Rev Iberoam Micol 1997; 14: 23-25

Diagnosis of candidosis by amplification of small subunit of 18S rRNA gene

Miren Josebe Unzaga[1], Lucía Gallego[2], Pilar Berdonces[1], Jose Ramón Donés[2] and Ramón Cisterna[1,2]

[1]Servicio de Microbiología, Hospital de Basurto, Bilbao, Vizcaya, Spain & [2]Departamento de Microbiología de la Universidad del País Vasco, Vizcaya, Spain.

Summary

A PCR assay for the diagnosis of infection produced by *Candida* sp. was developed. The primers, designated 520 and 522, were selected from highly-conserved areas of the small subunit (ssu) 18S rRNA gene of *Candida* spp. To check the value of the results a *Candida albicans* oligonucleotide probe, digoxigenin-labeled, and a general *Candida* probe were used in hybridization experiments with the amplified products. We were able to detect a *Candida*- specific fragment of 1800bp from different clinical samples. The procedure described could provide an interesting complement to present diagnostic methods of detecting *Candida* sp in clinical samples.

Key words *Candida,* DNA, PCR, Fungi

Diagnóstico de la candidosis mediante amplificación de la subunidad pequeña del gen 18s rRNA

Resumen

El objetivo del trabajo fue diagnosticar la infección por *Candida* sp. mediante la reacción en cadena de la polimerasa (PCR) amplificando el gen de la 18S rRNA y utilizando los iniciadores 520 y 522 en 28 muestras clínicas con cultivo positivo para *Candida* sp. A fín de comprobar su especificidad, los productos de la amplificación, se hibridaron con dos sondas, una específica de especie y otra general de *Candida.* Se amplificó una banda de 1800pb en todas las muestras. La PCR se presenta como un método complementario a las actuales técnicas diagnósticas de la infección por *Candida* sp.

Palabras clave *Candida,* DNA, PCR, Hongos

Infections by *Candida* sp are emerging as a common complication in immunocompromised patients. Several studies describe an up to tenfold increase in the incidence rates of candidemia, with a reported mortality range of between 26 and 80% [1,2]. Standard diagnostic procedures are not always successful: cultures take several days, and thus severe disseminated disease can occur with negative blood cultures; serologic methods are problematic, the production of antibodies in immunocompromised patients is reduced, a positive result can be misleading, and finally the sensitivity of antigen detection tests is not sufficient for diagnosis [3]. To avoid this problem, and to increase the sensitivity and reduce the time for identification, DNA-based diagnostic methods have been developed [4].

We developed a PCR assay to improve the diagnosis of *Candida* infection using the eukariotic small subunit ribosomal RNA (18S rRNA), which is the most highly conserved in the genome and thus offers regions of sequence that are identical in all fungi [5,6].

Dirección para correspondencia:
Dra. MJ Unzaga, Servicio de Microbiología, Hospital de Basurto, Avda. Montevideo 18, 48013 Bilbao, Bizkaia, Spain.

Aceptado para publicación el 28 de agosto de 1996

MATERIALS AND METHODS

Specimens: Clinical specimens were obtained from patients admitted to the Hospital of Basurto, Bilbao (Spain): 9 urine samples belonged to six patients, 11 pus samples belonged to three patients, 1 peritoneal fluid, 5 blood samples (Bactec NR6A culture vials Becton Dickinson, USA), these samples from different patients. As positive control (numbers 26 and 27 in Table 1), we used two blood-heparined samples (seeded with *C. albicans*) from a healthy volunteer. All samples were yeast culture positive. Routine culture was performed on aerobic and anaerobic media according to laboratory protocol, including Sabouraud's + chloramphenicol agar. The yeast were identified by germ tube production and API AUX (bioMérieux, France).

DNA extraction procedure: Samples (1-2 ml), were lysed by a method previously described [7], consisting of a non-ionic detergent cocktail (1% Tween 20, 1% Triton-X-100, and 1% NP-40 in 0.05 mol/l Tris, pH5). To digest yeast cell walls, Zymolyase (50.000 Lyticase-Sigma, USA) was used. The samples were subsequently solubilized with Proteinase K (15µg/ml), incubated for 5 min at 37ºC, and finally extracted with phenol/chloroform/isoamyl alcohol. After ethanol precipitation the pellet was resuspended in 50 µl water.

