Rapid Diagnosis of Fungal Infection in Patients with Acute Necrotizing Pancreatitis by Polymerase Chain Reaction

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OBJECTIVE: This study was conducted to assess the rapid diagnosis of fungal infections in patients with acute necrotizing pancreatitis by polymerase chain reaction (PCR) using universal primers targeting the 18S rRNA gene.

METHODS: In this study, a PCR assay was developed to identify clinically isolated fungi, and both PCR technique and conventional culture were used to detect fungi in 37 samples from patients with acute necrotizing pancreatitis.

RESULTS: A 197-bp fragment was amplified by PCR from all the clinically isolated fungal strains. This fragment was not isolated from gram-positive, gram-negative bacteria or human leucocytes. Thirty-seven samples of necrotic tissue or peripancreatic fluid from 11 patients were also analyzed, and eight samples were positive for fungi by PCR, six of which were also positive by conventional culture. The whole PCR procedure was completed within 7 hours.

CONCLUSION: PCR can be used to diagnose fungal infection secondary to acute necrotizing pancreatitis rapidly and sensitively. (Asian J Surg 2002;25(3):209–13)

INTRODUCTION

The natural course of acute necrotizing pancreatitis progresses in two phases. The first 14 days are characterized by the systemic inflammatory response syndrome resulting from the release of inflammatory mediators. The second phase, beginning approximately 2 weeks after the onset of the disease, is dominated by sepsis-related complications resulting from infection of pancreatic and peripancreatic necrosis; and late in the second phase, about 4 weeks after the onset, systemic fungal infection ensues in a certain percentage of patients.1,2 The clinical presentation of fungal infection in acute necrotizing pancreatitis is often nonspecific and laboratory diagnosis is often delayed due to the relatively slow growth of these yeasts from clinical specimens. As a result, the current culture-based diagnostic methods are often limited to detection of the organism in patients at an advanced stage of disease. The clinician must often make an empiric therapeutic decision and administer prophylactic antifungal drugs before culture results are obtained.3 Earlier detection of infection would permit prompt initiation of antifungal therapy with a greater likelihood for improved survival and reduced morbidity. Rapid identification of fungal infection would, therefore, be clinically helpful. Identification of minute quantities of fungus-specific DNA has been made possible by using polymerase chain reaction (PCR) techniques. The current study was conducted to evaluate the technique of using PCR with universal primers targeting fungal 18S rRNA genes to facilitate earlier diagnosis of fungal infection in patients with acute necrotizing pancreatitis.
MATERIALS AND METHODS

Patients and clinical specimens

Patients were enrolled in this study when they met both of the following conditions: 1) acute necrotizing pancreatitis confirmed by computed tomography (CT) or surgery according to the Atlanta criteria, and 2) clinical course more than 4 weeks. Between May 1998 and May 1999, 11 patients conforming to the inclusion criteria were admitted into the surgical department of Ruijin Hospital, Shanghai, and nine patients underwent necrosectomy. Thirty-seven clinical samples including 32 peripancreatic fluid and five necrotic tissue samples were obtained intraoperatively, during wound debridement or by fine needle aspiration under CT guidance. The average age of the patients was 56.0 ± 9.3 years. Eight of the patients were male and three were female; their average APACHE II score was 9.8 ± 3.3 points.

DNA extraction of fungal isolates

Fungal isolates including Candida species, Aspergillus species, Pseudallescheria boydii and Cryptococcus neoformans from the Department of Clinical Diagnosis of the Ruijin Hospital were obtained. These fungal isolates were recovered from patients with acute necrotizing pancreatitis, and were cultured onto Sabouraud dextrose agar slants, incubated at 37°C overnight and maintained at 4°C. Cultured fungal cells (50 µL) were prepared in 100 µL of spheroplast buffer (1.0 M sorbitol, 50.0 mM sodium phosphate monobasic, 0.1% 2-mercaptoethanol, 10 µ/mL lyticase). Pellets were suspended for 30 minutes at 56°C. After centrifugation for 5 minutes at 10,000 rpm, the supernatant was discarded and the sediment was preserved. The sediment was then lysed in 10% chelex-100 (Sigma, St. Louis, MO, USA), 0.03% sodium dodecyl sulfate, 1% Tween 20, 1% Nonidetp-40 for 5 minutes at 95°C for DNA extraction.

DNA extraction of clinical samples

Peripancreatic fluid or necrotic tissue samples were diluted with double-distilled H₂O. Fungal DNA (0.5ml) samples were transferred into 1.5 ml sterile Eppendorf tubes, and centrifugated at 3,000 rpm for 1 minute. The resulting sediment was preserved and lysed in 1 ml of spheroplast buffer for further DNA extraction.

DNA extraction of human leucocytes

First, 2 ml of blood from healthy volunteers were drawn into Na₂EDTA tubes, then 1 ml of blood was transferred from the Na₂EDTA tubes to sterile 1.5 ml Eppendorf tubes. Red cells were lyzed in 0.32 M of sugar, 5 mmol of MgCl₂, 0.01 M of Tris-Cl, 1% Triton-X for 10 minutes at room temperature. After centrifugation for 5 minutes at 5,000 rpm, the supernatant was discarded and the sediment was treated as above.

