures for blood cultures monitored by BACTEC 9240. J. Clin. Microbiol. 33: 1385-1388
15. **Tan, C.S., R.G. Wintermans, G.S. de Hoog, H.W. Engel, and E.P. IJzerman.** 1992. Shifts in the species spectrum of mycoses in the Netherlands from 1970-1990. Ned. Tijdschr. Geneeskd. 136: 631-637
16. **Taylor, G.D., M. Buchanan-Chell, T. Kirkland, M. McKenzie, and R. Wiens.** 1994. Trends and sources of nosocomial fungaemia. Mycoses 37: 187-190
17. **Tinghitella, T.J. and M.D. Lamagdeleine.** 1995. Assessment of Difco ESP 384 blood culture system by terminal subcultures: failure to detect *Cryptococcus neoformans* in clinical specimens. J. Clin. Microbiol. 33: 3031-3033
18. **Wenzel, R.P.** 1995. Nosocomial candidemia: risk factors and attributable mortality. Clin. Infect. Dis. 20: 1531-1534
19. **Wey, S.B., M. Mori, M.A. Pfaller, R.F. Woolson, and R.P. Wenzel.** 1988. Hospital-acquired candidemia. The attributable mortality and excess length of stay. Arch. Intern. Med. 148: 2642-2645
20. **Widjojoatmodjo, M. N., A. Borst, R.A.F. Schukkink, A.T.A. Box, N.M.M. Tacken, B. van Gemen, J. Verhoef, B. Top, and A.C. Fluit.** 1999. Nucleic acid sequence-based amplification (NASBA) detection of medically important *Candida* species. J. Microbiol. Meth. 38: 81-90

V: The Basic Kit amplification module for the detection of *Candida* spp.: fungal RNA contamination of kit components

Annemarie Borst

Eijkman-Winkler Institute, University Medical Center, Utrecht, the Netherlands

Nucleic Acid Sequence-Based Amplification (NASBA) is an isothermal RNA amplification method based on the simultaneous action of three enzymes: Avian Myeloblastosis Virus Reverse Transcriptase (AMV-RT), RNase H and T7 RNA polymerase[1]. The method is extremely sensitive. When rRNA is used as a target, as many as 10,000 copies can be present per cell. Furthermore, hundreds of RNA copies are generated in each amplification 'cycle', each of which serve as a target for the next round (in comparison: with PCR only two copies are generated in each cycle). This results in a large amount of product in a short period of time.

NASBA was successfully used in our laboratory for the detection of *Candida* spp. in blood and blood cultures[2,3]. Primers and probes for the detection of several *Candida* spp. were developed and used in an in-house NASBA assay[3]. Yeast RNA was extracted by using RNAzol (Campro Scientific, Veenendaal, the Netherlands), and amplification products were detected using the Basic Kit electrochemiluminescence (ECL) detection module (Organon Teknika, Boxtel, the Netherlands)[2]. The aim of this study was to replace our in-house NASBA assay by the Basic Kit amplification module (Organon Teknika).

The Basic Kit amplification module contains a reagent sphere (comprised of a.o. dNTP's and NTP's), reagent sphere diluent, a separate stock of KCl for optimization of the assay, enzyme mix, and NASBA-water. The primers are not included but have to be designed by the user (in our case, we could use the primers from our in-house NASBA assay).

We spiked a mixture of blood from a healthy volunteer and aerobic blood culture medium (BacT/Alert FAN medium, Organon Teknika) with a 10-fold dilution of *Candida albicans* cfu. RNA was extracted as described[2]. After amplification using the Basic Kit amplification module (according to the manufacturer; 70 mM KCl), the NASBA products were hybridized with a probe for *C. albicans* and a universal yeast/fungi probe[3]. Amplification products were diluted 1:20 before hybridization with the albicans probe, and a 1:200 dilution was used when amplicons were hybridized with the yeast/fungi probe. Hybridization took place at 41°C for 30 minutes. For detection, the Basic Kit ECL detection module was used as described[2]. ECL-signals were considered positive when ≥ 17% of the Instrument Reference Solution (IRS) signal, and increased, but not positive when < 17% of the IRS, but > 3x the signal of the Assay Negative (AN: probe + detection diluent). The results are depicted in Table 1a. Although all negative controls were correct when the albicans probe was used, both negative controls and the 0 cfu sample hybridized with the yeast/fungi probe.

We then used the Basic Kit amplification module to detect yeast RNA in a mixture of blood with either FAN-aerobic or standard anaerobic blood culture medium (BacT/Alert, Organon Teknika) spiked with a 10-fold dilution of *C. albicans* cfu (Table 1b). In this experiment, two of the four negative controls showed increased signals after hybridization with the albicans probe. For comparison, we performed an in-house NASBA (Table 1c). Although the signal for the positive control was low when the albicans probe was used, there were no problems with the negative controls.

A number of experiments were performed in order to find the cause of these contaminations. First, the water from the kit (NASBA-water) was exchanged with water that was treated with UV-light for two hours. This UV-treated water had proved to be free of contaminations in our in-house NASBA assay. The results are depicted in Table 2a. When UV-treated water was used in combination with the Basic Kit amplification module, problems occurred with the yeast/fungi probe. When the NASBA-water was used, both the albicans as

Table 1

a: NASBA with the Basic Kit amplification module on a 10-fold dilution of *C. albicans* cfu in blood + aerobic blood-culture medium

	AN	neg.	0	1	10	10^2	10^3	10^4	10^5	10^6	pos.	neg.
albicans	-	-	-	-	-	-	+	+	+	+	+	-
yeast/fungi	-	+	+	+	+	+	+	+	+	+	+	+

b: NASBA with the Basic Kit amplification module on a 10-fold dilution of *C. albicans* cfu in blood + aerobic and anaerobic blood-culture medium

	AN	neg.	neg.	0	1	10	10^2	10^3	10^4	pos.	neg.	neg.
albicans, aerobic	-	+/-	-	-	+	-	+/-	+	+	+	-	+/-
albicans, anaerobic				-	-	-	+	-	+			

c: In house-NASBA

	AN	neg.	neg.	neg.	pos.	neg.	neg.	neg.
albicans	-	-	-	-	+/-	-	-	-
yeast/fungi	-	-	-	-	+	-	-	-

AN: assay negative (probe + detection diluent)
neg.: negative control (no template added to NASBA)
pos.: positive control (0.70 fg *C. albicans* RNA added to NASBA)
albicans: probe for detection of *C. albicans; C. tropicalis; C. parapsilosis; C. viswanathii and C. guilliermondii*
yeast/fungi: universal probe for detection of yeasts and fungi
+: positive after ECL detection
-: negative after ECL detection
+/-: increased, but not positive, signal after ECL detection

well as the yeast/fungi probe showed hybridization with negative controls. To further examine the NASBA-water, we used this water in our in-house NASBA assay (Table 2b). False positive results occurred in 2 of the 4 negative controls. In conclusion: the NASBA-water is a source of contaminations, but it is not the only source.

To examine the role of the enzyme mix, we performed an experiment with the Basic Kit amplification module on two series of positive and negative controls. In one series, the enzyme mix of the kit was exchanged with our in-house enzyme mix (Table 2c). When the Basic Kit enzymes were used, all negative and positive controls were correct when the albicans probe was used. However, all negative controls were positive after hybridization with the yeast/fungi probe. When the in-house enzymes were used, one of the negative controls showed an increased (but not positive) signal after hybridization with the albicans probe, and all negative controls showed an enhanced or positive signal after hybridization with the yeast/fungi probe. We then performed the same experiment with the in-house NASBA assay (Table 2d). When the Basic Kit enzyme mix was used, all negative and positive controls were correct with both probes. When the in-house enzyme mix was used, three of the negative controls showed enhanced (but not positive) signals after hybridization with the yeast/fungi probe. Therefore, it seems like the enzyme mix from the Basic Kit amplification module is 'cleaner' than the in-house enzyme mix.

Table 2

a: NASBA with the Basic Kit amplification module: one series with NASBA-water (kit), one series with UV-treated water

		UV-treated water					NASBA-water					
	AN	neg.	neg.	neg.	pos.	neg.	neg.	neg.	neg.	pos.	neg.	neg.
albicans	-	-	-	-	+	-	+/-	-	+	+	-	-
yeast/fungi	-	+	+	+	+	+	+	+	+	+	+	+

b: In-house NASBA: NASBA-water (kit) instead of UV-treated water

	AN	neg.	neg.	pos.	neg.	neg.
albicans	-	-	-	+	-	-
yeast/fungi	-	+	+	+	-	-

c: NASBA with the Basic Kit amplification module: one series with enzyme mix (kit), one series with in-house assay enzymes

		Basic Kit enzymes					In-house assay enzymes					
	AN	neg.	neg.	pos.	neg.	neg.	neg.	neg.	pos.	neg.	neg.	neg.
albicans	-	-	-	+	-	-	-	+/-	+	-	-	-
yeast/fungi	-	+	+	+	+	+	+/-	+	+	+	+	+

d: In-house NASBA: one series with enzyme mix (kit), one series with in-house assay enzymes

		Basic Kit enzymes					In-house assay enzymes				
	AN	neg.	neg.	pos.	neg.	neg.	neg.	neg.	pos.	neg.	neg.
albicans	-	-	-	+	-	-	-	-	+	-	-
yeast/fungi	-	-	-	+	-	-	+/-	+/-	+	-	+/-

AN: assay negative (probe + detection diluent)
neg.: negative control (no template added to NASBA)
pos.: positive control (0.70 fg *C. albicans* RNA)
albicans: probe for detection of *C. albicans; C. tropicalis; C. parapsilosis; C. viswanathii and C. guilliermondii*
yeast/fungi: universal probe for detection of yeasts and fungi
+: positive after ECL detection
-: negative after ECL detection
+/-: increased, but not positive, signal after ECL detection

To further examine the source of the contaminating RNA, all available probes were used to hybridize with amplification products obtained with the Basic Kit amplification module (Table 3). Amplification products were diluted 1:20 before hybridization with the albicans, glabrata, lusitaniae, and krusei probes, and a 1:200 dilution was used when amplicons were hybridized with the tropicalis or the yeast/fungi probe. It is obvious that although some problems occur when the albicans or the tropicalis probe are used, numerous false positive results are obtained when the yeast/fungi probe is used. Therefore, the source of the contaminating RNA remains unclear.

Table 3
NASBA with the Basic Kit amplification module

	AN	neg.	neg.	neg.	neg.	pos.	neg.	neg.	neg.	neg.
glabrata	-	-	-	-	-	-	-	-	-	-
lusitaniae	-	-	-	-	-	-	-	-	-	-
krusei	-	-	-	-	-	-	-	-	-	-
tropicalis	-	-	-	-	+/-	-	-	-	-	+
albicans	-	+	-	-	-	+	-	-	-	-
yeast/fungi	-	+	+	+	+	+	+	-	+	+

AN: assay negative (probe + detection diluent)
neg.: negative control (no template added to NASBA)
pos.: positive control (0.70 fg *C. albicans* RNA)
glabrata: probe for detection of *C. glabrata*
lusitaniae: probe for detection of *C. lusitaniae*
krusei: probe for detection of *C. krusei*
tropicalis: probe for detection of C. tropicalis (cross-hybridizes with *Kluyveromyces marxianus*, *K. lactis*, *Saccharomyces cerevisiae)*
albicans: probe for detection of *C. albicans; C. tropicalis; C. parapsilosis; C. viswanathii and C. guilliermondii*
yeast/fungi: universal probe for detection of yeasts and fungi
+: positive after ECL detection
-: negative after ECL detection
+/-: increased, but not positive, signal after ECL detection

In conclusion: components of the Basic Kit amplification module are contaminated with fungal RNA. The water from the kit, NASBA-water, is part of the problem, but some other components are contaminated as well. The enzymes of the kit, however, are free of contaminations, and even cleaner than the in-house enzyme mix that was used in our laboratory. It was decided to continue the use of the in-house NASBA assay, but the enzyme mix was replaced by Basic Kit enzymes.

Because of the complicated production process of the reagent spheres, these spheres may very well be a source of contaminations. It is our experience, that companies apply the concept that a room or manufacturing hall is 'clean', unless it is used by people working with nucleic acids. However, microorganisms, cells and nucleic acids are everywhere. Therefore, it is advised to consider a room contaminated, and limit work to small areas that can easily be cleaned. Furthermore, all reagents (including water) have to be free of contaminating nucleic acids.

ACKNOWLEDGEMENTS

We would like to thank Peter Haima, Peter Sillekens and Margot Peeters of Organon Teknika for their support, and for providing the Basic Kit amplification modules and enzyme mix.

REFERENCES

1. **Compton, J.** 1991. Nucleic acid sequence-based amplification. Nature 350: 91-92
2. **Borst, A., M.A. Leverstein-Van Hall, J. Verhoef, and A.C. Fluit.** 2001. Detection of *Candida* spp. in blood cultures using nucleic acid sequence-based amplification (NASBA). Diagn. Microbiol. Infect. Dis. 39: 155-160
3. **Widjojoatmodjo, M.N., A. Borst, R.A. Schukkink, A.T.A. Box, N.M. Tacken, B. van Gemen, J. Verhoef, B. Top, and A.C. Fluit.** 1999. Nucleic acid sequence-based amplification (NASBA) detection of medically important *Candida* species. J. Microbiol. Methods 38: 81-90

VI: False-positive results and contaminations in nucleic acid amplification assays. Suggestions for a 'prevent and destroy'-strategy

Annemarie Borst, Adrienne Box, Ad Fluit

Eijkman-Winkler Institute, University Medical Center, Utrecht, the Netherlands

Submitted for publication.

Since the first publication in 1985 on primer-mediated enzymatic amplification of DNA sequences, better known as the Polymerase Chain Reaction (PCR), the number of papers describing the use of this technique has increased exponentially until 1999, and seems to have reached a more or less stable level of about 15.000 papers each year (PubMed bibliographic database search on 'polymerase chain reaction')[65]. Within a few years, other nucleic acid amplification methods were developed, e.g. Nucleic Acid Sequence-Based Amplification (NASBA)[13], Ligation Chain Reaction (LCR)[84], and Transcription-Mediated Amplification (TMA)[35].

Very soon after the introduction of the PCR, people realized that the advantage of this nucleic acid amplification assay, its great sensitivity, is also its drawback: even the smallest amount of contaminating DNA can be amplified. In 1988, Lo et al. reported the first false-positive results: PCR primers directed against hepatitis B virus (HBV) were contaminated with plasmid DNA containing a full length HBV insert[39]. This observation resulted in numerous reports on how to recognize and avoid false-positive results caused by contaminations, and how to eliminate contaminating DNA. Most of these papers were published between 1990 and 1993. Does this mean that we have tackled this problem? Unfortunately: no. Of all papers on PCR, the percentage of papers dealing with contaminations or false-positive results has been about 2% over the years, and is not declining. Also, despite the great sensitivity and speed of the amplification methods, they are still not generally used as standard methods in routine laboratories.

In this review, we would like to focus on the implications of contaminations in diagnosis and research on infectious diseases. Although most researchers using nucleic acid amplification methods will be familiar with carry-over contaminations, where DNA fragments from previous experiments are re-amplified, other sources of contamination can be very unexpected. Furthermore, we will review literature on different methods for prevention and destruction of contaminating DNA. We will discuss the functionality and draw-backs of these methods, and give recommendations on how to improve laboratory practice.

FALSE-POSITIVE RESULTS AND CONTAMINATIONS

False-positive results of nucleic acid amplification assays can have several causes, including contaminations. Because terms like 'false-positive' and 'contamination' will be used frequently in this review, it is necessary to emphasize our interpretation of these words.

False-positive results caused by a 'true contaminant'. This type of contamination will generally affect every sample in the assay. It occurs when unwanted target DNA is introduced in the assay through e.g. reagents, laboratory disposables, equipment, or the environment (including carry-over contaminations between tests).

False-positive results caused by a 'sample contaminant'. This type of contamination will generally only affect a limited number of samples in an assay. It occurs when unwanted target DNA is introduced in certain samples due to e.g. sample to sample contamination, or leakage between samples on agarose gels.

Other false-positive results. False-positive results that are not caused by the presence of target DNA, but e.g. by nonspecific products due to sub-optimal assay conditions.

In conclusion: a contamination will always lead to a false-positive result, but a false-positive result is not always caused by a contamination.

FALSE-POSITIVE RESULTS AND THEIR IMPLICATIONS

False-positive results can have considerable implications, both in research as well as in the clinic. The following examples show, that amplification assays are not always as reliable as is sometimes believed.

In search of causes of infectious diseases, PCR has been used as a tool to demonstrate an association between infectious disease and the presence of microbial DNA. Boyd et al. used PCR and in situ hybridization to study the involvement of human papilloma virus (HPV) in cutaneous lichen planus[9]. Initial results on archival paraffin-embedded biopsy material were encouraging. However, more in-depth evaluation revealed nucleic acid contamination, probably due to sample contamination from HPV-positive material or adjacent wells, and a correlation between cutaneous lichen planus and HPV could not be verified.

A case where a false-positive result almost led to the assumption that an HIV-1 vaccine-induced immune response led to an abortive infection with abrogation of seroreactivity (a very tempting theory) was described by Schwartz et al.[72]. A plasma-sample of an HIV-1 seronegative patient who had participated in an HIV-1 vaccine trial tested positive in an RT-PCR assay. Although this result was not confirmed by other assays, retrospective analysis of serum RNA samples obtained from earlier occasions in the vaccine trial showed a cluster of positive results over a limited period, convincing some investigators of the validity of the original positive result and leading to the hypothesis mentioned above. Eventually, all previously reactive samples were retested by RT-PCR in a quality-controlled laboratory. All samples were now negative for HIV-1 RNA, including the cluster that had previously been reported as positive and the original positive plasma sample. It is not clear what caused the false-positive results in the first RT-PCR assays.

A number of papers report cases where contaminations had far-reaching consequences for the patients involved. In one case, PCR analyses of pleural fluid of a patient diagnosed with chronic lymphocytic leukemia (CLL) were positive for *Mycobacterium tuberculosis* on two different occasions. Therefore, antituberculosis therapy was commenced, while treatment for CLL was postponed. Staining and cultures for *M. tuberculosis* were negative. After 9 months, PCR for *M. tuberculosis* was still positive even though there was no evidence of tuberculosis with standard diagnostic tests. Antituberculous treatment was discontinued and high-dose chemotherapy was begun. Active tuberculosis was never ascertained, and the postponement of chemotherapy was apparently based on false-positive results. Again, the source of this contamination is not clarified[76].

One well-known case of false-positive results in diagnostic tests even led to the patient's death[53]. A 30-year old woman was diagnosed with chronic Lyme disease based on one PCR assay of blood positive for *Borrelia burgdorferi*. MRI of the brain and CSF examination were unremarkable, and several EIAs, Western blot assays, and PCR assays on blood, urine and CSF were negative or indeterminate. A Groshong catheter was placed and the patient was treated with intravenous antibiotic drugs for 27 months. This therapy was discontinued when

an impaired liver function and thrombocytopenia were observed. EIAs, Western blot- and PCR assays performed in another hospital were all negative for *B. burgdorferi*. One month later the patient died as a result of a large *Candida parapsilosis* septic thrombus located on the tip of the catheter, obstructing the tricuspid valve. At autopsy, there were no indications of Lyme disease. The one positive PCR assay on which the whole therapy was based, was probably the result of a DNA contamination. Interestingly, the laboratory that reported this false-positive result reported another positive *B. burgdorferi* PCR result which proved to be false-positive as well. Luckily, in this case the patient was referred for a second opinion, and did not receive unnecessary therapy[47].

Quality control studies. In none of the cases described above, a cause of the false-positive results is identified. It is striking that in many cases the results of the amplification assays differ between laboratories[47,53], sometimes even when the same samples are used[72]. Because false-positive results can have very unfortunate effects on research and especially in the clinic, extensive quality control of amplification tests is essential. This quality control should be executed continuously by the technicians or researchers themselves, but also at regular intervals by an independent organization. Surprisingly, only a limited number of such independent, multi-center studies have been published, and the results were generally alarming.

Four examples of multi-center quality control studies (PCRs on hepatitis B-, C and G virus, GB virus C, and *Mycobacterium tuberculosis*) show a false-positive rate of 9% up to 57%[5,49,60,86]. Interestingly, in all cases there was no association between good results and the methods used for nucleic acid extraction, the primers used in the amplification, the use of nested PCR, detection by Southern blot analysis with or without radioactive probes, or the use of standardized commercially available kits. This indicates that the way in which the technique is handled is more critical than the assay itself. Besides continuing with and increasing the number of multi-center quality control studies, more attention should be paid to the in-house aspects of quality control, before amplification assays can be used reliably in the diagnosis of infectious diseases.

Comparisons with other test methods. The examples describing the implications of false-positive results have one remarkable similarity: in all cases there were results of several other (non-amplification) tests contradicting the (false-) positive results on which the theory or diagnosis was based. From these examples, and from the results of quality control studies as described above, clinicians should be aware that interpretation of PCR test results should be done with great care. In some cases, even though the amplification assay is truly positive, the result may not be of clinical significance: the sensitivity of nucleic acid amplification assays may lead to detection of microorganisms in patients that have no clinical consequences, and DNA derived from dead or degrading microorganisms may yield positive results. And of course, there is always a chance of false-positive results caused by contaminating DNA. Therefore, PCR results should always be validated by comparing them with conventional diagnostic methods as well as clinical data.

Several people reported situations where extensive retesting was performed because clinical findings and results of standard diagnostic methods did not agree with PCR results[37,47,72,74]. It is needless to say that besides the discomforting uncertainty for patients and clinicians, this results in a considerable increase in costs. Therefore, all possible efforts should be made to

improve the reliability of amplification assays.

Although we unequivocally recommend to compare PCR results with other available data, we would like to make one comment. As was observed by Jehuda-Cohen for HIV-1, it is sometimes claimed that all positive PCR results that are not matched by positive ELISA serology are false-positive[30]. However, although the 'golden standard' is always the best diagnostic method that is available, that does not mean there is no room for improvement. For example, blood culture is considered the golden standard for the detection of disseminated yeast infections. However, in many cases automated blood cultures fail to detect yeasts (up to 65%)[44]. We have shown by using NASBA that we were able to improve the detection rate of yeasts in blood cultures[6]. It is therefore possible that a positive amplification result which is not confirmed by other tests, is in fact of clinical significance. In summary: regard all available test results and clinical data, but also be aware of the limitations of the tests that are used.

PREVENTION: REDUCTION OF RISK FACTORS

The prevention of false-positive results in nucleic acid amplification assays can be divided into two parts. First: the risks of contamination should be kept as low as possible. Second: if contamination occurs, it should be destroyed. Several factors form a risk of causing false-positive results, some of which may be very unexpected. Below, we describe some of these risk factors and methods for risk reduction. In the next chapter, we will focus on the destruction of contaminating DNA.

