Comparison of Different Methods for Extraction of DNA of Fungal Pathogens from Cultures and Blood

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Received 9 June 1997/Returned for modification 8 July 1997/Accepted 5 September 1997

Five commercially available extraction kits and an in-house DNA extraction method for the release of DNA from Candida albicans and Aspergillus niger cells were assessed for sensitivity, purity, duration, and cost. All commercially available kits helped to shorten the duration of DNA extraction. However, the sensitivity varied from 1 to 1,000 fungal cells/ml and costs varied from $0.10 to 2.30. The QIAmp Tissue kit was the commercially available assay that yielded the same sensitivity and purity of fungal DNA release as the in-house protocol but was the most expensive method. In comparing these two extraction protocols, a 99% concordance of PCR results for 125 blood samples analyzed could be demonstrated.

Invasive fungal infection is an increasing cause of morbidity and mortality in the immunocompromised patient. As culture-based detection methods show low sensitivity and poor specificity, PCR-based assays have been recently developed to amplify DNA of fungal pathogens (1, 7, 8).

In order to perform a sensitive, specific, and reliable PCR-based diagnostic test, availability of pure DNA lacking PCR inhibitors as well as a rapid and easy-to-perform DNA extraction protocol is essential.

Protocols for extraction of DNA of fungal cells either are very time-consuming (3) or show poor release of fungal DNA (10) compared to methods of extraction of DNA of human cells or viruses. Other protocols require additional lysis steps like mechanical disruption or sonification or awkward toxic chemicals such as phenol-chloroform (4) or guanidine thiocyanate (9).

Zymolyase, a β-1,3-glucan laminaripentaohydrolase, may be used for converting cells to spheroplasts. The spectrum of this enzyme includes Candida, Torulopsis, and Saccharomyces strains (6). To release DNA of filamentous fungi such as Aspergillus niger, further lysis steps are necessary. Thus, mechanical destruction with glass beads (5) and freeze-thaw steps with liquid nitrogen or a heat alkali treatment have been successfully applied.

Because of daily clinical practice, we had to speed up our conventional DNA extraction method (2). Thus, we compared different DNA extraction kits to determine their sensitivity, reliability, cost per sample, and duration for DNA release of the most common pathogenic yeast, Candida albicans, and A. niger, a mold with a very complex cell wall. The method giving the best results in the tests was adapted for the extraction of DNA from clinical blood samples.

Prior to DNA extraction, Aspergillus isolates were precultured on Sabouraud agar for 72 h at 30°C and Candida isolates were precultured for 48 h at 30°C. Plates were washed with 10 ml of saline (0.9%) to obtain Aspergillus conidia. Candida colonies were inoculated in sterile saline. Thereafter, fungal suspensions were adjusted photometrically (A530; McFarland standard of 0.5) to a concentration of 1 × 10^6 to 5 × 10^6 cells/ml. Defined numbers of serially diluted fungal cells (10^6 to 10^8) were spiked into uninfected EDTA (anticoagulant)-treated blood samples to test the sensitivity of the assays.

As many pathogenic fungi (e.g., A. niger) are resistant to lysis enzymes, we established a combined heat-alkali enzymatic DNA extraction protocol. After the hypotonic lysis of the erythrocytes with erythrocyte lysis buffer (10 mM Tris [pH 7.6], 5 mM MgCl₂, 10 mM NaCl) and enzymatic lysis of the leukocytes with leukocyte lysis buffer (10 mM Tris [pH 7.6], 10 mM EDTA, 50 mM NaCl, 0.2% sodium dodecyl sulfate, 200 μg of proteinase K [Boehringer, Mannheim, Germany] per ml), the samples were incubated with 50 mM NaOH at 95°C for 10 min. Samples were neutralized with 1 M Tris, pH 7.0. In order to produce spheroplasts, pellets were incubated with 500 μl of Zymolyase solution (300 μg of Zymolyase [ICN, Costa Mesa, Calif.] per ml, 50 mM Tris [pH 7.5], 10 mM EDTA, 28 mM β-mercaptoethanol [Sigma, Deisenhofen, Germany]) for 45 min at 37°C. After an additional centrifugation step, the different DNA extraction kits (QIAamp Tissue [Qiagen, Los Angeles, Calif.], GeneReleaser [BioVentures, Murfreesboro, Tenn.], Puregene D 6000 [Genta, Minneapolis, Minn.], DNAbeads DNA DIRECT [Dynal, Oslo, Norway] and DNAzol [Molecular Research Center, Cincinnati, Ohio]) were used according to the manufacturer’s manual. Samples extracted by the in-house method were treated with 1 M Tris-EDTA–10% sodium dodecyl sulfate at 65°C for 45 min for plast lysis. Then, samples were incubated with 5 M potassium acetate for 40 min at −20°C for protein precipitation. After an additional centrifugation step (1,000 × g, 30 min), DNA precipitation was carried out with cold isopropanol. DNA was purified with 70% ethanol, air dried for 1 h, and resuspended in 40 μl of ddH₂O.

