Application of the polymerase chain reaction to the diagnosis of candidosis by amplification of an HSP 90 gene fragment

A. C. CRAMPIN and R. C. MATTHEWS*

Department of Medical Microbiology, Manchester University Medical School, Oxford Road, Manchester M13 9PT

Summary. A 317-base pair (bp) fragment of the Candida albicans heat shock protein 90 (HSP 90) gene was amplified by the polymerase chain reaction (PCR) for detection of C. albicans DNA in clinical specimens. One hundred specimens were examined including swabs (39), urines (36), peritoneal fluid (9), pus (8) and blood or serum (8): 23\% gave positive results with routine culture, 31\% with extended broth culture and 37\% with PCR. The amplified product was identified by hybridisation with a radiolabelled internal probe and their restriction enzyme digest patterns (SspI, HaeIII, EcoRI, Rsal and XhoI), which could be predicted from the known sequence of HSP 90. C. albicans DNA gave the characteristic 317-bp band and specifically hybridised with restriction enzyme-digested candidal DNA. DNA from other sources intermittently gave multiple faint bands especially in the presence of high concentrations of DNA, but these could be readily distinguished. The method was sensitive to 50 pg of DNA (5 pg with radiolabelled probing) and 100 cfu of C. albicans.

Introduction

The incidence of serious infection with Candida albicans is increasing,1-3 and clinical diagnosis is often difficult because of non-specific symptoms and signs. Delayed diagnosis is probably a crucial contributory factor in the high mortality of systemic candidosis. A recent study documented a 38\% mortality attributable to candidosis over and above that associated with equally sick but yeast-free control patients.2 C. albicans is the species responsible for > 70\% of yeast infections.4 Blood cultures take 48–72 h to become positive and in one series were persistently negative in 56\% of patients despite extensive invasive disease at necropsy.5 Blood cultures are of limited value in assessing effectiveness of therapy or disease endpoint, which would be helpful because of the toxicity and expense of current anti-fungal therapy.5 Greater sensitivity can be achieved by measuring circulating candidal antigens, such as enolase or HSP 90, or metabolites.6-8 Even here, sensitivity is only in the order of 64–85\%, depending on the type of infection, the species, the patient population (neutropenic or post-operative) as well as the assay system. The polymerase chain reaction (PCR) may be a useful addition to these methods because of its intrinsically high sensitivity.

A PCR method for detecting C. albicans has been developed in which a fungus-specific gene coding for the cytochrome P450L1A1, a major constituent of yeast cell walls, was amplified.8 In the present study, the gene coding for C. albicans HSP 90, which has already been cloned and sequenced,10 was amplified by PCR as a method for detecting candidal DNA in clinical specimens. Primers were selected from species-specific fragments of the HSP 90 gene, which were not present in the sequenced human or Saccharomyces cerevisiae HSP 90 genes.11,12

Materials and methods

Specimens

Clinical specimens, provided by the Bacteriology Department of the Manchester Royal Infirmary and Saint Mary’s Hospital, Manchester, were processed in three ways. Routine culture was performed on Sabouraud’s agar for 48 h at 30°C and yeasts were identified by germ tube production and API C AUX (bioMérieux, Basingstoke). For extended culture, Sabouraud’s broth was inoculated and incubated for 72 h at 30°C before subculture on to Sabouraud’s agar. DNA extraction was performed on the third portion (usually 0.5 ml of a liquid specimen or 0.5 ml of sterile water inoculated with a swab) for PCR. Specimens consisted of 36 urine samples, eight pus samples, nine peritoneal dialysates and 39 high vaginal swabs from 92 different patients. Four blood specimens from a healthy volunteer, anticoagulated with lithium heparin, were also processed; two were seeded with C. albicans, (5000 cells in 100 \( \mu \)l of blood)
and two were unadulterated. A portion of each was centrifuged at 4000 rpm and the serum was processed separately, giving four whole blood and four serum samples.

Isolates

Yeast (C. albicans, C. guillermondii, C. krusei, C. parapsilosis and Torulopsis glabrata) were isolated from clinical specimens and identified by API C AUX and germ tube production. The strain of C. albicans for Southern blotting was that responsible for the London Hospital outbreak. DNA from Cryptococcus neoformans, Aspergillus fumigatus and Enterococcus faecalis was prepared from stock cultures held by the Department of Medical Microbiology, Manchester University Medical School.

