Value of 16S rRNA microarray detection in early diagnosis of neonatal septicemia

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***Background*: Neonatal septicemia is a serious life-threatening infectious disease. Early diagnosis will improve the effectiveness of intervention, prognosis, and will reduce complications. This study was undertaken to explore the value of 16S rRNA oligonucleotide microarray in the early diagnosis of neonatal septicemia.**

***Methods*: Primers and oligonucleotide probe against the 16S rRNA gene were designed, then the probe was fixed on a specially designed glass slide to make a microarray. Venous blood was drawn from 285 neonates who were suspected of bacterial infection for blood culture and gene analysis of bacterial 16S rRNA, respectively. The DNA extracted from blood sample and cerebrospinal fluid was amplified by PCR, and the positive products were applied to the microarray for hybridization. Finally, the chip was scanned with laser, and the results were analyzed.**

***Results*: The positive rate of PCR detection was 5.96% (17/285), significantly higher than that of blood culture (2.81%, 8/285) ($P < 0.01$). In contrast to the confirmed diagnosis of septicemia in these patients, the sensitivity, specificity and correct diagnosis index of PCR were 100%, 96.75%, and 0.968, respectively. Microarray hybridization was carried out for the 17 samples of PCR-positive reaction. Positive results were detected in all samples by universal probes: G^+ probe in 12 samples, and G^- probe in 5 samples. In the 8 samples with positive results shown by PCR and blood culture, the results of microarray hybridization were consistent with those of blood culture in detecting bacterial species.**

***Conclusions*: Microarray technique can be used to rapidly diagnose neonatal septicemia by specially detecting the 16S rRNA gene in clinical samples. The sensitivity and specificity of this technique are much higher than those of blood culture, PCR, and other methods. It also can quickly identify the pathogen of the disease.**

World J Pediatr 2005;2:121-126

***Key words*: septicemia; ribonucleic acid; bacteria; polymerase chain reaction; microarray**

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Introduction

Neonatal septicemia is a serious life-threatening infectious disease. Although the methods of bacterial culture and histological and immunological examination are widely used, they all have some shortcomings in detection of the pathogens.[1,2] To explore a quick and reliable diagnostic tool, we designed primers within the conserved region of the 16S rRNA gene, and developed a PCR method for detecting the 16S rRNA gene in all kinds of bacteria. We analyzed the 16S rRNA microarray for the samples from 285 neonates suspected by bacterial infection from June to November 2003.

Methods

Subjects

A total of 285 neonates hospitalized from June to November 2003 were suspected to have bacterial infection because of susceptible factors and clinical manifestations.[3] These neonates aged from 2 hours to 28 days included 25 premature infants and 8 twin infants. Thirty neonates with noninfectious diseases served as controls in this study. One ml venous blood was taken from each neonate for blood culture and bacterial analysis of the 16S rRNA gene. Blood culture showed positive results in one neonate whose cerebrospinal fluid was drawn subsequently for bacterial analysis of the 16S rRNA gene.

Materials

Bacterial strain

A total of 110 strains were selected from 48 subspecies and 26 species, including the bacteria commonly detected in neonatal septicemia and purulent meningitis and those which frequently cause false positive results in PCR assay. Among them, 19 were standard strains (Table 1).