PCR reaction: DNA amplification was carried out with primers: 520 5'-ACTGCTGCAGCACCTACGGA AACCTTGTTACG-3' and 522 5'-ATATGGATCCTAT CTGGTTGATCCTGCCAG-3' [6]. The reaction volume

was 100 µl, the amount of DNA being 2µl (30 ng). Forty cycles were performed under the following conditions: 95ºC for 1 min, 2 min at 42ºC, and 3 min at 74ºC. The expected product of 1800 bp was visualized using electrophoresis on 0.8% agarose gel in 1X TBE.

Hybridization assays: Two internal oligonucleotides probes, localized in a variable sequence within the V4 domain, were used: a *C. albicans* probe 497 5'-GTAGCC ATTTATGGCGAACC-3' and a general *Candida* probe 733 5'-GCCTTTCCTTCTGGC T-3'[6]. Probes were labeled by incorporation of a digoxigenin ddUTP in accordance with the manufacturer's instructions (Boehringer Mannheim, Germany). The experiment was developed by dot blot using the amplification products (20 µl) as a target. The hybridization conditions selected involved a temperature of 56ºC overnight. Hybridized products were detected by a colorimetric procedure: Nitro blue tetrazolium salt +X phosphate (Boehringer Mannheim)

RESULTS

25 *C. albicans*, 1 *C. parapsilopsis* and 2 *Candida* spp. were identified in clinical sample cultures. In urine, peritoneal, pus and blood samples, *C. albicans* were isolated. In a blood sample only, a yeast was identified as *C. parapsilosis* by API AUX, and in two pus samples the yeast could not be identified to species level. The yeasts were isolated in pure culture in urine, peritoneal and blood samples. Six urine yeast had counts >100000 cfu/ml, in two >10000 and <25000 cfu/ml and one <10000 cfu/ml. Pus specimens were mixed culture. The microorganisms isolated together with the yeast were *Acinetobacter baumanii, Enterococcus faecalis, Escherichia coli* and *Stenotrophomonas maltophilia* (Table 1).

The DNA extraction procedure was efficient, since the average amount was 1000 µg/ml. We had to dilute the DNA to get an optimum concentration of yeast DNA (30 ng), enabling good amplification and no inhibition due to an excessive quantitiy of DNA.

Positive PCR results according to the growth culture on Sabouraud´s agar with chloramphenicol were obtained with all samples.

Table 1. Results of culture, DNA amplification and hybridization experiments in 28 clinical samples.

Specimens	Culture	PCR	*C. albicans* probe	*Candida* spp. probe
1. Urine	*C. albicans*	+	+	+
2. Urine	*C. albicans*	+	+	+
3. Urine	*C. albicans*	+	+	+
4. Urine	*C. albicans*	+	+	+
5. Urine	*C. albicans*	+	+	+
6. Urine	*C. albicans*	+	+	+
7. Urine	*C. albicans*	+	+	+
8. Urine	*C. albicans*	+	+	+
9. Urine	*C. albicans*	+	+	+
10. Pus	*C. albicans*	+	+	+
11. Pus	*C. albicans*	+	+	+
12. Pus	*C. albicans*	+	+	-
13. Pus	*C. albicans*	+	+	-
14. Pus	*C. albicans*	+	+	-
15. Pus	*C. albicans*	+	-	+
16. Pus	*C. albicans*	+	+	+
17. Pus	*C. albicans*	+	-	+
18. Pus	*C. albicans*	+	-	+
19. Pus	*C. albicans*	+	-	+
20. Pus	*C. albicans*	+	+	+
21. Blood	*C. albicans*	+	+	+
22. Blood	*C. albicans*	+	+	-
23. Blood	*C. albicans*	+	+	+
24. Blood	*C. albicans*	+	-	+
25. Blood	*C. albicans*	+	+	+
26. Blood	*C. albicans*	+	+	+
27. Blood	*C. albicans*	+	+	+
28. Peritoneal F	*C. albicans*	+	+	+

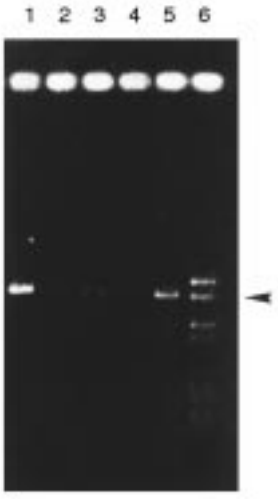

Figure 1. Electrophoresis on 0.8% agarose gel showing the PCR products. Lanes 1 and 3 : fragment of 1800 bp obtained from clinical samples. Lane 4: negative control. Lane 5: positive control. Lane 6: molecular weight marker pGEM made by digesting pGEM-3DNA to completion with HinfI, Rsa I, SinI (Promega) with the following sizes 2645, 1605, 1198, 676, 517, 460, 396, 350, 22, 179, 126, 75, 65, 41 and 36 bp.