DNA extraction from bacterial isolates

Bacterial isolates including Escherichia coli and Staphylococcus aureus were provided by the Department of Clinical Diagnosis and cultured conventionally. The cells were prepared in 10% chelex-100 (Sigma), 0.03% sodium dodecyl sulfate, 1% Tween-20, 1% Nonidetp-40 for 5 minutes at 95°C for DNA extraction.

Positive and negative controls

DNA from clinical isolates of Candida albicans was extracted in the same manner as outlined previously. This DNA was used to determine if the PCR reaction was successful. In addition to a positive control, each PCR experiment contained a reagent, negative control that consisted of all PCR reagents, but without DNA, to determine the presence of contamination.

Amplification of extracted DNAs

PCR was performed in 50 µL of reaction mixture (MJ, Research, INC, New York, USA). The final PCR mixture consisted of 35.5 µL of double-distilled water, 4.0 µL of a deoxynucleoside triphosphate (dNTP) mixture (0.08 mM for each dNTP), 2.0 µL of primer 1, 2.0 µL of primer 2, 5.0 µL of 10 x concentrated PCR buffer (15 mM MgCl₂, 500 mM KCl, 100mM Tris-Cl, and 0.01% gelatin), and 0.5 µL of Taq polymerase (Sigma, Shanghai, PRC). Amplification by PCR was performed under standard reaction conditions: one cycle of 94°C for 5 minutes, 55°C for 1 minute, and 72°C for 7 minutes; and a 4°C soak. Oligonucleotides used as primers in this study were AGC TCT TTC TTG ATT TTG TGG and GCA TCA CAG ACC TGT TAT TGC CTC, which amplify a 197-bp segment of a small nuclear rRNA fungal gene.
Identification of PCR products

After amplification, 2 µL of PCR products were electrophoresed on 2% agarose gel at 60 V for 60 minutes. DNA bands were detected by ethidium bromide staining and visualized by UV light photography.

Culture of clinical specimens

Necrotic tissues were minced in a sterile environment and peripancreatic fluid specimens over 2 ml in volume were concentrated by centrifugation. Following this, the specimens were inoculated onto Sabouraud agar slants and incubated at 37°C for an average of 5 days.

RESULTS

PCR detection of fungal isolates

Isolates of Candida spp. including albicans (n = 30), tropicalis (n = 23) and parapsilosis (n = 3), isolates of Aspergillus spp. including A. fumigatus (n = 14), A. flavus (n = 2), isolates of P. boydii (n = 2) and C. neoformans (n = 1) (provided by Department of Clinical Diagnosis, Ruijin Hospital) were studied. DNA samples were extracted, PCR amplification performed and a 197-bp band was identified (Figure).

PCR detection of bacterial and human leucocyte DNA

DNA from human leucocytes, S. aureus and E. coli were extracted and PCR amplification was completed. No 197-bp band was identified.

Fungal detection in clinical specimens

Thirty-two samples of peripancreatic fluid and five samples of necrotic tissue (37 in total) were collected from

Table. Detection results of 14 clinical samples by PCR and culture

<table>
<thead>
<tr>
<th>Case</th>
<th>Samples</th>
<th>Culture</th>
<th>PCR</th>
<th>Clinical outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Peripancreatic fluid</td>
<td><em>Candida albicans</em></td>
<td>+</td>
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<tr>
<td></td>
<td>Peripancreatic fluid</td>
<td>-</td>
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<td>survived</td>
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<td></td>
<td>Peripancreatic fluid</td>
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<tr>
<td>2</td>
<td>Peripancreatic fluid</td>
<td><em>Candida albicans</em></td>
<td>+</td>
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<tr>
<td></td>
<td>Peripancreatic fluid</td>
<td><em>Candida albicans</em></td>
<td>+</td>
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<tr>
<td></td>
<td>Peripancreatic fluid</td>
<td><em>Candida albicans</em></td>
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<td></td>
<td>Peripancreatic fluid</td>
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<td></td>
<td>Peripancreatic fluid</td>
<td>-</td>
<td>-</td>
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<tr>
<td>3</td>
<td>Necrotic tissue</td>
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<td>-</td>
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</tr>
<tr>
<td></td>
<td>Peripancreatic fluid</td>
<td><em>Candida albicans</em></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Peripancreatic fluid</td>
<td><em>Candida albicans</em></td>
<td>+</td>
<td>died</td>
</tr>
</tbody>
</table>

Data were time-ordered in the same patient. The remaining 23 samples from eight patients were both PCR and culture negative.
11 patients. Fungal DNA was extracted and amplified as above and clinical samples were sent for conventional cultures simultaneously. The necrotic tissues were negative for fungus by both conventional culture and PCR. Eight out of 32 peripancreatic fluid samples were positive for fungus on PCR, yielding a 197-bp band. Six were culture-positive and two culture-negative. The fungi recovered were \textit{C. albicans}. The eight samples positive for fungus on PCR were derived from three patients. Six of the culture-positive samples also came from the same three patients (Table). The remaining 24 peripancreatic fluid samples were both PCR- and culture-negative. Sensitivity and specificity of PCR detection were 100% and 93.5%, respectively.

