16S rDNA PCR-DGGE and sequencing in the diagnosis of neonatal late-onset septicemia

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Abstract. The 16S rDNA polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE) and sequencing method has been demonstrated to be valuable in detecting pathogens in the blood of patients suffering from fever or neutropenia. However, its use in the diagnosis of neonatal late-onset septicemia (LOS) has not yet been reported. The aim of the present study was to investigate the efficiency of this method in detection of the type of bacterial infection in neonatal LOS. Blood specimens from 60 neonates in whom LOS was suspected were collected. Fourteen culture positive blood samples and 24 spiked 'infected' blood samples were analyzed by the 16S rDNA PCR-DGGE and sequencing method or by pathogen-specific PCR. Only in 5 of the 14 cases did the results of 16S rDNA PCR-DGGE and sequencing match with the results of blood culturing. In the other 9 cases, the blood culture failed to detect bacteria, such as *Neisseria* sp. and *Moraxella* sp., which were detected by 16S rDNA PCR-DGGE and sequencing. Furthermore, the 16S rDNA PCR-DGGE and sequencing failed to detect blood culture-proven bacteria, such as *Klebsiella pneumonia*. A competitive inhibitory effect in 16S rDNA PCR amplification may lead to the discrepancy between pathogen-specific PCR and spiked 'infected' blood samples. When a certain species of bacteria was detected by 16S rDNA PCR, the competitive inhibitory effect presented a higher sensitivity in detecting this species in the blood samples that contained bacterial DNA only from this species compared with the blood samples that were blended with other bacterial DNAs. In conclusion, 16S rDNA PCR-DGGE and sequencing can detect a more comprehensive spectrum of pathogens than blood culture. However, the competitive inhibitory effect, which may lead to false negative results should be taken into consideration when the 16S rDNA PCR-DGGE and sequencing method is applied to the diagnosis of neonatal LOS.

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Introduction

Neonatal late-onset septicemia (LOS) is a common complication of infants under prolonged hospitalization in the neonatal intensive care unit (1). LOS occurs in ~10% of all neonates in neonatal intensive care units and up to 21% of very low birth weight infants experience an episode of LOS (2,3). Fast and accurate diagnosis is important for reduction of the mortality of LOS. Although blood culture remains to be the gold standard in the diagnosis of bacterial bloodstream infections, this method has certain limitations, such as a long waiting time for results (at least 48 h), poor sensitivity (10-20%) in detecting fastidious microbes, and the use of antibiotics before blood specimens are drawn, which may affect the results obtained from the blood culture (4,5). In addition, the blood culture method is flawed in detection of polymicrobial infection (5,6).

The 16S rDNA polymerase chain reaction (PCR), based on the amplification of 16S rDNA in bacteria, is fast with high sensitivity and can fully detect the whole bacterial spectrum in the experimental samples (7). In combination with denaturing gradient gel electrophoresis (DGGE) and sequencing, 16S rDNA PCR has been used to detect the pathogens in the blood of patients suffering from fever or neutropenia (8). However, the use of 16S rDNA PCR in the diagnosis of neonatal LOS has not yet been reported. The aim of the present study was to investigate the efficiency of 16S rDNA PCR-DGGE and sequencing in the detection of bacteria in neonatal LOS.

Materials and methods

The study protocol was approved by the Institutional Review Board of the Children's Hospital, Chongqing Medical University (Chongqing, China). Informed written consent was obtained from the guardians of the enrolled neonates.

Diagnostic criteria. Signs and symptoms suggestive of clinical sepsis were: Unstable temperature, lethargy, irritability, gastrointestinal dysfunction with milk intolerance, vomiting, abdominal distension or bloody stool, respiratory dysfunction, sudden increase in respiratory rate or persistent tachypnoea, and tachycardia or bradycardia. These signs and symptoms were described in detail in a previous study (9).