A band of 1.8 kbp was observed when DNA from *Candida* species was amplified. In clinical specimens containing the yeast and other microorganisms (pus samples), the mentioned band was also visualized in electrophoresis gel. Multiple bands in mixed cultures did not occur (Figure 1). The amplified products were hybridized with both probes (results are shown in Table 1).

In urine samples the hybridization with the *C. albicans* probe was positive in all samples (9/9), while using the *Candida* sp. probe eight positive samples (8/9) were obtained. In blood samples and peritoneal fluid, the *C. albicans* probe also hibridized with all samples, except in the sample in which *C. pararapsilopsis* was identified, as we expected. With the *Candida* sp. probe, seven positive hybridization signals (7/8) were obtained.

In pus samples, the *Candida* sp. probe gave eight positive results (8/11) and three negative ones (numbers 12-14). The *C. albicans* probe gave two negative signals (numbers 15 and 18), despite *C. albicans* being the yeast identified in culture.

The negative hybridization with the *Candida* sp. probe assays (sample numbers 3,12,13,14,22) may be considered as a false negative, since we obtained a positive signal with the *C. albicans* probe.

DISCUSSION

The PCR has had a major impact on our ability to detect infections agents. The design of new diagnostic laboratory methods are essential for *Candida* species infections, as delayed diagnosis is important in the high mortality of systemic candidosis. PCR assays to detect *Candida* sp. in clinical samples, including blood samples, have been carried out over recent years [7-20]. Since the first PCR assay which amplified a segment of fungal DNA coding for cytochrome P450L1A1 [7-9], multiple targets have been used: the (ssu) 18S rRNA gene [5,6,10-14], a multicopy DNA fragment, EO3 [15,16] , *Candida* actin gen [17], heat shock protein 90 [18], 5SrRNA gene [19] and chitin syntethase gene [20].

The target used in our assay described above has proved its efficacy in detecting medically important *Candida* spp; moreover, the use of this gene as target has been capable of detecting other pathogenic fungi, such as *Blastomyces dermatitidis, Histoplasma capsulatum*,

Coccidiodes immitis, *Cryptococcus neoformans* [5,12]. Furthermore, this gen is present in high copy in fungal genome and finally, species-specific oligonucleotides for hybridization assays in order to confirm the identification can be selected.

The specificity of the reaction was high to detect *Candida* spp. as DNA from other microorganisms present in pus samples did not interfere with the reaction, and multiple bands did not occur in the presence of DNA from other sources.

The sensitivity of the assay is good, and indeed could be improved if a smaller fragment were used [10,11,13,14], the utilization of a whole gen amplification having the advantage that restriction enzyme analysis may be used to provide molecular typing of closely-related yeast [12].

A long target sequence has been used by others authors, but not in clinical specimens directetly as we did. Maiwald amplified a 1090bp fragment of the 18S rRNA [12] and a multicopy DNA fragment EO3 specific for *C. albicans*, unable to detect other medically important *Candida* species, was used by Miyakawa [15].

Compared with results obtained by culture, the sensitivity and specifity of the *Candida* spp. and species-specific probe were 82.1% (5 false negative) and 100% for *Candida* spp. probe and 92% (2 false-negative) and 100% for *C. albicans* probe, respectively. The negative hybridizations with *C. albicans* probe in pus samples may be due to misidentification of the strains in the germ tube production. This conclusion may be explained by the fact that the two samples belonged to the same patient as numbers 17 and 19, in which *C. albicans* was not identified. The false negative hybridization results with 733 probe may considered as mistakes in hybridization procedure or in the specifity of the probe.

Fungi cause 7.9% of nosocomial infections and *Candida* species account for 79% of the cases of clinical mycology infections reported through National Nososcomial Infections Surtveillance System [21]. Therefore, for clinical purposes and due to the increasing incidence of fungal infections, firstly the ability to amplify and detect any fungi with an all-fungi probe is crucial, and secondary with species-specific probes allow direct identification of the yeast.

This new technology may increase sensitivity and shorten detection time in comparison with conventional diagnostic methods. The time spent on the PCR assay, including the DNA yeast extraction, is about 6 h compared with the days needed for growth culture.

We conclude that this method could provide an important complement to present diagnostic methods used in the diagnosis of pathogenic fungi infections, so important in clinical practice nowadays. With this work, we would like to contribute our own experience to the diagnosis by PCR of yeast in different types of clinical specimens.