Table 1. Detection results of different strains and species of bacteria

Bacteria species (26)	Bacteria (48)	No. of strain (110)	Universal probe (110)	G^+ probe (55)	G^- probe (55)
G^- bacteria					
Acinetobacter brison	*Acinetobacter lwoffi**	1	+	−	+
	A. calcoaceticus	4	+	−	+
Aeromonas	*Aeromonas hydrophila**	1	+	−	+
Alcaligenes	*Alcaligenes faecalis*	2	+	−	+
Bacteroides	*Bacteroides fragilis*	1	+	−	+
Citrobacter	*Citrobacter freundii**	1	+	−	+
Edwardsiella	*Edwardsiella tarda*	1	+	−	+
Enterobacter	*Enterobacter aerogenes**	1	+	−	+
	E. cloacae	3	+	−	+
Escherichia	*Escherichia coli**	7	+	−	+
Chryseo bacterium	*Flavobacterium meningosepticum*	1	+	−	+
Haemophilus	*Haemophilus ducreyi*	1	+	−	+
	*H. influenzae**	1	+	−	+
Klebsiella	*Klebsiella pneumoniae**	8	+	−	+
Neisseria	*Neisseria meningitidis*	1	+	−	+
Proteus vulgaris	*Proteus mirabilis**	1	+	−	+
	P. vulgaris	1	+	−	+
	P. penneri	3	+	−	+
Providencia	*Providencia stuartii*	1	+	−	+
Pseudomonas	*Pseudomonasaeruginosa**	5	+	−	+
	P. putidabiovars	3	+	−	+
	P. cepacia	1	+	−	+
Salmonella	*Samonella typhimurium**	1	+	−	+
	*Salmonella enteritidis**	1	+	−	+
Serratia	*Serratia marcescens**	3	+	−	+
Yersinia	*Yersinia enterocolitica*	1	+	−	+
G^+ bacteria					
Spore-forming bacillus	*Bacillus subtilis**	3	+	+	−
Corynebacterium	*Corynebacterium pseudotuberculosis*	1	+	+	−
Listeria	*Listeria monocytogenes*	1	+	+	−
Micrococcus	*Micrococcus luteus**	1	+	+	−
Geneus mycobacterium	*Mycobacterium bovis*	1	+	+	−
	M. tuberculosis	1	+	+	−
Propionibacterium	*Propionibacterium acnes*	1	+	+	−
Staphylococcus	*Staphylococcus aureus**	9	+	+	−
	*S. epidermidis**	11	+	+	−
	S. capitis	1	+	+	−
	S. cohnii,	1	+	+	−
	S. haemolyticus	6	+	+	−
	S. hominis	3	+	+	−
	S. saprophyticus	1	+	+	−
	S. warneri	2	+	+	−
	S. sciuri	1	+	+	−
Streptococcus	*Streptococcus agalactiae**	3	+	+	−
	S. bovis	1	+	+	−
	S. mitis	1	+	+	−
	*S. pneumoniae**	4	+	+	−
	*S. pyogenes**	1	+	+	−
	S. salivarius	1	+	+	−

*: one of the total 19 standard strains in the bacteria species.

Viral strain

The virus included cytomegalovirus, EB virus and hepatitis B virus. Cryptococcus neoformans and candida albicans, and human genome DNA were also used in this study.

Standard bacterial strains were provided by the Clinical Test Center of Zhejiang Province and the Research Laboratory of Microorganism and Epidemiology, Academy of Military Medical Sciences, China. Common clinical bacterial strains, viral strains, cryptococcus neoformans and candida albicans were provided by the Bacterial Laboratory and Infectious Laboratory of this hospital. Human genome DNA was provided by the Chinese Academy of Preventative Medicine, China.

Methods

PCR amplification

Primer was designed within the ultra-conserved regions of the bacterial 16S rRNA gene. The amplified fragment was analyzed with the MegAlign software to identify G^-, G^+ and other specific bacteria. Then different primer sequences were designed. 5-15 polydT was added to the 5′end of the above unique probe sequences as a junction arm to connect the 5′ end of amino acid (NH2) and specific probe. In sample DNA extraction, 1 ml whole blood was collected and placed in a 1.5-ml sterile centrifuge tube containing 150 μl anticoagulant fluid of 3.8% sodium citrate. About 200 μl of white blood cells were extracted. One ml of cerebrospinal fluid was collected and centrifuged at 15 000 RPM for 10 minutes. The supernatant was discarded. Bacterial DNA was extracted with the boiling method developed at our laboratory.[4] Approximate 5 μl of extract was used in the PCR system (PCR-200, MJ, USA). Fluorescence-labeled PCR system was used (Cy5 fluorescence-labeled primer was synthesized by TAKARA).

