# Development and Evaluation of 16S rDNA Microarray for Detecting Bacterial Pathogens in Cerebrospinal Fluid

YI LIU,*,† JIN-XIANG HAN,*,†,[1] HAI-YAN HUANG,* AND BO ZHU*

*Key Laboratory for Biotech-Drugs Ministry of Health, Shandong Medicinal Biotechnology Center, Jinan 250062, China; and †Medical College of Shandong University, Jinan 250012, China

**The rapid identification of bacteria in cerebrospinal fluid (CSF) is very important for patient management and antimicrobial therapies. We developed a 16S DNA microarray–based method that targets 16S rDNA and can directly detect bacteria from CSF without cultivation. Universal primers and specific probes were designed from the 16S rDNA sequence data retrieved directly from the GenBank database. The specificity of the assay is obtained through a combination of microarray hybridization and enzymatic labeling of the constructed specific probes. Cultivation-dependent assays were used as reference methods in the development and evaluation of the method. With the exception of *Mycobacterium tuberculosis* and *Proteus mirabilis*, forty-five positive blood culture media were successfully differentiated. When this procedure was applied directly to 100 CSF specimens, 29 specimens from 16 patients were positive by bacterial culture and 3 culture-positive CSF specimens produced no hybridized signals. The remaining 26 specimens were correctly identified, including one with mixed infection. The accuracy, sensitivity, and specificity of the assay can be increased further by designing more oligonucleotides for the microarray. This method is versatile and makes it possible to detect more bacteria in a single assay and discriminate different bacterial genera.** Exp Biol Med 230:587–591, 2005

**Key words:** 16S rDNA; microarray; cerebrospinal fluid; bacteria

## Introduction

Bacterial meningitis is a serious disease with high morbidity and mortality (1). An accurate and rapid diagnosis is, therefore, important for effective treatment and care. With the emergence of antibiotic resistance, identifying the pathogen that is involved in an infectious disease is becoming more and more important. Among the various diagnosis methods currently used in clinical laboratories, the most sensitive method is based on the successful culture and identification of bacteria from cerebrospinal fluid (CSF). But, at least 2 days are required for the cultivation and biochemical and/or immunologic tests. The time required to obtain a positive culture result would be longer for patients infected with slowly growing organisms or for those with low bacterial counts. Sometimes the cultures may also remain negative if the disease is caused by fastidious and slowly growing microorganisms.

Assays based on nucleic acid detection are more rapid and sensitive than traditional assays. In previous studies, polymerase chain reaction (PCR) analyses were used to detect pathogens, and many primer sets have been developed to detect species-specific genes (2–5). The use of different primers for different species is impractical for the routine analysis of cultures that may contain one or more of many possible pathogens. But, this can be avoided by using a single pair of universal primers designed to amplify conserved stretches of DNA from any bacterium, followed by sequence analysis or hybridization to determine the species (6–7). Using available sequence data from GenBank, we designed such primers and oligonucleotide probes to develop a 16S rDNA microarray with the potential to simultaneously detect the wide range of bacterial pathogens that cause meningitis. We describe here the validation of this microarray by using 45 positive blood culture media, as well as 100 CSF specimens.

## Materials and Methods

**Bacterial Strains.** The reference strains purchased from the National Institute for the Control of Pharmaceutical and Biological Products and used in the study as positive controls included *Acinetobacter calcoaceticus* 12004, *Escherichia coli* ATCC 35218 and ATCC 25922, *Haemophi-*

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[1] To whom correspondence should be addressed at Shandong Medicinal Biotechnology Center, Jinan 250062, China. E-mail: jxhan@sdu.edu.cn

