Rapid detection of sepsis complicating acute necrotizing pancreatitis using polymerase chain reaction

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INTRODUCTION
Acute necrotizing pancreatitis usually takes a severe clinical course and is associated with multiple organ dysfunction. With the further understanding of pathophysiological events of acute pancreatitis and the therapeutic measures taken by the clinicians, the patients can pass through the critical early stages, and then the septic complication caused by translocated bacteria, mostly gram-negative microbes from the intestines ensues[1]. During this stage, the clinical manifestation is not specific and is characterized by systemic inflammatory response, but bacterial cultures are often negative.

Identification of minute quantities of microbial-specific DNA has been made possible by using polymerase chain reaction techniques[2-19] and this method has been used to detect and identify specific pathogen in clinical specimens. It has been shown that PCR method is more sensitive than conventional blood cultures for detecting microbial products in blood[20-22].

The current study was performed to evaluate the technique of PCR with the universal primers targeting bacterial 16S rRNA genes in diagnosing the systemic infection secondary to acute necrotizing pancreatitis.

MATERIALS AND METHODS

Patients and sample collection
Between May 1998 and May 1999, 22 blood samples were obtained from 13 patients with CT or surgically confirmed acute necrotizing pancreatitis who were admitted consecutively to surgical ICU in Ruijin Hospital, Shanghai. There were 8 men and 5 women, the average age was 56.6 ± 8.9 years, and the average APACHE II scores were 10.5 ± 2.2 points.

The blood samples were drawn if the patients presented two or more of the following conditions: (1) temperature more than 38°C or less than 36°C, (2) elevated heart rate more than 90 beats per minute, (3) respiratory rate more than 20 breaths per minute or PaCO₂ less than 32 mmHg, and (4) white blood cell count more than 12 000/cu mm, less than 4 000/cu mm, or more than 10% immature band forms. And the foci of infection were documented[23-26].

Twelve mL of blood was drawn from each patient, of which 2 milliliter was collected in sterile Na₂EDTA anticoagulant Eppendorf tubes and stored at 4°C until DNA extraction was performed, 10 milliliter was sent for conventional blood cultures.

At the same time, 10 blood samples were obtained from 10 healthy volunteers for controlled study.

Bacterial strains
The bacterial strains used were clinical isolates collected from Ruijin Hospital and identified by automated Vitek system. The strains were cultured at 37°C on blood agar plates until DNA extraction was performed, 10 milliliter was sent for conventional blood cultures.

DNA extraction
Blood was transferred from Na₂EDTA tubes to sterile 1.5 mL Eppendorf tubes, red cells were lysed in 0.32M sugar-5 mmol MgCl₂-0.01M 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 sediment was preserved for DNA extraction.

The sediment was lysed in 10% Chelex-100 (Sigma) - 0.03% Sodium dodecyl sulfate - 1% Tween 20-1% Nonidetp-40 for 5 minutes at 95°C. After centrifugation (5 000 rpm) for 10 seconds, 5 µL of the supernatant was directly used for PCR amplification[2].
Oligonucleotide primers for PCR
One set of oligonucleotide primer pair was synthesized by the Promega Company, Shanghai Office. The target DNA sequence was the 16S rRNA gene. This set of primers was 5’-GGC GGA CGG GTG AGT AA-3’ and 5’-ACT GCT GCC TCC CGT AG-3’ to amplify a 255 bp region.

Positive and negative controls
DNA from clinical isolates of {E.coli} was extracted in the same manner as outlined previously. This DNA was used in PCR reactions 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 whether the potential contamination was present.

Microbial DNA amplification
PCR assay was established according to the protocols described by Widjojoatmodjo et al[2]. The PCR mixture (50 µL) contained 50 mM Tris-HCl, 200 mM each deoxynucleoside triphosphate (dNTP), 0.4 µM each primer and 1.0 u of Super-Taq Polymerase (Promega Company, Shanghai Office ) and 7 mM MgCl₂.

The PCR was performed in a DNA Thermal Controller (MJ, Research, INC, USA) as follows. The first step of 5 minutes at 94 °C was followed by 30 cycles of 30 seconds at 94 °C, 10 seconds at 72 °C and 1 minute at 55 °C; and extension period of 2 minutes at 55 °C completed the cycling sequence.

Identification of PCR products
After amplification, 5 µL of PCR products was run on a 1% agarose gel in 0.5 × TBE. DNA bands were detected by ethidium bromide staining and visualized by UV light photography.

Blood cultures
Blood obtained for culture was collected from patients in a sterile manner and inoculated directly into aerobic and anaerobic bottles. The procedure was performed in the department of clinical diagnosis, Ruijin Hospital.

Statistical analysis
Statistical analysis was done by using the Chi-square test. The difference was considered significant at P<0.05.