Yeast DNA extraction procedure

Cells from clinical specimens or, for estimation of sensitivity, from serially diluted broth of C. albicans were pelleted and resuspended in 50 mM Tris, pH 7.5, 10 mM EDTA, 2-mercaptoethanol 1% v/v and "Lyticase" (Boehringer Mannheim) 1% v/v, and incubated at 30°C for 60 min. Sodium dodecyl sulphate was added to a final concentration at 1% and proteinase K to 15 μg/ml. The sample was incubated for a further 30 min at 56°C. Phenol-chloroform extraction was performed (at which stage any supernate constituent from the original specimen was added). DNA was harvested by ethanol precipitation. Purification and removal of inhibitors was performed on any sample containing an excess of red blood cells by a modification of the glass adhesion method described by Vogelstein and Gillespie in the form of the "Bioclean" kit (United States Biochemical Corporation, Cleveland, OH, USA). The DNA precipitate was resuspended in de-ionised water and used for PCR. All stock reagents were DNA-free and prepared and distributed with dedicated DNA-free pipettes.

PCR primers

Primers were selected from the sequenced HSP 90 gene of C. albicans from areas which were highly specific for C. albicans. Primer 1 was 5'-GAC ACC ACC ATG TCT TCT TAC-3' (852 bp from the carboxy terminal) and primer 2, 5'-GCA GAT TCT CCA GCT GGT TCG TC-3' (1146 bp from the carboxy terminal). The expected product was thus 317 bp in length. Primers were synthesised on a "PCR-Mate" DNA synthesiser (Applied Biosystems, Warrington) by standard phosphoramidite chemistry. Stocks were stored in ammonium hydroxide at -20°C and samples were dried down and resuspended in de-ionised water as required.

PCR reaction

Preliminary tests were performed to optimise the PCR reaction with regard to magnesium concentra-
tion, nucleotide concentration and cycling temperatures.\textsuperscript{16,17} The optimal reaction mixture was found to be 10 mM Tris pH 8.8, 50 mM KCl, 1.5 mM MgCl\textsubscript{2} and Triton X100 0.1% with 20 nmol of each nucleotide, and 50 pmol of each primer/100 \( \mu l \) of reaction volume. Mixtures were prepared in a DNA-free environment with dedicated DNA-free pipettes and exposed to 1000 mJ/cm\textsuperscript{2} of ultraviolet radiation to nick contaminating DNA\textsuperscript{18} before storage at \(-20^\circ\)C. As required, mixtures were thawed, 15 \( \mu l \) of test sample were added, and the reaction mixture was overlaid with two drops of mineral oil. The reaction was heated to 95\(^\circ\)C for 10 min to ensure denaturation of template DNA, then 1 \( \mu l \) of Taq polymerase (Northumbria Biologics Ltd), diluted to 2.5 U/\( \mu l \) with de-ionised water, was added. Thirty amplification cycles were performed: 94\(^\circ\)C for 90 s, 55\(^\circ\)C for 45 s, 72\(^\circ\)C for 90 s and a single final extension time of 7 min at 72\(^\circ\)C, before cooling to 4\(^\circ\)C. Negative and positive controls were included with each batch, the former for early identification of contamination.

\textbf{Visualisation and confirmation of product}

A 25 \( \mu l \) volume of the amplified reaction mixture was examined by electrophoresis in Tris-borate buffer examined on a UV transilluminator and photographed. To confirm that the 317-bp product was the result of amplification of \textit{C. albicans} DNA, the product was cut from the gel, radiolabelled (see below) and used as a probe for in-situ hybridisation with \textit{C. albicans}, \textit{Cr. neoformans}, \textit{A. fumigatus}, \textit{E. faecalis} and human DNA (Oncogene Science, NY, USA). DNA from \textit{A. fumigatus} and \textit{E. faecalis} was extracted by previously described methods\textsuperscript{19,20} and from \textit{Cr. neoformans} as described above for yeasts. The DNA was digested with EcoRI and HaeIII and transferred to a nylon membrane by Southern blotting on a vacuum blotter (BioRad). The radiolabelled PCR product was also used as a probe on 24 selected specimens with the intention of improving specificity and sensitivity. An internal oligonucleotide probe (see below) was synthesised, radiolabelled and hybridised to six of the products as further confirmation of specificity.