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*lus influenzae* 58534, *Listeria monocytogenes* 54005, *Mycobacterium tuberculosis* Hv37r, *Neisseria meningitidis* 29019, *Proteus mirabilis* 11527, *Pseudomonas aeruginosa* ATCC 27312, *Salmonella typhimurium* 50515, *Salmonella enteritidis* 50041, *Staphylococcus aureus* ATCC 29213 and ATCC 25923, and *Streptococcus pneumoniae* 11526. The collections of blood culture and clinical isolates from the microbial laboratory of Jinan Fourth Hospital (JFH) and PLA Jinan Military General Hospital (PJMGH) included *Bacteroides fragilis* (1 strain), *E. coli* (1 strain), *Enterococcus* (6 strains), *Flavobacterium meningosepticum* (1 strain), *Fusobacterium necrophorum* (1 strain), *H. influenzae* (1 strain), *Moraxella catarrhalis* (4 strains), *Peptostreptococcus* (1 strain), *Pasteurella haemolytica* (1 strain), *P. mirabilis* (4 strains), *P. aeruginosa* (1 strain), *S. typhimurium* (1 strain), *S. aureus* (1 strain), *Staphylococcus epidermidis* (2 strains), *Staphylococcus saprophyticus* (1 strain), *Streptococcus agalactiae* (2 strains), and *S. pneumoniae* (3 strains). These organisms were identified by conventional methods and the API test system (bioMerieux, France). Forty-five strains were chosen to represent 20 species and many of the common organisms causing infection of the central nervous system (8). Prior to DNA extraction, each strain was streaked on chocolate or blood agar and examined for the proper colony morphology.

**Source of CSF Samples.** Between October 2000 and May 2003, 100 CSF samples from 87 patients were collected for the diagnosis of bacterial meningitis in the JFH and PJMGH. Patient age ranged from 5 to 81 years. Specimens of CSF were immediately cultured, and 29 CSF specimens from 16 patients were positive for bacteria.

**Microarray Design and Construction.** Bacteria sequence data were obtained directly from the GenBank database. The results of these alignments were tabulated by using the best BLAST hit for each species of bacteria. A set of primers and 40 oligonucleotides with similar melting temperatures was designed (Table 1) and synthesized by the BioAsia Biotechnology Corporation (Shanghai, China). The reverse primer was modified by adding a digoxin molecular to 5 end.

The 40 oligonucleotide probes were suspended in distilled water at a concentration of 25 m*M* and printed on nylon membranes (Pharmacia, Uppsala, Sweden) with a contact-printing robot (PixSys5500; Cartesian Inc., Newton, MA). Two dots were printed on each probe. Digoxin-labeling PCR products were used as positive controls, human genome DNA and hepatitis B virus (HBV) DNA were used as negative controls, and double-distilled water was used as blank control; each of the controls was also spotted with two dots. The size of each array was 13.0 mm × 8.0 mm (the probes arranged in membrane are displayed in Fig. 1). A UV crosslinker (UVP Inc., Upland, CA) fixed the DNA to the membrane for 3 mins.

**DNA Extraction.** The DNA was extracted from CSF samples according to van der Vliet *et al.* (9). Briefly, 500 μl of CSF specimen was centrifuged at 12,000 *g* for 5 mins. The pellet was resuspended in 180 μl of lysis buffer containing 1% Triton X-100, 10 m*M* Tris (pH 8.0), and 1 m*M* EDTA. The mixture was boiled for 10 mins and centrifuged at 12,000 *g* for 5 mins. The supernatant was saved for PCR analysis.

Alternatively, DNA extraction was performed according to the phenol-chloroform-isoamyl alcohol method (10).

**PCR Amplification.** The PCR analysis was initially done on DNA extracts from CSF specimens using Pba1 primer (5′-ACT CCT ACG GGA GGC AGC AGT-3′) and Pba2 primer (5′-digoxin-TCA CCG GCC GTG TGT ACA AG-3′) that amplify a 1086-bp region of the 16S rRNA gene, which is highly conserved among all bacteria (6). The amplification of the PCR analysis was carried in a 25.0-μl reaction mixture containing 14.8 μl of sterile distilled water, 2.0 μl of deoxyribonucleoside triphosphate (0.25 m*M*), 4.5 μl of 10 × PCR buffer (100 m*M* Tris-HCl [pH 8.3], 500 m*M* KCl, and 15 m*M* $MgCl_2$), 1 μl of each primer-pair (Pba1, 1 μ*M*; Pba2, 10 μ*M*), 0.2 μl of Taq DNA polymerase (5 U/μl), and 2 μl of the DNA extract from the CSF specimens. The PCR reaction was performed in a DNA Engine Tetrad thermal cycler (MJ Research, South San Francisco, CA).

The PCR program involved 30 cycles. Each cycle consisted of denaturation at 94°C for 40 secs, annealing at 56°C for 40 secs, extension at 72°C for 50 secs, and final extension for 8 mins. The presence of a PCR product was confirmed by 1% agarose electrophoresis and visualization with ethidium bromide. The DNA of *Candida albicans,* HBV, and human genomic DNA were used as negative controls for PCR analysis.