Risk factors. (i): Reagents. It is known that some reagents may be contaminated with DNA. For many applications involving yeasts or fungi, a pretreatment protocol is necessary to lyse the cells before DNA extraction. In most cases, lyticase, lysing enzymes and/or zymolyase are used. Rimek et al. have found that different batches of Zymolyase-20T from two different companies contained fungal DNA[62]. The fragment that was amplified in the negative controls of their panfungal PCR assay showed 100% sequence identity to the *Saccharomyces* sensu stricto complex (*S. cerevisiae, S. pastorianus, S. paradoxus* and *S. bayanus*) and to *Kluyveromyces lodderae*. The same contamination was described by Loeffler et al.[40]. They found fungal DNA in specific lot numbers of zymolyase powder, but also in batches of lyticase and lysing enzymes. The zymolyase appeared to be contaminated with DNA from *S. cerevisiae*. The origin of the DNA found in lyticase and lysing enzymes could not be specified.

Several components of PCR mixtures have also been shown to contain contaminating DNA. Different tubes of one lot number of 10x PCR buffer were contaminated with DNA from *Acremonium* spp.[40]. Furthermore, commercial primer preparations used by Goldenberger and Altwegg were the source of contaminations in an assay using broad range primers directed against 16S rDNA[24]. The origin of this contamination was not further specified. Another example of contaminated primers was reported by Lo et al.[39]. Their primers were contaminated by plasmid containing a full length HBV insert. Although it is not mentioned, it is most likely that this contamination originated from their own laboratory, as opposed to the other examples which can all be traced back to the manufacturers.

Contamination of *Taq* DNA polymerase with bacterial DNA has been reported several times[8,16,28,43,61,71]. All researchers have used several polymerases from different suppliers, often

including low-DNA *Taq* DNA polymerase, and although quantitative differences between the products from different companies are observed, all preparations yielded false-positive results. In three of the studies, universal primer systems directed against rDNA sequences were used. It is, however, important to note that false-positive results were also obtained when more specific primer systems were used (16S rDNA sequences of mycobacteria[28], 5S rDNA sequences of *Legionella*[43], rDNA sequences of *Escherichia coli* [61]). When an attempt was made to specify the identity of the contaminating DNA, all authors agreed that the bacteria in which the *Taq* polymerase is produced (*E. coli* and *Thermus aquaticus*) can be ruled out as a source. However, the exact identity could not be revealed. It is generally believed that more than one strain or species is responsible for the occurrence of false-positive results when using *Taq* DNA polymerases, which is most likely the main reason why identification is difficult. Even though it is not unequivocally proven, all findings point to the involvement of either the buffers, the chromatography columns, or the water used in the purification of the enzyme. Thus, it is likely that other biological products are also contaminated, and indeed the purification is also assumed to be the source of the contamination in some primer preparations[24].

(ii): Laboratory disposables and equipment. Besides the reagents, laboratory disposables and equipment can also be the source of contaminating DNA. An obvious example is the need to disinfect the rubber septum of evacuated sample tubes or blood culture bottles before drawing blood. Besides leading to false-positive cultures, this can also cause problems when blood is used for diagnostic amplification methods[27].

Another problem can occur when people disregard the fact that 'sterile' does not necessarily mean 'DNA-free'. In a study performed by Kaul et al., it was shown that 3.6% of sterilized bronchoscopes used for broncho-alveolar lavages (BAL) contained amplifiable *Mycobacterium tuberculosis* DNA[32]. When looking at 277 *M. tuberculosis* PCR results in retrospect (validation- and clinical samples), 5 false-positive samples were detected, 4 of which were BAL samples.

Even the single use plasticware, that has taken over the washable glassware in our laboratories, is not always free of contaminating DNA. For example, according to Schmidt et al. reaction tubes show contamination rates of 20% up to 80-90%, depending on the supplier[70]. The vast majority of these contaminations is of human origin, although in one case the sample did not show any similarity with a human reference sequence.

(iii): Environment. Microorganisms and their DNA are present everywhere around us. For example, fungal spores like conidia from *Aspergillus* spp. can be present in the air. This can lead to false-positive results due to airborne spore inoculation during DNA extraction, as was detected by Loeffler et al.[40]. However, the following examples show that it is also very important to be aware of all activities that takes place in your laboratory, and even in your building. Situations that are routine for one person, can turn out to be an unexpected and huge problem for the other.

Porter-Jordan and Garrett found false-positives when using a PCR for human cytomegalovirus (CMV)[58]. Upon further examination, they realized that this contamination could originate from a laboratory situated one floor below theirs, where CMV culture material was autoclaved before disposal. It turned out that autoclaved positive material included small DNA fragments contaminating the environment, which may have produced positive signals in their PCR

assays.

A second example was described by Taranger et al.[74]. They found a discrepancy of 57% between PCR (91% positive) and culture results (34% positive) for *Bordetella pertussis* in one pediatric outpatient clinic, while in another clinic no PCR-positive and culture-negative samples were seen. All tested surfaces in the two rooms where vaccinations and diagnostic work-ups were done (e.g. laboratory benches, steel tables for equipment, the staff's clothes and the skin of the hands of the staff), were contaminated with *B. pertussis* positive material. However, even though the same environmental findings were made in the vaccination room of the second clinic, in this clinic vaccinations were given in rooms located far away from the examination rooms where patient samples were taken for diagnostic purposes. This environmental contamination was caused by droplets from a whole cell pertussis vaccine.

In our own laboratory we recently encountered a problem with contamination of a diagnostic test in which PCR is used to detect TEM ß-lactamases in clinical isolates (unpublished observations). At a certain time, all negative controls started to become false-positive, and this problem could not be resolved by extensive cleaning of the working areas and the use of new reagents. A research group sharing the same laboratory areas used cloning- and expression systems for the production of proteins. It became clear that the vector applied in their cloning- and expression systems contains a commonly used ampicillin-resistance selection marker, which is a TEM ß-lactamase-gene. Since the focus of this research was to study proteins, this work was done in a regular laboratory room. Therefore, the researchers were not restricted in entering areas where PCR-premixes were prepared, and often, after purification and analysis of expressed proteins, non-related PCRs were performed. No one realized that the protein preparations were highly contaminated with vector-associated DNA, resulting in high copy TEM ß-lactamase-gene contamination of the environment.

Risk reduction. (i): Communication. From these examples we can conclude that communication is very important, especially in larger laboratories where several research groups make use of the same rooms and equipment. Even non-molecular biologists may be working with large amounts of DNA (culturing, plasmids, etc.), and form an unexpected risk of contaminations. The same holds true for researchers using species-specific amplification assays: these tests may not be very sensitive for contaminations, other tests performed in the same area may be. All researchers using the same laboratory space and equipment should conduct themselves to the precautions necessary for the assay that is most sensitive to contaminations, without any exceptions.

The difficulty with contaminated reagents is that it is impossible for a laboratory to prevent such contaminations. It is therefore very important to communicate such problems with the manufacturer. In our experience, companies are not always aware of the very diverse range of sources that can cause contamination of their products. It is often believed that a room (or manufacturing hall) is 'clean', unless it is used by people working with DNA. However, microorganisms, cells and DNA are everywhere. Even though this will be difficult to implement in a production process, it may be sensible for companies to apply the concept that a room is 'dirty', except for small areas that can easily be cleaned.

(ii): Separation of workflow. The amount of contaminating DNA/RNA varies: in general, during sample preparation only low amounts of nucleic acids are generated (so called 'low copy', corresponding with low risk), whereas handling of recombinant plasmids or phages and

amplification techniques reveal high amounts of DNA (so called 'high copy', corresponding with high risk). Therefore, attention must be paid to the work-flow. Laboratories should be subdivided in 'no copy', 'low copy' and 'high copy' working areas, if possible equipped with overpressure (no copy, i.e. clean areas) or underpressure (high copy, i.e. contaminated areas). These working areas can comprise separate rooms, biosafety hoods or for this purpose designed cabinets[26,36,54]. Also, flowcabinets can be used in which the direction of flow ensures that aerosols are pushed down to the base of the cabinet, away from the top of the reaction tubes[51]. Activities in which no DNA is involved (preparation of PCR-mixes) should be performed first, after which activities can be build up via low copy (extraction of low copy nucleic acids, adding template to premixes) to high copy operations (analysis of amplification-products or other sources of high copy DNA). Subsequently, the no- and low copy work area should not be entered again that particular day. Furthermore, it is recommended to aliquot reagents in an area that is free of nucleic acids, and to use different freezers and refrigerators for storage of reagents and samples.

Providing every work area with separate sets of supplies and equipment like racks, pipettes, test tube holders, centrifuges, etc. substantially reduces the risk of carry-over contamination. Appropriate disposable clothing should be present in each room, and these should be changed frequently (e.g. gloves, masks, coats, mob caps, goggles)[34,36,79]. Additionally, it is recommended to clean the working areas, equipment, and literally everything which is routinely touched by hands like doorknobs, handles of freezers, telephones etc. on a regular base. Sodium hypochlorite and HCl are mentioned as cleaning agents for work areas and equipment. Prince et al. recommend a concentration of 0.08% sodium hypochlorite (w/v, 5 min) for fragments as small as 76 bp[59]. This concentration should be stable for 1 week. However, our own observations revealed that a 0.4% (w/v) hypochlorite solution of 1 week old needs an incubation time of 30 minutes before an RNA target of 200 bases is not detectable by NASBA anymore. Better results were obtained after a 5 minute incubation with a daily prepared 0.4% (w/v) solution (unpublished data).

Results for decontamination by depurination with 1 M HCl[15,36,59], possibly in combination with detergent to reduce surface tension and/or UV treatment[70], are controversial. Schmidt et al. described that long-term treatment (2.5 h) with HCl did not yield complete destruction[70] and even 2 M HCl did not completely destroy DNA detectable by PCR[59]. UV irradiation of surfaces like laboratory benches, floors, instruments, microcentrifuge tubes and racks is recommended, but it is much less effective in eliminating dried DNA. Destruction of contaminating DNA by UV-irradiation is described in more detail below. Cleaning agents, like HNO_3, ethanol, and commercial cleaners as *Extran*© (cleaner for laboratory use) are not effective[70].

Furthermore, it is advised to monitor DNA contamination by routine wipe tests as described by Cone et al.[15]. However, the wipe test can be false negative due to inhibitory substances originating from laboratory surfaces, so routinely test a duplicate of each wipe test sample with an amplification positive control[45].

(iii): Prevention of aerosol formation. To prevent contamination by aerosols containing nucleic acids, the use of positive displacement pipettes[26,36] or disposable filtertips/plugged pipette tips[26,46,55] is advised. However, differences in the quality of filtertips from different suppliers is observed[55] (personal observation). To avoid aerosols, reaction tubes should be

opened with caution[26,36]. Furthermore, it is advisable to use the minimum number of cycles during amplification reactions, to produce only as much amplicon as is needed to obtain the desired results.

Another approach to reduce the risk of contaminations, is to carefully handle waste disposal in order to prevent aerosol formation. Disposables which contain RNA/DNA, like pipette tips and reaction tubes, but also electrophoresis buffer, are important potential sources of contaminations. It is recommended to collect nucleic acid contaminated plastic ware in disposable bags and to close the bags directly after activities are finished. Electrophoresis buffer can be decontaminated with sodiumchlorite directly after use (see above).

PREVENTION: DESTRUCTION OF CONTAMINATING DNA

Prevention of DNA contamination is part of the job, but if contamination occurs, one has to try to eliminate this problem. In course of time a lot of suggestions have been published. The conditions for using a protocol should be established and evaluated for each target system. In general, destruction of contaminating DNA can be performed before amplification, to avoid false-positive results in the experiment that is performed, and/or after amplification, to reduce the risk of contaminating following experiments.

In this chapter we will focus on different methods for destruction of contaminating DNA and their draw-backs. Procedures which achieve elimination of contaminating DNA in general are: irradiation with or without the addition of (iso)psoralen, enzymatic treatment, and the use of hydroxylamine hydrochloride. Two other methods specifically destroy amplification products: the use of modified primers and the Uracil DNA glycosylase-dUTP approach. In addition, we will pay attention to specific decontamination methods for reagents and disposables.

Destruction of contaminating DNA. (i): UV-irradiation. UV-irradiation is mainly used to treat PCR premixes and to decontaminate work areas or equipment, and is extensively described. Both single wavelength (254, 300 or 365 nm) and double wavelength (254 nm and 300 nm) treatments have been examined[11,20,22,24,46,50,64,67-69]. The mechanism is based on oxidation of bases, induction of single and double strand breaks, and formation of cyclobutane rings between neighboring pyrimidine bases. The cyclobutane rings form intrastrand pyrimidine dimers that inhibit polymerase mediated chain elongation[7,25].

UV-treatment is not always effective[11,20,46,51,66]. Its efficiency depends on the intensity, wavelength, and exposure-time[20,51,64,67]. In case of treatment of PCR premixes: the presence of nucleotides influences the elimination of contaminating DNA in a dose dependent manner[51]. Furthermore, size and internal sequence of the contaminating DNA fragment are important[11,25,29,46,50,64,67-69]. However, these findings are contradicted[22,68,69].

UV-irradiation may influence the activity of PCR reagents. Although nucleotides and primers are relatively resistant[24], the activity of primers[20,22,50] and undoubtedly of *Taq* polymerase[11,24,46,50,69] and uracil N glycosylase[24] is affected. It may be wise to add these components after UV-irradiation of the premixes. This illustrates an important drawback of this decontamination technique: after sterilization, cups have to be reopened to add reagents and template DNA, which enlarges the risk of contaminations. Besides, reagents like *Taq*

polymerase can contain contaminants themselves (as described above), which will remain unaffected. Furthermore, Niederhauser et al. showed that degraded amplification products and primer artifacts account for a decreased amplification sensitivity[48]. Another note of concern is that Linquist et al. reported that UV-irradiation of polystyrene pipettes releases PCR inhibitors[38].

UV-irradiation is also recommended for elimination of contaminating DNA/RNA from surfaces like laboratory benches, floors, instruments, microcentrifuge tubes and racks[36,46], but it is much less effective in eliminating dried DNA[66,68,69]. Guidelines for eliminating dried DNA by UV-irradiation are clearly described by Cone et al.[14]. A point of consideration for eliminating dried DNA is that the surface must be perpendicular to the light source to achieve maximal light intensity. Additionally, other materials dried with the target DNA such as irrelevant DNA and nucleotides can shield the target, making inactivation less efficient. It is also important to note that UV lamps will still look blue even though their UV output has decreased.

In summary: the efficacy of this method has not been uniform. UV-irradiation should be seen as an additional precaution rather than a replacement for careful laboratory practice. Intensity, wavelength, duration of exposure and effects on the sensitivity of the PCR have to be determined empirically.

(ii): UV-irradiation in combination with (iso)psoralen. Several publications describe the combination of (iso)psoralen and long wavelength UV photoactivation (320-400 nm) for sterilization of PCR amplicons[12,29,29,31,33,46,54,64,79]. (Iso)psoralens are known to intercalate between base pairs of nucleic acids. This results in the formation of cyclobutane adducts with pyrimidine bases and cross-links when excited by 320-400 nm light[12,31], which inhibit the extension by polymerases[56,57,73]. In general, (iso)psoralens are added prior to amplification while photoactivation by UV takes place after amplification.

Isopsoralen modified PCR-products can be probed by hybridization and are therefore favored above psoralen[12,29]. However, lower hybridization stringencies may be required to compensate for the presence of isopsoralen in the amplified DNA[12,29,54] and a significant loss of sensitivity is observed at high concentrations[64]. This loss of sensitivity can be corrected by adding glycerol or DMSO[12,29,64]. Isopsoralen inactivation of contaminating DNA depends on the length and nucleotide base composition of the amplicon[54].

In summary: (iso)psoralen treatment in combination with long wavelength UV irradiation is an effective method for sterilizing PCR amplicons. Since each amplicon has its own base sequence and length, optimal sterilization conditions must be evaluated on a test by test basis. Note that (iso)psoralen must be handled with care due to their mutagenic properties.

(iii): γ-Irradiation. Inactivation of DNA templates by γ-irradiation is described by Deragon et al.[18]. The efficiency depends on several factors including the length of the DNA fragment and the precise composition of the PCR mixture. Irradiation conditions (like dose) have to be established for each amplification system. Unfortunately, laboratories routinely have no γ sources available.

(iv): Enzymatic treatment. Several kinds of enzymes have been described for sterilizing PCR-reagent mixtures like DNase I, exonuclease III, and restriction enzymes[10,17,23,63,82,83,87]. DNA is degraded whereas other components like the *Taq* polymerase remain unaffected. After inactivation of the nucleases by heating, target DNA must be added. This implies that cups

have to be reopened, which enlarges the risk of environmental contaminations. Another disadvantage is that an enzyme combination is used (*Taq* polymerase and a nuclease). Therefore, reaction conditions have to be optimal for both enzymes used.

(v): Hydroxylamine hydrochloride treatment. Aslanzadeh describes the use of hydroxylamine hydrochloride as an effective alternative for the destruction of amplicons after amplification[1]. Hydroxylamine is a mutagenic agent, which disrupts normal nucleic acid pairing. Hydroxylamine hydrochloride modified PCR-products do not appear to bind to and modify other PCR reagents such as *Taq* polymerase.

Destruction of amplification products. (i): Primer modification. A PCR sterilization method which only affects amplification products is the use of primers containing a 3'-terminal ribose residue. *Taq* DNA polymerase is able to both extend and copy a single ribose residue efficiently, which generates a cleavable ribonucleotide linkage within the amplified product[81]. Cleavage can be established either by RNase A treatment (prior amplification) or NaOH treatment (after amplification). In case of RNase treatment, addition of a sulfhydryl reducing agent and thermal denaturation is necessary to inactivate the enzyme, which do not affect the activity of *Taq* DNA polymerase. Efficiency of treatment with NaOH varies, depending on the number and position of the 3' ribose residues[64]. Also, cups have to be opened after amplification to add the base, risking the possibility of aerosol formation. Amplicons generated with 3'-ribose primers can be used for sequencing, cloning, and all other research applications of PCR-products.

(ii): Uracil DNA glycosylase. The use of the uracil DNA glycosylase (UDG) or uracil N glycosylase (UNG)-dUTP approach is another method to combat carry-over contamination of amplification products. The method is introduced by Longo et al.[41] and nowadays commercial diagnostic tests which use this system are available[26]. UDG removes the uracil residues from the sugar moiety of either single- or double stranded DNA, creating abasic sites in the phosphodiester backbone[41]. This method involves substituting dUTP for dTTP in all PCRs to ensure that all DNA arising from these amplifications will contain dUTP. Since UDG does not function on dT-containing DNA, dUTP, UTP or RNA, amplification of natural target RNA or DNA is not affected. If all amplification products contain dUTP, contamination can be eliminated prior amplification without reopening of cups to add polymerase or template. However, fidelity of incorporation of dUTP in place of dTTP is not known for all polymerases. In some cases, PCR does not proceed with quite the same efficiency when dTTP is completely replaced with dUTP[41]. This inefficiency appears to be sequence specific and is not necessarily related to the length of the fragment to be amplified[26]. Poor reaction efficiency is probably due to lower incorporation efficiency of dUTP by *Taq* polymerase or to changes in primer annealing on dUTP substituted templates. Higher concentrations of dUTP with compensating magnesium concentrations can increase product yields[26,41,52,54].

Although there is no significant activity during typical PCR thermal cycling[75], UNG is not completely inactivated at the elevated temperatures in the amplification procedure. So following thermal cycling, prolonged incubation at either 4 or 25°C increases the risk of degradation. Therefore it is recommended to set soak files at 72°C to protect amplified dUTP containing products, or to use UDG inhibitor protein[75]. According to others, dUTP sites are heat labile and break during temperature cycling[26].

DNA containing dUTP is normal in most respects (e.g., it is cut by many restriction

enzymes and hybridizes to oligonucleotide probes)[4], although Bebee et al. found that restriction endonuclease cleavage is dependent on the specific endonuclease used as well as the sequences flanking the endonuclease recognition site[3]. For cloning, amplification products must be introduced into an UDG-deficient *E. coli* host to avoid destruction of the amplified DNA. Last but not least: high degrees of contamination can not be destroyed completely by this system, which results in false-positive signals[52].

Effectiveness and combinations. A comparison of three different methods for elimination of amplification products (pre-PCR treatment of a dU-containing PCR product with UNG, post PCR UV-irradiation in combination with isopsoralen, and post-PCR alkaline primer hydrolysis) showed that all three methods are effective and were able to eliminate up to 10^9 copies of the product[64]. Also, the combination of different protocols like treatment with UV (amplification reactions excluding polymerase, primers, and template) followed by DNase I treatment of polymerase and primers, appear to be practicable[63]. Methods which sterilize the whole PCR-mixture directly before amplification starts are preferable, because reopening of cups enlarges the risk of cross-contamination. For this, Udaykumar et al. have introduced the use of a wax-barrier[77].

Decontamination of reagents and disposables. Methods which have been described to eliminate contaminating DNA from buffers, primers, and disposables are ultrafiltration [70,80] and autoclaving under conditions that provide bacterial decontamination[36,46]. However, effectiveness is controversial[20,70]. Several preparative methods have been described to eliminate contaminating DNA from the *Taq* polymerase as anion exchange chromatography[19], polyethyleneimine precipitation followed by centrifugation and dialysis[21], and an aqueous/organic biphasic system[42].

CONTROLS

In the end it is worthwhile to check whether precautions and sterilizing protocols have functioned. Therefore, it is recommended to run negative sample- and assay controls with every test. Negative sample controls should be similar to the tested samples, but should not contain any target DNA. For example, blood from healthy individuals or culture medium can be used. These control samples should be subjected to all preparation steps in parallel with the extracted samples. Assay-controls should consist of all PCR components except template DNA. Negative controls should be added for every batch of 10 samples analyzed[79]. It is desirable to place one negative control at the beginning of a series of samples (to check whether the sample itself, the reagents, and the environment are free of contaminations), and other negative controls in-between and at the end of a series of samples (to check whether cross-contamination between samples has occurred). However, although the presence of contaminated reagents or gross contaminations of the environment should be observed by using these controls, sporadic contaminations can occur and will be more difficult to recognize.

When there is a suspicion of the environment being contaminated, negative controls prepared in the different laboratory spaces can be used. These controls are similar to sample- or assay controls, but differ with respect to the preparation area. At least one control should be

prepared in each area used for the assay: sample controls where DNA is extracted, assay controls in the areas where premixes are made (no-copy area) and where DNA is added to the premixes (low-copy area)[78]. If the contaminated room or area is located, wipe tests can be used to check whether the source can be localized to specific benches or other surfaces, equipment, or even labcoats[15].