As a rapid and simple method of confirmation, the amplified products from two specimens were digested with restriction endonucleases \textit{SspI}, \textit{HaeIII}, \textit{EcoRI}, \textit{Rsal} and \textit{Xhol} (Northumbria Biologics Ltd) and the resulting digests were run on an agarose gel to separate any fragments. A single site for \textit{SspI} is known to lie within the HSP 90 sequence between the two primers. No such sites exist for the other restriction endonucleases listed.

\textbf{Radiolabelling of PCR product}

The product (317 bp band) was excised from a low melting point agarose gel and melted at 95\(^\circ\)C for 10 min. A 2 \( \mu l \) volume was added to a mixture of ATP, TTP, GTP nucleotides, random hexamer primers, Klenow fragment enzyme and buffer (Promega "Prime-a-gene" kit), and 1\textsuperscript{32}P CTP\textsuperscript{21} and incubated at room temperature for 60 min. The solution was run through a G-50 Sephadex column to remove unincorporated nucleotides.

\textbf{Radiolabelling of oligonucleotide probe}

An internal oligonucleotide probe (5'-GAA ACC GAT GGA GCT GAA G-3') was selected from an area specific for \textit{C. albicans} (936 bp from the carboxy end), synthesised as were the primers, and 5'-OH end labelled with 3\textsuperscript{32}P ATP, by means of T4 polynucleotide kinase and buffer.\textsuperscript{14} The reaction was run through a G-25 Sephadex column to remove unincorporated nucleotides.

\textbf{Hybridisation of probe}

The nylon membrane was incubated for 2 h in a standard pre-hybridisation fluid at a temperature compatible to the melting temperature (Tm) of the probe (50\(^\circ\)C and 65\(^\circ\)C for internal probe and PCR product, respectively). The radiolabelled probe was then added and allowed to hybridise for 4 h (internal probe), or overnight (labelled PCR product). The membrane was washed and exposed to X-ray film (Kodak X-OMAT AR) overnight at \(-70^\circ\)C. The film was developed and compared to the photograph of the original agarose gel.

\textbf{Results}

\textbf{Results from clinical samples}

Positive PCR results were gained with 10 of 36 urine samples, 3 of 8 pus samples, 3 of 9 peritoneal dialysates and 17 of 39 swabs (fig. 1). These results were compared with those of culture: one or more colonies of \textit{C. albicans} had been isolated from 3 of 36, 3 of 8, 3 of 9 and 10 of 39 samples, respectively, on routine culture and from 6 of 36, 3 of 8, 3 of 9 and 15 of 39 samples, respectively, on extended culture (table). No routine culture-positive samples were negative on

\textbf{Table. Summary of results}

<table>
<thead>
<tr>
<th>Nature of specimen</th>
<th>Total number</th>
<th>Routine culture positive</th>
<th>Extended culture positive</th>
<th>PCR positive</th>
<th>PCR false negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine</td>
<td>36</td>
<td>3</td>
<td>6</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Swab</td>
<td>39</td>
<td>10</td>
<td>15</td>
<td>17</td>
<td>5</td>
</tr>
<tr>
<td>Pus</td>
<td>8</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Peritoneal fluid</td>
<td>9</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Whole blood*</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Serum*</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>23</td>
<td>31</td>
<td>37</td>
<td>5</td>
</tr>
</tbody>
</table>

\*Specimens seeded in laboratory.
extended culture. Generally, specimens that were culture-negative or from which germ-tube negative yeasts or bacteria were grown gave negative PCR results. The exceptions were four urine samples from female patients and seven high vaginal swabs from women with vaginal discharges that were PCR-positive but gave negative results on routine or extended broth culture for *C. albicans*. Five culture-positive swabs failed to give positive results on PCR. Swabs were processed for PCR only after plates had been
inoculated for routine culture; therefore, in some cases there may have been little of the original specimen left. Whole blood seeded with C. albicans gave positive results only when inhibitors were removed by the glass adhesion method. Overall, 23% of specimens gave positive results for C. albicans on routine culture, 31% on extended culture and 37% on PCR.

