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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 measuses 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℃ or less than 36℃, ② elevated heart rate more than 90 beats per minute, ③ respiratory rate more than 20 breaths per minute or $PaCO_2$ 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 Na_2 EDTA anticoagulant Eppendorf tubes and stored at 4℃ 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℃ on blood agar plates until DNA extraction was performed.

DNA extraction

Blood was transferred from Na_2EDTA tubes to sterile 1.5 mL Eppendorf tubes, red cells were lysed in 0.32M sugar-5 mmol $MgCl_2$-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℃. After centrifugation (5 000 rpm) for 10 seconds, 5 μL of the supernatant was directly used for PCR amplification[2].

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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 $MgCl_2$.

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

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 (℃)	HR (beats / min)	R (breaths / min)	WBC (10^9/L)
1	53	M	-	+	39	120	20	13.5
2*	36	M	+	+	39.1	116	22	14.3
			-	+	38.6	130	25	17.9
			-	-	38.1	114	22	9.8
3*	51	M	-	+	39.1	130	24	17.1
			-	-	39.1	140	24	18.3
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
			-	-	37.9	116	22	12.2
			-	-	37.2	95	18	18.7
			-	-	37.9	118	23	18.3
7	57	M	-	-	39.5	120	26	18.6
8*	51	M	-	+	39.7	180	Mechanic	19.2
			-	-	39.2	130	Mechanic	13.4
9*	54	F	-	+	39.6	128	Mechanic	37.7
			-	-	41.3	130	Mechanic	18.5
10	60	F	-	-	39.3	170	Mechanic	9.3
11*	61	F	-	-	39.1	126	28	20.4
			-	+	38.5	116	22	8.9
12	60	M	-	-	38.5	108	22	16
13	74	F	-	-	38	116	Mechanic	18.6

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

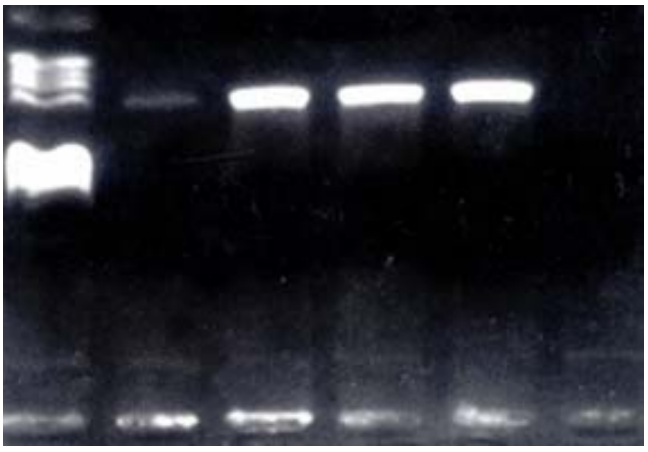

Figure 1 Agarose gel electrophoresis of amplified DNA from clinical samples and controls and volunteers.
Lane 1:DNA marker; 2: Case 2; 3: Case 8; 4: *Escherichia Coli*; 5: *Staphylococcus aureus*; 6:human leucocyte

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 Widjojoatmodjo *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 reagants 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.

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