Sample collection. From January to May 2012, 60 neonates who were suspected of neonatal septicemia according to the above diagnostic criteria in the Department of Neonatology, Children's Hospital of Chongqing Medical University were enrolled in the present study. Paired blood samples were collected after careful skin disinfection and sent for blood culture and molecular analysis. Blood samples from 10 neonates diagnosed with jaundice (caused by ABO hemolytic disease, without any evidence of infection or antibiotic treatment) were collected and served as negative controls. Venous blood (3 ml) was collected, of which 1 ml was inoculated in the corresponding culture bottles for aerobic blood culture in the BACTEC 9120 system (BD Diagnostics, Bergen, NJ, USA), and another 1 ml was used for anaerobic blood culture in the BacT/Alert system (BioMérieux, Marcy-l'Etoile, France). The remaining 1 ml venous blood was collected in a sterile blood collection tube containing EDTA (Shanghai Kehua Bio-engineering, Shanghai, China) and stored at -70˚C until molecular processing.

DNA extraction. DNA extraction was performed according to the manufacturer's instructions using the QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany) with sterile water as a negative control. The blood samples were frozen, thawed for 3 cycles, and incubated at 37˚C for 1 h with mixing every 20 min followed by the addition of 180 μl (40 mg/ml) lysozyme (Sigma-Aldrich, St. Louis, MO, USA). All DNA extraction reagents except the lysozyme solution were filtered through a 0.22-μm filter prior to bacterial DNA extraction. Whole genomic DNA (100 ml), including bacterial genomic DNA, was dissolved in buffer AE (10 mM Tris·Cl, 0.5 mM EDTA, pH 9.0) and stored at -20˚C. PCR amplification was performed using a PCR amplifier (Eppendorf, Humburg, Germany), with previously described thermocycling conditions (8).

16S rDNA nested PCR amplification. Primers for amplification of the variable region of 16S rDNA for reaction 1 were as follows: B5, 5'-TCAGATTGAACGCTGGCGGC-3' and B4, 5'-TATTACCGCGGCTGCTGGCA-3' (8). The amplified 493-bp products from reaction 1 were then amplified in reaction 2 with primers P2 (5'-CCTACGGGAGGCAGCAG-3') and P3 (5'-ATTACCGCGGCTGCTGG-3') starting from nucleotide 341 and 534 of the 16S rDNA, respectively (8). A 40-bp GC clamp was attached to the 5' end of the P2 primer to prevent complete separation of PCR amplicons during DGGE analysis. The sterile water was filtered through a 0.22-μm filter to avoid possible contamination. The PCR mixture of the first amplification was adjusted to a final volume of 25 μl with sterile water after 12.5 μl of Premix Taq (Takara Bio Inc., Otsu, Japan), 0.5 μl each primer (10 μM), and 5 μl DNA template were added. The PCR mixture of the second amplification was adjusted to a final volume of 50 μl with sterile water after 1 μl of PCR product from the first amplification, 25 μl Premix Taq (Takara Bio Inc.), and 1 μl each primer (10 μM) were added, with sterile water used as a negative control.

DGGE analysis. DGGE analysis was performed using the Dcode Universal Mutation system (Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions. The products from PCR amplification were first electrophoresed on a 2% (wt/vol) agarose gel and stained with 4S nucleic acid (Sangon Biotech Co. Ltd., Shanghai, China). Only samples with visible target bands were adopted for further DGGE analysis. Electrophoresis was performed on 8% polyacrylamide gels with a denaturing gradient ranging from 35 to 65% in 1X TAE buffer (0.04 M Tris-acetate, 0.00l M EDTA; pH 8.0) at 85 V and 60˚C for 16 h. Then the gels were incubated in 1X TAE buffer containing SYBR Green I (Biotech, Beijing, China) for 30 min and scanned using a Benchtop 3UV Transilluminator (UVP, Inc., Upland, CA, USA). Each visible band was excised from the DGGE polyacrylamide gels, placed in 30 μl of sterile water, and incubated at 4˚C overnight. PCR-DGGE analysis of each sample was repeated twice.