**Hybridization Protocol.** The membrane microarray was washed using 5 × SSC/0.5% SDS for 15 mins to remove any unbound oligonucleotides. Ten microliters of digoxin-labeled PCR products were heated to 98°C for 10 mins in a thermal cycler followed by quick cooling in an ice bath for 5 mins. After it was preheated to 55°C, 200 μl of the hybridized fluid (5 × SSC, 0.5% SDS, and 0.5% blocking reagent) was mixed with amplicons and added to the hybridized chamber. Hybridization was continued for 30 mins at 55°C. Then, the hybridized fluid was discarded. The membrane was washed two times in 0.5 ml of 2 × SSC/0.1% SDS for 5 mins and then washed two more times in 0.5 ml of 0.2 × SSC/0.1% SDS for 5 mins. This was followed by detection using a colorimetric detection system (Roche, Penzberg, Germany) that included an alkaline phosphatase (ALP)-covalent antidigoxin antibody and ALP substrate, following the manufacturer's instructions. Color development was visible between 15 mins and 1 hr after the start of the reaction.

**Interpretation of Hybridization Results.** All CSF samples were processed on the day on which they were identified, and the resulting membranes were visually compared with the results previously obtained from the bacterial strains. A report prediction concerning the presence of bacterial species was then produced, and the

**Table 1.** Universal Primers and Oligonucleotide Probes Designed

| Primers or organism | GenBank number | Oligonucleotide primer or probe | Location |
|---|---|---|---|
| Forward primer Pba1 | | 5′-ACT CCT ACG GGA GGC AGC AGT-3′ | 358–378 |
| Reverse primer Pba2 | | 5′-digoxin-TCA CCG GCC GTG TGT ACA AG-3′ | 1444–1425 |
| *A. calcoaceticus* | Z93434 | AC1: 5′-CAC TTT AAG CGA GGA GGA GG-3′ | 456–475 |
| | | AC2: 5′-GGC CTT TGA GGC TTT AGT GGC-3′ | 862–884 |
| *B. fragilis* | X83947 | BF1: 5′-TAT GTA TAA TAT GAA TAA GGA T-3′ | 506–527 |
| | | BF2: 5′-GAG AGA CTG CCG TCG TAA GAT-3′ | 1185–1205 |
| *E. coli* | E05133 | EC1: 5′-CAG CGG GGA GGAAGG GAG TAA AGT-3′ | 461–485 |
| | | EC2: 5′-GAT GAG AAT GTG CCT TCG GGA A-3′ | 1042–1067 |
| *Enterococcus* | AJ291732 | EET1: 5′-GAA CAA GGA TGA GAG TAA CTG T-3′ | 471–492 |
| | | EET2: 5′-GAA GTA CAA CGA GTC GCG AAG T-3′ | 1278–1301 |
| *F. meningosepticum* | D14018 | FM1: 5′-GGT CCG TAG GCG GAC TAG TAA GTC-3′ | 600–623 |
| | | FM2: 5′-GTC GAA CTG CCA TTG ATT CTG C-3′ | 649–670 |
| *Fusobacterium* | AF044948 | FN1: 5′-TCT AGG CGG CAA GGA AAG TCT GAT-3′ | 605–627 |
| | | FN2: 5′-GAC GAA CGG AGC AGA GAT GCG-3′ | 1026–1047 |
| *H. influenzae* | X87977 | HI1: 5′-TCT TTC GGT TAT TGA GGA AGG TTG-3′ | 456–480 |
| | | HI2: 5′-GTA ACT AGA GTA CTT TAG GGA GGG G-3′ | 671–693 |
| *L. monocytogenes* | U84150 | LM1: 5′-CAA GGA TAA GAG TAA CTG CTT GTC C-3′ | 474–497 |
| | | LM2: 5′-TAA AGT GAC TGC CGG TGC AAG CC-3′ | 1183–1207 |
| *M. catarrhalis* | AF005185 | MCA1: 5′-GCT TAT GGT TAA TAC CCA TAA GCC-3′ | 477–503 |
| | | MCA2: 5′-CTT TTA AAG ACT TAG TGA CGC AGT-3′ | 865–890 |
| *M. tuberculosis* | X52917 | MT1: 5′-GAC GAA GGT CCG GGT TCT CT-3′ | 468–502 |
| | | MT2: 5′-ACA TGC ACA GGA CGC GTC TAG AG-3′ | 1020–1043 |
| *N. meningitidis* | AJ239290 | NM1: 5′-AAA AGG CTG TTG CTA ATA TCA GCG-3′ | 472–498 |
| | | NM2: 5′-CTG GGA CAA CAC TGA CGT TCA TGC-3′ | 654–678 |
| *Peptostreptococcus* | L04168 | PEP1: 5′-ACG GTA CCC TGT GAG GAA GCC-3′ | 506–527 |
| | | PEP2: 5′-GAC CGG TGT TTA ATC A CA CCT T-3′ | 1030–1054 |
| *P. haemolytica* | AF224287 | PH1: 5′-GAA CAG TCG ATT GAC GTT AAT CA-3′ | 493–516 |
| | | PEP2: 5′-GAC CGG TGT TTA ATC A CA CCT T-3′ | 853–876 |
| *P. mirabilis* | AF008582 | PM1: 5′-GGA GGA AGG TGA TAA GGT TAA TAC-3′ | 467–490 |
| | | PM2: 5′-TGT GGT CTT GAA CCG TGG CTT CT-3′ | 859–884 |
| *Pseudomonas* | AF094720 | PSE1: 5′-TAG GTG GTT CAG CAA GTT GGA T-3′ | 606–627 |
| | | PSE2: 5′-GCA TCC AAA ACT ACT GAG CTA GAG-3′ | 657–680 |
| *S. aureus* | D83353 | SA1: 5′-GAA CAT ATG TGT AAG TAA CTG TGC-3′ | 471–495 |
| | | SA2: 5′-CTA GAG ATA GAG CCT TCC CCT T-3′ | 1037–1059 |
| *Staphylococcus* | AY030340 | S1: 5′-GGT TTC CGC DCCC TTA GTG CTG-3′ | 439–459 |
| | D83371 | S2: 5′-CGT AAA ACT CTG TTA TTA GGG-3′ | 854–874 |
| *S. agalactiae* | AB023574 | SAG1: 5′-CGT TGG TAG GAG TGG AAA ATC TA-3′ | 474–496 |
| | | SAG2: 5′-TTC TGA CCG GCC TAG AGA TAG GC-3′ | 1026–1048 |
| *Salmonella* | AF227869 | SAL1: 5′-CAG CGG GGA GGA AGG TGT TGT-3′ | 461–483 |
| | | SAL2: 5′-TTG TGG TTA ATA ACC GCA GCA-3′ | 479–501 |
| *S. pneumoniae* | AF003930 | SPN1: 5′-AAG TTC ACA CTG TGA CGG TAT C-3′ | 491–515 |
| | | SPN2: 5′-TTT AAC TTG AGT GCA AGA GG-3′ | 670–689 |