After DNA extraction, spectral photometry was performed to measure DNA content and purity. PCR was carried out with universal fungal primers binding within the 18S subunit rRNA multicyclic gene (5'-ATTGGAGGGAAGCTGGTG, 5'-CC GATCCCTAGTCGGCATAG) as published previously (2). Amplification reactions were performed with standard buffers. PCR products were transferred onto nylon membranes (Amersham, Braunschweig, Germany) by Southern slot blot technique. A species-specific probe for C. albicans and a genus-specific probe detecting most of the pathogenic Aspergillus spp. (30 pM each) were 5’ labeled with digoxigenin (Roth, Karlsruhe, Germany). After hybridization, PCR products were detected by antidigoxigenin-alkaline phosphatase (150 mU/ml;
Boehringer) and visualized by nitroblue tetrazolium and bromochlorindoylphosphate (Boehringer).

Extractions and PCR were repeated five times. To check the reliability of the assays and the presence of inhibitors, a 129-bp fragment from the human DR-β gene was coamplified (2).

The use of commercial kits did shorten the extraction procedure, but quality and quantity of released DNA varied widely. Table 1 compares the sensitivity, duration, and cost per sample of the five different kits and the in-house protocol. QIAmp Tissue and GeneReleaser proved to be as sensitive as our in-house protocol (Fig. 1). All other kits lacked sensitivity for DNA compared to our in-house method. Kits resulting in a DNA release of 2 to 3 log units below our standard method are not sensitive enough for reliable detection of low amounts of fungal DNA in patients with febrile neutropenia.

We performed our test with C. albicans cells, as this is the most common pathogenic yeast in neutropenic patients. Furthermore, extraction of DNA of the filamentous fungus A. niger was performed. Previous tests showed that DNA release from A. niger, especially, is very difficult to obtain.

Smears in the agarose gel occurred when GeneReleaser or DNAzol was used, indicating that the samples contained impure DNA. This could potentially lead to a lower sensitivity in the PCR assay or to false-negative samples because of inhibition of DNA polymerization. In contrast, QIAmp Tissue yielded good sensitivity and pure DNA. By using internal controls to check the presence of PCR inhibitors, no false-negative results were obtained.

In order to perform the PCR technique routinely, low costs per assay are very important. We established our PCR assay with a volume of 3 ml of blood, as this comparatively large amount of blood might help to increase the sensitivity of the assay because of the higher yield of fungal DNA. Therefore, we took 3 ml as the uniform level for all calculations. Thus, higher costs than those calculated by the manufacturers occurred, especially with QIAmp Tissue; we calculated costs 23 times higher than those for a conventional method.

The kit resulting in highest sensitivity and purity was adapted in order to test its value in clinical routine. A total of 95 EDTA (anti-coagulant)-treated blood samples from a selected group of nine neutropenic patients with proven or presumed invasive fungal infection were tested. Moreover, 30 blood samples from 10 healthy control persons were taken as negative controls.

Blood samples were cultured by bedside inoculation into BACTEC fungal medium and were tested daily for microbial growth by infrared detection (BACTEC 860; Becton Dickinson). All blood samples were culture negative.

DNA extraction was performed by our in-house protocol and with the QIAmp spin columns in parallel. All 30 blood samples from the 10 negative controls showed a negative PCR result in both assays, irrespective of the DNA extraction protocol applied.

Of the 95 samples from neutropenic patients, 52 were positive and 43 were negative by the in-house method, and 51 were positive and 44 were negative when QIAmp Tissue was used. Thus, a 99% correspondence between the two tests could be documented.

We thank T. Rogers, Hammersmith Hospital, London, United Kingdom, for critical review of the manuscript.

REFERENCES