TA cloning. DNA recovered from excised DGGE bands was amplified by PCR as described previously (10), and the PCR products were electrophoresed on a 2% agar gel and purified using the Gel DNA Extraction kit (Takara Bio Inc.). The purified PCR products (2 ml) were used to perform TA cloning using the pMD 18-T Vector kit (Takara Bio Inc.) according to the manufacturer's instructions. Then the pMD 18-T plasmids containing the PCR amplicons were transformed to *Escherichia coli* DH5α-competent cells (TIANGEN Biotech (Beijing), Co., Ltd., Beijing, China) and cultured on ampicillin-resistant Luria-Bertani (LB) broth at 37˚C overnight.

Confirmation of the cloned DNAs by colony PCR. Clones on ampicillin-resistant LB media were collected and blended in 10 μl sterile water. Then colony PCR was performed to confirm the cloned DNAs using primers M13-RV (5'-CAGGAAACAGCTATGAC-3') and M13-M3 (5'-GTAAAACGACGGCCAGT-3'). The PCR mixture containing 1 μl template (the monoclone blended in sterile water), 1 μl of each primer (10 μM), and 12.5 μl Premix Taq (Takara Bio Inc.) was adjusted to a final volume of 25 μl with sterile water. The PCR mixture was first incubated at 94˚C for 7 min followed by a total of 30 cycles of 30 sec at 94˚C, 30 sec at 60˚C, and 30 sec at 72˚C, with a final step at 72˚C for 5 min. The length of each amplicon was confirmed by agarose gel electrophoresis.

Sequencing of PCR products. The sequencing of the PCR products was performed on an ABI 3730xl DNA Analyzer with M13+(-47) primers and BigDye terminator v3.1 (Applied Biosystems, USA) at Sangon Biotech (Shanghai, China),

Table I. Comparison of sequencing results of blood culture.

Patient	Identified bacterial species	
	Blood culture	16S rDNA PCR-denaturing gradient gel electrophoresis and sequencing method
1	*Klebsiella pneumoniae*	*Neisseria* sp., *Moraxella* sp., *Enterobacter* sp., *Micrococcus* sp.
2	*Klebsiella pneumoniae*	*Moraxella* sp., *Acinetobacter* sp., *Bacillus* sp., Aeromonas sp.
3	*Escherichia coli*	***Escherichia coli***[a], *Vibrio* sp.
4	Group B *Streptococcus*	*Stenotrophomonas* sp., *Acinetobacter* sp., *sphingobacterium*
5	*Enterococcus faecalis*	*Micrococcus* sp., *Klebsiella* sp., *Enterobacter* sp., *Acinetobacter* sp.
6	*Serratia marcescens*	*Acinetobacter* sp., *Enterobacter* sp.
7	*Staphylococcus epidermidis*	***Staphylococcus epidermidis*****,** *Bacillus* sp., *Halomonas* sp., *Propionibacterium* sp.
8	*Staphylococcus aureus*	*Acinetobacter* sp., *Klebsiella* sp., *Enterobacter* sp., *Micrococcus* sp.
9	*Enterobacter cloacae, Klebsiella pneumoniae*	***Klebsiella* sp., *Enterobacter* sp.,** *Acinetobacter* sp., *Corynebacterium* sp.
10	*Enterococcus faecium, Staphylococcus haemolyticus*	*Dietzia* sp., *Enterobacter* sp., *Klebsiella* sp., *Proteobacterium*, *Bacillus* sp.
11	*Enterobacter aerogenes*	***Enterobacter* sp.**, unknown bacteria
12	*Klebsiella pneumoniae*	***Klebsiella* sp.**
13	Group B streptococcus	*Vibrio* sp., *Escherichia coli*, *Burkholderia* sp.
14	*Streptococcus agalactiae*	*Acinetobacter* sp., *Klebsiella* sp., *Enterobacter* sp.