data were compared with the subsequent identification of organisms by routine laboratory testing.

## Results

**Specificity of Universal Bacterial Primers.** The primers Pba1 and Pba2 correspond to regions of the 16S rDNA that are highly conserved in all eubacteria were designed in 2001 and, therefore, would be expected to amplify DNA from almost all pathogenic bacteria. The primer locations were chosen to be relatively specific for eubacterial genes (Table 1). The specificity of the universal primers was assessed with DNA extracts from 45 bacterial strains representing 20 different bacterial species. All tested bacterial strains produced PCR products of approximately 1086 base pairs. No positive bands were seen in the negative controls.

The results of the PCR analyses performed with the CSF specimens are as follows. The 29 samples from 16 patients that were positive by bacterial culture were also positive by PCR analysis, and the 71 samples that were negative by culture were also negative by PCR analysis.

**Sensitivity of Universal PCR.** The sensitivity of universal PCR was tested with purified *E. coli* DNA and *E. coli* cells (ATCC 35218). The DNA was fixed in quantity and serially diluted to the appropriate concentration. The cells were grown in Luria-Bertani (LB) fluid media to log phase and serially diluted 10-fold in sterile water. Aliquots from the same dilutions were removed for amplification and for plating onto LB agar. The CFUs were counted after overnight growth at 37°C. Reactions testing the sensitivity of amplification were performed, and the sensitivity of PCR

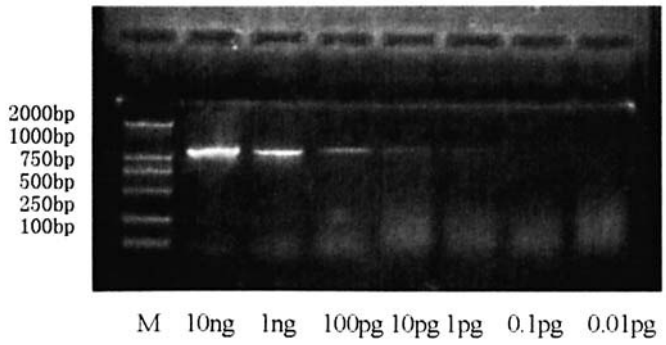


**Figure 1.** The sensitivity of a universal PCR assay. Serial dilutions of *E. coli* DNA and cells were amplified with primers Pba1 and Pba2 and run on an agarose gel. Molecular marker sizes are given on the left in base pairs.