RESULTS
There was only 1 positive blood culture in the 22 blood samples of 13 patients (4.55%). The organism was {Escherichia coli} (Table 1). But PCR amplification was positive for 8 samples (36.36% P<0.05 vs culture) from patients and all clinical isolates, yielding the 255 bp band (Figure 1). No DNA amplification occurred in the blood samples from volunteers.

Table 1 Results of PCR and blood culture data for ANP patients

| Sample No | Age (yr) | Gender | Blood culture | PCR | T (°C) | HR (beats /min) | WBC (10⁹/L) |
|-----------|---------|--------|---------------|-----|--------|----------------|-------------|
| 1         | 53      | M      | -             | +   | 39     | 120            | 20          | 13.5        |
| 2*        | 36      | M      | +             | +   | 39.1   | 116            | 22          | 14.3        |
| 3*        | 51      | M      | -             | +   | 39.1   | 130            | 24          | 17.1        |
| 4         | 55      | M      | -             | +   | 38.3   | 100            | 22          | 17.5        |
| 5         | 67 F    | -      | -             | -   | 38.2   | 110            | 24          | 12.8        |
| 6*        | 54      | M      | -             | -   | 38.5   | 116            | 34          | 14.4        |
| 7         | 57      | M      | -             | -   | 37.9   | 116            | 22          | 12.2        |
| 8*        | 51      | M      | +             | -   | 39.7   | 180            | 26          | 18.6        |
| 9*        | 54      | F      | -             | +   | 39.6   | 128            | 37          | 17.7        |
| 10        | 60 F    | -      | -             | -   | 41.3   | 130            | 18          | 18.5        |
| 11*       | 61      | F      | -             | -   | 39.3   | 170            | 26          | 20.4        |
| 12        | 60      | M      | -             | -   | 38.5   | 116            | 22          | 8.9         |
| 13        | 74      | F      | -             | -   | 38.5   | 116            | 22          | 16          |

The data are time-ordered in the same patient; Mechanic- means mechanical ventilation and ANP is the abbreviation of acute necrotizing pancreatitis.

DISCUSSION
During the late course of acute necrotizing pancreatitis, starting from the second week, local and systemic complication caused by translocated bacteria from intestines are dominant. The infection occurs in 30% to 40% of patients with acute necrotizing pancreatitis. Around 80% of deaths in patients with acute necrotizing pancreatitis are caused by septic complication[1]. But during this stage, the clinical manifestation is not specific and...
characterized by systemic inflammatory reaction and the blood culture is usually negative; this will levy a heavy toll on the clinician for the prompt management of the patients.

Recent studies showed that blood culture techniques, such as volume of inoculated blood, culture media could significantly influence the recovery of bacteria in clinically suspected septic patients and culture is more time-consuming[27-30].

Molecular biology techniques, such as PCR have been used in making a specific and sensitive diagnosis of bacterial infection[2,8,9,12,13,15,16]. The 16S rRNA sequence is highly conserved through the phylogenetic tree. The conserved sequences of the 16S rRNA have led to the development of conserved primers for PCR for the detection of eubacteria. Recently the PCR with universal primers targeting 16S rRNA genes has been used widely to define bacteria[2,15,16,31-41].

With the protocol described by Widjooatojmodjo et al[2,31], we developed PCR assay by using the 16S rRNA genes as the amplification targets. In this assay, we found no DNA amplification in healthy blood cells, suggestive of high specificity of these primer pairs. The disadvantage of PCR technique is the contamination of DNA templates, and therefore we employed negative controls at each PCR experiment to safeguard against the potential contamination of stock PCR reagents with microbial DNA products in the environment, and this study showed no false-positive results (Figure 1).

The gold standard of identifying sepsis is blood culture; however, the clinical sepsis is observed in the absence of documented infection in more than 50% of patients with MOF[21] and the prevalence of positive blood culture is around 12%. The positive rate of blood cultures in our study was 4.55% (propably due to small sample), whereas the PCR-positive rate was 36.36% (P<0.05), which signifies that this detection method has higher sensitivity than blood culture.

Another advantage of this PCR assay is its ability to perform serial measurements in the same patient for detection of bacterial DNA in the blood, as shown in patients 2, 3, 6, 8, 9 and 11 (Table 1), because PCR is time-saving (less than 8 hours) and blood cultures usually take much longer time (at least 2 days).

However, this detection method cannot identify whether it represents living invading organisms or dead ones engulfed by phagocytes, so this approach cannot differentiate between controlled and invasive infections. Until methods that quantitate bacterial DNA are developed[36], we should combined the results of PCR assays with relevant clinical information to determine whether the sepsis is present. Furthermore, if we apply multiple oligonucleotide primers in the PCR assay[21], there would be a higher PCR-positive rate.

