Comparison Between Two PCR-Based Bacterial Identification Methods Through Artificial Neural Network Data Analysis

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The 16S ribosomal ribonucleic acid (rRNA) and 16S-23S rRNA spacer region genes are commonly used as taxonomic and phylogenetic tools. In this study, two pairs of fluorescent-labeled primers for 16S rRNA genes and one pair of primers for 16S-23S rRNA spacer region genes were selected to amplify target sequences of 317 isolates from positive blood cultures. The polymerase chain reaction (PCR) products of both were then subjected to restriction fragment length polymorphism (RFLP) analysis by capillary electrophoresis after incomplete digestion by *Hae III*. For products of 16S rRNA genes, single-strand confor-

mation polymorphism (SSCP) analysis was also performed directly. When the data were processed by artificial neural network (ANN), the accuracy of prediction based on 16S-23S rRNA spacer region gene RFLP data was much higher than that of prediction based on 16S rRNA gene SSCP analysis data (98.0% vs. 79.6%). This study proved that the utilization of ANN as a pattern recognition method was a valuable strategy to simplify bacterial identification when relatively complex data were encountered. *J. Clin. Lab. Anal.* 22:14–20, 2008. © 2008 Wiley-Liss, Inc.

Key words: 16S rRNA gene; 16S-23S rRNA spacer region gene; capillary electrophoresis; artificial neural network (ANN); single-strand conformation polymorphism (SSCP); restriction fragment length polymorphism (RFLP)

INTRODUCTION

Infectious diseases are still a major threat to human beings. Usually, the presence of living microbes in the blood of a patient is a sign of serious invasive infection taking place. The exact mortality associated with bloodstream infections varied depending on several factors, and might range from 20% to 50% (1). Blood culture is therefore essential to enable physicians to accurately determine the etiology of an infection, and prescribe the optimum antibiotic treatment. Routine microbiological detection of bacteremia relies on enrichment of the causative pathogen using automated continuous-monitoring blood culture systems followed by Gram stain, subculture on agar, and subsequent biochemical identification. This time-consuming procedure often results in the unavailability of rapid definitive identification of pathogens earlier than 24–72 hr, even when automated identification systems and commercial blood culture systems are at hand.

To facilitate rapid identification of pathogens, circumventing or shortening the culture procedure is

crucial to developing methods. Molecular diagnosis is playing an increasingly important role in the rapid detection and identification of pathogenic organisms in clinical samples (2). The genetic variation of ribosomal genes in bacteria offers an alternative target for bacterial detection and identification. The ribosomal ribonucleic acid (rRNA) genetic loci are found in all prokaryotic organisms, and highly conservative within families, genera, or species. Even so, relatively stable variations are found frequently amid different bacteria. 16S rRNA and 16S-23S rRNA spacer region genes are the commonly used targets for polymerase chain reaction (PCR)-based identification (3–6). Although most of the

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reports claimed to successfully identify certain microorganisms, many data were judged arbitrarily. When multiple bacteria data were presented simultaneously, the differential abilities were not very satisfying (7). Microarray technology can overcome the above problems to some extent, but it is an uneconomical strategy that may not be very practical for common laboratory users. In this study, first we utilized a fluorescent PCR technique to amplify 16S rRNA and 16S-23S rRNA spacer region genes, and then performed restriction fragment length polymorphism analysis by capillary electrophoresis for the PCR products incompletely digested by *Hae III* (in short as RFLP-CE-ID). Capillary electrophoresis single-strand conformation polymorphism (CE-SSCP) analysis for 16S rRNA gene products was also performed directly. Last, those data were processed by an artificial neural network (ANN) to evaluate and compare their application in bacterial identification.