It is recommended to record the lot numbers of reagents used so that if contamination occurs, it can more easily be traced. A possible way to check the reagents for the presence of contaminating DNA is to prepare PCR mixes lacking individual components, and to treat these mixes with UV-light. After UV-treatment, the lacking component is added, and a PCR performed. If a product is formed, the component which was not exposed to UV-light is contaminated. Since *Taq* DNA polymerase is sensitive to treatment with UV-light (see above), it may be wise to add this enzyme only after the UV-treatment, together with the other missing component[24].

The enzymes used for cell-lysis can be examined by dissolving them in (clean) water, followed by heat-inactivation at 95°C, DNA extraction and concentration by standard methods, and amplification[62]. Obviously, using a DNA extraction- and amplification assay to look for contaminating DNA is risky: besides the risk of introducing contaminating DNA from yet another source, it is possible that the contaminant remains undetected because the amount of contaminating DNA in the sample is too low, or some of it is lost during sample preparation.

To prevent recurrence of the same problem, it is often important to not only localize but also identify the source of the contamination. At this point, it is wise to check with other researchers using the same or neighboring laboratory spaces which microorganisms, plasmids or DNA sequences are used. If plasmids containing target DNA are commonly used, an amplification with primers that span the vector-insert junction can help identifying the plasmid as the source of the contamination[39]. Otherwise, sequencing of the PCR product, if necessary preceded by cloning into a vector, followed by sequence similarity searches in sequence data banks may be essential.

Contamination or? Besides contaminating DNA, positive results of negative controls may also be caused by sub-optimal assay-conditions. Sub-optimal PCR assays may lead to nonspecific bands after gel analysis. Optimizing the annealing temperature may help avoiding this problem. However, instead of using gel analysis, based only on the size of the product obtained, it is advised to use a more specific detection assay using template-specific probes (e.g. Southern- or Northern blotting) when possible, to circumvent problems with nonspecific products. In some cases, a simple RFLP analysis will be sufficient to distinguish false-positive results from targets. More complex techniques like SSCP or sequencing may also be helpful[28,61,85]. However, this will only work when nonspecific products are formed, or when the primers recognize more species or strains than anticipated. In case of carry-over contaminations, these methods will not be of any help.

Another problem occurring during gel analysis is 'leaking': when a slot contains a large amount of target DNA, this may leak into neighboring slots in the gel and cause a false-positive result in an adjacent lane. Obviously, in this case blotting of the gel and hybridizing with a specific probe will still lead to a false-positive result. When high amounts of PCR product are expected, it is best to skip lanes between samples[9].

When equipment is re-used, the cleaning method may not always be sufficient. In some

cases additional testing may be necessary to distinguish false-positives from positives. Upon realizing that sterilized bronchoscopes were the source of contaminations, Kaul et al. started to request a sterile prewash of the bronchoscope for analysis along with the actual patient sample when BAL samples are submitted for PCR testing[32].

Balfe described a statistical method which can be used to determine whether positive results of PCR reactions carried out in a microtiter plate are unlikely to have occurred by chance, and hence whether these results are false. A similar method for tube-based PCRs is also available. These methods are based on expected probability distributions[2].

CONCLUDING REMARK

The most important conclusion is the fact that although most nucleic acid amplification assays are basically useful both in research as well as in the clinic, problems can occur when procedures for prevention of nucleic acid contaminations are insufficient. This review may be a guide to improve laboratory practice and reduce the number of false-positive results.

REFERENCES

1. **Aslanzadeh, J.** 1993. Application of hydroxylamine hydrochloride for post-PCR sterilization. Mol. Cell Probes 7: 145-150
2. **Balfe, P.** 1992. A statistical method for the detection of false positives and false negatives in microtitre format PCR assays. J. Virol. Methods 39: 69-82
3. **Beebe, R.L., C.G. Thornton, J.L. Hartley, and A. Rashtchian.** 1992. Contamination-free polymerase chain reaction: endonuclease cleavage and cloning of dU-PCR products. Focus 14: 53-56
4. **Bodnar, J.W., W. Zempsky, D. Warder, C. Bergson, and D.C. Ward.** 1983. Effect of nucleotide analogs on the cleavage of DNA by the restriction enzymes *Alu*I, *Dde*I, *Hinf*I, *Rsa*I, and *Taq*I. J. Biol. Chem. 258: 15206-15213
5. **Bogard, M., C. Buffet-Janvresse, J.F. Cantaloube, P. Biagini, G. Duverlie, S. Castelain, J. Izopet, M. Dubois, C. Defer, I. Lepot, J. Coste, P. Marcellin, M. Martinot-Peignoux, P. Halfon, V. Gerolami, L. Frangeul, J.M. Pawlotsky, F. Roudot-Thoraval, E. Dussaix, P. Loiseau, N. Ravera, P. Lewin, J. Lamoril, J. Lerable, and P. Lebon .** 1997. GEMHEP multicenter quality control study of PCR detection of GB virus C/hepatitis G virus RNA in serum. J. Clin. Microbiol. 35: 3298-3300
6. **Borst, A., M.A. Leverstein-Van Hall, J. Verhoef, and A.C. Fluit.** 2001. Detection of *Candida* spp. in blood cultures using nucleic acid sequence-based amplification (NASBA). Diagn. Microbiol. Infect. Dis. 39: 155-160
7. **Bottema, C.D., D.D. Koeberl, R.P. Ketterling, E.J. Bowie, S.A. Taylor, D. Lillicrap, A. Shapiro, G. Gilchrist, and S.S. Sommer.** 1990. A past mutation at isoleucine 397 is now a common cause of moderate/mild haemophilia B. Br. J. Haematol. 75: 212-216
8. **Bottger, E.C.** 1990. Frequent contamination of *Taq* polymerase with DNA. Clin. Chem. 36: 1258-1259
9. **Boyd, A.S., M. Annarella, R.P. Rapini, K. Adler-Storthz, and M. Duvic.** 1996. False-positive polymerase

chain reaction results for human papillomavirus in lichen planus. Potential laboratory pitfalls of this procedure. J. Am. Acad. Dermatol. 35: 42-46

10. **Carroll, N.M., P. Adamson, and N. Okhravi.** 1999. Elimination of bacterial DNA from *Taq* DNA polymerases by restriction endonuclease digestion. J. Clin. Microbiol. 37: 3402-3404
11. **Cimino, G.D., K. Metchette, S.T. Isaacs, and Y.S. Zhu.** 1990. More false-positive problems. Nature 345: 773-774
12. **Cimino, G.D., K.C. Metchette, J.W. Tessman, J.E. Hearst, and S.T. Isaacs.** 1991. Post-PCR sterilization: a method to control carryover contamination for the polymerase chain reaction. Nucleic Acids Res. 19: 99-107
13. **Compton, J.** 1991. Nucleic acid sequence-based amplification. Nature 350: 91-92
14. **Cone, R.W. and M.R. Fairfax.** 1993. Protocol for ultraviolet irradiation of surfaces to reduce PCR contamination. PCR Methods Appl. 3: S15-S17
15. **Cone, R.W., A.C. Hobson, M.L. Huang, and M.R. Fairfax.** 1990. Polymerase chain reaction decontamination: the wipe test. Lancet 336: 686-687
16. **Corless, C.E., M. Guiver, R. Borrow, V. Edwards-Jones, E.B. Kaczmarski, and A.J. Fox.** 2000. Contamination and sensitivity issues with a real-time universal 16S rRNA PCR. J. Clin. Microbiol. 38: 1747-1752
17. **DeFilippes, F.M.** 1991. Decontaminating the polymerase chain reaction. Biotechniques 10: 26, 28, 30
18. **Deragon, J.M., D. Sinnett, G. Mitchell, M. Potier, and D. Labuda.** 1990. Use of gamma irradiation to eliminate DNA contamination for PCR. Nucleic Acids Res. 18: 6149
19. **Desai, U.J. and P.K. Pfaffle.** 1995. Single-step purification of a thermostable DNA polymerase expressed in *Escherichia coli*. Biotechniques 19: 780-2, 784
20. **Dwyer, D.E. and N. Saksena.** 1992. Failure of ultra-violet irradiation and autoclaving to eliminate PCR contamination. Mol. Cell Probes 6: 87-88
21. **Engelke, D.R., A. Krikos, M.E. Bruck, and D. Ginsburg.** 1990. Purification of *Thermus aquaticus* DNA polymerase expressed in *Escherichia coli*. Anal. Biochem. 191: 396-400
22. **Fox, J.C., M. Ait-Khaled, A. Webster, and V.C. Emery.** 1991. Eliminating PCR contamination: is UV irradiation the answer? J. Virol. Methods 33: 375-382
23. **Furrer, B., U. Candrian, P. Wieland, and J. Luthy.** 1990. Improving PCR efficiency. Nature 346: 324
24. **Goldenberger, D. and M. Altwegg.** 1995. Eubacterial PCR: contaminating DNA in primer preparations and its elimination by UV light. J. Microbiol. Methods 21: 27-32
25. **Gordon, L.K. and W.A. Haseltine.** 1982. Quantitation of cyclobutane pyrimidine dimer formation in double- and single-stranded DNA fragments of defined sequence. Radiat. Res. 89: 99-112
26. **Hartley, J.L. and A. Rashtchian.** 1993. Dealing with contamination: enzymatic control of carryover contamination in PCR. PCR Methods Appl. 3: S10-S14
27. **Hauman, J.H., P.D. Van Helden, and C.H. Hauman.** 1995. Evacuated tubes and possible false-positive PCR results with blood samples. S. Afr. Med. J. 85: 119
28. **Hughes, M.S., L.A. Beck, and R.A. Skuce.** 1994. Identification and elimination of DNA sequences in *Taq* DNA polymerase. J. Clin. Microbiol. 32: 2007-2008
29. **Isaacs, S.T., J.W. Tessman, K.C. Metchette, J.E. Hearst, and G.D. Cimino.** 1991. Post-PCR sterilization: development and application to an HIV-1 diagnostic assay. Nucleic Acids Res. 19: 109-116
30. **Jehuda-Cohen, T.** 1995. The false-positive polymerase chain reaction and the ostrich. J. Infect. Dis. 172: 1420-1421
31. **Jinno, Y., K. Yoshiura, and N. Niikawa.** 1990. Use of psoralen as extinguisher of contaminated DNA in

PCR. Nucleic Acids Res. 18: 6739

32. **Kaul, K., S. Luke, C. McGurn, N. Snowden, C. Monti, and W.A. Fry.** 1996. Amplification of residual DNA sequences in sterile bronchoscopes leading to false-positive PCR results. J. Clin. Microbiol. 34: 1949-1951
33. **Keum, W.K., C.E. Park, J.H. Lee, L.Y. Khil, I. Kang, S.S. Kim, J.C. Jung, S.M. Oh, H.J. Woo, J.H. Lee, Y.C. Kim, Y. Yoon, J.W. Choi, and J. Ha.** 1997. Primers determine the sensitivity of PCR-mediated hepatitis B virus DNA detection and pretreatment of PCR mixture with 8-methoxypsoralen eliminates false-positive results. Mol. Cells 7: 244-250
34. **Kitchin, P.A., Z. Szotyori, C. Fromholc, and N. Almond.** 1990. Avoidance of PCR false positives. Nature 344: 201
35. **Kwoh, D.Y., G.R. Davis, K.M. Whitfield, H.L. Chappelle, L.J. DiMichele, and T.R. Gingeras.** 1989. Transcription-based amplification system and detection of amplified human immunodeficiency virus type 1 with a bead-based sandwich hybridization format. Proc. Natl. Acad. Sci. U. S. A. 86: 1173-1177
36. **Kwok, S. and R. Higuchi.** 1989. Avoiding false positives with PCR. Nature 339: 237-238
37. **Landry, M.L.** 1995. False-positive polymerase chain reaction results in the diagnosis of herpes simplex encephalitis. J. Infect. Dis. 172: 1641-1643
38. **Linquist, V., C.A. Stoddart, and J.M. McCune.** 1998. UV irradiation of polystyrene pipets releases PCR inhibitors. Biotechniques 24: 50-52
39. **Lo, Y.M., W.Z. Mehal, and K.A. Fleming.** 1988. False-positive results and the polymerase chain reaction. Lancet 2: 679
40. **Loeffler, J., H. Hebart, R. Bialek, L. Hagmeyer, D. Schmidt, F.P. Serey, M. Hartmann, J. Eucker, and H. Einsele.** 1999. Contaminations occurring in fungal PCR assays. J. Clin. Microbiol. 37: 1200-1202
41. **Longo, M.C., M.S. Berninger, and J.L. Hartley.** 1990. Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reactions. Gene 93: 125-128
42. **Louwrier, A.** 1999. Nucleic acid removal from *Taq* polymerase preparations using an aqueous/organic biphasic system. Biotechniques 27: 444-445
43. **Maiwald, M., H.J. Ditton, H.G. Sonntag, and D.M. von Knebel.** 1994. Characterization of contaminating DNA in *Taq* polymerase which occurs during amplification with a primer set for *Legionella* 5S ribosomal RNA. Mol. Cell Probes 8: 11-14
44. **Maksymiuk, A.W., S. Thongprasert, R. Hopfer, M. Luna, V. Fainstein, and G.P. Bodey.** 1984. Systemic candidiasis in cancer patients. Am. J. Med. 77: 20-27
45. **McCormack, J.M., M.L. Sherman, and D.H. Maurer.** 1997. Quality control for DNA contamination in laboratories using PCR-based class II HLA typing methods. Hum. Immunol. 54: 82-88
46. **Meier, A., D.H. Persing, M. Finken, and E.C. Bottger.** 1993. Elimination of contaminating DNA within polymerase chain reaction reagents: implications for a general approach to detection of uncultured pathogens. J. Clin. Microbiol. 31: 646-652
47. **Molloy, P.J., D.H. Persing, and V.P. Berardi.** 2001. False-positive results of pcr testing for lyme disease. Clin. Infect. Dis. 33: 412-413
48. **Niederhauser, C., C. Hofelein, B. Wegmuller, J. Luthy, and U. Candrian.** 1994. Reliability of PCR decontamination systems. PCR Methods Appl. 4: 117-123
49. **Noordhoek, G.T., J.D. van Embden, and A.H. Kolk.** 1996. Reliability of nucleic acid amplification for detection of *Mycobacterium tuberculosis*: an international collaborative quality control study among 30 laboratories. J. Clin. Microbiol. 34: 2522-2525
50. **Ou, C.Y., J.L. Moore, and G. Schochetman.** 1991. Use of UV irradiation to reduce false positivity in

polymerase chain reaction. Biotechniques 10: 442, 444, 446

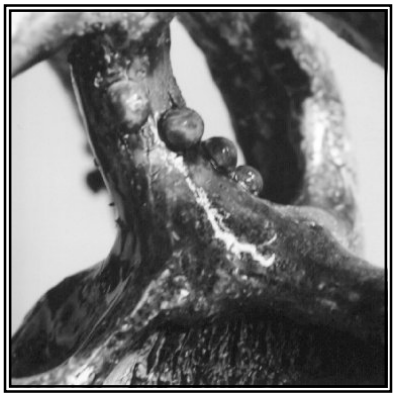
51. **Padua, R.A., A. Parrado, J. Larghero, and C. Chomienne.** 1999. UV and clean air result in contamination-free PCR. Leukemia 13: 1898-1899
52. **Pang, J., J. Modlin, and R. Yolken.** 1992. Use of modified nucleotides and uracil-DNA glycosylase (UNG) for the control of contamination in the PCR-based amplification of RNA. Mol. Cell Probes 6: 251-256
53. **Patel, R., K.L. Grogg, W.D. Edwards, A.J. Wright, and N.M. Schwenk.** 2000. Death from inappropriate therapy for Lyme disease. Clin. Infect. Dis. 31: 1107-1109
54. **Persing, D.H.** 1991. Polymerase chain reaction: trenches to benches. J. Clin. Microbiol. 29: 1281-1285
55. **Peters, R.** 1992. Elimination of PCR carryover. Am. Biotechnol. Lab 10: 42
56. **Piette, J. and J. Hearst.** 1985. Sites of termination of in vitro DNA synthesis on psoralen phototreated single-stranded templates. Int. J. Radiat. Biol. Relat Stud. Phys. Chem. Med. 48: 381-388
57. **Piette, J.G. and J.E. Hearst.** 1983. Termination sites of the in vitro nick-translation reaction on DNA that had photoreacted with psoralen. Proc. Natl. Acad. Sci. U. S. A. 80: 5540-5544
58. **Porter-Jordan, K. and C.T. Garrett.** 1990. Source of contamination in polymerase chain reaction assay. Lancet 335: 1220
59. **Prince, A.M. and L. Andrus.** 1992. PCR: how to kill unwanted DNA. Biotechniques 12: 358-360
60. **Quint, W.G., R.A. Heijtink, J. Schirm, W.H. Gerlich, and H.G. Niesters.** 1995. Reliability of methods for hepatitis B virus DNA detection. J. Clin. Microbiol. 33: 225-228
61. **Rand, K.H. and H. Houck.** 1990. *Taq* polymerase contains bacterial DNA of unknown origin. Mol. Cell Probes 4: 445-450
62. **Rimek, D., A.P. Garg, W.H. Haas, and R. Kappe.** 1999. Identification of contaminating fungal DNA sequences in Zymolyase. J. Clin. Microbiol. 37: 830-831
63. **Rochelle, P.A., A.J. Weightman, and J.C. Fry.** 1992. DNase I treatment of *Taq* DNA polymerase for complete PCR decontamination. Biotechniques 13: 520
64. **Rys, P.N. and D.H. Persing.** 1993. Preventing false positives: quantitative evaluation of three protocols for inactivation of polymerase chain reaction amplification products. J. Clin. Microbiol. 31: 2356-2360
65. **Saiki, R.K., S. Scharf, F. Faloona, K.B. Mullis, G.T. Horn, H.A. Erlich, and N. Arnheim.** 1985. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230: 1350-1354
66. **Sarkar, G. and S. Sommer.** 1990. More light on PCR contamination. Nature 347: 340-341
67. **Sarkar, G. and S.S. Sommer.** 1990. Shedding light on PCR contamination. Nature 343: 27
68. **Sarkar, G. and S.S. Sommer.** 1991. Parameters affecting susceptibility of PCR contamination to UV inactivation. Biotechniques 10: 590-594
69. **Sarkar, G. and S.S. Sommer.** 1993. Removal of DNA contamination in polymerase chain reaction reagents by ultraviolet irradiation. Methods Enzymol. 218: 381-388
70. **Schmidt, T., S. Hummel, and B. Herrmann.** 1995. Evidence of contamination in PCR laboratory disposables. Naturwissenschaften 82: 423-431
71. **Schmidt, T.M., B. Pace, and N.R. Pace.** 1991. Detection of DNA contamination in *Taq* polymerase. Biotechniques 11: 176-177
72. **Schwartz, D.H., O.B. Laeyendecker, S. Arango-Jaramillo, R.C. Castillo, and M.J. Reynolds.** 1997. Extensive evaluation of a seronegative participant in an HIV-1 vaccine trial as a result of false-positive PCR. Lancet 350: 256-259
73. **Shi, Y.B., H. Gamper, and J.E. Hearst.** 1987. The effects of covalent additions of a psoralen on transcription by *E. coli* RNA polymerase. Nucleic Acids Res. 15: 6843-6854

74. **Taranger, J., B. Trollfors, L. Lind, G. Zackrisson, and K. Beling-Holmquist.** 1994. Environmental contamination leading to false-positive polymerase chain reaction for pertussis. Pediatr. Infect. Dis. J. 13: 936-937
75. **Thornton, C.G., J.L. Hartley, and A. Rashtchian.** 1992. Utilizing uracil DNA glycosylase to control carryover contamination in PCR: characterization of residual UDG activity following thermal cycling. Biotechniques 13: 180-184
76. **Trinker, M., G. Hofler, and H. Sill.** 1996. False-positive diagnosis of tuberculosis with PCR. Lancet 348: 1388
77. **Udaykumar, J.S. Epstein, and I.K. Hewlett.** 1993. A novel method employing UNG to avoid carry-over contamination in RNA-PCR. Nucleic Acids Res. 21: 3917-3918
78. **Urban, C., F. Gruber, M. Kundi, F.G. Falkner, F. Dorner, and T. Hammerle.** 2000. A systematic and quantitative analysis of PCR template contamination. J. Forensic Sci. 45: 1307-1311
79. **Victor, T., A. Jordaan, T.R. du Toit, and P.D. Van Helden.** 1993. Laboratory experience and guidelines for avoiding false positive polymerase chain reaction results. Eur. J. Clin. Chem. Clin. Biochem. 31: 531-535
80. **Wages, J.M., Jr., D. Cai, and A.K. Fowler.** 1994. Removal of contaminating DNA from PCR reagents by ultrafiltration. Biotechniques 16: 1014-1017
81. **Walder, R.Y., J.R. Hayes, and J.A. Walder.** 1993. Use of PCR primers containing a 3'-terminal ribose residue to prevent cross-contamination of amplified sequences. Nucleic Acids Res. 21: 4339-4343
82. **Widjojoatmodjo, M.N.** 1995. Sample preparation for the detection of bacteria in blood using the polymerase chain reaction. In: Diagnosis of Infections Based on DNA Amplification. Ph. D. thesis, Utrecht University, the Netherlands: 65-72
83. **Widjojoatmodjo, M.N., A.C. Fluit, and J. Verhoef.** 1994. Rapid identification of bacteria by PCR-single-strand conformation polymorphism. J. Clin. Microbiol. 32: 3002-3007
84. **Wu, D.Y. and R.B. Wallace.** 1989. The ligation amplification reaction (LAR)-amplification of specific DNA sequences using sequential rounds of template-dependent ligation. Genomics 4: 560-569
85. **Yap, E.P., Y.M. Lo, K. Cooper, K.A. Fleming, and J.O. McGee.** 1992. Exclusion of false-positive PCR viral diagnosis by single-strand conformation polymorphism. Lancet 340: 736
86. **Zaaijer, H.L., H.T. Cuypers, H.W. Reesink, I.N. Winkel, G. Gerken, and P.N. Lelie.** 1993. Reliability of polymerase chain reaction for detection of hepatitis C virus. Lancet 341: 722-724
87. **Zhu, Y.S., S.T. Isaacs, C.D. Cimino, and J.E. Hearst.** 1991. The use of exonuclease III for polymerase chain reaction sterilization. Nucleic Acids Res. 19: 2511

VII: AFLP as an identification method for medically important *Candida* spp., including *C. dubliniensis*

A. Borst[1], B. Theelen[2], E. Reinders[3], T. Boekhout[2], A.C. Fluit[1] and P.H.M. Savelkoul[3]

[1] Eijkman-Winkler Center, University Medical Center, Utrecht, the Netherlands
[2] Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands
[3] Department of Medical Microbiology and Infection Control, VU University Medical Center, Amsterdam, the Netherlands



Submitted for publication.