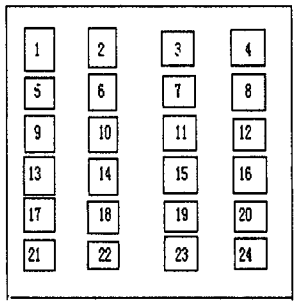


**Figure 2.** The order of probes arranged. 1–20 oligonucleotide probes arranged in order: 1. *A. calcoaceticus*, 2. *B. fragilis*, 3. *E. coli*, 4. *Enterococcus*, 5. *F. meningosepticum*, 6. *F. necrophorum*, 7. *H. influenzae*, 8. *L. monocytogenes*, 9. *M. catarrhalis*, 10. *M. tuberculosis*, 11. *N. meningitidis*, 12. *Pseudomonas*, 13. *Peptostreptococcus*, 14. *P. haemolytica*, 15. *P. mirabilis*, 16. *S. aureus*, 17. *Staphylococcus* sp, 18. *S. agalactiae*, 19. *Salmonella*, and 20. *S. pneumoniae*. 21–24 represent positive control, HBV DNA negative control, human DNA negative control, and blank, respectively.

was tested as $10^{-12}$ g, while the lowest number of bacterial cells that gave a positive result was 7 CFUs (Fig. 1).

**Hybridization From Bacterial Strains and Clinical Specimens.** After gene amplification, bacteria were hybridized with microarray. It was shown that *A. calcoaceticus, E. coli, Enterococcus, F. meningosepticum, F. necrophorum, H. influenzae, L. monocytogenes, M. catarrhalis, N. meningitidis, Peptostreptococcus, P. haemolytica, Pseudomonas, S. aureus, Staphylococcus* sp., *S. agalactiae, Salmonella*, and *S. pneumoniae* were hybridized to their own probes, but there were unspecific hybridization phenomena such as probes NM1 and NM2 hybridized to *M. catarrhalis* and *L. monocytogenes* DNA in addition to *N. meningitidis* DNA. In addition to *M. catarrhalis*, MCA2 hybridized to *N. meningitidis* DNA. Both EC1 and EC2 were tested against other bacteria such as *Pseudomonas, Salmonella*, and *P. mirabilis*. Standard strain *M. tuberculosis* and *P. mirabilis* did not present any hybridized results. The most unspecific hybridization dots appeared with *L. monocytogenes*. Examples of the obtained microarray hybridization are shown in Figure 3.

Twenty-nine CSF samples from 16 patients identified as positive by bacterial culture were performed by PCR, and 29 CSF samples produced PCR products (100%), followed by hybridization with microarray. The results are as follows. Ten CSF specimens from four patients identified as *S. epidermidis* by culture hybridized to probe S1 and S2. Seven CSF specimens from three patients were identified as *A. calcoaceticus* by culture and microarray. Four CSF samples from two patients identified as *P. aeruginosa* by culture appeared as positive dots with the probes PSE1 and PSE2. There were six CSF samples from four patients identified as *Enterococcus*, two of which did not have hybridized dots. One CSF sample identified as *S. aureus* could hybridize to probes SA1, SA2, S1, and S2. Furthermore, one CSF sample that was a mixture of *Enterococcus* and *A. calcoaceticus* was correctly identified by microarray.