Fluorescence-labeled primers 1 and 2 were 5′(Cy5)-TGCGGTTGGATCACCTCCT-3′ (1539-1521) and 5′(Cy5)-TCCCCACCTTCCTCCAGTT-3′ (1169-1187), respectively.

The two primers with Cy5 fluorescence labeled at the 5′end were cycled: at 94℃ for 3 minutes; at 94℃ and 55℃ for 30 seconds respectively (35 cycles); at 72℃ for 30 seconds; and at 72℃ for 5 minutes.

Positive, negative and blank controls were set each time. *E. coli* ATCC11775 and *Staphylococcus aureus* ATCC25923 were used as positive controls. One kind of virus (*Cyclomegalovirus*, EB virus, or hepatitis B virus), fungus (*Cryptococcus neoformans* or *Candida albicans*), and one human genome DNA were used as negative controls. There was no DNA in the blank control.

Microarray hybridization

Amino-modified glass slides were treated by 0.2% 1, 4- phenylene-dibutyl isosulfocyanate (pyridine/DMF 1:9) for 2 hours. Oligonucleotide probe with amino-modified 5′ end was fixed on the treated aldehyde glass slides (CEL, USA) with a GMS417 sample applicator, and dried for 30 minutes. Later, it was washed out with 0.2% sodium dodecyl sulfate (SDS) twice, air-dried, and stored at 4℃.

Alogether 72 points on each microarray included 18 localization points and 54 points of 18 specific probes (3 points for each probe) (Fig. 1). The 18 probes included a general probe for all bacteria; a G^+ probe of G^+ bacteria-specific; a G^- probe of G^- bacteria-specific; probes for diagnosis of meningitis, for pathogen identification in epidemic, purulent, and virus meningitis, and for detection of common pathogens in purulent meningitis like *Streptococcus pneumoniae*, *Staphylococcus*, *Haemophilus influenza*, *E. coli*, *Streptococus agalactiae*, and *Listeria monocytogenes*; and probes for contaminating bacteria in the diagnosis of sepsis and purulent meningitis including coagulase-negative *Staphylococcus* (CoNS), *Bacillus*, *Corynebacterium*, *Propionibacterium*, etc. The equivalent volume of PCR products was added into 8 μl of 2 × hybridization fluid containing 5 × SSPE and 1% SDS. Denatured at 95℃ for 5 minutes and then placed on ice immediately, all fluid was extracted and spotted on the chip with a transfer pipette. The chip was covered with glass slide and placed in a box at 48℃ for 0.5-hour hybridization. After hybridization, the chip was washed twice with 0.01% SDS and then air-dried for screening.

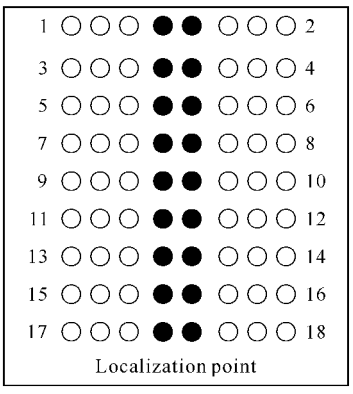

Fig. 1. The maxtrix in sample on microarray of 16S rRNA. 1: universal primer 1; 2: universal primer 2; 3: G^+ primer; 4: G^- primer 1; 5: *Staphylococcus aureus*; 6: G^- primer 2; 7: CoNS1; 8: *E. coli*; 9: CoNS2; 10: *Haemophilus influenzae*; 11: *Listeria monocytogenes*; 12: *Streptococcus pneumoniae*; 13: *Streptococcus agalactiae*; 14: *Bacteroides fragilis*; 15: Spore-forming bacillus; 16: *Meningococcus*; 17: *Corynebacterium*; 18: *Propionibacterium*.