ABSTRACT

Non-*C. albicans Candida* species are isolated in increasing frequencies. These species show differences in resistance to antimycotic agents and virulence. Therefore, it is important to be able to correctly identify the causative organism to the species level. Phenotypic identification methods are not always reliable. Especially identification of *C. dubliniensis* remains problematic, due to the high degree of phenotypic similarity between this species and *C. albicans*. Molecular identification methods may be more reliable. The use of Amplified Fragment Length Polymorphism (AFLP™) analysis as an identification method for medically important *Candida* species was investigated. Our results show very clear differences between medically important *Candida* species. Furthermore, when screening a large collection of clinical isolates previously identified on CHROMagar as *C. albicans* we found a misidentification rate of 6%. AFLP is universally applicable and the patterns can easily be stored in a general accessible database. Therefore, AFLP might prove to be a reliable method for the identification of medically important *Candida* species.

INTRODUCTION

In the past decade, life-threatening forms of candidiasis increased dramatically[1]. The attributable mortality of these infections is as high as 38%[34], whereas crude mortality rates exceed 50%[10,27,33]. For a long time, *Candida albicans* was the main cause of invasive fungal infections. However, the number of infections by this species is declining, whereas non-*albicans Candida* species like *C. glabrata, C. krusei* and *C. parapsilosis* are increasingly isolated. At present, non-*albicans Candida* species account for approximately 50% of all *Candida* infections[14].

In case of candidiasis, it is important to be able to correctly identify the causative organism to the species level. Different species show differences in resistance to antimycotic agents. *C. krusei* is innately resistant to fluconazole, and *C. glabrata* is able to acquire resistance to this drug rapidly. Furthermore, *C. glabrata* infections have been associated with a high mortality[11]. A particular problem is formed by the recently recognized species *Candida dubliniensis*. Like *C. glabrata*, this species is capable of acquiring stable fluconazole resistance rapidly[22,23]. Identification of *C. dubliniensis* remains difficult, due to the high degree of phenotypic similarity between this species and *C. albicans*. However, it is known that genotypically there is more variation between the species[30]. Therefore, molecular identification methods may be more reliable than methods based on phenotypic characteristics.

Amplified Fragment Length Polymorphism (AFLP™) analysis is a relatively new technique which discriminatory power makes it suitable for identification as well as strain typing[29,32]. In short: genomic DNA is digested with two restriction enzymes (e.g. *Eco*RI and *Mse*I), and double-stranded oligonucleotide adapters are ligated to the fragments. These adapters serve as targets for the primers during PCR amplification. To increase the specificity, it is possible to elongate the primers at the 3' end with 1-3 selective nucleotides. One of the primers is labeled with a fluorescent dye. The fragments are separated and analyzed using software packages like BioNumerics (Applied Maths, Sint-Martens-Latem, Belgium). The advantage of AFLP is that

only a limited amount of DNA is needed, since the fragments are PCR-amplified. Furthermore, since stringent annealing temperatures are used during amplification, the technique is more reproducible and robust compared to other methods such as Randomly Amplified Polymorphic DNA analysis (RAPD)[13,29]. This paper describes the use of AFLP as an identification method for medically important *Candida* species, including *C. dubliniensis*.

MATERIALS AND METHODS

Yeast strains. The yeast strains and isolates used are listed in Table 1. Reference strains were obtained from the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands, and the American Type Culture Collection (ATCC), Manassas, USA. Clinical isolates were obtained from the European SENTRY collection (Eijkman-Winkler Center, Utrecht, the Netherlands)[26] and from the Haematology ward of the VU University Medical Center (VUMC), Amsterdam, the Netherlands. The SENTRY isolates were identified using CHROMagar plates (CHROMagar, Paris, France). The isolates from the VUMC were identified using the Germ-tube test. Isolates that were negative in this assay were further identified using Vitek YBC (bioMérieux, Boxtel, the Netherlands).

Table 1

Reference strains and clinical isolates used in this study

Spp.[1]	Strains/isolates	Origin	Source
C. albicans	CBS 562	Uruguay	skin of man with interdigital mycosis
	CBS 1905	unknown	man
	CBS 1912	Norway	sputum of asthma patient
	ATCC 90028	Iowa, USA	blood
	ATCC 90029	Iowa, USA	blood
	04A080 (SENTRY)	Paris, France	blood
	06A309 (SENTRY)	Lille, France	blood
	07C069 (SENTRY)	Freiburg, Germany	pneumonia
	08E058 (SENTRY)	Dusseldorf, Germany	wound/skin/soft tissue
	10A173 (SENTRY)	Genoa, Italy	blood
	10C007 (SENTRY)	Genoa, Italy	pneumonia
	11A134 (SENTRY)	Roma, Italy	blood
	11C034 (SENTRY)	Roma, Italy	pneumonia
	12E033 (SENTRY)	Utrecht, the Netherlands	wound/skin/soft tissue
	15A020 (SENTRY)	Coimbra, Portugal	blood
	15A206 (SENTRY)	Coimbra, Portugal	blood
	15A561 (SENTRY)	Coimbra, Portugal	blood
	16A232 (SENTRY)	Sevilla, Spain	blood
	16A438 (SENTRY)	Sevilla, Spain	blood
	16C088 (SENTRY)	Sevilla, Spain	pneumonia
	17A381 (SENTRY)	Madrid, Spain	blood
	19A164 (SENTRY)	Lausanne, Switzerland	blood
	19A519 (SENTRY)	Lausanne, Switzerland	blood

C. albicans	19A567 (SENTRY)	Lausanne, Switzerland	blood
(contin.)	19A568 (SENTRY)	Lausanne, Switzerland	blood
	23D045 (SENTRY)	Ankara, Turkey	urinary tract
	TY727 (VUMC)	Amsterdam, the Netherlands	oral cavity
	TY728 (VUMC)	Amsterdam, the Netherlands	oral cavity
	TY729 (VUMC)	Amsterdam, the Netherlands	oral cavity
	TY732 (VUMC)	Amsterdam, the Netherlands	faeces (human)
C. dubliniensis	CBS 7987	Dublin, Ireland	oral cavity of HIV-infected patient
	CBS 7988	Melbourne, Australia	oral cavity of HIV-infected patient
	CBS 8500	Nijmegen, the Netherlands	blood of 38-year-old woman with chronic myelogenous leukaemia
	CBS 8501	Nijmegen, the Netherlands	child with neutropeny induced by chemotherapy
	02A038 (SENTRY)	Brussels, Belgium	blood
	05C118 (SENTRY)	Lyon, France	pneumonia
	05C121 (SENTRY)	Lyon, France	pneumonia
	18A221 (SENTRY)	Barcelona, Spain	blood
	20C149 (SENTRY)	London, UK	pneumonia
	23A137 (SENTRY)	Ankara, Turkey	blood
C. glabrata	CBS 138	unknown	faeces (human)
	ATCC 90030	Iowa, USA	blood
	TY714 (VUMC)	Amsterdam, the Netherlands	oral cavity
	TY715 (VUMC)	Amsterdam, the Netherlands	faeces (human)
	TY716 (VUMC)	Amsterdam, the Netherlands	oral cavity
	TY717 (VUMC)	Amsterdam, the Netherlands	oral cavity
	TY718 (VUMC)	Amsterdam, the Netherlands	oral cavity
	TY719 (VUMC)	Amsterdam, the Netherlands	oral cavity
	TY731 (VUMC)	Amsterdam, the Netherlands	oral cavity
C. guilliermondii	CBS 566	unknown	sputum (human)
	CBS 2024	Berlin, Germany	ulcer on horse
	14A097 (SENTRY)	Cracow, Poland	blood
C. krusei	CBS 573	Colombo, Sri Lanka	sputum of bronchitic convict
	TY722 (VUMC)	Amsterdam, the Netherlands	oral cavity
	TY723 (VUMC)	Amsterdam, the Netherlands	oral cavity
	TY726 (VUMC)	Amsterdam, the Netherlands	faeces (human)
C. lusitaniae	CBS 4413	Portugal	caecum of pig
C. parapsilosis	CBS 604	Puerto Rico	case of sprue (human)
	CBS 2195	Austria	infected nail (11-year-old boy)
	ATCC 90018	Virginia, USA	blood
	07A212 (SENTRY)	Freiburg, Germany	blood
	10A120 (SENTRY)	Genoa, Italy	blood
	10A311 (SENTRY)	Genoa, Italy	blood
	14A161 (SENTRY)	Cracow, Poland	blood
	TY735 (VUMC)	Amsterdam, the Netherlands	oral cavity
	TY736 (VUMC)	Amsterdam, the Netherlands	unknown
C. pseudotropicalis	CBS 607	Sri Lanka	bronchitic patient
C. tropicalis	CBS 94	unknown	bronchitic patient
	CBS 2310	unknown	unknown
	11D028 (SENTRY)	Roma, Italy	urinary tract
	TY737 (VUMC)	Amsterdam, the Netherlands	oral cavity
	TY739 (VUMC)	Amsterdam, the Netherlands	oral cavity

[1] Identification of SENTRY isolates based on AFLP patterns

Extraction of DNA. DNA was extracted from approximately 10^7 cfu using the DNeasy Tissue kit (Qiagen, West Sussex, England) according to the manufacturer (protocol for isolation of genomic DNA from yeasts). DNA was eluted in 100 µl elution buffer (buffer AE of the kit) and stored at -20°C.

AFLP. The sequences of the adapters and primers used for AFLP are depicted in Table 2. DNA was extracted from approximately 10^7 cfu *C. albicans* as described above. Five µl of the DNA samples were added to 5 µl restriction-ligation reaction mixture (1x T_4 DNA ligase buffer; 0.05 M NaCl; 0.5 µg BSA; 2 pmol *Eco*RI-adapter; 20 pmol *Mse*I-adapter; 80 U T_4 DNA ligase; 1 U *Eco*RI; 1 U *Mse*I, and incubated over night at 37°C. All enzymes were obtained from New England BioLabs (Beverly, USA). The mixture was diluted 1:5 with 0.1x TE (5 mM Tris-HCl (pH 7.5); 1 mM EDTA). Pre-selective PCR was performed using the core sequences, i.e. primers without extensions. The AFLP primers, core mix, and internal size standard were supplied by Applied Biosystems (Nieuwerkerk a/d IJssel, the Netherlands). Four µl of diluted restriction-ligation product was added to 15 µl of AFLP amplification core mix, 0.5 µl *Eco*RI core sequence and 0.5 µl *Mse*I core sequence. The mixture was amplified in a GeneAmp® PCR System 9700 machine under the following conditions: 2 min. at 72°C, followed by 20 cycles of 20 sec. at 94°C, 30 sec. at 56°C and 2 min. at 72°C each. The PCR product was diluted by adding 25 µl sterile double distilled water. In a second PCR reaction more selective primers were used: *Eco*RI-AC (FAM-labeled) and *Mse*I-C. The conditions were: 2 min. at 94°C, followed by 10 cycles consisting of 20 sec. at 94°C, 30 sec. at 66°C decreasing 1°C every step of the cycle, and 2 min. at 72°C, followed by 25 cycles consisting of 20 sec. at 94°C, 30 sec. at 56°C and 2 min. at 72°C. After a final incubation of 30 min. at 60°C the samples were prepared for capillary electrophoresis by adding 2 µl of the selective PCR product to 24 µl of deionized formamide and 1 µl of GeneScan-500 (ROX-labeled) as an internal size standard. They were run on the ABI 310 Genetic Analyzer for 30 min. each. Data were analyzed with the BioNumerics software package, version 2.5 (Applied Maths, Sint-Martens-Latem, Belgium) using the Pearson correlation as a similarity coefficient in combination with Unweighted Pair Group Method with Arithmatic Mean (UPGMA) cluster analysis.

Table 2

The adapter- and primer-sequences used for AFLP

Adapter	**Sequence**
*Eco*RI	5'-CTCGTAGACTGCGTACC-3' 3'-CATCTGACGCATGGTTAA-5'
*Mse*I	5'-GACGATGAGTCCTGAG-3' 3'-CTACTCAGGACTCAT-5'
Primer	**Sequence**[1]
*Eco*RI	5'-GACTGCGTACCAATTC**AC**-3'
*Mse*I	5'-GATGAGTCCTGAGTAA**C**-3'

[1] **bold:** selective nucleotides (used only in the second PCR reaction)

RESULTS AND DISCUSSION

A dendogram representing all reference strains and clinical isolates is depicted in Figure 1. The AFLP-patterns of the reference strains clearly show that each species forms a distinct cluster, with the following cophenetic values: *C. albicans*: 78; *C. dubliniensis*: 92; *C. glabrata*: 99; *C. krusei*: 84; *C. pseudotropicalis*: 98; *C. tropicalis*: 85; *C. parapsilosis*: 91; *C. lusitaniae*: 98; *C. guilliermondii*: 94. These results were highly reproducible.

The *C. albicans* isolates show two main clusters. One cluster contains clinical isolates from the VUMC and the SENTRY collection as well as reference strains from the CBS. The other cluster only contains isolates from the SENTRY collection. There is no clear relation between these clusters and the geographical origin or source of the isolates. North American *C. albicans* isolates show a three-part division by several typing methods, such as RAPD, multilocus enzyme electrophoresis (MLEE), and Southern blot hybridization with the moderately repetitive *C. albicans* specific Ca3 probe. In South-Africa, an additional cluster is found besides these same three clusters[4,18,28]. It will be interesting to investigate whether the two AFLP clusters of *C. albicans* correspond with the North American or South-African clusters.

The *C. dubliniensis* isolates also show two clusters, with remarkable high similarities (91% and 98%) of the isolates within the clusters. One cluster contains all reference strains used and one SENTRY clinical isolate, the other cluster is composed of SENTRY isolates only. Using the *C. dubliniensis*-specific fingerprinting probe Cd25 on a panel of 98 isolates Gee et al. also recognized two different clusters, one of which contained mainly isolates from HIV-infected individuals while the other cluster contained mainly isolates derived from HIV-negative individuals[9]. Strains CBS 7987 and CBS 7988, both part of the same AFLP cluster, are isolated from an HIV-infected individual. However, data on the HIV-status of the patients of which the other isolates were obtained (CBS 8500, CBS 8501, and SENTRY isolates) is lacking. Further investigations are necessary to examine whether the AFLP clusters correspond with the Cd25 clusters.

Another noteworthy finding is that all AFLP patterns for the *C. glabrata* isolates are very similar (90%), except for the CBS reference strain (58%). This reference strain (CBS 138) was isolated from human faeces in 1936. The fact that all other isolates studied were clinical isolates which were isolated fairly recently may account for this difference.

The AFLP patterns of the 18 isolates from the VUMC all corresponded with the results of the phenotypic identification (Germ-tube test and Vitek). The clinical isolates from the European SENTRY collection were all originally identified on CHROMagar as being *Candida albicans*. However, based on the AFLP patterns shown in Figure 1, some of these strains presumably were misidentified and belong to different species. When the total collection of isolates previously identified as *C. albicans* (n = 213) was screened with AFLP, a misidentification rate of 6% was observed. Six strains are now identified as *C. dubliniensis*, four as *C. parapsilosis*, one as *C. tropicalis*, and one as *C. guilliermondii* (results partly shown in Figure 1).

CHROMagar identification of *Candida* species is based on differences in colony color. It has been shown, that the reliability of this method depends on the incubation time and temperature used[2,24,35]. However, even when optimum conditions are used, the method is

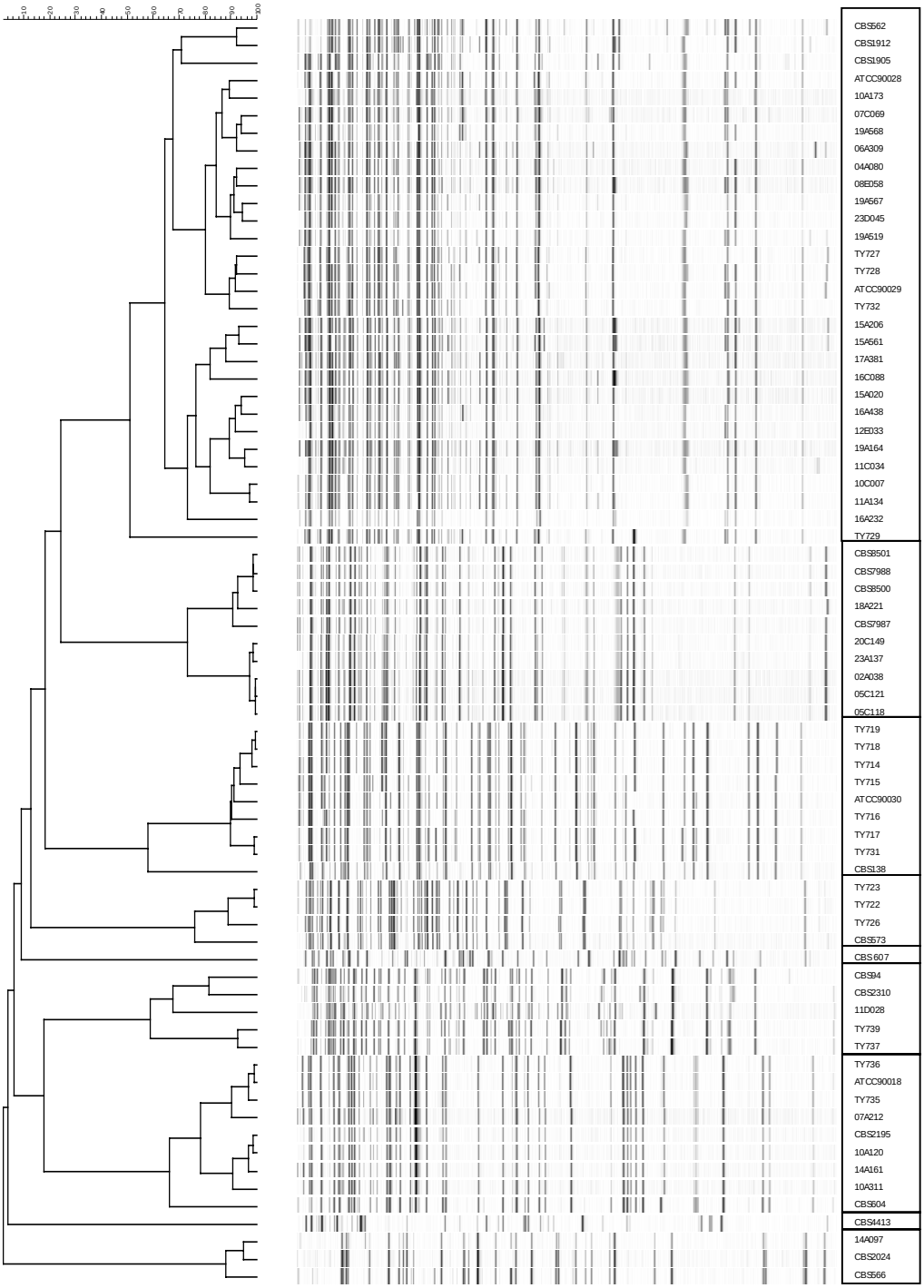


Figure 1

Dendogram representing all reference strains and clinical isolates (see also Table 1)

not ideal, and especially the differentiation between *C. albicans* and *C. dubliniensis* is problematic. Kurzai et al. reported that only 81% of their *C. dubliniensis* isolates showed the dark green color on CHROMagar, which is considered indicative for *C. dubliniensis*[17]. Furthermore, 15.9% of their *C. albicans* isolates also showed a dark green coloration, instead of the usual lighter green. Tintelnot et al. reported an even lower number of 57% of *C. dubliniensis* isolates that showed the dark green coloration on CHROMagar, and only 48% of the isolates of Kirkpatrick et al. showing the dark green coloration turned out to be *C. dubliniensis*[15,31].

Other commercial tests that allow (presumptive) identification of *C. albicans* as well as non-*albicans Candida* species usually show high sensitivities and specificities for *C. albicans*, but are less reliable or need further testing for the identification of other, less common, species[3,5,8,12]. *C. dubliniensis*-specific PCR assays as well as generic PCR assays in combination with species-specific probes have been developed[6,7,16,19,25]. The advantage of AFLP, however, is that this method is based on ligation of known sequences (adapters) to restriction fragments, which function as targets for the PCR primers. Therefore, the technique is universally applicable. In the current assay we made use of two subsequent amplifications, but similar results were obtained when only the second amplification was used (unpublished observations). The use of an internal size standard with every sample for normalization purposes greatly enhances the reproducibility between tests. Storing all patterns, including those of the reference strains, in a general accessible database will provide a screening library for identification of species.

Two other universally applicable methods for identification of *Candida* species have been described: PCR fingerprinting and reference strand-mediated conformational analysis (RSCA)[20,21]. However, whereas PCR fingerprinting uses mini- and microsatellite sequences as targets for the primers and RSCA is based on 18S rRNA sequences, AFLP patterns are a representation of the whole genome. Our results show very clear differences between medically important *Candida* species. Therefore, AFLP might prove to be a reliable method for the identification of medically important *Candida* species, including *Candida dubliniensis*.

ACKNOWLEDGEMENTS

Annemarie Borst is supported by a grant from bioMérieux (formerly Organon Teknika).