Analysis of PCR product

A band of 317 bp was observed when DNA from C. albicans was amplified. When radiolabelled, the product specifically hybridised with digested C. albicans DNA and not with the DNA from A. fumigatus, Cr. neoformans, E. faecalis or human DNA. The PCR products specifically hybridised with the radiolabelled internal oligonucleotide probe and one further positive sample was identified from the serial dilutions, improving the sensitivity by a factor of 10. When digested with Ssp1, the products were separated into the two expected bands at 234 bp and 83 bp. When digestion was attempted with other restriction endonucleases (HaeIII, EcoRI, Rsal and Xhol), the products remained intact, as predicted, as their recognition sites did not occur within the sequence of interest (fig. 2).

C. guillermondii, C. krusei, C. parapsilosis, T. glabrata and human DNA extracts intermittently gave multiple faint bands on amplification if high concentrations (e. 0.5 µg) were used. T. glabrata and C. parapsilosis gave a faint band of approximately the same size as C. albicans (fig. 3). However, clinical specimens containing either of these other yeast species or human cells (e.g., pus samples) did not give bands after PCR. Negative controls included in both the DNA extraction process and the PCR remained clear.

The method was sensitive to as little as 50 pg of genomic C. albicans DNA (determined spectrophotometrically) (fig. 4), or 100 cfu of C. albicans/ml of broth. Sensitivity was increased to 5 pg of DNA by Southern blotting and in-situ hybridisation with the radiolabelled PCR product.

Discussion

Amplification of the 317-bp fragment of C. albicans HSP 90 gene with primers directed against species-specific sequences provided a sensitive and specific means of detecting candidal DNA in clinical specimens. Of the 100 specimens examined, 39% gave positive results by PCR compared to 25% by routine culture methods and 33% by extended broth culture. It is difficult to assess the clinical significance of the extra four urine samples (all from female patients) and seven high vaginal swabs (from women with vaginal discharges) that gave positive results by PCR but negative results even with extended broth culture. Some may have been "false" positive results due to detection of non-viable C. albicans. Great care was exercised to avoid cross-contamination and the success of this was shown by including negative controls at all stages.

The specificity of the reaction was high. Faint multiple bands occurred occasionally in the presence of high concentrations of DNA from other sources, particularly from other Candida species, but these could be distinguished easily from the bright 317 bp band produced with C. albicans. In contrast, Buchman et al.,9 amplifying the cytochrome P450L. A1 gene, found that species of Candida other than C. albicans...
often produced alternate bands which were preferentially amplified, probably because of ectopic primer annealing sites. In the study by Buchman et al.,9 relatively few specimens were examined and the identity of the PCR product was not confirmed by hybridisation. In the present study, the identity of the PCR product was confirmed in three ways: restriction enzyme digests, hybridisation with a radiolabelled species-specific internal probe, and radiolabelling the PCR product for hybridisation with fungal, bacterial and human DNA.

The level of sensitivity of this HSP 90 PCR assay (100 cells or 50 pg of DNA) was not as great as that reported by Buchman et al.,9 (12 cells) or Miyakawa et al.10 (10 cells). The latter group were amplifying the EO3 DNA fragment which is present in multiple copies in the C. albicans genome. They applied this to yeast isolates but not to clinical specimens. All three methods appear to be more sensitive than the use of DNA probes without amplification such as EOB1 and EOB2, against repetitive sequences, which detected 500 yeast cells.10

The PCR assay described here missed five culture-positive swabs. Sensitivity could be increased 10-fold by probing the PCR product with a radiolabelled probe. Kan and Bennett24 obtained six positive results by applying PCR amplification of the actin gene to eight patients with blood-culture positive deep candidal infections. The sensitivity of their assay system was five cells or 25 fg of DNA. They found sufficient candidal DNA in the serum, thereby avoiding the problem of inhibitors associated with processing whole blood.

In conclusion, PCR with primers directed against species-specific fragments of the HSP 90 gene provided a sensitive and specific means of detecting candidal DNA in clinical specimens. Nevertheless, some false negative results did occur, and some "false" positive results may have reflected the presence of non-viable yeasts. PCR has the advantage of providing an answer in 8 h, compared to 24–96 h for culture. In the routine diagnostic laboratory, PCR can be justified only if it enables earlier diagnosis of systemic candidosis, and this is still the subject of investigation.

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References