MATERIALS AND METHODS

Bacterial Strains

The 317 pathogenic bacteria were isolated from clinical positive blood culture samples. All the isolates were identified by an ATB expression system (bioMérieux, France) with the kits ID 32GN, API 20E, API 20NE, API staph, and API 20 Strep. All their identities were confirmed without any doubt. Three American Type Culture Collection strains, *E. coli* (ATCC25922), *S. aureus* (ATCC25923), and *P. aeruginosa* (ATCC27853), were utilized as controls. Detailed information on the clinical isolates is given in Table 1. All organisms were subcultured onto 5% sheep blood agar (bioMérieux, Marcy l'Etoile, France) and incubated at 35°C for 20 hr prior to template preparation. A total of two strains of fungi (*C. albicans* and *C. neoformans*) were utilized as negative controls when PCR amplification was performed.

Template Preparation

Fresh colonies of the bacteria were suspended in properly sterilized distilled water to the turbidity of 2.5 Mcfarland using a bioMérieux DENSIMAT transmissometer. The suspensions were then subjected to template preparing using a TaKaRa (Dalian, China) MiniBEST Bacterial Genomic DNA Extraction Kit Ver.2.0 according to the instructions provided by the manufacturer.

PCR Amplification Utilizing Fluorescent Primers

A total of two pairs of universal fluorescent labeled primers for 16S rRNA genes (synthesized by TaKaRa) used in the PCR were as follows: 5'FAM-GGCGGACGGGTGAGTAA-3' (ER10) and 5'JOE-GGACTGCTGCCTCCCGTAG-3' (ER11G) (in *E. coli*, their complementary regions in 16S rRNA gene are 103–119 and 341–357); 5'JOE-GAGGAAGGTGGGGATGACGT-3' (P11P) and 5'ROX-AGGCCCGGGAACGTATTCAC-3' (P13P) (in *E. coli*, they are complementary to the 1173–1192 and 1370–1389 regions of the 16S rRNA gene). The primers for 16S-23S rRNA spacer region genes were 5'FAM-GAAGTCGTAACAAGG-3' (G1) and 5'JOE-CAAGGCATCCACCGT-3' (L1) (synthesized by TaKaRa). The complementary positions in genomic DNA are shown in Fig. 1. The 50 µL PCR amplification mixture contained 5 µL Ex Taq buffer (TaKaRa), 5 µL deoxyribonucleotide triphosphate (dNTP) mixture, 2 µL primer mixture (final concentration of 0.2–1.0 µM for each forward and reverse primer), 4 µL 25 mM MgCl₂, 0.25 µL Ex Taq polymerase (TaKaRa), and the rest was water. PCR was performed using a GeneAmp PCR system 2700 (Applied Biosystems, Foster City, CA) with one cycle at 94°C for 5 min and 30 cycles at 98°C 15 sec, 55°C for 1 min, and 72°C for 1 min. The last cycle was ended by 72°C for 7 min. The whole products were kept at –20°C until further processing.

TABLE 1. Strains isolated from positive blood cultures

Name	Numbers	Name	Numbers	Name	Numbers
<i>S. aureus</i>	11	<i>P. fluorescens</i>	15	<i>S. paratyphi-A</i>	5
<i>S. epidermidis</i>	9	<i>P. stutzeri</i>	2	<i>E. faecalis</i>	8
<i>K. pneumoniae</i>	38	<i>C. testosteroni</i>	2	<i>E. faecium</i>	5
<i>K. oxytoca</i>	11	<i>S. maltophilia</i>	5	<i>S. pneumoniae</i>	9
<i>A. junii</i>	4	<i>S. putrefaciens</i>	2	<i>E. coli</i>	42
<i>A. baumannii</i>	26	<i>F. meningosepticum</i>	2	<i>E. tarda</i>	8
<i>A. lwoffii</i>	3	<i>F. indologenes</i>	1	<i>C. freundii</i>	2
<i>P. aeruginosa</i>	23	<i>E. sakazakii</i>	17	<i>P. mirabilis</i>	8
<i>P. putida</i>	14	<i>E. aerogenes</i>	13	<i>P. vulgaris</i>	3
<i>B. cepacia</i>	7	<i>E. cloacae</i>	13		
<i>M. morgani</i>	3	<i>S. typhi</i>	6		

Fig. 1. Schematic representation of rRNA genes showing approximate priming sites for the PCR amplification.