[a]Bold indicates stronger detection compared with the blood culture. PCR, polymerase chain reaction.

and the sequences were analyzed and blasted on NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome).

Confirmation of the efficiency of the DNA extraction method in spiked samples using the 16S rDNA PCR-DGGE and sequencing method. E. coli and *Staphylococcus epidermidis* cultures at $1x10^5$ CFU/ml (10 ml) each were blended in blood samples (200 μl) from neonates diagnosed with jaundice to prepare spiked, 'infected' blood samples, in order to imitate the bacterial load in septicemia (4). The bacterial DNA was then extracted following the procedures described above and analyzed by the 16S rDNA PCR-DGGE and sequencing method.

Pathogen-specific PCR of blood culture-proven samples. Pathogen-specific PCR was conducted to detect the DNA of the bacteria confirmed by blood culture but not by the 16S rDNA PCR-DGGE and sequencing method. Two patients who were diagnosed with *Klebsiella pneumoniae* septicemia were selected. The primers for amplification of *K. pneumoniae* were as follows: Forward: 5'-GCGTGGCGGTAGATCTAAGTCATA-3' and reverse: 5'-TTCAGCTCCGCCACAAAGGTA-3'. The PCR conditions and method used to confirm the length of the PCR products were the same as described above.

16S rDNA PCR-DGGE and sequencing of spiked samples. Enterococcus faecalis, S. epidermidis and Pseudomonas aeruginosa, often observed in septicemia, were used to imitate the situation present in infected blood by a pyrosequencing method. Genomic DNA of each bacterium was extracted as described above. The concentration of the DNA was quantified using NanoDrop 1000 (Nanodrop, Wilmington, DE, USA) and diluted to 65 ng/μl. The DNA of *E. faecalis* was 5-fold serially diluted (served as less dominant bacteria) and blended with a constant concentration of DNA from the other two bacteria (both served as dominant bacteria). The set of the mixed DNA served as the template and was subjected to a series of 16S rDNA PCR amplifications as described previously (10). In addition, the set of diluted DNA of *E. faecalis* alone served as

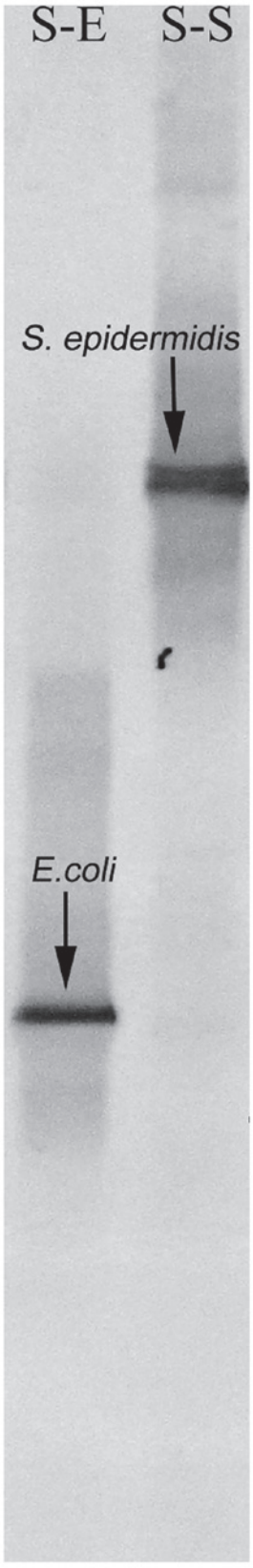

Figure 1. Denaturing gradient gel electrophoresis fingerprint after DNA extraction and 16S rDNA polymerase chain reaction of spiked blood samples. S-E, blood sample spiked with *E. coli*; S-S, blood sample spiked with *S. epidermidis*.

the template and was subjected to 16S rDNA PCR amplification.