Before administration of antibiotics, 1 ml of blood was routinely taken from 285 hospitalized neonates with suspected bacterial infection and injected into culture bottles containing 20 ml of blood bacteria-increasing fluid. Being mixed evenly, they were cultured with a VITAL 200 blood culture device. If bacteria were detected, they were further identified with the VITEK-60 system. Culture bottles, the device of blood culture, and the bacteria identification system were all purchased from Biomerieux Co., Ltd., France.

Results

Sensitivity and specificity of PCR and microarray hybridization of the 16S rRNA gene

The 16S rRNA gene sequences of 19 standard strains were amplified with PCR. A 371 bp DNA band was seen after electrophoresis. No positive band was observed after amplification with human genome DNA and cyclomegavirus. The 16S rRNA gene was highly specific to bacteria and no cross-reaction with human genome or virus was expected. With DNA extracted from *E. coli* as template with a dilution of 1:10, the minimum detected volume was 1 pg (10^{-12}) in PCR and the sensitivity of the detection was very high (Fig. 2). The results of microarray hybridization with PCR products of 110 bacteria strains (Table 1) were as follows: for 55 G^+ bacteria: positive for both G^+ and universal probes, and negative for G^- probes; for 55 G^- bacteria: positive for both G^- and universal probes, and negative for G^+ probes. Each strain was hybridized by its specific probe, and the microarray hybridization results of *Staphylococcus aureus* (Fig. 3) were positive for universal probes 1 and 2, as well as the probe for *Staphylococcus aureus*. Microarray hybridization showed positive results for universal probes, G^- probes 1 and 2, and the probe for *E. coli* (Fig. 4). These results suggested that microarray hybridization could not only differentiate G^+/G^- bacteria, but could identify special bacterial strains as well.

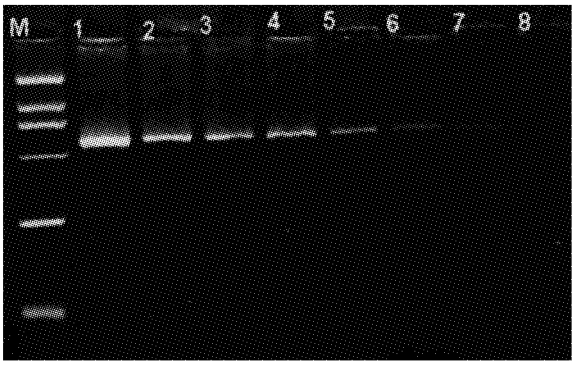

Fig. 2. Sensitivity analysis of 16S rDNA PCR. M: Pgem-7Zf(+) DNA/Hae Ⅲ DNA markers.

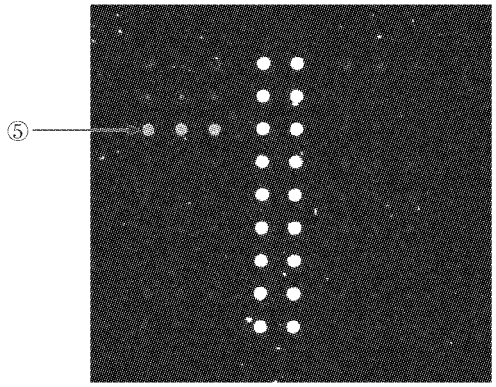

Fig. 3. Results of microarray hybridization in *Staphylococcus aureus*. ⑤: *Staphylococcus aureus*.

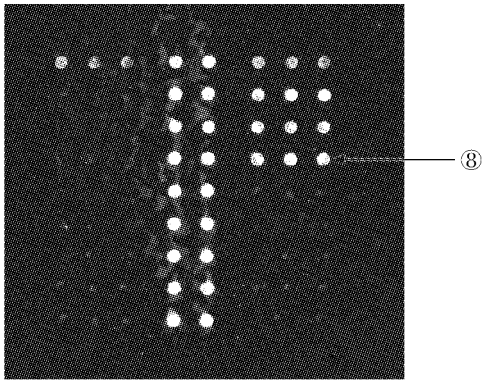

Fig. 4. Results of microarray hybridization in *E. coli*. ⑧: *E. coli*.