REFERENCES

1. **Abi Said, D., E. Anaissie, O. Uzun, I. Raad, H. Pinzcowski, and S. Vartivarian.** 1997. The epidemiology of hematogenous candidiasis caused by different *Candida* species. Infect. Dis. 24: 1122-1128
2. **Baumgartner, C., A.M. Freydiere, and Y. Gille.** 1996. Direct identification and recognition of yeast species from clinical material by using albicans ID and CHROMagar *Candida* plates. J. Clin. Microbiol. 34: 454-456

3. **Bernal, S., M.E. Martin, M. Chavez, J. Coronilla, and A. Valverde.** 1998. Evaluation of the new API *Candida* system for identification of the most clinically important yeast species. Diagn. Microbiol. Infect. Dis. 32: 217-221
4. **Blignaut, E., C. Pujol, S. Lockhart, S. Joly, and D.R. Soll.** 2002. Ca3 Fingerprinting of *Candida albicans* Isolates from Human Immunodeficiency Virus-Positive and Healthy Individuals Reveals a New Clade in South Africa. J. Clin. Microbiol. 40: 826-836
5. **Campbell, C.K., K.G. Davey, A.D. Holmes, A. Szekely, and D.W. Warnock.** 1999. Comparison of the API *Candida* system with the AUXACOLOR system for identification of common yeast pathogens. J. Clin. Microbiol. 37: 821-823
6. **Donnelly, S.M., D.J. Sullivan, D.B. Shanley, and D.C. Coleman.** 1999. Phylogenetic analysis and rapid identification of *Candida dubliniensis* based on analysis of ACT1 intron and exon sequences. Microbiology 145 (Pt 8): 1871-1882
7. **Elie, C.M., T.J. Lott, E. Reiss, and C.J. Morrison.** 1998. Rapid identification of *Candida* species with species-specific DNA probes. J. Clin. Microbiol. 36: 3260-3265
8. **Espinel-Ingroff, A., L. Stockman, G. Roberts, D. Pincus, J. Pollack, and J. Marler.** 1998. Comparison of RapID yeast plus system with API 20C system for identification of common, new, and emerging yeast pathogens. J. Clin. Microbiol. 36: 883-886
9. **Gee, S.F., S. Joly, D.R. Soll, J.F. Meis, P.E. Verweij, I. Polacheck, D.J. Sullivan, and D.C. Coleman.** 2002. Identification of four distinct genotypes of *Candida dubliniensis* and detection of microevolution in vitro and in vivo. J. Clin. Microbiol. 40: 556-574
10. **Giamarellou, H. and A. Antoniadou.** 1996. Epidemiology, diagnosis, and therapy of fungal infections in surgery. Infect. Control Hosp. Epidemiol. 17: 558-564
11. **Gumbo, T., C.M. Isada, G. Hall, M.T. Karafa, and S.M. Gordon.** 1999. *Candida glabrata* Fungemia. Clinical features of 139 patients. Medicine (Baltimore) 78: 220-227
12. **Hoppe, J.E. and P. Frey.** 1999. Evaluation of six commercial tests and the germ-tube test for presumptive identification of *Candida albicans*. Eur. J. Clin. Microbiol. Infect. Dis. 18: 188-191
13. **Jones, C.J., K.J. Edwards, S. Castaglione, M.O. Winfield, F. Sala, C. VandeWiel, G. Bredemeijer, B. Vosman, M. Matthes, A. Daly, R. Brettschneider, P. Bettini, M. Buiatti, E. Maestri, A. Malcevschi, N. Marmiroli, R. Aert, G. Volckaert, J. Rueda, R. Linacero, A. Vazquez, and A. Karp.** 1997. Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories. Molecular Breeding 3: 381-390
14. **Kao, A.S., M.E. Brandt, W.R. Pruitt, L.A. Conn, B.A. Perkins, D.S. Stephens, W.S. Baughman, A.L. Reingold, G.A. Rothrock, M.A. Pfaller, R.W. Pinner, and R.A. Hajjeh.** 1999. The epidemiology of candidemia in two United States cities: results of a population-based active surveillance. Clin. Infect. Dis. 29: 1164-1170
15. **Kirkpatrick, W.R., S.G. Revankar, R.K. Mcatee, J.L. Lopez-Ribot, A.W. Fothergill, D.I. McCarthy, S.E. Sanche, R.A. Cantu, M.G. Rinaldi, and T.F. Patterson.** 1998. Detection of *Candida dubliniensis* in oropharyngeal samples from human immunodeficiency virus-infected patients in North America by primary CHROMagar candida screening and susceptibility testing of isolates. J. Clin. Microbiol. 36: 3007-3012
16. **Kurzai, O., W.J. Heinz, D.J. Sullivan, D.C. Coleman, M. Frosch, and F.A. Muhlschlegel.** 1999. Rapid PCR test for discriminating between *Candida albicans* and *Candida dubliniensis* isolates using primers derived from the pH-regulated PHR1 and PHR2 genes of *C. albicans*. J. Clin. Microbiol. 37: 1587-1590
17. **Kurzai, O., H.C. Korting, D. Harmsen, W. Bautsch, M. Molitor, M. Frosch, and F.A. Muhlschlegel.** 2000. Molecular and phenotypic identification of the yeast pathogen *Candida dubliniensis*. J. Mol. Med. 78:

521-529

18. **Lott, T.J. and M.M. Effat.** 2001. Evidence for a more recently evolved clade within a *Candida albicans* North American population. Microbiology 147: 1687-1692
19. **Martin, C., D. Roberts, M. Van der Weide, R. Rossau, G. Jannes, T. Smith, and M. Maher.** 2000. Development of a PCR-based line probe assay for identification of fungal pathogens. J. Clin. Microbiol. 38: 3735-3742
20. **McIlhatton, B.P., C. Keating, M.D. Curran, M.F. McMullin, J.G. Barr, J.A. Madrigal, and D. Middleton.** 2002. Identification of medically important pathogenic fungi by reference strand-mediated conformational analysis (RSCA). J. Med. Microbiol. 51: 468-478
21. **Meyer, W., K. Maszewska, and T.C. Sorrell.** 2001. PCR fingerprinting: a convenient molecular tool to distinguish between *Candida dubliniensis* and *Candida albicans.* Med. Mycol. 39: 185-193
22. **Moran, G.P., D. Sanglard, S.M. Donnelly, D.B. Shanley, D.J. Sullivan, and D.C. Coleman.** 1998. Identification and expression of multidrug transporters responsible for fluconazole resistance in *Candida dubliniensis*. Antimicrob. Agents Chemother. 42: 1819-1830
23. **Moran, G.P., D.J. Sullivan, M.C. Henman, C.E. McCreary, B.J. Harrington, D.B. Shanley, and D.C. Coleman.** 1997. Antifungal drug susceptibilities of oral *Candida dubliniensis* isolates from human immunodeficiency virus (HIV)-infected and non-HIV-infected subjects and generation of stable fluconazole-resistant derivatives in vitro. Antimicrob. Agents Chemother. 41: 617-623
24. **Odds, F.C. and A. Davidson.** 2000. "Room temperature" use of CHROMagar *Candida*. Diagn. Microbiol. Infect. Dis. 38: 147-150
25. **Park, S., M. Wong, S.A. Marras, E.W. Cross, T.E. Kiehn, V. Chaturvedi, S. Tyagi, and D.S. Perlin.** 2000. Rapid identification of *Candida dubliniensis* using a species-specific molecular beacon. J. Clin. Microbiol. 38: 2829-2836
26. **Pfaller, M.A., R.N. Jones, G.V. Doern, A.C. Fluit, J. Verhoef, H.S. Sader, S.A. Messer, A. Houston, S. Coffman, and R.J. Hollis.** 1999. International surveillance of blood stream infections due to *Candida* species in the European SENTRY Program: species distribution and antifungal susceptibility including the investigational triazole and echinocandin agents. SENTRY Participant Group (Europe). Diagn. Microbiol. Infect. Dis. 35: 19-25
27. **Pittet, D., N. Li, and R.P. Wenzel.** 1993. Association of secondary and polymicrobial nosocomial bloodstream infections with higher mortality. Eur. J. Clin. Microbiol. Infect. Dis. 12: 813-819
28. **Pujol, C., S. Joly, S.R. Lockhart, S. Noel, M. Tibayrenc, and D.R. Soll.** 1997. Parity among the randomly amplified polymorphic DNA method, multilocus enzyme electrophoresis, and Southern blot hybridization with the moderately repetitive DNA probe Ca3 for fingerprinting *Candida albicans*. J. Clin. Microbiol. 35: 2348-2358
29. **Savelkoul, P.H., H.J. Aarts, J. de Haas, L. Dijkshoorn, B. Duim, M. Otsen, J.L. Rademaker, L. Schouls, and J.A. Lenstra.** 1999. Amplified-fragment length polymorphism analysis: the state of an art. J. Clin. Microbiol. 37: 3083-3091
30. **Sullivan, D. and D. Coleman.** 1998. *Candida dubliniensis*: characteristics and identification. J. Clin. Microbiol. 36: 329-334
31. **Tintelnot, K., G. Haase, M. Seibold, F. Bergmann, M. Staemmler, T. Franz, and D. Naumann.** 2000. Evaluation of phenotypic markers for selection and identification of *Candida dubliniensis*. J. Clin. Microbiol. 38: 1599-1608
32. **Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. Van de Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper, and M. Zabeau.** 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res. 23:

4407-4414

33. **Wenzel, R.P.** 1995. Nosocomial candidemia: risk factors and attributable mortality. Clin. Infect. Dis. 20: 1531-1534
34. **Wey, S.B., M. Mori, M.A. Pfaller, R.F. Woolson, and R.P. Wenzel.** 1988. Hospital-acquired candidemia. The attributable mortality and excess length of stay. Arch. Intern. Med. 148: 2642-2645
35. **Willinger, B., C. Hillowoth, B. Selitsch, and M. Manafi.** 2001. Performance of *Candida* ID, a new chromogenic medium for presumptive identification of *Candida* species, in comparison to CHROMagar *Candida*. J. Clin. Microbiol. 39: 3793-3795

VIII: High levels of hydrolytic enzymes secreted by *Candida albicans* isolates involved in pneumonia

A. Borst and A.C. Fluit

Eijkman-Winkler Center, University Medical Center, Utrecht, the Netherlands

Submitted for publication.

ABSTRACT

The differences in production of two putative virulence factors of *Candida albicans*, (phospho)lipase and proteinase, were determined for a large (n = 186) panel of clinical *C. albicans* isolates obtained from the European SENTRY program. Seventy-two percent of the isolates produced detectable amounts of (phospho)lipase, 95% of the isolates produced detectable amounts of proteinase. There was no clear correlation between the results of the (phospho)lipase- and proteinase assays and the geographical distribution of the isolates. However, isolates which originated from pneumonia produced significantly higher amounts of (phospho)lipase than isolates obtained from blood, the urinary tract or wound/skin/soft tissue, and also appeared to produce more proteinase. It is hypothesized that these virulent isolates involved in pneumonia originate from the oral cavity. Whether these results are caused by selection for these high virulent isolates remains to be solved.

INTRODUCTION

The opportunistic pathogen *Candida albicans* is considered to be the most virulent of the *Candida* species. Several putative virulence factors of *C. albicans* have been described, including phenotypic switching, host recognition biomolecules (adhesins), morphogenesis (the reversible transition between unicellular yeast cells and filamentous, growth forms), and secreted hydrolytic enzymes[1]. Two types of secreted enzymes have been described extensively: phospholipases and secreted aspartyl proteinases.

C. albicans strains show phospholipase B as well as lysophospholipase-transacylase activities. Both activities are performed by a single enzyme, *C. albicans* phospholipase B (caPLB). Besides this secreted phospholipase, *C. albicans* also shows a phospholipase D activity which appears to be membrane-associated[12]. The data on phospholipases in pathogenic fungi has been reviewed by Ghannoum[6].

Several researchers have found indications that phospholipases are virulence factors of *C. albicans*. Ibrahim et al. compared the ability to produce phospholipases of clinical blood isolates with oral strains from healthy volunteers. Significantly more phospholipase activity was detected in the clinical isolates. Furthermore, in a mouse-model of haematogenously disseminated candidiasis, a *C. albicans* strain which produced high amounts of phospholipases was invasive whereas a low-producing strain was not, and phospholipase-activity was the only putative virulence factor tested that predicted mortality[9]. In another animal pathogenicity study, a significant correlation was found between phospholipase activity and the severity of kidney-infections[10]. The ultimate proof was delivered by Leidich et al., who cloned and disrupted a gene encoding for PLB and showed that the null mutant significantly attenuated virulence in mice and dramatically reduced the ability of the yeast to penetrate host cells[11]. Disruption of the gene did not affect adherence of the yeast cells to human endothelial or epithelial cells. Thus, phospholipases most likely contribute to the pathogenicity of *C. albicans* by damaging host cell membranes, aiding the fungus by invasion of host tissues. This role in invasion is also implied by the finding that phospholipases are mainly concentrated at the tips of the fungal hyphae[15]. Besides phospholipases, *C. albicans* strains also secrete lipases. It is

likely that these enzymes, like phospholipases, are involved in virulence of *C. albicans*[5,8].

The role of *C. albicans* secreted aspartyl proteinases in pathogenicity has recently been reviewed by De Bernardis et al.[4]. These enzymes are encoded by a family of at least nine genes, and are capable of degrading epithelial and mucosal barrier proteins like collagen, keratin and mucin, as well as antibodies, complement and cytokines. Gene disruption experiments showed altered adherence of yeast cells and attenuation of virulence in different animal models[2,7,17,18].

The expression of virulence factors may be associated with specific characteristics of *Candida* isolates such as geographic origin or the type of infection. Knowledge of such correlations may help to understand the epidemiology of these infections, which may result in improved therapeutic regimens. Price et al. developed a simple egg yolk agar plate assay for the detection of (phospho)lipase activity. Hydrolysis of lipid substrates present in the egg yolk results in the formation of a calcium complex with the fatty acids released by the action of the secreted enzymes. The diameter of this zone of precipitation around the colonies is very constant for any given isolate, and correlates well with a biochemical assay for hydrolysis of phosphatidylcholine[14]. Although this method does not detect (phospho)lipase activity in fungal isolates that produce very low levels of phospholipase[6], it is an excellent screening method for large numbers of isolates. Therefore, we used this method to investigate the differences in (phospho)lipase activity of a large collection of clinical *C. albicans* isolates obtained from 12 European countries, and the results were linked to data on the geographic origin of the isolates and the site of infection. For the detection of proteinase activity we incorporated bovine serum albumin (BSA) into YCB-agar plates and measured the clearing zone after staining with Coomassie blue.

MATERIALS AND METHODS

Yeast strains. *Candida albicans* isolates were obtained from the European SENTRY program. Only one isolate per patient was included. A total of 186 isolates derived from 19 medical centers in 12 European countries were studied (Table 1). One-hundred-and-thirty-one isolates (70%) originated from infections from blood, 7 (4%) from wounds/skin/soft tissue, 25 (13%) from the urinary tract, and 23 (12%) from pneumonia. Most isolates were derived from the intensive care (36%), internal medicine (15%), surgery (14%), pediatrics (12%) or oncology ward (6%). The number of isolates derived from the most relevant hospital wards in relation to the site of infection is depicted in Table 2. Identification of the isolates was performed using CHROMagar plates (CHROMagar, Paris, France). The isolates were cultured on Blood Agar and subcultured on Sabouraud Dextrose Agar (SDA) at 37°C.

(Phospho)lipase assay. SDA plates supplemented with 1 M NaCl, 5 mM $CaCl_2$ and 8% sterile egg yolk (Oxoid, Basingstoke, England) were inoculated with 1 µl sterile saline containing approximately 10^5 cfu *C. albicans*, and incubated at 37°C for three days. Each isolate was tested in duplicate. The diameter of the colonies and the total diameter of the colonies plus precipitation zones were measured. (Phospho)lipase activity was determined by the ratio of the diameter of the colony plus precipitation zone to the diameter of the colony alone, and scored as follows: - = no precipitation zone; +/- = ratio between 1.01 and 1.25; + =

ratio between 1.26 and 1.50; ++ = ratio between 1.51 and 1.75; +++ = ratio between 1.76 and 2.00; ++++ = ratio between 2.01 and 2.25.

Proteinase assay. YCB-BSA plates (1.5% agar; 1.17% Yeast Carbon Base powder (Becton Dickinson, Le Pont de Claix, France); 0.2% Bovine Serum Albumin (Instruchemie, Hilversum, the Netherlands); 0.2% glucose; 100 µl/l Vitox solution (Oxoid)) were inoculated with 1 µl sterile saline containing approximately 10^5 cfu *C. albicans*, and incubated at 25°C for three weeks. Several isolates were tested twice or more. The plates were stained with Coomassie brilliant blue (0.5% Coomassie brilliant blue R250 (Pierce, Rockford, USA); 10% v/v acetic acid; 45% v/v ethanol) for 20 min. at room temperature, and destained three times with destaining solution (10% v/v acetic acid; 45% v/v ethanol) for 20 min. at 37°C and one time with water for 20 min. at 37°C. The diameter of the colonies was measured before Coomassie staining, the diameter of the clear zones was measured after staining. Proteinase activity was determined by the ratio of the diameter of the clear zone to the diameter of the colony, and scored as follows: - = no clear zone; +/- = ratio < 0.9 (clear zone smaller than colony, limited proteinase activity); + = ratio between 0.9 and 1.1 (clear zone and colony of similar size); ++ = ratio > 1.1 (clear zone clearly larger than colony).

Table 1

The geographic origin of the isolates used in this study

Country	Center	No. of isolates (%)
Austria	Linz	3 (2)
France	Paris	6 (3)
	Lille	7 (4)
Germany	Freiburg	9 (5)
	Dusseldorf	7 (4)
Greece	Athens	3 (2)
Italy	Genoa	24 (13)
	Roma	17 (9)
the Netherlands	Utrecht	6 (3)
Poland	Warsaw	1 (1)
	Cracow	2 (1)
Portugal	Coimbra	23 (12)
Spain	Sevilla	21 (11)
	Madrid	4 (2)
	Barcelona	1 (1)
Switzerland	Lausanne	14 (8)
Turkey	Ankara	20 (11)
	Istanbul	6 (3)
United Kingdom	London	12 (6)
	Total:	186 (100)

Table 2

The number of isolates derived from the different hospital wards in relation to the site of infection

	No. of isolates (%)						
Source	**IC**	**Int. med.**	**Surgery**	**Pediatrics**	**Oncology**	**Other**	**Total**
blood	39 (30)	22 (17)	22 (17)	18 (14)	11 (8)	19 (15)	131 (100)
pneumonia	18 (78)	0 (0)	0 (0)	0 (0)	(0)	5 (22)	23 (100)
urinary tract	8 (32)	4 (16)	3 (12)	4 (16)	0 (0)	6 (24)	25 (100)
wound/s/st	2 (29)	1 (14)	2 (29)	0 (0)	0 (0)	2 (29)	7 (100)

IC: intensive care
Int. med.: internal medicine
wound/s/st: isolates originating from wounds, skin or soft tissue

RESULTS

(Phospho)lipase assay. One-hundred-and-eighty-six isolates were tested in the (phospho)lipase assay. The number of isolates and the different scores are depicted in Table 3. No (phospho)lipase activity was detected in 28% of the isolates. Duplicate testing of the isolates only showed minor differences (average difference between duplicate tests: 0.08). There was no clear correlation between the results of the (phospho)lipase assay and the geographical distribution of the isolates.

The results of the (phospho)lipase assay in relation with the site of infection are shown in Table 4. Of all strains obtained from blood (n = 133), the urinary tract (n = 25), or wounds/skin/soft tissue (n = 7) that were tested in the (phospho)lipase assay, most isolates were either negative or produced only low amounts of (phospho)lipase (-, +/-, or +)(blood: 64%, urinary tract: 72%, wound/sst: 85%). However, 61% of the isolates obtained from pneumonia (n = 23) produced high amounts of lipase (++, +++, or ++++). This difference was statistically significant (p = 0.042; Pearson chi-square test (exact)).

Table 3

Results of the (phospho)lipase assay

Score	**-**	**+/-**	**+**	**++**	**+++**	**++++**
No. isolates (%)	53 (28)	13 (7)	51 (27)	33 (18)	28 (15)	8 (4)

Table 4

Results of the (phospho)lipase assay in relation with the site of infection

	No. of isolates (%)						
Source	**-**	**+/-**	**+**	**++**	**+++**	**++++**	**Total**
blood	38 (29)	10 (8)	36 (27)	22 (17)	19 (15)	6 (5)	131 (100)
pneumonia	3 (13)	1 (4)	5 (22)	8 (35)	5 (22)	1 (4)	23 (100)
urinary tract	7 (28)	2 (8)	9 (36)	3 (12)	3 (12)	1 (4)	25 (100)
wound/s/st	5 (71)	0 (0)	1 (14)	0 (0)	1 (14)	0 (0)	7 (100)

wound/s/st: isolates originating from wounds, skin or soft tissue

Proteinase assay. One-hundred-and-eighty-five isolates were tested in de proteinase assay. The number of isolates and the different scores are depicted in Table 5. In all but nine isolates proteinase activity was detected (95%). Several isolates were tested in duplicate or triplicate, and showed only minor differences (average difference between duplicate tests: 0.03). There was no clear correlation between the results of the proteinase assay and the geographical distribution of the isolates.

Of all isolates obtained from pneumonia that were tested in the proteinase assay (n = 23), 96% produced considerable amounts of proteinase (+ or ++), whereas for isolates obtained from the other sources (blood (n = 131), the urinary tract (n = 24), or wound/sst (n = 7)) this percentage was 80, 79, and 73% respectively (Table 6). However, this difference was not statistically significant.

Table 5

Results of the proteinase assay

Score	**-**	**+/-**	**+**	**++**
No. isolates (%)	9 (5)	25 (14)	118 (64)	33 (18)

Table 6

Results of the proteinase assay in relation with the site of infection

	No. of isolates (%)				
Source	**-**	**+/-**	**+**	**++**	**Total**
blood	5 (4)	21 (16)	83 (63)	22 (17)	131 (100)
pneumonia	0 (0)	1 (4)	14 (61)	8 (35)	23 (100)
urinary tract	2 (8)	3 (13)	17 (71)	2 (8)	24 (100)
wound/s/st	2 (29)	0 (0)	4 (57)	1 (14)	7 (100)

wound/s/st: isolates originating from wounds, skin or soft tissue

DISCUSSION

Previous studies on the (phospho)lipase activity of *C. albicans* isolates reported a large variation in activity among different isolates, but a remarkably constant degree of activity of individual isolates which was fairly independent of inoculum size[10,14,16]. This is in agreement with our results: the ratio of the diameter of the colony plus precipitation zone and the colony alone ranged from 1.05 to 2.36 in the positive isolates, with an average difference between duplicate tests of 0.08.

When looking at (phospho)lipase activity in relation to the site of infection, Price et al. found that 55% of the blood isolates studied were positive in the assay[14]. These isolates were also among the highest producers. Furthermore, 50% of the isolates cultured from wounds, and 30% of the isolates from the urinary tract were also positive. Our results show different percentages: 71% of the blood isolates, 72% of the isolates from the urinary tract, and 29% of the isolates from wound/skin/soft tissue were positive in the assay. However, whereas Price et

al. examined substantially more wound isolates than we did (n = 28 versus n = 7), we tested larger numbers of isolates from blood and the urinary tract (blood: n = 131 versus n = 11; urinary tract: n = 25 versus n = 13). These differences may account for the different results.

In addition to the sources described above, we also examined strains which originated from pneumonia. It appeared that this group showed the highest number of positive isolates in the (phospho)lipase assay (87%, n = 23). Furthermore, 61% of these isolates were among the higher producers (in comparison with the other sources: blood 37%; urinary tract 28%; wound/skin/soft tissue 14%)(Table 4). Although not statistically significant, a similar trend was observed for the proteinase assay: all isolates obtained from pneumonia were positive in the proteinase assay, and 96% of these isolates were high producers (other sources: blood 80%; urinary tract 79%; wound/skin/soft tissue 73%)(Table 6). According to fingerprinting data obtained with amplified fragment length polymorphism analysis (AFLP) only two isolates originating from pneumonia (from Genoa, Italy) were identical. The patterns of all other pneumonia isolates showed clear differences (results not shown). Therefore, there is no bias due to hospital outbreaks.