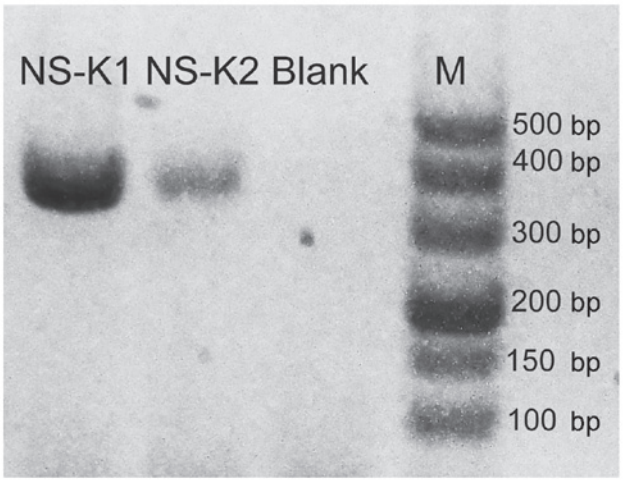

Figure 2. Pathogen-specific PCR of DNA from positive blood culture. Visible bands were observed in NS-K1 and NS-K2 (neonatal septicemia with *K. pneumonia*) after repeated PCR amplification; the number correspond to patient numbers. PCR, polymerase chain reaction; blank, blank control; M, DNA marker.

The PCR mixture containing 25 μl Premix Taq (Takara Bio Inc.), 1 μl of each primer (10 μM), 1 μl diluted DNA of *E. faecalis*, or 3 μl (mixed DNA with 1 μl of each bacterial DNA) of bacterial DNA was adjusted to a final volume of 50 μl with sterile water. The PCR products were then analyzed by the DGGE and sequencing method as described above.

Results

Blood culture. From January to May 2012, 60 neonates who were suspected of neonatal septicemia were enrolled in the present study. Positive blood culture results occurring >72 h after birth were reported for 14 of these 60 neonates (Table I). Of these 14 neonates with positive blood culture, 12 neonates were diagnosed with mono-bacterial infection and 2 neonates were confirmed to be infected by two species of bacteria.

Bacterial spectrum screened by molecular methods. The sequencing results showed diverse bacterial species in the blood samples, the majority of which were not detected by blood culture (Table I). Only in five cases did the sequencing results match partly or wholly with the blood culture results. One of these five patients was diagnosed with *K. pneumoniae* bloodstream infection, which was confirmed by molecular methods and blood culture. In the other four of these five cases, the molecular method detected a more complex bacterial spectrum, which contained the blood culture proven bacteria (Table I). In the other 9 cases, blood culture failed to detect bacteria, such as *Neisseria* sp., *Moraxella* sp., that were detected by the 16S rDNA PCR-DGGE and sequencing method. In addition, the 16S rDNA PCR-DGGE and sequencing method also failed to detect the blood culture-proven bacteria, such as *Klebsiella pneumonia* and *Enterococcus faecalis*.

Investigation of the reasons leading to poor correlation between results of blood culture and the molecular method. The three steps investigating the possible reason for failure of the molecular method in detecting culture-proven bacteria