Results of PCR for the 16S rRNA gene and bacterial detection for blood culture samples

The positive rates of PCR blood culture were 5.96% (17/285) and 2.81% (8/285), respectively. The positive rate of PCR was significantly higher than that of blood culture ($P<0.01$) (Table 2). PCR in 30 neonates with noninfectious diseases showed negative results. In this series, 17 neonates were diagnosed as having septicemia, 155 clinical septicemia, 83 bacterial infection (respiratory tract infection, enteritis, omphalitis and diaper rash), and 32 noninfectious diseases (intracranial hemorrhage, ischemic and anoxic cerebral diseases, and ABO hemolysis) according to *Diagnosis and Therapy of Neonatal septicemia*.[3] In contrast to the final diagnosis of septicemia, PCR as a diagnostic tool, had a sensitivity of 100%, a specificity of 96.75%, and a correct diagnosis index of 0.968.

Table 2. Results of PCR for the 16S rRNA gene and culture of blood bacteria

Results	Positive results in blood culture	Negative results in blood culture	Sum
Positive in PCR	8*	9	17
Negative in PCR	0	268	268
Total	8	277	285

*: One of the samples showed positive PCR results of cerebrospinal fluid at the same time; $\chi^2=128.97$, $P<0.01$.

Results of microarray hybridization for blood samples

Microarray hybridization was carried out in 17 samples with positive PCR results for universal probes: G^+ probes (12 samples) and G^- probes (5 samples) [*E. coli* + probes, *Staphylococcus epidermidis* + probes (4), bacillaceae + (2) and propionibacterium + probes (2), and CoNS + probes (4)]. In the 8 samples with positive results of blood culture, 7 were positive for G^+ probes and 1 was positive for G^- probe. These results could be hybridized with the specific

Table 3. Results of microarray hybridization and bacterial culture of blood

Code number	Blood culture	PCR	Microarray hybridization			
			Universal primer	G^+ primer	G^- primer	Specific primer
1	*S. epidermidis*	+	+	+	−	*S. epidermidis*
2	*S. epidermidis*	+	+	+	−	*S. epidermidis*
3	*S. epidermidis*	+	+	+	−	*S. epidermidis*
4	*S. epidermidis*	+	+	+	−	*S. epidermidis*
5	*S. hominis*	+	+	+	−	*CoNS staphylococcus*
6	*S. haemolyticus*	+	+	+	−	*CoNS staphylococcus*
7	*S. sciuri*	+	+	+	−	*CoNS staphylococcus*
8*	*E. coli*	+	+	−	+	*E. coli*
9	−	+	+	−	+	*E. coli*
10	−	+	+	−	+	*E. coli*
11	−	+	+	−	+	*E. coli*
12	−	+	+	−	+	*E. coli*
13	−	+	+	+	−	*CoNS staphylococcus*
14	−	+	+	+	−	*Bacillaceae*
15	−	+	+	+	−	*Bacillaceae*
16	−	+	+	+	−	*Propionibacterium*
17	−	+	+	+	−	*Propionibacterium*
Total	8	17	17	12	5	17

*: PCR of cerebrospinal fluid was also positive, and microarray hybridization of cerebrospinal fluid involved *E. coli*.

probe. The results of microarray hybridization matched with those of blood culture. In the 9 samples with negative results of blood culture, 5 were positive for G^+ probes and 4 were positive for G^- probes (Table 3). For the sample from one neonate who had *E. coli* positive results of blood culture and microarray hybridization, the results of PCR and G^- probe were all positive in the cerebrospinal fluid. And microarray hybridization showed *E. coli* positive results.