It is possible that our findings are related to earlier reports by Samaranayake et al. and Kothavade and Panthaki which mention relatively high numbers of (phospho)lipase producers among clinical oral *C. albicans* isolates (79% and 89% respectively)[10,16]. An exceptionally high number of 78% of our pneumonia isolates were derived from patients in the intensive care. For the three other sources this percentage was approximately 30% (Table 2). Albeit this data is lacking, it seems legitimate to assume that many of these patients were mechanically ventilated. In that case the *C. albicans* isolates causing the pneumonia may very well originate from the patient's own oral cavity. It is interesting to note that whereas oral *C. albicans* isolates from healthy volunteers show a relatively low phospholipase activity, clinical isolates from the oral cavities of patients suffering from oral candidosis produce relatively high amounts of this enzyme[9,10,16]. Furthermore, oral *C. albicans* isolates of HIV-positive individuals are known to cause unusual severe infections. These isolates also produce extremely high amounts of proteinases[3,13]. It is hypothesized that these infections are attributable to selection of commensal *C. albicans* isolates which are characterized by a higher virulence. It is a tempting idea that these more virulent isolates also have an increased potential of causing pneumonia in intensive care patients. The underlying mechanisms behind the selection of these high virulent strains are undetermined.

SAP production by *C. albicans* not only depends on strain type or type of infection, but also on phenotypic switch type, environmental conditions, and even the stage of infection[4]. Therefore, caution must be employed in the interpretation of proteinase assays. However, although especially the proteinase assay is a crude screening method, it is noteworthy that the results of both assays indicate a possible higher virulence for isolates involved in pneumonia. Whether this is caused by selection of more virulent isolates which are part of the commensal flora of the patients remains to be solved.

ACKNOWLEDGEMENTS

We would like to thank Christine Morrison for her advice on the proteinase assay. Annemarie Borst is supported by a grant from bioMérieux (formerly Organon Teknika).

REFERENCES

1. **Calderone, R.A. and W.A.** Fonzi. 2001. Virulence factors of *Candida albicans*. Trends Microbiol. 9: 327-335
2. **De Bernardis, F., S. Arancia, L. Morelli, B. Hube, D. Sanglard, W. Schafer, and A. Cassone.** 1999. Evidence that members of the secretory aspartyl proteinase gene family, in particular SAP2, are virulence factors for *Candida* vaginitis. J. Infect. Dis. 179: 201-208
3. **De Bernardis, F., P. Chiani, M. Ciccozzi, G. Pellegrini, T. Ceddia, G. D'Offizzi, I. Quinti, P.A. Sullivan, and A. Cassone.** 1996. Elevated aspartic proteinase secretion and experimental pathogenicity of *Candida albicans* isolates from oral cavities of subjects infected with human immunodeficiency virus. Infect. Immun. 64: 466-471
4. **De Bernardis, F., P.A. Sullivan, and A. Cassone.** 2001. Aspartyl proteinases of *Candida albicans* and their role in pathogenicity. Med. Mycol. 39: 303-313
5. **Fu, Y., A.S. Ibrahim, W. Fonzi, X. Zhou, C.F. Ramos, and M.A. Ghannoum.** 1997. Cloning and characterization of a gene (LIP1) which encodes a lipase from the pathogenic yeast *Candida albicans*. Microbiology 143 (Pt 2): 331-340
6. **Ghannoum, M.A.** 2000. Potential role of phospholipases in virulence and fungal pathogenesis. Clin. Microbiol. Rev. 13: 122-43
7. **Hube, B., D. Sanglard, F.C. Odds, D. Hess, M. Monod, W. Schafer, A.J. Brown, and N.A. Gow.** 1997. Disruption of each of the secreted aspartyl proteinase genes SAP1, SAP2, and SAP3 of *Candida albicans* attenuates virulence. Infect. Immun. 65: 3529-3538
8. **Hube, B., F. Stehr, M. Bossenz, A. Mazur, M. Kretschmar, and W. Schafer.** 2000. Secreted lipases of *Candida albicans*: cloning, characterisation and expression analysis of a new gene family with at least ten members. Arch. Microbiol. 174: 362-374
9. **Ibrahim, A.S., F. Mirbod, S.G. Filler, Y. Banno, G.T. Cole, Y. Kitajima, J.E. Edwards, Jr., Y. Nozawa, and M.A. Ghannoum.** 1995. Evidence implicating phospholipase as a virulence factor of *Candida albicans*. Infect. Immun. 63: 1993-1998
10. **Kothavade, R.J. and M.H. Panthaki.** 1998. Evaluation of phospholipase activity of *Candida albicans* and its correlation with pathogenicity in mice. J. Med. Microbiol. 47: 99-102
11. **Leidich, S.D., A.S. Ibrahim, Y. Fu, A. Koul, C. Jessup, J. Vitullo, W. Fonzi, F. Mirbod, S. Nakashima, Y. Nozawa, and M.A. Ghannoum.** 1998. Cloning and disruption of caPLB1, a phospholipase B gene involved in the pathogenicity of *Candida albicans*. J. Biol. Chem. 273: 26078-26086
12. **McLain, N. and J.W. Dolan.** 1997. Phospholipase D activity is required for dimorphic transition in *Candida albicans*. Microbiology 143 (Pt 11): 3521-3526
13. **Ollert, M.W., C. Wende, M. Gorlich, C.G. McMullan-Vogel, M. Borg-von Zepelin, C.W. Vogel, and H.C. Korting.** 1995. Increased expression of *Candida albicans* secretory proteinase, a putative virulence

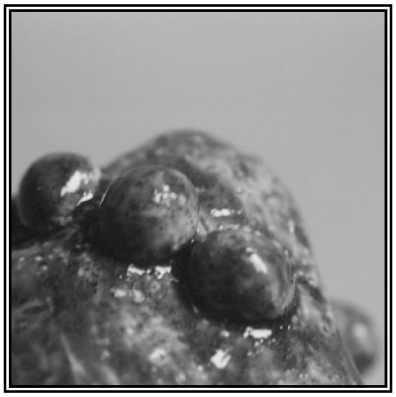
factor, in isolates from human immunodeficiency virus-positive patients. J. Clin. Microbiol. 33: 2543-2549

14. **Price, M.F., I.D. Wilkinson, and L.O. Gentry.** 1982. Plate method for detection of phospholipase activity in *Candida albicans*. Sabouraudia. 20: 7-14
15. **Pugh, D. and R.A. Cawson.** 1975. The cytochemical localization of phospholipase a and lysophospholipase in *Candida albicans*. Sabouraudia. 13 Pt 1: 110-115
16. **Samaranayake, L.P., J.M. Raeside, and T.W. MacFarlane.** 1984. Factors affecting the phospholipase activity of *Candida* species in vitro. Sabouraudia. 22: 201-207
17. **Sanglard, D., B. Hube, M. Monod, F.C. Odds, and N.A. Gow.** 1997. A triple deletion of the secreted aspartyl proteinase genes SAP4, SAP5, and SAP6 of *Candida albicans* causes attenuated virulence. Infect. Immun. 65: 3539-3546
18. **Watts, H.J., F.S. Cheah, B. Hube, D. Sanglard, and N.A. Gow.** 1998. Altered adherence in strains of *Candida albicans* harbouring null mutations in secreted aspartic proteinase genes. FEMS Microbiol. Lett. 159: 129-135

IX: AFLP typing of European *Candida albicans* isolates shows geographical specificities

A. Borst[1], B. Theelen[2], T. Boekhout[2], A.C. Fluit[1]

[1] Eijkman-Winkler Center, University Medical Center, Utrecht, the Netherlands
[2] Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands



Submitted for publication.

ABSTRACT

Amplified Fragment Length Polymorphism (AFLP™) analysis was used to fingerprint a large panel of European clinical *C. albicans* isolates obtained from the SENTRY antimicrobial surveillance program, and the correlation between AFLP type and geographical origin of the isolates, the site of infection, and the production of (phospho)lipases and proteinases was studied. The isolates show a subdivision into two main clusters, cluster 1 and cluster 2. Isolates from most countries can be found in both clusters. However, isolates from Spain and Portugal (n = 49) are restricted to cluster 1, whereas isolates from the United Kingdom and Germany (n = 27) seem restricted to cluster 2. The differences between the two clusters in relation to the site of infection and the production of hydrolytic enzymes are probably due to sampling bias. In conclusion: our results suggest the presence of a clone specific for the Iberian peninsula, and possibly also a specific Northern European clone.

INTRODUCTION

Candida albicans is a commensal yeast which can cause severe infections in immunocompromised individuals. Relatively little is known about the epidemiology of *Candida* infections. Several typing methods have been used for *Candida* species, but none of them is considered the golden standard[3,8,13-15,18]. Amplified Fragment Length Polymorphism (AFLP™) analysis, first described by Vos et al. in 1995, has several advantages over other fingerprinting methods[16,19]. The technique is based on the restriction of chromosomal DNA and ligation of known sequences (adapters) to the restriction fragments, which serve as targets for PCR amplification. Therefore, AFLP is universally applicable without the need to design specific primers and probes. Depending on the organism under study, the primers are extended with one or more selective nucleotides to increase the specificity. Since the primer binding sites are known, stringent annealing temperatures can be used. This makes this technique more reproducible compared to Randomly Amplified Polymorphic DNA (RAPD) analysis[10]. Furthermore, only a limited amount of DNA is needed. This greatly reduces the risk of partial digestion, a major source of irreproducibility with Restriction Fragment Length Polymorphism (RFLP) analysis. Another difference with many other typing methods is that AFLP patterns are a representation of the whole genome. The patterns can easily be stored in general accessible databases, which may greatly facilitate the exchange of results between laboratories.

We have previously demonstrated the value of AFLP for the identification of different *Candida* species[5]. In the present study, AFLP was used to fingerprint a large panel of European clinical *C. albicans* isolates obtained from the SENTRY antimicrobial surveillance program. Furthermore, we studied whether specific AFLP types of *C. albicans* correlated with the geographical origin of the isolates, with the type of infection, and with the production of two putative virulence factors, (phospho)lipases and proteinases[7,9]. Knowledge of such correlations may help to understand the epidemiology of *C. albicans* infections, which may result in improved therapeutic regimens.

MATERIALS AND METHODS

Yeast strains. *Candida albicans* isolates were obtained from the European SENTRY Program. Only one isolate per patient was included. A total number of 189 isolates derived from 19 medical centers in 12 European countries were studied. One-hundred-and-thirty-two isolates (70%) originated from infections from blood, 6 (3%) from wounds/skin/soft tissue, 26 (14%) from the urinary tract, and 25 (13%) from pneumonia. Most isolates were derived from the intensive care (37%), internal medicine (15%), surgery (13%), pediatrics (11%) or oncology (6%). Identification of the isolates was performed using CHROMagar plates (CHROMagar, Paris, France). The isolates were cultured on Blood Agar and subcultured on Sabouraud Dextrose Agar (SDA) at 37°C.

Extraction of DNA. DNA was extracted from approximately 10^7 cfu *C. albicans* using the DNeasy Tissue kit (Qiagen, West Sussex, England) according to the manufacturer (protocol for isolation of genomic DNA from yeasts). DNA was eluted in 100 µl elution buffer (buffer AE of the kit) and stored at -20°C.

AFLP. The sequences of the adapters and primers used for AFLP are depicted in Table 1. DNA was extracted from approximately 10^7 cfu *C. albicans* as described above. Five µl of the DNA samples were added to 5 µl restriction-ligation reaction mixture (1x T_4 DNA ligase buffer; 0.05 M NaCl; 0.5 µg BSA; 2 pmol *Eco*RI-adapter; 20 pmol *Mse*I-adapter; 80 U T_4 DNA ligase; 1 U *Eco*RI; 1 U *Mse*I, and incubated over night at 37°C. All enzymes were obtained from New England BioLabs (Beverly, USA). The mixture was diluted 1:5 with 0.1x TE (5 mM Tris-HCl (pH 7.5); 1 mM EDTA). Pre-selective PCR was performed using the core sequences, i.e. primers without extensions. The AFLP primers, core mix, and internal size standard were supplied by Applied Biosystems (Nieuwerkerk a/d IJssel, the Netherlands). Four µl of diluted restriction-ligation product was added to 15 µl of AFLP amplification core mix, 0.5 µl *Eco*RI core sequence and 0.5 µl *Mse*I core sequence. The mixture was amplified in a GeneAmp® PCR System 9700 machine under the following conditions: 2 min. at 72°C, followed by 20 cycles of 20 sec. at 94°C, 30 sec. at 56°C and 2 min. at 72°C each. The PCR product was diluted by adding 25 µl sterile double distilled water. In a second PCR reaction more selective primers were used: *Eco*RI-AC (FAM-labeled) and *Mse*I-C. The conditions were: 2 min. at 94°C, followed by 10 cycles consisting of 20 sec. at 94°C, 30 sec. at 66°C decreasing 1°C every step of the cycle, and 2 min. at 72°C, followed by 25 cycles consisting of 20 sec. at 94°C, 30 sec. at 56°C and 2 min. at 72°C. After a final incubation of 30 min. at 60°C the samples were prepared for capillary electrophoresis by adding 2 µl of the selective PCR product to 24 µl of deionized formamide and 1 µl of GeneScan-500 (ROX-labeled) as an internal size standard. They were run on the ABI 310 Genetic Analyzer for 30 min. each. Data were analyzed with the BioNumerics software package, version 2.5 (Applied Maths, Sint-Martens-Latem, Belgium) using the Pearson correlation as a similarity coefficient in combination with Unweighted Pair Group Method with Arithmatic Mean (UPGMA) cluster analysis.

(Phospho)lipase assay. (Phospho)lipase activity of the isolates was determined by using the egg yolk agar plate assay developed by Price et al. as described before[4,12]. The activity was determined by the ratio of the diameter of the colony plus precipitation zone to the diameter of the colony alone. The isolates were classified as 'low producers' (ratio < 1.50) or 'high

producers' (ratio ≥ 1.50).

Proteinase assay. Proteinase activity of the isolates was determined by using agar plates supplemented with bovine serum albumin (BSA) as described before[4]. The activity was determined by the ratio of the diameter of the clear zone to the diameter of the colony. The isolates were classified as 'low producers' (ratio < 0.9) or 'high producers' (ratio ≥ 0.9).

Table 1

The adapter- and primer-sequences used for AFLP

Adapter	**Sequence**
*Eco*RI	5'-CTCGTAGACTGCGTACC-3' 3'-CATCTGACGCATGGTTAA-5'
*Mse*I	5'-GACGATGAGTCCTGAG-3' 3'-CTACTCAGGACTCAT-5'
Primer	**Sequence**[1]
*Eco*RI	5'-GACTGCGTACCAATTC**AC**-3'
*Mse*I	5'-GATGAGTCCTGAGTAA**C**-3'

[1] **bold:** selective nucleotides (used only in the second PCR reaction)

RESULTS

A dendogram representing 50% of the results of the AFLP analysis, the (phospho)lipase assay, and the proteinase assay is shown in Figure 1. The complete dendogram can be obtained from the corresponding author. Identical isolates (similarity > 90%, based on duplicate testing) were found within one hospital as well as in different countries. The isolates show two main clusters, 1 and 2, and a few miscellaneous types (cophenetic values: cluster 1: 76; cluster 2: 72). Cluster 1 contains isolates from France (n = 5), Germany (n = 1), Italy (n = 16), the Netherlands (n = 6), Poland (n = 1), Portugal (n = 23), Spain (n = 26), Switzerland (n = 7), and Turkey (n = 4). Cluster 2 contains isolates from Austria (n = 3), France (n = 10), Germany (n = 15), Greece (n = 3), Italy (n = 25), Switzerland (n = 6), the United Kingdom (n = 11), and Turkey (n = 20). There is no correlation between AFLP type and hospital ward (results not shown). The relation between the clusters and the site of infection is depicted in Table 2. Cluster 1 contains significantly more isolates from infections of the urinary tract compared to cluster 2, whereas cluster 2 contains significantly more isolates from pneumonia compared to cluster 1 (p = 0.001, Pearson chi-square test (exact)).

Table 2

The relation between the AFLP clusters and the site of infection

	No. of isolates (%)			
cluster	**blood**	**pneumonia**	**urinary tract**	**wound/skin/soft tissue**
1	64 (74)	4 (5)	17 (20)	1 (1)
2	65 (69)	18 (19)	7 (7)	4 (4)

Isolate[1]:	P[2]:	PL[2]:	
16A107	n.d.	n.d.	
16A232	L	L	
15A020	L	L	
15A237	H	L	
10A343	H	L	
11A384	H	L	
16ED14	H	L	
16A551	L	L	
16ED26	n.d.	L	
17A381	H	L	
12ED70	H	L	
12ED36	L	H	
10A139	H	L	
12A103	H	L	
11A182	H	L	
18ED14	n.d.	n.d.	
23D046	L	L	
16ED33	H	L	
18A220	L	L	
16CD67	H	H	
15A257	H	H	Cluster 1
13A145	H	H	
15A093	L	H	
10A138	n.d.	n.d.	
11CD34	H	H	
06A267	L	L	
19A298	H	L	
16ED50	H	L	
15A375	H	H	
15E106	H	L	
16A308	H	L	
06A063	H	L	
15A629	n.d.	n.d.	
15A647	L	L	
19A355	L	L	
15A206	H	L	
15A561	H	L	
17A184	H	L	
11A301	L	L	
16CD88	n.d.	n.d.	
19A260	L	H	
20CL08	H	H	
20CL47	H	L	
01A120	H	L	
20CL10	H	H	
07CD73	H	H	
08A240	H	H	
07CD60	H	L	
07A524	H	L	
10CD21	H	H	
20A156	H	H	
19A568	H	H	
10A506	H	H	
10A570	H	H	
10A535	H	H	
09A263	L	L	
23ED55	H	H	
23A080	H	H	
19A566	H	H	
04A080	H	L	
07CD45	H	L	
07A301	H	H	
23ED03	L	H	Cluster 2
07CD66	H	L	
23A005	n.d.	n.d.	
23A078	H	L	
07A621	L	L	
06A125	H	H	
06A309	H	L	
04A360	H	H	
06A154	H	H	
24A028	H	L	
04A122	H	L	
08ED58	H	L	
08A573	L	L	
09A437	H	L	
23ED95	L	L	
08A424	H	H	
10CD07	H	H	
11A134	H	H	
10CD26	H	H	
10A144	L	H	
10A555	H	L	
10A600	H	H	
20CD72	H	H	
11A219	H	H	
06A038	H	L	
15D013	H	L	
10A614	H	L	
16CD81	H	L	
16A380	H	H	
15A160	H	H	
17A462	H	L	
24ED07	H	L	

Figure 1 (previous page)

Dendogram representing 50% of all *C. albicans* isolates studied

[1] Isolate codes starting with '01' are derived from Linz (Austria); 04: Paris (France); 06: Lille (France); 07: Freiburg (Germany); 08: Dusseldorf (Germany); 09: Athens (Greece); 10: Genoa (Italy); 11: Roma (Italy); 12: Utrecht (the Netherlands); 13: Warsaw (Poland); 15: Coimbra (Portugal); 16: Sevilla (Spain); 17: Madrid (Spain); 18: Barcelona (Spain); 19: Lausanne (Switzerland); 20: London (United Kingdom); 23: Ankara (Turkey); 24: Istanbul (Turkey).
Isolates with an 'A' in the code are derived from blood; C: pneumonia; D: wound/skin/soft tissue; E: urinary tract

[2] P: proteinase; PL: (phospho)lipase; H: high producer; L: low producer; n.d.: no data

The relation between the clusters and the production of hydrolytic enzymes is depicted in Table 3. Significantly more isolates in cluster 2 were among the high producers of (phospho)lipases and proteinases compared to the isolates in cluster 1 ($p = 0.001$ and $p = 0.025$ respectively, Pearson chi-square test (exact)).

Table 3

The relation between the AFLP clusters and production of hydrolytic enzymes

	(Phospho)lipase		**Proteinase**	
cluster	**low**	**high**	**low**	**high**
1	59 (76)	19 (24)	20 (26)	57 (74)
2	44 (49)	45 (51)	10 (11)	78 (89)

No. of isolates (%)

DISCUSSION

Strain typing is important in various situations, e.g. to find the source of a hospital outbreak or to elucidate the relationship between commensal and infecting strains. Furthermore, strain typing is essential to study the dynamics of a microorganism in a human population. Linking genotypic fingerprinting data to data on the geographic origin of certain isolates, the site of infection, or the expression of virulence factors will improve our understanding of the epidemiology of *Candida* infections. Furthermore, because *Candida albicans* reproduction is predominantly clonal, it may be the first step in identifying genetic markers for specific traits, a process which ultimately may lead to improved therapeutic regimens.

When we only take into account the countries of which ten or more isolates were studied, there seems to be a division between countries represented exclusively in cluster 1 (Portugal, Spain), countries represented exclusively (United Kingdom) or almost exclusively (Germany) in cluster 2, and countries of which the isolates are represented more or less equally in both clusters (France, Italy, Switzerland, Turkey). The possibility that our results demonstrate the presence of separate 'Iberian' and 'Northern-European' *C. albicans* clones which coexist in Central-European countries is appealing. Clemons et al. showed that 86 clinical isolates from three medical centers in the United Kingdom and six medical centers in the United States form two clusters, IA and IB, when typed by restriction endonuclease analysis using *Eco*RI[6]. We

examined 11 isolated from one medical center in the United Kingdom. Our results implying a clone specific for Northern Europe may be biased by the limited number of isolates and medical centers studied. Clusters IA and IB may correspond with the AFLP clusters 1 and 2. However, it is also possible that a third cluster exists, which is as yet undiscovered by AFLP. Forty-nine isolates from four medical centers throughout Spain and Portugal were included in our experiments, and none of them was found in cluster 2. Therefore, the existence of an Iberian clone is more convincing.

The finding that cluster 1 contains significantly more isolates from infections of the urinary tract compared to cluster 2 (Table 2) can at least in part be explained by the relatively large number of urinary tract-derived isolates obtained from Portugal and Spain. The same holds true for the abundance of pneumonia-derived isolates in cluster 2: the isolates obtained from the Germany and the United Kingdom show relatively high numbers of isolates from this source. Whether there is an additional explanation for this relation between AFLP cluster and site of infection remains to be solved.

Cluster 2 contains significantly more isolates which produce high amounts of (phospho)lipases and/or proteinases, both putative virulence factors of *C. albicans* (Table 3). We have shown previously that pneumonia-derived isolates show a relatively high production of hydrolytic enzymes[4]. Compared to cluster 1, cluster 2 contains a relatively high number of isolates which originate from pneumonia (Table 2). This may account for the difference in production of these hydrolytic enzymes between isolates in the two groups.