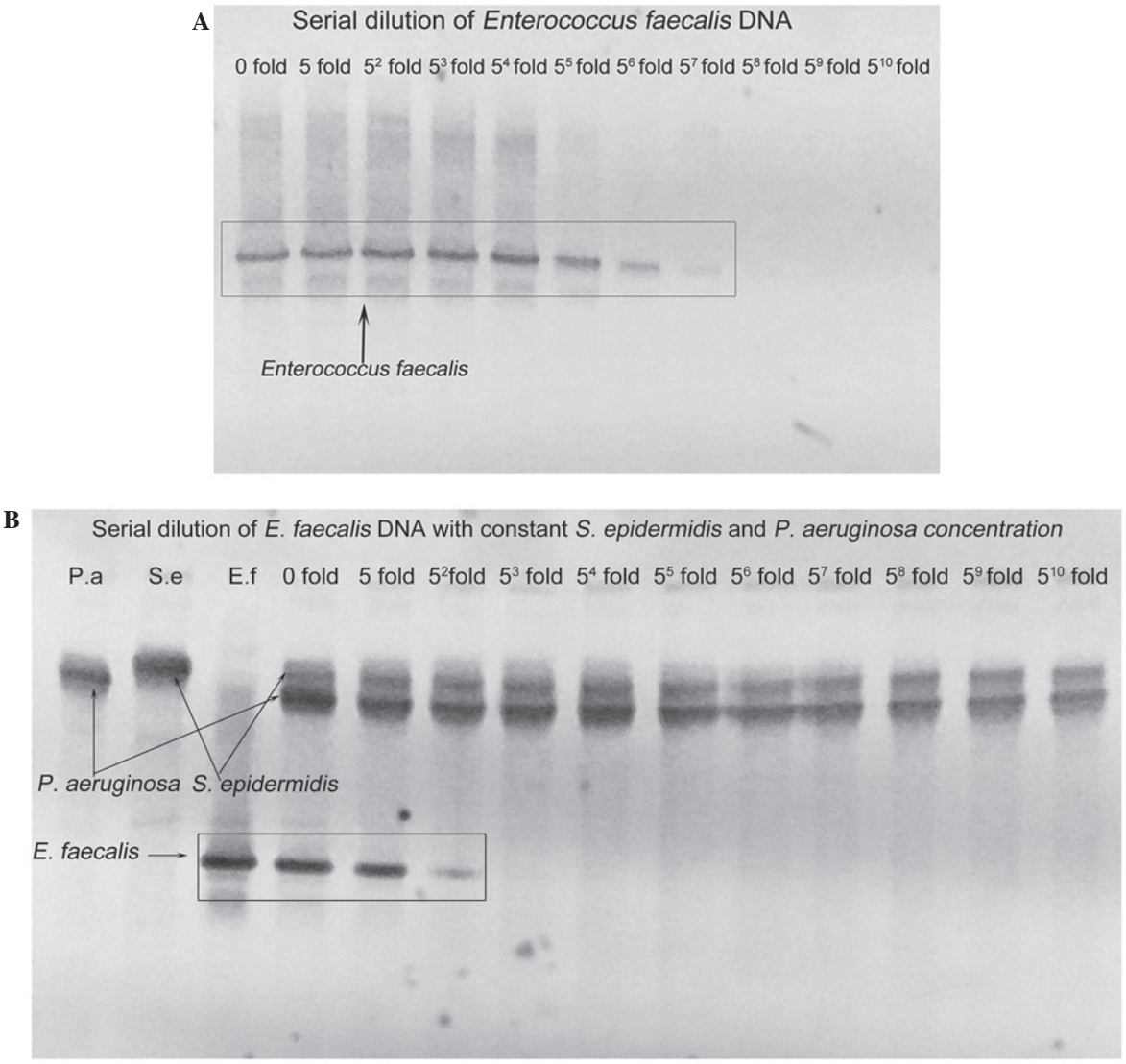

Figure 3. Competitive inhibitory effect in 16S rDNA PCR-DGGE. (A) Detection limit of PCR-DGGE when *E. faecalis* DNA was amplified alone. The genomic DNA of *E. faecalis* was serially diluted 5-fold, and a visible DNA band was observed even at a 57-fold dilution. (B) Detection limit of PCR-DGGE when *E. faecalis* DNA was amplified together with *S. epidermidis* and *P. aeruginosa* DNA. The genomic *E. faecalis* DNA was serially diluted by 5-fold, while the concentrations of DNA from *S. epidermidis* and *P. aeruginosa* remained constant. The bands corresponding to PCR amplification of *E. faecalis* DNA disappeared when the DNA template was diluted by 53-fold. P. a, *P. aeruginosa*; S.e, *S. epidermidis*; E.f, *E. faecalis*. The numbers indicate fold dilution. PCR-DGGE, polymerase chain reaction-denaturing gradient gel electrophoresis.