Discussion

In recent years the technique of microarray has been widely used in analysis of gene sequence, gene mutation, polypeptide analysis, mapping of genome bank, and gene diagnosis of diseases.[5,6] With this technique, large quantities of different probes are fixed on the same holder, and the sample can be used in the analysis of many sequences and nucleic acid simultaneously.[7]

Neonatal septicemia is common but lacks specific signs and clinical symptoms. Blood culture as an important diagnostic method is often affected by many factors. Because of the low detection rate and time-consuming, it is not suitable for early diagnosis.[8-10] To avoid false negative result in the diagnosis of septicemia by blood culture as "golden standard", we designed a probe in the conserved region of the 16S rRNA gene, and developed a PCR method that can detect the 16S rRNA gene in all bacteria.[11] The PCR results of standard strains, clinical specimens, human genome DNA, and cyclomegalovirus showed that there was a 371 bp positive band in all standard strains but no cross reaction with human genome DNA and virus. This finding indicated that the method has a high specificity in bacterial determination. The minimum detection volume of PCR was 1 pg, and the positive test rate was significantly higher than that of blood culture. PCR has a sensitivity of 100%, a specificity of 96.75%, and a correct diagnosis rate of 0.968. Approximate 1/3 of neonates with septicemia will develop purulent meningitis, presenting no typically clinical features, and need culture of cerebrospinal fluid and routine examination for a correct diagnosis. Cell number and levels of sugar and protein vary greatly in neonatal cerebrospinal fluid. Usually, the culture of cerebrospinal fluid takes 3 days but the positive rate is low. Bacterial culture and PCR for one sample of cerebrospinal fluid showed positive results, and the results of microarray hybridization were consistent with those of blood culture. Normal cerebrospinal fluid is sterile and can be examined with PCR of the 16S rRNA gene to detect bacteria, thus improving the early diagnosis rate of purulent meningitis.

It is difficult to diagnose common bacterial infection clinically. Despite the mentioned advantages, PCR can only determine whether there is bacterial infection without identification of the specific bacteria. PCR and microarray hybridization of the 16S rRNA gene can directly detect all bacterial DNA and strains in sterile body fluid like blood, cerebrospinal fluid, pleuritic fluid, and ascites.[12] Based on common pathogens in neonatal septicemia and purulent meningitis, and bacteria that easily causes false positive results in PCR due to contamination, we arranged 18 different probes on each chip. The multipoint matrix makes it possible to detect an extended spectrum of bacteria, not only those that can be detected in blood culture, but those which need special culture like L-type bacteria and anaerobs or are difficult and unable to culture like actinomycetes. The sensitivity of this method can be as high as 3 bacterial cells per reaction.[13] The amount of PCR products was relatively higher but the hybrid time was only 4 hours, which is less than those for filter membrane hybridization, e. g, reversed phase hybridization. To avoid false positive results, we designed probes for commonly contaminated strains including spore-forming bacillus and corynebacterium. In case of positive results for both universal probes and probes of contaminating bacteria, further analysis should be made according to clinical findings. In our 17 patients demonstrating microarray-positive, 9 showed negative results of blood culture although they all met the diagnostic criteria for septicemia. The results may be due to 2 strains of anaerobs, undetectable in normal culture (4 neonates); a small quantity of bacteria or a low sensitivity to blood culture (5).

In conclusion, PCR in combination with microarray hybridization of the 16S rRNA gene is quick, and reliable for the etiological diagnosis of neonatal septicemia. Combined with clinical findings, it can greatly improve the early diagnosis rate of the disease and prognosis and also reduce complications.

Funding: None.
Ethical approval: Not needed.
Competing interest: None declared.
Contributors: TMQ wrote the first draft of this paper. All authors contributed to the intellectual content and approved the final version.

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Received May 30, 2005
Accepted after revision August 13, 2005