The results of several typing methods for *C. albicans*, such as RAPD, multilocus enzyme electrophoresis (MLEE), and Southern blot hybridization with the moderately repetitive *C. albicans* specific Ca3 probe, show a good agreement[13]. To validate the results of AFLP typing, the technique should be compared with at least one of these other typing methods, using the same collection of isolates. However, AFLP typing has been used successfully for many organisms including *Saccharomyces cerevisiae*, a close relative of *C. albicans*[1,16,19]. Geographical specificities of certain *C. albicans* strains have been demonstrated before for isolates from South-Africa, Singapore, and the North-Eastern United States[2,11,17]. Our results suggest the presence of a clone specific for the Iberian peninsula, and possibly also a specific Northern European clone.

ACKNOWLEDGEMENTS

We would like to thank Paul Savelkoul for technical advice.
Annemarie Borst is supported by a grant from bioMérieux (formerly Organon Teknika).

REFERENCES

1. **Azumi, M. and N. Goto-Yamamoto.** 2001. AFLP analysis of type strains and laboratory and industrial strains of *Saccharomyces* sensu stricto and its application to phenetic clustering. Yeast 18: 1145-1154

2. **Blignaut, E., C. Pujol, S. Lockhart, S. Joly, and D.R. Soll.** 2002. Ca3 Fingerprinting of *Candida albicans* Isolates from Human Immunodeficiency Virus-Positive and Healthy Individuals Reveals a New Clade in South Africa. J. Clin. Microbiol. 40: 826-836
3. **Boerlin, P., F. Boerlin-Petzold, J. Goudet, C. Durussel, J.L. Pagani, J.P. Chave, and J. Bille.** 1996. Typing *Candida albicans* oral isolates from human immunodeficiency virus-infected patients by multilocus enzyme electrophoresis and DNA fingerprinting. J. Clin. Microbiol. 34: 1235-1248
4. **Borst, A., and A.C. Fluit.** 2002. High levels of hydrolytic enzymes secreted by *Candida albicans* isolates involved in pneumonia. Submitted for publication.
5. **Borst, A., B. Theelen, E. Reinders, T. Boekhout, A.C. Fluit, and P.H. Savelkoul.** 2002. AFLP as an identification method for medically important *Candida* spp., including *C. dubliniensis.* Submitted for publication.
6. **Clemons, K.V., F. Feroze, K. Holmberg, and D.A. Stevens.** 1997. Comparative analysis of genetic variability among *Candida albicans* isolates from different geographic locales by three genotypic methods. J. Clin. Microbiol. 35: 1332-1336
7. **De Bernardis, F., P.A. Sullivan, and A. Cassone.** 2001. Aspartyl proteinases of *Candida albicans* and their role in pathogenicity. Med. Mycol. 39: 303-313
8. **Diaz-Guerra, T.M., J.V. Martinez-Suarez, F. Laguna, and J.L. Rodriguez-Tudela.** 1997. Comparison of four molecular typing methods for evaluating genetic diversity among *Candida albicans* isolates from human immunodeficiency virus-positive patients with oral candidiasis. J. Clin. Microbiol. 35: 856-861
9. **Ghannoum, M.A.** 2000. Potential role of phospholipases in virulence and fungal pathogenesis. Clin. Microbiol. Rev. 13: 122-43, table
10. **Jones, C.J., K.J. Edwards, S. Castaglione, M.O. Winfield, F. Sala, C. VandeWiel, G. Bredemeijer, B. Vosman, M. Matthes, A. Daly, R. Brettschneider, P. Bettini, M. Buiatti, E. Maestri, A. Malcevschi, N. Marmiroli, R. Aert, G. Volckaert, J. Rueda, R. Linacero, A. Vazquez, and A. Karp.** 1997. Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories. Molecular Breeding 3: 381-390
11. **Pfaller, M.A., S.R. Lockhart, C. Pujol, J.A. Swails-Wenger, S.A. Messer, M.B. Edmond, R.N. Jones, R.P. Wenzel, and D.R. Soll.** 1998. Hospital specificity, region specificity, and fluconazole resistance of *Candida albicans* bloodstream isolates. J. Clin. Microbiol. 36: 1518-1529
12. **Price, M.F., I.D. Wilkinson, and L.O. Gentry.** 1982. Plate method for detection of phospholipase activity in *Candida albicans*. Sabouraudia. 20: 7-14
13. **Pujol, C., S. Joly, S.R. Lockhart, S. Noel, M. Tibayrenc, and D.R. Soll.** 1997. Parity among the randomly amplified polymorphic DNA method, multilocus enzyme electrophoresis, and Southern blot hybridization with the moderately repetitive DNA probe Ca3 for fingerprinting *Candida albicans*. J. Clin. Microbiol. 35: 2348-2358
14. **Robert, F., F. Lebreton, M.E. Bougnoux, A. Paugam, D. Wassermann, M. Schlotterer, C. Tourte-Schaefer, and J. Dupouy-Camet.** 1995. Use of random amplified polymorphic DNA as a typing method for *Candida albicans* in epidemiological surveillance of a burn unit. J. Clin. Microbiol. 33: 2366-2371
15. **Sadhu, C., M.J. McEachern, E.P. Rustchenko-Bulgac, J. Schmid, D.R. Soll, and J.B. Hicks.** 1991. Telomeric and dispersed repeat sequences in *Candida* yeasts and their use in strain identification. J. Bacteriol. 173: 842-850
16. **Savelkoul, P.H., H.J. Aarts, J. de Haas, L. Dijkshoorn, B. Duim, M. Otsen, J.L. Rademaker, L. Schouls, and J.A. Lenstra.** 1999. Amplified-fragment length polymorphism analysis: the state of an art. J. Clin. Microbiol. 37: 3083-3091

17. **Schmid, J., S. Herd, P.R. Hunter, R.D. Cannon, M.S. Yasin, S. Samad, M. Carr, D. Parr, W. McKinney, M. Schousboe, B. Harris, R. Ikram, M. Harris, A. Restrepo, G. Hoyos, and K.P. Singh.** 1999. Evidence for a general-purpose genotype in *Candida albicans*, highly prevalent in multiple geographical regions, patient types and types of infection. Microbiology 145 (Pt 9): 2405-2413
18. **Thanos, M., G. Schonian, W. Meyer, C. Schweynoch, Y. Graser, T.G. Mitchell, W. Presber, and H.J. Tietz.** 1996. Rapid identification of *Candida* species by DNA fingerprinting with PCR. J. Clin. Microbiol. 34: 615-621
19. **Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. Van de Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper, and M. Zabeau.** 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res. 23: 4407-4414

Discussion

Despite the fact that the yeast *Candida* is the number 4 cause of bloodstream infections in the United States and ranks number 8 in Europe[9,16], adequate detection methods are lacking. Furthermore, relatively little is known about the epidemiology of *Candida*, including the main *Candida* species, *C. albicans*. In both fields, knowledge has increased in the past decade due to the rapid development and improvements of molecular biological techniques. This is illustrated by the fact that a clinically relevant species, *C. dubliniensis*, was misidentified as *C. albicans* until the discovery of the genetic differences between the species led to its recognition in 1995[33]. Our aim was to improve the detection of *Candida* infections and to study the epidemiology of these infections in Europe. Most research was performed by using two relatively new molecular biological tools: Nucleic Acid Sequence-Based Amplification (NASBA™)[6] and Amplified Fragment Length Polymorphism analysis (AFLP™)[35].

DETECTION OF *CANDIDA* INFECTIONS

The current routine detection method for *Candida* infections, automated blood culture, is inadequate. In many cases the blood cultures remain negative, even when the patient suffers from candidaemia[20]. It is hypothesized that at least in some cases this is a technical problem of the monitoring system. Shigei et al. and Tinghitella and Lamagdeleine have shown, that some automated blood culture instruments may fail to detect yeasts in spite of good growth of the organisms in the culture bottles, as was demonstrated by confluent growth after subculturing[31,34]. Other authors, however, claim that all important pathogens (including yeasts) are detected within the standard incubation time, without the need for terminal subculture[18,26,37]. In **Chapter 1** we examined whether terminal subculture of negative blood culture bottles improves the detection rate for patients with candidaemia[3]. For three of the ten patients studied, subculturing resulted in extra information. For one patient, this information was clinically relevant: yeast was detected up to 7 days after the last positive blood culture. Since the patient was treated with antifungal agents, blood culturing alone would have suggested that the infection was adequately treated. Therefore, we believe that routine terminal subculturing of negative blood cultures from patients with suspected candidaemia and patients under treatment for candidaemia might be valuable.

Chapters 2, 3 and **4** describe the development of a NASBA assay for the detection of *Candida* species in blood and blood cultures. Preliminary experiments on clinical material showed that it is possible to detect the yeast in blood and serum samples, without the prior need for culture (Chapter 2)[36]. However, since only a very small number of *Candida* cells may be present in the blood during candidaemia[25], we also investigated whether the NASBA assay can be used to improve the detection rate after blood culturing, by testing samples from blood culture bottles which remained negative in the blood culture system (Chapter 3). Furthermore, we examined whether a short pre-culture step of two days could improve the detection rate (Chapter 4). The results were encouraging in both cases. When the NASBA assay was used to detect *Candida* in blood cultures which were negative in the BacT/Alert monitoring system, the number of positive blood cultures increased with 62% (from 21% to 34%)[4]. Furthermore, we demonstrated that a substantial increase in detection rate can already be obtained with a 2 day pre-culture step: 80% of all samples positive in the NASBA assay in Chapter 4 were pre-

cultured for two days. In the same study, *Candida* RNA was detected in the blood of a patient, whereas no yeast was detected by the automated blood culturing system. In another patient the NASBA assay detected the infection two days earlier than the blood culture system[5]. Although the number of patients included in these studies was limited, the results indicate that improved detection of *Candida* infections (detection rate as well as speed) is possible. Improved detection will lead to a reduced morbidity and mortality.

One of the main advantages of the NASBA technology is also its disadvantage. The high sensitivity compared to other amplification methods makes it more prone to problems with contaminations. This was illustrated in **Chapter 5**, where an attempt to replace our in-house NASBA amplification protocol by a commercial kit failed due to contaminated kit components. Based on a literature review (**Chapter 6**) and our own experiences it was concluded that in many cases problems with contaminations occur because people are unaware of the impact which their undertakings have on the environment. Even non-molecular biologists may be working with large amounts of DNA and form an unexpected risk of contaminations, e.g. protein chemists who study recombinant proteins by using plasmids as expression systems. Therefore, all researchers using the same laboratory space and equipment should conduct themselves to stringent precautions, without any exceptions. The problem described in Chapter 5 originated from the manufacturer. Although it is difficult to take stringent measures for contamination control in a large-scale production process, the increasing request for standardized nucleic acid amplification assays obligates diagnostic companies to reevaluate their procedures.

The NASBA assay is able to detect most medically important *Candida* species. However, it is unfeasible to implement probes for all the different species in the assay. It is highly likely that uncommon species are encountered, which cannot be identified with the NASBA assay. Therefore, the availability of an identification tool which is universally applicable is desired. This is especially important considering the fact that less common *Candida* species are emerging and a new species (*C. dubliniensis*) was recognized only recently[17,32]. In **Chapter 7** of this thesis we show that AFLP is an excellent method for the identification of *Candida* species. The different species show very distinct clusters, and by using this technique we discovered that 6% of our (phenotypically identified) collection of clinical *Candida* isolates was misidentified. The potential of storing AFLP patterns in general accessible databases will greatly enhance the chances of a correct identification.

EPIDEMIOLOGY OF *CANDIDA* INFECTIONS

The second objective of this thesis was to study the epidemiology of *Candida albicans* infections in Europe. Some strains of the same species may be associated with a specific type of disease or may be restricted to a certain geographic region. It is important to recognize such correlations, and to identify the underlying mechanisms. Although *Candida albicans* is capable of sexual reproduction and recombination occurs to some extent, the yeast reproduces mainly clonally[11,12,14,19]. Therefore, all genes are associated and research on the epidemiology of clinically important traits such as the expression of virulence factors may lead to the identification of genetic markers for these traits. This may result in improved therapeutic regimens.

In **Chapter 8** we investigated whether the expression of two putative virulence factors of *C. albicans*, (phospho)lipases and proteinases[8,10], are associated with a certain type of infection or with the geographic origin of the isolates. It appeared that compared to infections of blood, the urinary tract or wound/skin/soft tissue, a relatively high number of isolates which were involved in pneumonia produced (phospho)lipases. Also, a significantly higher number of these isolates were among the higher producers of this enzyme. A similar trend was observed for the production of proteinases: all isolates obtained from pneumonia were positive in the proteinase assay, and 96% of these isolates were high producers. These results suggest that isolates involved in pneumonia are more virulent than isolates obtained from the other types of infection that were studied. It is hypothesized that selection for more virulent isolates in debilitated individuals occurs in HIV-positive patients[7,7,23]. Also, oral *C. albicans* isolates from healthy volunteers show a relatively low phospholipase activity, whereas clinical isolates from the oral cavities of patients suffering from oral candidosis produce relatively high amounts of this enzyme[15,27]. It will be interesting to study whether the enhanced (phospho)lipase and proteinase production in pneumonia-derived isolates is caused by a positive selection of more virulent isolates, and to elucidate the mechanisms behind such a selection.

Another interesting epidemiological finding is described in **Chapter 9**. By using AFLP as a fingerprinting method, we typed a large collection of European *C. albicans* isolates. It was discovered, that isolates from Portugal and Spain all belonged to the same AFLP cluster (cluster 1), whereas isolates from the United Kingdom and all but one isolate from Germany belonged to another cluster (cluster 2). Isolates from France, Italy, Switzerland, and Turkey were represented in both clusters. These results indicate the presence of an Iberian and a Northern European clone. Geographical specificities of certain *C. albicans* strains have been demonstrated before for isolates from South-Africa, Singapore, and the North-Eastern United States[2,24,29]. To our knowledge, this is the first time that such specificities have been demonstrated for Europe.

Although AFLP has been established as a typing method for several microorganisms including the yeast *Saccharomyces cerevisiae*[1,28], we will need to compare AFLP with another fingerprinting method on the same group of isolates to validate the feasibility of this technique as a typing method for *C. albicans*. The difficulty, however, is that no other typing method has been accepted as the golden standard for typing of *Candida*.

It can be concluded that our first objective, improved detection of *Candida* infections, was feasible. Although the implementation of the NASBA assay in a routine laboratory needs further efforts, we have demonstrated that this assay can lead to a more rapid detection as well as increased detection rates. The second objective, to study the epidemiology of *C. albicans* infections in Europe, resulted in two interesting discoveries: *C. albicans* isolates involved in pneumonia seem to be more virulent than isolates involved in other types of infection, and European *C. albicans* isolates can be distinguished in an Iberian and a Northern European AFLP-type, which are mingled in Central European countries. These last two findings can be the onset of extensive epidemiological studies.

REFERENCES

1. **Azumi, M. and N. Goto-Yamamoto.** 2001. AFLP analysis of type strains and laboratory and industrial strains of *Saccharomyces* sensu stricto and its application to phenetic clustering. Yeast 18: 1145-1154
2. **Blignaut, E., C. Pujol, S. Lockhart, S. Joly, and D.R. Soll.** 2002. Ca3 Fingerprinting of *Candida albicans* Isolates from Human Immunodeficiency Virus-Positive and Healthy Individuals Reveals a New Clade in South Africa. J. Clin. Microbiol. 40: 826-836
3. **Borst, A., M. Leverstein-Van Hall, J. Verhoef, and A. Fluit.** 2000. Value of terminal subculture of automated blood cultures in patients with candidaemia. Eur. J. Clin. Microbiol. Infect. Dis. 19: 803-805
4. **Borst, A., M.A. Leverstein-Van Hall, J. Verhoef, and A.C. Fluit.** 2001. Detection of *Candida* spp. in blood cultures using nucleic acid sequence-based amplification (NASBA). Diagn. Microbiol. Infect. Dis. 39: 155-160
5. **Borst, A., J. Verhoef, E. Boel, and A.C. Fluit.** 2002. Clinical evaluation of a NASBA-based assay for detection of *Candida* spp. in blood and blood cultures. Clin. Lab. (In press)
6. **Compton, J.** 1991. Nucleic acid sequence-based amplification. Nature 350: 91-92
7. **De Bernardis, F., P. Chiani, M. Ciccozzi, G. Pellegrini, T. Ceddia, G. D'Offizzi, I. Quinti, P.A. Sullivan, and A. Cassone.** 1996. Elevated aspartic proteinase secretion and experimental pathogenicity of *Candida albicans* isolates from oral cavities of subjects infected with human immunodeficiency virus. Infect. Immun. 64: 466-471
8. **De Bernardis, F., P.A. Sullivan, and A. Cassone.** 2001. Aspartyl proteinases of *Candida albicans* and their role in pathogenicity. Med. Mycol. 39: 303-313
9. **Fluit, A.C., M.E. Jones, F.J. Schmitz, J. Acar, R. Gupta, and J. Verhoef.** 2000. Antimicrobial susceptibility and frequency of occurrence of clinical blood isolates in Europe from the SENTRY antimicrobial surveillance program, 1997 and 1998. Clin. Infect. Dis. 30: 454-460
10. **Ghannoum, M.A.** 2000. Potential role of phospholipases in virulence and fungal pathogenesis. Clin. Microbiol. Rev. 13: 122-43, table
11. **Gow, N.A., A.J. Brown, and F.C. Odds.** 2000. *Candida*'s arranged marriage. Science 289: 256-257
12. **Graser, Y., M. Volovsek, J. Arrington, G. Schonian, W. Presber, T.G. Mitchell, and R. Vilgalys.** 1996. Molecular markers reveal that population structure of the human pathogen *Candida albicans* exhibits both clonality and recombination. Proc. Natl. Acad. Sci. U. S. A 93: 12473-12477
13. **Gumbo, T., C.M. Isada, G. Hall, M.T. Karafa, and S.M. Gordon.** 1999. *Candida glabrata* Fungemia. Clinical features of 139 patients. Medicine (Baltimore) 78: 220-227
14. **Hull, C.M., R.M. Raisner, and A.D. Johnson.** 2000. Evidence for mating of the "asexual" yeast *Candida albicans* in a mammalian host. Science 289: 307-310
15. **Ibrahim, A.S., F. Mirbod, S.G. Filler, Y. Banno, G.T. Cole, Y. Kitajima, J.E. Edwards, Jr., Y. Nozawa, and M.A. Ghannoum.** 1995. Evidence implicating phospholipase as a virulence factor of *Candida albicans*. Infect. Immun. 63: 1993-1998
16. **Jarvis, W.R.** 1995. Epidemiology of nosocomial fungal infections, with emphasis on *Candida* species. Clin. Infect. Dis. 20: 1526-1530
17. **Kao, A.S., M.E. Brandt, W.R. Pruitt, L.A. Conn, B.A. Perkins, D.S. Stephens, W.S. Baughman, A.L. Reingold, G.A. Rothrock, M.A. Pfaller, R.W. Pinner, and R.A. Hajjeh.** 1999. The epidemiology of candidemia in two United States cities: results of a population-based active surveillance. Clin. Infect. Dis. 29: 1164-1170
18. **Kennedy, G.T., J.G. Barr, and C. Goldsmith.** 1995. Detection of bacteraemia by the continuously

monitoring BacT/Alert system. J. Clin. Pathol. 48: 912-914

19. **Magee, B.B. and P.T. Magee.** 2000. Induction of mating in *Candida albicans* by construction of MTLa and MTLalpha strains. Science 289: 310-313
20. **Maksymiuk, A.W., S. Thongprasert, R. Hopfer, M. Luna, V. Fainstein, and G.P. Bodey.** 1984. Systemic candidiasis in cancer patients. Am. J. Med. 77: 20-27
21. **Moran, G.P., D. Sanglard, S.M. Donnelly, D.B. Shanley, D.J. Sullivan, and D.C. Coleman.** 1998. Identification and expression of multidrug transporters responsible for fluconazole resistance in *Candida dubliniensis*. Antimicrob. Agents Chemother. 42: 1819-1830
22. **Moran, G.P., D.J. Sullivan, M.C. Henman, C.E. McCreary, B.J. Harrington, D.B. Shanley, and D.C. Coleman.** 1997. Antifungal drug susceptibilities of oral *Candida dubliniensis* isolates from human immunodeficiency virus (HIV)-infected and non-HIV-infected subjects and generation of stable fluconazole-resistant derivatives in vitro. Antimicrob. Agents Chemother. 41: 617-623
23. **Ollert, M.W., C. Wende, M. Gorlich, C.G. McMullan-Vogel, M. Borg-von Zepelin, C.W. Vogel, and H.C. Korting.** 1995. Increased expression of *Candida albicans* secretory proteinase, a putative virulence factor, in isolates from human immunodeficiency virus-positive patients. J. Clin. Microbiol. 33: 2543-2549
24. **Pfaller, M.A., S.R. Lockhart, C. Pujol, J.A. Swails-Wenger, S.A. Messer, M.B. Edmond, R.N. Jones, R.P. Wenzel, and D.R. Soll.** 1998. Hospital specificity, region specificity, and fluconazole resistance of *Candida albicans* bloodstream isolates. J. Clin. Microbiol. 36: 1518-1529
25. **Rand, K.H., H. Houck, and M. Wolff.** 1994. Detection of candidemia by polymerase chain reaction. Mol. Cell Probes. 8: 215-221
26. **Reisner, B.S. and G.L. Woods.** 1999. Times to detection of bacteria and yeasts in BACTEC 9240 blood culture bottles. J. Clin. Microbiol. 37: 2024-2026
27. **Samaranayake, L.P., J.M. Raeside, and T.W. MacFarlane.** 1984. Factors affecting the phospholipase activity of *Candida* species in vitro. Sabouraudia. 22: 201-207
28. **Savelkoul, P.H., H.J. Aarts, J. de Haas, L. Dijkshoorn, B. Duim, M. Otsen, J.L. Rademaker, L. Schouls, and J.A. Lenstra.** 1999. Amplified-fragment length polymorphism analysis: the state of an art. J. Clin. Microbiol. 37: 3083-3091
29. **Schmid, J., S. Herd, P.R. Hunter, R.D. Cannon, M.S. Yasin, S. Samad, M. Carr, D. Parr, W. McKinney, M. Schousboe, B. Harris, R. Ikram, M. Harris, A. Restrepo, G. Hoyos, and K.P. Singh.** 1999. Evidence for a general-purpose genotype in *Candida albicans*, highly prevalent in multiple geographical regions, patient types and types of infection. Microbiology 145 (Pt 9): 2405-2413
30. **Schmid, J., P.R. Hunter, G.C. White, A.K. Nand, and R.D. Cannon.** 1995. Physiological traits associated with success of *Candida albicans* strains as commensal colonizers and pathogens. J. Clin. Microbiol. 33: 2920-2926
31. **Shigei, J.T., J.A. Shimabukuro, M.T. Pezzlo, L.M. de la Maza, and E.M. Peterson.** 1995. Value of terminal subcultures for blood cultures monitored by BACTEC 9240. J. Clin. Microbiol. 33: 1385-1388
32. **Sullivan, D. and D. Coleman.** 1998. *Candida dubliniensis*: characteristics and identification. J. Clin. Microbiol. 36: 329-334
33. **Sullivan, D.J., T.J. Westerneng, K.A. Haynes, D.E. Bennett, and D.C. Coleman.** 1995. *Candida dubliniensis* sp. nov.: phenotypic and molecular characterization of a novel species associated with oral candidosis in HIV-infected individuals. Microbiology 141 (Pt 7): 1507-1521
34. **Tinghitella, T.J. and M.D. Lamagdeleine.** 1995. Assessment of Difco ESP 384 blood culture system by terminal subcultures: failure to detect *Cryptococcus neoformans* in clinical specimens. J. Clin. Microbiol. 33: 3031-3033

35. **Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. Van de Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper, and M. Zabeau.** 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res. 23: 4407-4414
36. **Widjojoatmodjo, M.N., A. Borst, R.A. Schukkink, A.T. Box, N.M. Tacken, B. Van Gemen, J. Verhoef, B. Top, and A.C. Fluit.** 1999. Nucleic acid sequence-based amplification (NASBA) detection of medically important *Candida* species. J. Microbiol. Methods 38: 81-90
37. **Ziegler, R., I. Johnscher, P. Martus, D. Lenhardt, and H.M. Just.** 1998. Controlled clinical laboratory comparison of two supplemented aerobic and anaerobic media used in automated blood culture systems to detect bloodstream infections. J. Clin. Microbiol. 36: 657-661

Nederlandse samenvatting

Candida is een opportunistische gistsoort die ernstige infecties kan veroorzaken in patiënten met een verminderde afweer. *Candida albicans* komt het meest voor, maar andere *Candida* soorten zoals *C. glabrata* en *C. krusei* worden steeds vaker geïsoleerd. Risicogroepen voor *Candida* infecties zijn neutropene kankerpatiënten, beenmerg- en orgaantransplantatie-patiënten, AIDS-patiënten, diabetici, patiënten die worden behandeld met breedspectrum antibiotica en patiënten die parenterale voeding krijgen. Van alle micro-organismen die bloedbaan infecties veroorzaken komt *Candida* op de 4^{e} plaats in Noord-Amerika, en op de 8^{e} plaats in Europa. Toch is de standaard detectiemethode voor deze infecties, geautomatiseerde bloedkweek, niet toereikend. Veel infecties blijven ongedetecteerd of worden pas na meerdere dagen aangetoond. Daarna duurt het nog één of soms meerdere dagen voordat duidelijk is om welke soort *Candida* het gaat. Sommige onderzoekers zijn van mening dat de detectie verbeterd kan worden door de negatieve bloedkweken af te enten. Uit het onderzoek beschreven in hoofdstuk 1, waarin negatieve bloedkweken van patiënten met candidaemie werden afgeënt, blijkt dat dit afenten kan leiden tot extra (klinisch relevante) informatie: na afenten werden positieve bloedkweken gevonden op een moment dat de patiënt werd behandeld met antimycotica. Bloedkweken zonder afenten zou hebben gesuggereerd dat de behandeling aansloeg. Het is daarom aan te bevelen om negatieve bloedkweken van patiënten die verdacht worden van candidaemie of patiënten die behandeld worden voor deze infectie af te enten voor verder onderzoek.