were as follows. Firstly, efficiency of the DNA extraction method was tested. Then pathogen-specific PCR was applied to confirm the existence of culture-proven bacterial DNA in the sample DNA solutions. Thirdly, spiked samples were made to imitate the infection revealed by 16S rDNA PCR-DGGE and sequencing (presence of 'culture-proven pathogen' bacteria with other bacteria detected by molecular method). The hypothesis that the molecular method-proven bacterial DNA could interfere with the detection of blood culture-proven bacteria was tested in these spiked blood samples by the 16S rDNA PCR-DGGE and sequencing method as described in the materials and methods.

Efficiency of the DNA extraction method. The results of the PCR-DGGE and sequencing method for the spiked 'infected' blood samples demonstrated that the DNA of the corresponding bacteria in blood was successfully isolated. The bands on the polyacrylamide gel of the DGGE were identical to those of *E. coli* and *S. epidermidis*, which were confirmed by subsequent sequencing (Fig. 1).

Detection of blood culture-proven pathogens by pathogen-specific PCR. No visible band was found on the gels of two culture-proven cases after PCR-agarose gel electrophoresis. Hence, a second PCR amplification of the products from the first amplification was performed. After the second amplification, the target gene of *K. pneumoniae* was observed on the agarose gel (Fig. 2).

Competitive inhibitory effect of 16S rDNA PCR-DGGE. The detection limits of 16S rDNA PCR-DGGE for a single certain species of bacterial DNA versus mixed species of bacterial DNA (mixture of other bacterial DNA with this certain species of bacterial DNA) were completely different

with a higher sensitivity in detecting this certain species of bacterial DNA in the spiked samples which were free of other bacterial DNA (Fig. 3A and B). When the *E. faecalis* DNA was amplified alone even at a $1x5^7$-fold dilution, the faint band amplified from *E. faecalis* DNA was still observed (Fig. 3A). When the DNAs of the other two species of bacteria were introduced, no band was observed for *E. faecalis* DNA even at dilution by $1x5^3$-fold (Fig. 3B). The detection limit of *E. faecalis* was different under the above two situations where the amount of *E. faecalis* DNA and 16S rDNA were identical throughout, indicating a possible influence of the introduced bacterial DNA on detecting the *E. faecalis* DNA. As the amplifying condition of 16S rDNA PCR was consistent, the introduced bacterial DNA caused reduced capability in amplifying *E. faecalis* DNA. There was lower sensitivity of 16S rDNA PCR in detecting the same copies of *E. faecalis* DNA when other bacterial DNA was introduced, compared with detecting the *E. faecalis* DNA in the samples containing *E. faecalis* DNA alone. This suggests a competitive inhibitory effect of other bacterial DNA on the *E. faecalis* DNA during the 16S rDNA PCR amplification.

Discussion

The recognition of pathogens in the blood is a crucial aspect in the management of neonates with LOS (6). Although blood culture is regarded as a gold standard in the clinic, there is a requirement for a more rapid and sensitive strategy to detect the bacteria in the blood (5).

In the present study, although a broader bacterial spectrum was demonstrated by the 16S rDNA PCR-DGGE and sequencing method as compared with blood culture, it failed to detect culture-proven bacteria in 9 of the 14 blood culture-positive cases, indicating its poor correlation with blood culture.