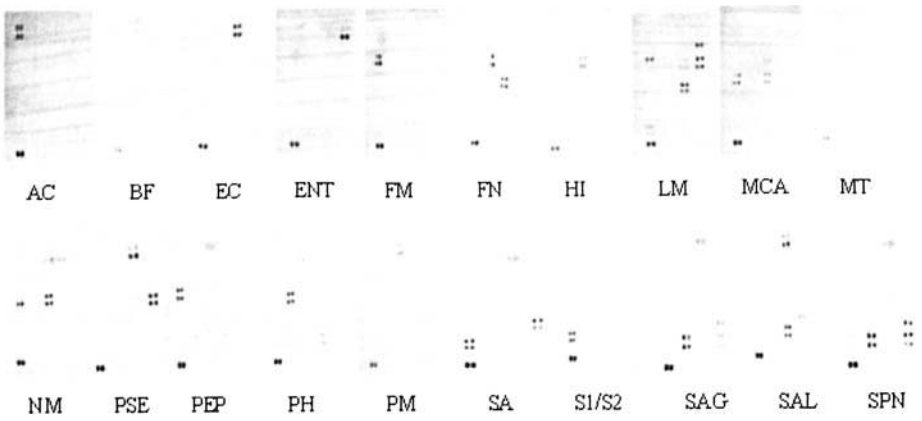


**Figure 3.** The results of hybridization of 20 species of bacteria from blood culture media.The strips of membrane *A. calcoaceticus* to SPN were PCR amplifications from blood culture media that subsequently grew bacteria identified as *A. calcoaceticus* (AC), *B. fragilis* (BF), *E. coli* (EC), *Enterococcus* (ENT), *F. meningosepticum* (FM), *F. necrophorum* (FN), *H. influenzae* (HI), *L. monocytogenes* (LM), *M. catarrhalis* (MC), *M. tuberculosis* (MT), *N. meningitides* (NM), *Pseudomonas* (PSE), *Peptostreptococcus* (PEP), *P. haemolytica* (PH), *P. mirabilis* (PM), *S. aureus* (SA), *Staphylococcus* sp. (S), *S. agalactiae* (SAG), *Salmonella* (SAL), and *S. pneumoniae* (SPN). No hybridization is visible on *M. tuberculosis* and *P. mirabilis* because the target genes of two bacteria are mismatched with their probes. The oligonucleotides are listed in Table 1 and were arranged as shown in Figure 2.

## Discussion

Currently, the 16S rRNA genes of almost all bacterial pathogens found in body fluids have been sequenced, and it is possible to design PCR primers that amplify all eubacteria based on the conservative nature of the 16S rRNA genes (11). Furthermore, 16S rDNA sequences constitute the largest gene-specific database, and the number of entries in generally accessible databases is continually increasing, making the 16S rDNA–based identification of unknown bacteria isolates more and more possible (12). The 16S rDNA has become an ideal target sequence of bacterial gene classification and has been used for more and more parallel detection of bacteria in body fluid. The use of universal PCR primers targeting DNA regions conserved in bacteria for the purpose of DNA amplification has been described (6, 11, 14). The ranges of these universal primers, however, are too narrow for us to design enough species-specific probes.

The purpose of this study was to develop a rapid and sensitive method to detect and identify bacteria in CSF specimens that are supposedly sterile. The PCR analysis was used, and a set of PCR primers was designed based on the conserved sequence of the 16S rRNA genes of various bacteria, which provide sensitivity 1.0 pg of *E. coli* DNA, corresponding to 7 CFUs. It would be adequate for detection of bacteria in CSF specimens because 85% of CSF samples with bacterial infection contained more than 1000 CFU/ml (14).

Each oligonucleotide probe should hybridize to only one bacterial species for an ideal microarray. In practice, two or more oligonucleotide probes are often required (15). Many probes designed by other researchers cannot be used with our target genes, so we designed two specific probes for each bacterium and found that there was still some cross-reaction among the related species. Recently, probeBase (www.microbial-ecology.net/probebase), an online resource for rRNA-targeted oligonucleotide probes, has provided the other way to obtain suitable and better probes (16). So, it is possible for us to use probeBase as a resource for further improving the microarray.

The membrane microarray method was used to detect a CSF of two bacteria infections (*Enterococcus* and *A. calcoaceticus*), which was successful in identifying mixtures of organisms in polymicrobial meningitis. The result represents an important advantage of using a single parallel identification procedure. We have not yet investigated sufficient numbers of bacteria and CSF specimens. As more isolates and CSF specimens are studied, interpretation of variations in hybridization will be possible and their taxonomic significance can be determined.

We have developed the membrane microarray technique where nylon membrane was used as a substrate. Various species of bacteria can be identified through hybridization at a time, and specialized detection equipment is not required, making the cost very low. Furthermore, identification can be achieved in a short period of time (5 hrs). In short, this represents a good prospect for clinical applications. We are sure that with the further development of the microarray technique, it will become a valuable tool for detecting clinical organisms.

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