In conclusion, the PCR assay with universal primers targeting 16S rRNA genes is more sensitive in detecting the sepsis secondary to acute necrotizing pancreatitis and this may prompt us to take more aggressive approach to the disease.

REFERENCES
1 Beger HG, Rau B, Mayer J, Pralle U. Natural course of acute pancreatitis. World J Surg 1997;21:130-135
2 Widjooatojmodjo MN, Fluit AC, Verhoef J. Molecular identification of bacteria by fluorescence based PCR single strand conformation polymorphism analysis of the 16S rRNA gene. J Clin Microbiol 1996;34:424-426
3 An P, Li SY, Han LX. The value PCR direct detection in HBsAg negative liver diseases. Xin Xiaohuabingxue Zazhi, 1996;4:385-386
4 Shi FJ, Li XZ, Wang CS. The detection of HBV DNA in digestive carcinomatous tissue by PCR. Xin Xiaohuabingxue Zazhi, 1996;4:408-409
5 Liang YR, Cheng JD. Nested PCR demonstrated: hepatocellular carcinoma had HBV-DNA replication. Xin Xiaohuabingxue Zazhi, 1996;4:424-426
6 Cheng JD, Liang YR. A study on HBVDNA in hepaticcellular carcinoma and para-carcinomatous tissue by location PCR. Xin Xiaohuabingxue Zazhi, 1996;4:427-429
7 Ou YH, Yao QX, Kang AN, Jiah ZH, Niu Y. Investigation of HBV infection in blood donors with PCR. Xin Xiaohuabingxue Zazhi, 1997;5:108-109
8 Li RP, Zhang ZK, Yao XX, Ren XL, Li LG. Evaluation of PCR method in detection of Helicobacter pylori infection. Xin Xiaohuabingxue Zazhi, 1997;5:301-302
9 Ji XH, Xu GM, Li ZS, Man XH, Zhang HF, Xu AF. Clinical diagnostic value of PCR in gastric H. pylori infection. Xin Xiaohuabingxue Zazhi, 1997;5:364-366
10 Tang W, Du SC, Tao QM, Zhu L. A study on anti contamination of RT-PCR in detection of HCV-RNA. Xin Xiaohuabingxue Zazhi, 1997;5:638-639
11 Zheng N, Yu ZY, Zha SN. Detection of hepatitis B virus DNA in liver cancer tissue by in situ polymerase chain reaction. Huaren Xiaohua Zazhi, 1998;6:371-373
12 Hua JS, Zheng PY, Bow H, Megraud F. Detection of Helicobacter pylori in gastric biopsy by polymerase chain reaction. Huaren Xiaohua Zazhi, 1998;6:377-379
13 Hua JS, Zheng PY, Bo B. Species differentiation and identification in the genus of Helicobacter. World J Gastroentero, 1999;5:7-9
14 Yamashita Y, Kohno S, Koga H, Tomono K, Kaku M. Detection of Bacteroides fragilis in clinical specimens by PCR. J Clin Microbiol, 1994;32:679-683
15 Greisen K, Loeffelholz M, Purohit A, Leong D. PCR primers and probes for the 16S rRNA gene of most species of pathogenic bacteria, including bacteria found in cerebrospinal fluid. J Clin Microbiol, 1995;33:355-356
16 Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC, Hiom SJ, Wade WG. Design and evaluation of useful bacterium specific PCR primers that amplify genes coding for bacterial 16S rRNA. Appl Environ Microbiol, 1998;64:795-799
17 Walsh TJ, Francesconi A, Kusai M, Chanock SJ. PCR and single strand conformational polymorphism for recognition of medically important opportunistic fungi. J Clin Microbiol, 1995;33:3216-3220
18 Mannarelli BM, Kurtzman CP. Rapid identification of Candida albicans and other human pathogenic yeasts by using short oligonucleotides in a PCR. J Clin Microbiol, 1998;36:1634-1641
19 Zhang WZ, Han TQ, Tang YQ, Wan ZM, Zhang SD. Study on rapid diagnosis of fungal infection in the patients with acute necrotizing pancreatitis by polymerase chain reaction. Acta Paediatria, 1997;86:1097-1099
20 Lafortuga N, Coppola B, Carbone R, Grassi A, Mautone A, Iolascon A. Rapid detection of neonatal septis using polymerase chain reaction. Zhongguo Shiyan Zhenduanxue, 2000;4:109-111
21 Kane TD, Alexander JW, Johannigman JA. The detection of microbial DNA in the blood. Ann Surg, 1998;227:1-9
22 Kane TD, Johnson SR, Alexander JW, Babcock GF, Ogle CK. Detection of intestinal bacterial translocation using PCR. J Surg Res,
American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference Committee. The ACCP/SCCM Consensus Conference. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. *Chest*, 1992;101:1644-1655