Restriction Endonuclease Digestion

The 20- μ L reaction mixture contained 2 μ L PCR product, 1 μ L *Hae III* (TaKaRa), 2 μ L 10 $\times$ M buffer (TaKaRa), and the rest was water. After incubation at 35°C for 30 min, the reaction tubes were frozen at -20°C immediately to stop the digestion.

RFLP-CE-ID Analysis

Unless otherwise stated, all the reagents and apparatus were from Applied Biosystems. A two-fold diluted digestion solution was denatured at 98°C for 4 min and then rapidly cooled in ice water. Sample preparation for capillary electrophoresis also involved the addition of 1 μ L sample to 24 μ L deionized formamide and 1 μ L GeneScan 500[ROX]. Size standard (Warrington, UK). Subsequent preparation, such as setting up of the ABI PRISM 310 Genetic Analyzer, was done in accordance with the manufacturer's instructions. The separation medium was 4% POP-4 GeneScan polymer. A 47 cm $\times$ 50 μ m (inner diameter) capillary was used for separation. Electrophoresis condition was set at a 5-sec injection time, a 15-kV injection voltage, a 15-kV electrophoresis voltage, a constant temperature of 60°C, and a collection time of 24 min. To ensure the accuracy and reproducibility, only fragments in lengths from 50 to 500 bp were taken into consideration.

CE-SSCP Analysis

Linear polyacrylamide (LPA) (Sigma-Aldrich, Steinheim, Germany) was dissolved in Tris/borate/ethylene diamine tetraacetic acid (EDTA) (TBE) buffer (89 mM Tris, 89 mM boric acid, and 2 mM EDTA, pH = 8.0) to the final concentration of 6% (m/v) and kept at 4°C for storage. Polymer preparation was just the same as described previously (8). A 47-cm-long fused-silica capillary with the inner diameter of 50 μ m (Yongnian Optical Fibre Work, Hundan, China) was rinsed by

0.2 M NaOH for 1 hr, and then by deionized water, methyl alcohol, and air respectively. The protocols of coating capillary were according to Hjerten (9) and Hjerten et al. (10). The setting of capillary electrophoresis instrument was similar to that of described above. All capillary analysis used TBE (pH = 8.0) buffer.

ANN Analysis

ANN analysis was performed using the software of EasyNN v7.0 (Neural Planner Software Ltd., Cheshire, UK). The number of units in the input layer was determined by the number of input variables. The corresponding number of units in the output layer depended on the species of the studied bacteria. The numbers of units in the middle layers of the constructed networks were optimized by the software. The training process employed learning rule of back propagation of error utilizing randomly selected 60% of the whole data. Training was terminated when the sum of squares error with respect to the validation data set was at a minimum in order to avoid overfitting. The remaining data were randomly divided into two parts, one for validation and the other for prediction, equally. To eliminate any possible bias, each network was trained and validated with 10 randomly selected training and validation data sets. The differences in network performance among these selections were not significant. All statistical analysis was done on Minitab v.14 for Windows (State College, PA).

RESULTS

PCR Results

For all the bacteria, their 16S rRNA gene products were 255 (ER10-ER11G) and 217bps (P11P-P13P) in length, respectively. But the spacer region gene primers could generate multiple bands for each strain in the agarose electrophoretograms. Fig. 2 shows some typical patterns of some isolates.