**DISCUSSION**

Late in the second phase of the natural course of acute necrotizing pancreatitis (i.e., 4 weeks after onset), systemic fungal infection occurs in some patients, mainly as a result of the prophylactic use of broad-spectrum antibacterial agents, including third-generation cephalosporins and imipenem, which suppress the intestinal bacterial flora and allow the proliferation of fungi within the gastrointestinal tract.\textsuperscript{1,2} However, the diagnosis of fungal infection is problematic, as the clinical presentation can be rather variable and nonspecific. Fever occurs in up to 80% of cases and leukocytosis in up to 50%. Patients with fungal infection may not immediately appear to be seriously ill, whilst others may present with septic shock.\textsuperscript{3} The diagnostic standard is conventional fungal culture, but for urine, aspirate, tissue and respiratory specimens, at least 95% of the isolates that were ultimately recovered had become evident by day 7, and the rate of recovery at 7 days was slightly lower for cerebrospinal fluid (93%), stool (91%), and nose, mouth, and sinus specimens (90%), due to the relatively slow growth of these yeasts from clinical specimens.\textsuperscript{6} Therefore, early detection and identification of fungi is critical for the proper targeting of antifungal therapy.

Universal primers common to all fungi have been used as a promising approach for clinical diagnosis. The 18S rRNA sequence is highly conserved through the phylogenetic tree. This conserved sequence of the 18S rRNA has led to the development of conserved primers for PCR for the detection of fungi.\textsuperscript{4,7} In this study, we developed a fungal PCR assay, including fungal DNA extraction, PCR amplification, identification of PCR products, PCR contamination monitoring via negative and positive controls. A heavy wall surrounds the fungal cell, and this cell wall should be destroyed before DNA extraction with lyticate at 56°C for 30 minutes. Fungal pellets were then lysed in 10% chelex-100 (Sigma), 0.03% sodium dodecyl sulfate, 1% Tween-20, 1% Nonidetp-40 for 5 minutes at 95°C for DNA extraction, and the prepared DNA was amplified directly.

The whole process of DNA extraction required less than 1 hour, while DNA amplification took 3.5 hours. One hour was needed for identification. The whole procedure was completed within 7 hours, in contrast to a mean of 5 days by routine identification methods. Other advantages of this PCR assay include a simple DNA extraction method, and no interference in the detection of yeasts in samples containing bacteria, compared with conventional culture which could be affected by overgrowth of bacteria. Because of the minute quantity of reaction reagents used in the detection, the cost of PCR diagnosis is comparable to that of conventional culture.

In our study, we detected by PCR assay the fungal isolates commonly identified in patients with acute necrotizing pancreatitis, positive PCR assays yielding the 197-bp DNA product. At the same time, samples from healthy human leucocytes, \textit{S. aureus} and \textit{E. coli} were negative by PCR and no detectable PCR amplicon was demonstrated by gel electrophoresis and EB staining, suggestive of high specificity.

Thirty-seven samples were collected and underwent both conventional culture and PCR assay. Eight samples were positive for fungal DNA on PCR, of which six were also culture-positive. The culture-positive samples were obtained from three patients, and antifungal drugs such as fluconazole or amphotericin B were initiated as soon as the PCR results were obtained. One patient died from multi-organ failure. With each PCR assay of clinical samples, a negative control was employed to safeguard against the potential contamination of the stock PCR reagent with fungal DNA products in the environment, and this study showed no false-positive results, which signified that the PCR results for clinical samples were reliable. Therefore, as long as the environmental contamination is controlled, it is practical that the PCR test can be used in peripheral non-teaching hospitals.

The two PCR-positive but culture-negative samples came from patients treated with amphotericin B. Before
antifungal treatment, both amplification and culture were positive. Two weeks following antifungal treatment, samples were again collected for PCR and culture detection: PCR was positive but culture was negative in one patient, while the other patient had positive results by both detection methods. Antifungals were continued for another 7 days, after which both PCR and culture were negative in the former patient. After another 10 days of amphotericin B administration, the second patient had both PCR- and culture-negative samples. Flahaut et al reported two false-positive PCR samples from patients treated with azole antifungal agents, both samples being positive for culture before the initiation of antifungal treatment. One may conclude from this that PCR is probably able to detect damaged or inhibited organisms resulting from antifungal treatment that would not otherwise be recovered by culture.

In conclusion, the PCR assay using universal primers targeting 18S rRNA gene can rapidly and sensitively diagnose fungal infection secondary to acute necrotizing pancreatitis. Further study using large samples is under way to confirm this conclusion.

REFERENCES