This research was supported by grants from the University of the Basque Country and Roussel Scientific Institute (Spain).

References

1. Banjee SN, Emori G, Culver DH *et al.* National Nosocomial Infections Surveillance. Secular trends in nosocomial primary bloodstream infections in the United States, 1980-1989. Am J Med 1991; 91(Suppl. 3B):86-89.
2. Pfaller M, Wenzel R. Impact of the changing epidemiology of fungal infections in the 1990s. Eur J Clin Microbiol Infect Dis 1992;11:287-291
3. Jones JM. Laboratory diagnosis of invasive candidiasis. Clin Microbiol Rev 1990;3:32-45.
4. Holmes AR, Lee YC, Cannon RD, Jenkinson HF, Shepherd MG. Yeast-specific DNA probes and their application for the detection of *Candida albicans*. J Med Microbiol 1992; 37:346-351.
5. Bowman BH. Designing a PCR/probe detection system for pathogenic fungi. Clin Immun Newsl 1992; 12:65-69.
6. Niesters HGM, Goessens WHF, Meis JFMG, Quint WGV. Rapid, polymerase chain reaction-based identification assays for *Candida* species. J Clin Microbiol 1993; 31: 904-910.
7. Buchman TG, Rossier M, Merz W, Charache P. Detection of surgical pathogens by *in vitro* DNA amplification. Part I. Rapid Identification of *Candida albicans* by *in vitro* amplification of a fungus-specific gene. Surgery 1990; 108:338-347.
8. Burgener-Kairuz P, Zuber JP, Jaunin P, Buchman TG, Bille J, Rossier M. 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-demethylase (L1A1) gene fragment. J Clin Microbiol 1994; 32:1902-1907.
9. Chryssanthou E, Andersson B, Petrini B, Löfdahl S, Tollemar J. Detection of *Candida albicans* DNA in serum by polymerase chain reaction. Scand J Infec Dis 1994; 26:479-485.
10. Deventer AJM, Goessens WHF, van Belkum A, van Vliet HJA, Etten EWM, Verbrugh HA. Improved detection of *Candida albicans* by PCR in blood of neutropenic mice with systemic candidiasis. J Clin Microbiol 1995; 33:625-628.
11. Hopfer RL, Walden P, Setterquist S, Highsmith WE. Detection and differentiation of fungi in clinical specimens using polymerase chain reaction (PCR) amplification and restriction enzyme analysis. J Med Mycol 1993;31: 65-75.
12. Maiwald M, Kappe R, Sonntag HG. Rapid Presumptive identification of medically relevant yeast to the species level by polymerase chain reaction and restriction enzyme analysis. J Med Vet Mycol 1994; 32: 115-132.
13 Makimura K, Murayama SY, Yamaguchi H. Detection of a wide range of medically important fungi by the polymerase chain reaction. J Med Microbiol 1994; 40:358-364.
14. Polanco AM, Rodríguez-Tudela JL, Martínez-Suárez JV. Detection of pathogenic fungi in human blood by the polymerase chain reaction. Eur J Clin Microbiol Dis 1995; 14: 618-621.
15. Miyakawa Y, Mabuchi T, Kagaya K, Fukazawa Y. Isolation and characterization of a species-specific DNA fragment for detection of *Candida albicans* by polymerase chain reaction .J Clin Microbiol 1992; 30: 894-900.
16. Miyakawa Y, Mabuchi T, Fukazawa Y. New method for detection of *Candida albicans* in human blood by polymerase chain reaction. J Clin Microbiol 1993; 31: 3344-3347.
17. Kan VL. Polymerase Chain Reaction for the diagnosis of candidemia. J Infect Dis 1993; 168:779-783.
18. Crampin AC, Matthews RC. Application of the polymerase chain reaction to the diagnosis of candidosis by amplification of an HSP 90 gene fragment. J Med Microbiol 1993; 39:233-238.
19. Holmes AR, Cannon RD, Shepherd MG, Jenkinson HF. Detection of *Candida albicans* and other yeast in blood by PCR. J Clin Microbiol 1994; 32: 228-231.
20. Jordan JA. PCR Identification of four medically important *Candida* species by using a single primer pair. J Clin Microbiol 1994; 32: 2962-2967.
21. Bullock W, Kozel T, Scherer S, Dixon D. Medical mycology in the 1990s: involvement of NIH and the wider community. ASM News 1993; 59: 182-185.