The 16S rDNA PCR technique involves broad-range PCR amplification of the 16S rDNA regions of different bacterial species and has been reported by Muyzer *et al* (11) to be sensitive enough to detect one species of bacterium in a bacterial community when the bacterium constitutes >1%. To determine whether the sensitivity is responsible for the failure of 16S rDNA PCR in the diagnosis of certain culture-proven cases of septicemia, pathogen-specific PCR was used in the samples in which blood culture-proven bacteria failed to be detected by 16S rDNA PCR-DGGE and sequencing method. Using this procedure the present study aimed to confirm whether the DNA of culture-proven bacteria was successfully extracted and existed in the sample DNA solutions. The results of pathogen-specific PCR demonstrated the presence of a low amount (only detected after the second amplification) culture-proven bacterial DNA in the sample DNA solutions. Given the fact that the 16S rDNA PCR used in the present study also went through the second PCR amplification in nested-PCR, which is similar to the second PCR amplification in the pathogen-specific PCR, 16S rDNA PCR had the same capability in detecting these trace amounts of bacterial DNA. The low load of culture-proven bacterial DNA may not account for the discrepancy between the 16S rDNA PCR and pathogen-specific PCR methods. The only difference between these two PCR methods is in that pathogen-specific PCR primers target a specific species of bacterial DNA, but 16S rDNA PCR primers target the whole bacterial spectrum of DNA in the samples. To investigate whether the detection of blood culture-proven bacterial DNA (such as *Klebsiella pneumonia*) interfered with the molecular method-proven bacterial DNA (such as *Neisseria* sp., *Moraxella* sp.) in the 16S rDNA PCR amplification among the patients with poor correlation between the two detection methods, spiked samples were used to imitate the situation (presence of other bacterial DNA with blood culture-proven bacterial DNA) and performed 16S rDNA PCR-DGGE.

The results obtained from the test on spiked samples in the present study supported the above hypothesis and demonstrated a competitive inhibitory effect, an unequal amplification of different bacterial DNAs in the 16S rDNA PCR. The 16S rDNA PCR-DGGE has a higher sensitivity in detecting the 'pathogen' bacterial DNA when the spiked samples were free of other bacterial 'infection'. However, the introduction of other bacterial DNAs interferes with the detection of the 'pathogen' bacteria by the 16S rDNA PCR-DGGE method.

Our findings demonstrate that the 16S rDNA PCR based molecular methods may cause bias in screening bacteria in LOS due to the competitive inhibitory effect. A previous study conducted by Muyzer *et al* (11) does not address the exact reason for the limited sensitivity of 16S rDNA PCR-DGGE. The present study demonstrates that the limited sensitivity of 16S rDNA PCR-DGGE in detecting bacteria which constitutes <1% of the whole bacterial community may be attributed to an unequal PCR amplification among the bacteria in the bacterial community. For this reason, any practice which leads to a change in the constitution of the bacterial community should be noticed and avoided when applying the 16S rDNA PCR based molecular method. For instance, numerous attempts have been made to enhance the sensitivity of 16S rDNA PCR, including pre-culture of blood (incubation before bacterial DNA extraction) to amplify the amount of the bacteria in the blood samples (12-14). Although pre-culture is useful for enhancing the detection limit of 16S rDNA PCR, it can induce a competitive inhibitory effect in 16S rDNA PCR amplification of easy-to-grow bacteria and fastidious/uncultivable bacteria under certain culture conditions. Therefore, pre-culture adversely affects the ability to detect fastidious/uncultivable bacteria with the 16S rDNA PCR based molecular methods.

Limitations of the present study include a small sample size and lack of more accurate quantitative methods. The competitive inhibitory effect was an obstacle to the application of 16S rDNA PCR in the diagnosis of neonatal LOS. Thus, the present study did not attempt to investigate its use in a large-scale neonatal population.

Overall, this preliminary investigation focused on the efficiency of the 16S rDNA PCR-DGGE and sequencing method in the diagnosis of neonatal LOS. It was demonstrated that a competitive inhibitory effect caused a bias in 16S rDNA PCR amplification to screen the bacterial spectrum of neonatal septicemia. To obtain a higher efficiency of 16S rDNA PCR-DGGE and sequencing for the diagnosis of LOS, protocols aiming to overcome the competitive inhibitory effect in 16S rDNA PCR amplification require development in the future.

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