Bone RC, Sibbald WJ, Sprung CL. The ACCP-SCCM consensus conference on sepsis and organ failure. *Chest*, 1992;101:1481-1482

Salvo I, de Cian W, Musico M, Langer M, Piadena R, Wolffler A, Montani C, Magni F. The SEPSIS study group. The Italian SEPSIS study: preliminary results on the incidence and evolution of SIRS, sepsis, severe sepsis and septic shock. *Intensive Care Med*, 1995;21:S244-S249

Rangel-Frausto MS, Costigan M, Hwang T, Davis CS, Wenzel RP. The natural history of the systemic inflammatory response syndrome (SIRS). *JAMA*, 1992;273:117-123

Murray PR, Traynor P, Hopson D. Critical assessment of blood culture techniques: analysis of recovery of obligate and facultative anaerobes, strict aerobic bacteria, and fungi in aerobic and anaerobic blood culture bottles. *J Clin Microbiol*, 1992;30:1462-1468

Weinstein MP, Mirrett S, Wilson ML, Reimer LG, Reller LB. Controlled evaluation of 5 versus 10 milliliters of blood cultured in aerobic BacT/Alert blood culture bottles. *J Clin Microbiol*, 1994;32:2103-2106

Wilson ML, Weinstein MP, Mirrett S, Reimer LG, Feldman RJ, Chuard CR, Reller LB. Controlled evaluation of BacT/Alert standard anaerobic and FAN anaerobic blood culture bottles for the detection of bacteremia and fungemia. *J Clin Microbiol*, 1995;33:2265-2270

McDonald LC, Fune J, Gaido LB, Weinstein MP, Reimer LG, Flynn TM, Wilson ML, Mirrett S, Reller LB. Clinical importance of increased sensitivity of BacT/Alert FAN aerobic and anaerobic blood culture bottles. *J Clin Microbiol*, 1996;34:2180-2184

Widjojoatmodjo MN, Flatt AC, Verhoef J. Rapid identification of bacteria by PCR single strand conformation polymorphism. *J Clin Microbiol*, 1994;32:3002-3007

Weiss J, Mecca J, Silva E, Gassner D. Comparison of PCR and other diagnostic techniques for detection of *Helicobacter pylori* infection in dyspeptic patients. *J Clin Microbiol*, 1994;32:1663-1668

Rdstem P, Bckman A, Qian N, Kragsbjerg P, Pilsen C, Olesen P. Detection of bacterial DNA in cerebrospinal fluid by an assay for simultaneous detection of Neisseria meningitidis, Haemophilus influenzae, and streptococci using a seminested PCR strategy. *J Clin Microbiol*, 1994;32:2738-2744

West B, Wilson SM, Changalucha J, Patel S, Mayaud P, Ballard RC, Mabey D. Simplified PCR for detection of Haemophilus ducreyi and diagnosis of chancroid. *J Clin Microbiol*, 1995;33:787-790

Battles JK, Williamson JC, Pike KM, Gorelick PL, Ward JM, Gonda MA. Diagnostic assay for Helicobacter hepaticus based on nucleotide sequence of its 16S rRNA gene. *J Clin Microbiol*, 1995;33:1344-1347

Wang RF, Cao WW, Cemuglia CE. PCR detection and quantitation of predominant anaerobic bacteria in human and animal fecal samples. *Appl Environ Microbiol*, 1996;62:1242-1247

Karttunen TJ, Genta RM, Yoffe B, Hachem CY, Graham DY, EL zaatari FAK. Detection of *Helicobacter pylori* in paraffin embedded gastric biopsy specimens by in situ hybridization. *Am J Pathol*, 1996;106:305-311

Goldenberger D, Knzli A, Vogt P, Zbinden R, Altwegg M. Molecular diagnosis of bacterial endocarditis by broad range PCR amplification and direct sequencing. *J Clin Microbiol*, 1997;35:2733-2739

Ley BE, Linton CJ, Longhurst S, Jalal H, Millar MR. Eubacterial approach to the diagnosis of bacterial infection. *Arch Dis Child*, 1997;77:148-149

Messick JB, Berent LM, Cooper SK. Development and evaluation of a PCR-based assay for detection of Haemobartonella felis in cats and differentiation of H. felis from related bacteria by restriction fragment length polymorphism analysis. *J Clin Microbiol*, 1998;36:462-466

Matar GM, Sidani N, Fayad M, Hadi U. Two step PCR based assay for identification of bacterial etiology of otitis media with effusion in infected Lebanese children. *J Clin Microbiol*, 1998;36:1185-1188