Het is belangrijk om te weten welke *Candida* soort de veroorzaker is van een infectie. *C. krusei* is bijvoorbeeld resistent tegen één van de meest gebruikte antimycotica, fluconazol, en het is bekend dat *C. glabrata* deze resistentie snel kan ontwikkelen. Om zo snel mogelijk met de juiste behandeling te kunnen starten is het dus belangrijk om een detectiemethode te hebben die niet alleen snel is, maar ook tot op soortniveau kan aangeven welke gist de infectie veroorzaakt.

Door hun snelheid en gevoeligheid zijn nucleïnezuur-amplificatiemethoden belangrijke kandidaten voor het verbeteren van de diagnostiek van infecties. De meest gebruikte techniek is de Polymerase Chain Reaction (PCR), waarin specifieke stukjes DNA worden vermeerderd tot een aantoonbaar niveau. In 1991 werd echter een nieuwe techniek beschreven die een aantal voordelen heeft boven PCR. Deze techniek, Nucleic Acid Sequence-Based Amplification (NASBA), is gebaseerd op het vermeerderen van specifieke stukjes RNA in plaats van DNA. In tegenstelling tot PCR vindt dit proces plaats bij een constante temperatuur waardoor er geen dure warmtewisselaar hoeft worden aangeschaft. Daarnaast wordt er per reactie veel meer product gevormd dan bij PCR, waardoor de techniek gevoeliger is.

De hoofdstukken 2, 3 en 4 beschrijven de ontwikkeling van een NASBA assay voor het aantonen van *Candida* RNA in bloed en bloedkweken. Uit hoofdstuk 2 blijkt dat het mogelijk is om de gist direct in bloed aan te tonen. Omdat er bij candidaemie echter vaak sprake is van zeer kleine aantallen gistcellen in het bloed, is er tevens gekeken naar de mogelijkheid om de detectie van bloedkweken te verbeteren door met NASBA gist RNA aan te tonen in negatieve bloedkweken (hoofdstuk 3). Daarnaast is in hoofdstuk 4 onderzocht of een korte voorkweek stap van twee dagen de detectie verbeterde. In beide gevallen waren de resultaten bemoedigend. Door de NASBA assay te gebruiken voor het aantonen van gist RNA in bloedkweken die negatief bleven in de geautomatiseerde monitor werden 62% meer positieve kweken gevonden (een stijging van 21% naar 34%). Daarnaast verbeterde de detectie

substantieel na een korte voorkweek stap: 80% van de monsters die positief waren in de NASBA assay in hoofdstuk 4 waren dat na twee dagen voorkweken. In dezelfde studie werd *Candida* RNA aangetoond in het bloed van een patiënt waarvan alle bloedkweken negatief waren gebleven. Bij een andere patiënt werd de infectie twee dagen eerder gedetecteerd in vergelijking met de bloedkweken. Hoewel maar een klein aantal patiënten geïncludeerd was in deze studies, blijkt uit de resultaten dat een verbetering van de detectie (zowel in aantal als in snelheid) mogelijk is. Dit kan uiteindelijk leiden tot minder morbiditeit en mortaliteit bij de patiënten.

Het belangrijkste voordeel van de NASBA is tevens ook een nadeel. Door de hoge gevoeligheid vergeleken met andere technologiën is er ook een grotere kans op contaminaties. Een duidelijk voorbeeld hiervan wordt beschreven in hoofdstuk 5, waar een poging om de NASBA assay te standaardiseren met behulp van een commerciële kit mislukte doordat bepaalde bestanddelen van de kit gecontamineerd waren met gist- of schimmel RNA. Naar aanleiding van dit onderzoek en een literatuuronderzoek (beschreven in hoofdstuk 6) werd geconcludeerd dat de meeste problemen met contaminaties zich voordoen omdat mensen zich niet bewust zijn van het effect van hun handelen op hun omgeving. Ook niet-moleculair biologen kunnen met grote hoeveelheden DNA werkzaam zijn en zo een onverwacht risico vormen, bijvoorbeeld tijdens onderzoek naar recombinant-eiwitten die door middel van plasmiden tot expressie gebracht worden. Het is daarom aan te raden dat alle onderzoekers die dezelfde laboratoria en benodigdheden gebruiken zich aan dezelfde strenge voorzorgsmaatregelen houden. Het in hoofdstuk 5 beschreven probleem stamde af van de fabrikant. Hoewel het moeilijk is om strenge voorzorgsmaatregelen te nemen in een grootschalig productieproces, zouden fabrikanten zich het belang van zulke maatregelen beter moeten realiseren.

Met de in dit proefschrift ontwikkelde NASBA assay kunnen de belangrijkste *Candida* soorten snel herkend worden. Het is echter hoogst aannemelijk dat er ook gistsoorten gevonden zullen worden die met deze methode niet gespecificeerd kunnen worden. Het is ondoenlijk om voor elke soort een extra probe aan de assay toe te voegen. Men zou daarom naast de NASBA assay ook de beschikking moeten hebben over een universeel toepasbare techniek, hoewel die doorgaans meer tijd in beslag nemen. In hoofdstuk 7 werd onderzocht of zo'n universele techniek, Amplified Fragment Length Polymorphism analysis (AFLP) geschikt is voor de identificatie van verschillende *Candida* soorten. De onderzochte soorten blijken allemaal een uniek bandenpatroon te hebben, en met behulp van deze methode werd aangetoond dat 6% van een grote collectie *C. albicans* isolaten in het verleden verkeerd geïdentificeerd was. AFLP patronen zijn gemakkelijk op te slaan in algemeen toegankelijke databestanden, hetgeen de kans op een correcte identificatie vergroot.

Naast het verbeteren van de detectie van *Candida* infecties wilden we in dit onderzoek ook kijken naar de epidemiologie van *C. albicans* in Europa. Binnen een soort kunnen er bepaalde stammen zijn de geassocieerd zijn met een bepaald type infectie of met een bepaalde geografische locatie. Het aantonen van zulke relaties leidt tot een beter begrip van de epidemiologie van een soort, en kan tevens een aanzet zijn tot bijvoorbeeld het vinden van moleculaire markers voor belangrijke eigenschappen als virulentie. Uiteindelijk kan dit leiden tot verbeterde therapeutische voorschriften. In hoofdstuk 8 werd onderzocht of er een relatie bestaat tussen de productie van twee virulentiefactoren van *C. albicans*, (fosfo)lipasen en

proteïnasen, en het type infectie of de geografische herkomst van de isolaten. In vergelijking met isolaten afkomstig uit bloed, de urinewegen of wond/huid-infecties bleken significant meer isolaten die betrokken waren bij pneumonieën (fosfo)lipasen te produceren. Tevens produceerde een significant hoger aantal van deze isolaten grote hoeveelheden van dit enzym. Vergelijkbare bevindingen werden gedaan voor proteïnasen: alle pneumonie-isolaten produceerden dit enzym, en 96% van deze isolaten produceerden grote hoeveelheden. Deze resultaten tonen aan dat isolaten betrokken bij pneumonie waarschijnlijk virulenter zijn dan isolaten van andere typen infecties. Mogelijk heeft dit te maken met selectie van virulente isolaten in patiënten met een verminderde afweer.

Een andere interessante bevinding werd gedaan on hoofdstuk 9. Een groot aantal Europese *C. albicans* isolaten werd getypeerd. Hiervoor werd opnieuw gebruikt gemaakt van AFLP, een techniek die naast identificatie van soorten ook geschikt is voor het aantonen van verschillen binnen een soort. De Europese isolaten bleken onderverdeeld te kunnen worden in twee clusters. Opvallend was, dat isolaten afkomstig uit Portugal en Spanje allen tot cluster 1 behoorden, terwijl isolaten uit het Verenigd Koninkrijk en Duitsland vrijwel allemaal tot cluster 2 behoorden. Isolaten uit Frankrijk, Italië, Zwitserland en Turkije waren verdeeld over de twee clusters. Deze resultaten impliceren het bestaan van een Iberische en een Noord-Europese kloon.

In conclusie: in dit proefschrift is aangetoond dat een verbetering van de detectie van *Candida* infecties mogelijk is, zowel in aantal als in snelheid. Daarnaast is ontdekt dat *C. albicans* isolaten betrokken bij pneumonie virulenter zijn dan isolaten uit andere typen infecties. Tevens lijkt het er op dat er binnen Europa sprake is van een Iberische- en een Noord-Europese *C. albicans*-kloon. Deze laatste twee bevindingen kunnen het begin zijn van een grootschalig epidemiologisch onderzoek.

Dankwoord

Na een onmogelijk lijkende eindsprint is het dan nu toch echt af. Het zal vreemd zijn om het EWI te verlaten. Ik heb de afgelopen 4½ jaar vrijwel altijd met veel plezier hier rondgelopen, en hoewel ik de enige was die met NASBA en *Candida* werkte zijn er heel wat mensen die hun steentje hebben bijgedragen aan dit boekje. Gelukkig is daarvoor het dankwoord uitgevonden.

Mijn grote dank gaat uit naar mijn co-promotor. Ad, het was soms bijna eng hoe goed wij op één lijn zaten. Bedankt voor de tijd die je nooit had maar altijd wist te vinden, voor de gezellige tijd op congressen, en vooral voor je vertrouwen in mijn kunnen de laatste twee maanden. Het wordt tijd voor een ASM meeting in Atlanta, dan kun je margherita's komen drinken en beren spotten in de Smokey's! Mijn promotor, professor Verhoef, wil ik bedanken voor de mogelijkheid om binnen het EWI mijn promotieonderzoek uit te voeren.

Adrienne, jij leerde mij de fijne kneepjes van het NASBA-gebeuren, en daarmee het belang van schoon. Daarnaast wist ik jouw hoekje altijd te vinden als het tijd was voor een klaagsessie over mislukte proeven of collega's die er geen bal van snapten. Dank voor je schouder.

Jeena, jij was mijn eerste logeeradres in Utrecht, waardoor ik niet meer met de laatste trein terug hoefde. Bedankt dus voor de oranje bank, maar ook voor de vele theepauzes, bij voorkeur onder het genot van een kinderei ("die is te zwaar, dat is zo'n stomme").

Ingrid, mijn oudste vriendinnetje, en dan bedoel ik natuurlijk dat ik je al héél lang ken, bedankt voor het buddende zeebeest. Ik vind hem prachtig!

De mensen van Organon zorgden voor meer dan alleen de financiën voor dit onderzoek. In den beginne waren er Bob, Rianne en Piet die mij naar Boxtel haalden en leerden dat PCR een vies woord is. Later volgden de beide Peters en Margot en de wondere wereld van de Basic Kit. Jullie allemaal bedankt voor de steun en ook voor de vrijheid om dit onderzoek naar eigen inzicht uit te voeren.

Het NASBA-onderzoek zou nooit hebben kunnen plaatsvinden zonder de bereidwillige medewerking van de patiënten en hun families. Bedankt dat u ook in moeilijke tijden open stond voor wetenschappelijk onderzoek. Nog veel meer mensen waren betrokken bij dit onderzoek. Maurine Leverstein-Van Hall, Edwin Boel, Henk van Leeuwen, Dr. Dekker, Dr. Vos, Dr. Bindels, de verpleegkundigen van de betrokken afdelingen en natuurlijk de analisten van de klinische bacteriologie, zowel in Utrecht als in Veldhoven. Jullie allemaal bedankt voor de medewerking.

Het AFLP-werk liep ook al uit op één groot samenwerkingsverband. In Amsterdam: Paul, Erik en Madelon, jullie bedankt voor het helpen opstarten, de brainstorms als alles weer eens mislukte, en de AFLP-stage in jullie eigen lab. De buren van het CBS: Teun, bedankt voor je kritische oog bij het lezen van mijn schrijfsels. Bart, bedankt voor de prachtige bomen en voor de uren dat we samen vloekend achter de computer zaten in een poging om Candy te koppelen aan Lolita. Misschien zien we elkaar komende winter aan de andere kant van de grote haringvijver.

Mijn 'kindjes': Wendy van de mega-PCRs, Marcel die het aandurfde om een jaar te blijven (interessant hè, die Candida!), Cindy die de ene na de andere carrière bedacht en nog steeds gezellig bij het EWI werkt, Mariette, één van de weinige geneeskunde-studenten met een pipetteer-duim, en Bregje die maar heel kort van mij was maar gelukkig heel flexibel bleek. Bedankt voor jullie hulp!

Ook in het lab waren velen onmisbaar. Mirjam, Stefan, Karlijn en Alice, altijd bereid om

kweken in te zetten of op mijn platen te passen. De mediaroom, en met name Martin voor het maken van de speciale platen die in mijn eindsprint zo belangrijk waren. Verder iedereen van de ARE groep en de collega's op 602, bedankt voor de gezelligheid.

Annemarie W. (de luidruchtige buuv), Bart (de harige bruid met het blauwe haar), Ben (bedankt voor de liften naar Amsterdam), Camiel (shoppen in Chicago), Desiree (die borrel lukt vast een keer), Dirk (kort maar gezellig), Gunnar (alias 'Gladiator'), Hessel (sorry voor mijn eeuwige zooi), James (tot ziens in de trein), Leonie (groeten van de toverdoos), Leontine (Happiness, weet je nog?), Mascha (broem-toet!), Miquel (sorry dat ik voor Bart koos), Mireille (de afwezige buuv), Monique (da's lang geleden), Pieter-Jan (wanneer bloeit je plant nou eindelijk?), Ruzena (het koekiemonster), Steven (IJS!), Wilco (mijn eerste buurman), Wouter (de kamerfilosoof), oftewel DE kamergenoten, onmisbaar in het leven als AIO. De vele koppen thee, koffie of chocolademelk op het onverwoestbare dienblaadje, het meedenken over woorden, zinnen, de kleur van een poster, de verkleedpartijen voor promoties en bruiloften, de steun in moeilijke tijden, ik zal jullie heel erg missen. Ik laat jullie mijn pennencollectie na. Laat ze niet gejat worden!

Roel, bedankt voor je steun en advies toen het allemaal even minder leuk was.

Chris, thanks for your enthusiasm which made writing the proposal almost fun. Your sight-seeing tour in Atlanta was truly a memorable event. I look forward to working with you this fall!

De dames van de betere lichamen, oftewel de Zeister sportclub, Adrienne, Ellen en Carla. Bedankt dat ik als semi(demi?)Zeistenaar ook lid mocht worden. Marcheer op rechts!

De zwemzwerm en alle vrienden in Amsterdam en daarbuiten, die het afgelopen half jaar niets meer van mij hoorden: bedankt voor jullie begrip.

Pim, die er voor zorgde dat ik ook in Utrecht fietsend door het leven ging.

Mijn ouders, die mij altijd alle vrijheid en mogelijkheden hebben gegeven om te worden wat ik wilde.

Mijn broer Dr. Ir. Pim, altijd een paar jaar eerder met alles en daarom toch een beetje een voorbeeld. Stuur je veel foto's van mijn prachtige nicht als ik weg ben?

En dan Martijn… Wat had ik zonder jou gemoeten? Je bent de enige die weet wat een enorme stresskip ik ben, en degene die altijd de volle laag kreeg. Bedankt voor je eindeloze geduld, brede schouders en knuffels. De dikste knuffel is nu voor jou: {{{Martijn}}

CURRICULUM VITAE

Annemarie Borst werd geboren op 20 mei 1972 te Castricum. Na het behalen van het eindexamen VWO aan het Bonhoeffer College te Castricum werd in 1990 gestart met de studie Medische Biologie aan de Vrije Universiteit te Amsterdam. Tijdens de studie verrichtte zij wetenschappelijk onderzoek bij de vakgroep Celbiologie van de Vrije Universiteit en het laboratorium voor Parasitologie en Mycologie van het Rijksinstituut voor Volksgezondheid en Milieu (RIVM). Als extra stageperiode werd een half jaar onderzoek uitgevoerd aan de Washington State University te Pullman, WA, Verenigde Staten. In 1996 werd het doctoraaldiploma behaald, waarna zij gedurende anderhalf jaar werkzaam was bij het Onderwijsbureau van de faculteit Biologie van de Vrije Universiteit.

Het in dit proefschrift beschreven onderzoek werd aangevangen in 1998 bij het Eijkman-Winkler Instituut, UMC Utrecht (Prof. J. Verhoef), onder begeleiding van Dr. A.C. Fluit. Haar werk werd in 2000 beloond met een Student Travel Grant Award van de American Society for Microbiology (ASM).

Een in samenwerking met Dr. C.J. Morrison geschreven onderzoeksvoorstel voor een post-doctoral fellowship werd gehonoreerd door de ASM en het National Center for Infectious Diseases (NCID), waardoor zij vanaf oktober 2002 gedurende twee jaar werkzaam zal zijn bij de Mycotic Disease Branch van de Centers for Disease Control and Prevention (CDC) in Atlanta, GA, Verenigde Staten.

LIST OF PUBLICATIONS

1. **Biewenga, J., M.B. van der Ende, L.F. Krist, A. Borst, M. Ghufron, and N. van Rooijen.** 1995. Macrophage depletion in the rat after intraperitoneal administration of liposome-encapsulated clodronate: depletion kinetics and accelerated repopulation of peritoneal and omental macrophages by administration of Freund's adjuvant. Cell Tissue Res. 280: 189-196

2. **Homan, W.L., L. Limper, M. Verlaan, A. Borst, M. Vercammen, and F. van Knapen.** 1997. Comparison of the internal transcribed spacer, ITS 1, from *Toxoplasma gondii* isolates and *Neospora caninum*. Parasitol. Res. 83: 285-289

3. **Widjojoatmodjo, M.N., A. Borst, R.A.F. Schukkink, A.T.A. Box, N.M.M. Tacken, B. Van Gemen, J. Verhoef, B. Top, and A.C. Fluit.** 1999. Nucleic acid sequence-based amplification (NASBA) detection of medically important *Candida* species. J. Microbiol. Methods 38: 81-90

4. **Borst, A., M. Leverstein-Van Hall, J. Verhoef, and A. Fluit.** 2000. Value of terminal subculture of automated blood cultures in patients with candidaemia. Eur. J. Clin. Microbiol. Infect. Dis. 19: 803-805

5. **Borst, A., M.A. Leverstein-Van Hall, J. Verhoef, and A.C. Fluit.** 2001. Detection of *Candida* spp. in blood cultures using nucleic acid sequence-based amplification (NASBA). Diagn. Microbiol. Infect. Dis. 39: 155-160

6. **Borst, A., J. Verhoef, E. Boel, and A.C. Fluit.** 2002. Clinical evaluation of a NASBA-based assay for detection of *Candida* spp. in blood and blood cultures. Clin. Lab. (In press)

7. **Borst, A., A.T.A. Box, and A.C. Fluit.** 2002. False-positive results and contaminations in nucleic acid amplification assays. Suggestions for a 'Prevent and Destroy'-strategy. Submitted for publication.

8. **Borst, A., B. Theelen, E. Reinders, T. Boekhout, A.C. Fluit, and P.H.M. Savelkoul.** 2002. AFLP as an identification method for medically important *Candida* spp., including *C. dubliniensis.* Submitted for publication.

9. **Borst, A. and A.C. Fluit.** 2002. High levels of hydrolytic enzymes secreted by *Candida albicans* isolates involved in pneumonia. Submitted for publication.

10. **Borst, A., B. Theelen, T. Boekhout, and A.C. Fluit.** 2002. AFLP typing of European *Candida albicans* isolates shows geographical specificities. Submitted for publication.