Fig. 2. The agarose gel electrophoresis patterns of the 16S-23S rRNA gene PCR products of some strains. A: *E. coli* (ATCC25922); B: *E. coli*; C: *E. coli* extended spectrum beta-lactanases (ESBLs) producing strains; D: *K. pneumoniae*; E: *K. pneumoniae* (ESBLs producing strains); F: *K. oxytoca*; G: *S. paratyphi-A*; H: *E. sakazakii*; I: *S. typhi*; J: *E. cloacae*; K: *S. putrefaciens*; L: *P. mirabilis*; M: *P. vulgaris*; N: *C. freundii*; O: *M. morganii*; P: *B. cepacia*; Q: *P. aeruginosa*; R: *A. baumannii*; S: *A. lwoffii*; T: *S. maltophilia*; U: *P. aeruginosa* (ATCC27853); V: *S. aureus*; W: *S. epidermidis*; X: *S. pneumoniae*; Y: *C. testosteronei*; Z: *E. faecalis*; 1: *C. albicans*; 2: *C. neoformans*; 3: absence of template; 4: marker, from top to the bottom, 2,000, 1,000, 750, 500, 250, and 100 bp.

TABLE 2. Performances of ANNs on prediction data set

Data source	Average error for training ANN	Validation (%) ^a	Overall accuracy for prediction (%) (95% CI)
16S rRNA genes	0.002	85.1	79.6 (65–90)
16S-23S rRNA spacer region genes	0.0008	95.9	98.0 (89–100)

^aCorrect validation rate when sum of squares error was at a minimum. CI, confidence interval.

RFLP-CE-ID Analysis

All the PCR products, after incomplete digestion by *Hae III*, were separated by capillary electrophoresis. Under most circumstances, raw PCR products stemming from each pair of 16S rRNA gene primers gave two digested fragments for each strain. Typical digested product patterns of 16S-23S rRNA spacer region genes were involved in multiple fragments.

Basically, for every primer, the detectable fragment number was more than two for each strain.

Capillary Electrophoresis SSCP Analysis

For each isolate, 16S rRNA gene products were mingled together and analyzed simultaneously. Each strain was characterized with four detectable peaks with different retention times, as expected.

ANN Analysis Results

ANN analysis results are shown in Table 2. The wrongly predicted strains by ANNs based on different kinds of data are shown in Table 3.

DISCUSSION

Traditionally, identification of bacteria is mainly depended on biochemical characteristics. Commercial identification systems only can achieve the identification rate of 57–80% (11). Molecular diagnosis gains more

TABLE 3. Confusion matrixes of ANNs based on the two kinds of data

Data source of ANN	Bacterium in reality	Predicted by ANN	Total number of incorrectly predicted
16S rRNA genes	<i>K. pneumoniae</i>	<i>E. cloacae</i>	10
	<i>K. pneumoniae</i>	<i>S. pneumoniae</i>	
	<i>K. oxytoca</i>	<i>E. coli</i>	
	<i>P. aeruginosa</i>	<i>P. fluorescens</i>	
	<i>E. cloacae</i>	<i>E. coli</i>	
	<i>E. cloacae</i>	<i>E. coli</i>	
	<i>E. cloacae</i>	<i>E. sakazakii</i>	
	<i>E. sakazakii</i>	<i>E. coli</i>	
	<i>E. sakazakii</i>	<i>E. coli</i>	
	<i>E. coli</i>	<i>E. sakazakii</i>	
16S-23S rRNA spacer region genes	<i>P. aeruginosa</i>	<i>P. fluorescens</i>	1

application in rapid detection and identification of pathogenic organisms in clinical samples. In theory, since each bacterial species has unique 16S rRNA sequences, microorganisms can be differentiated from each other thereby. 16S rRNA genes have been utilized as targets for identification of microorganisms including *chlamydia*, *mycoplasma*, *spirochetes*, and *rickettsia* for many years. With the rapid development of modern molecular biology, many fastidious bacteria can be identified easily. DNA sequence-based analysis of 16S rRNA genes has been used as a new taxonomic and nomenclature method; many new isolates have been given proper names accordingly (12). The primers of 16S rRNA genes used in our research were chosen from V2 and V8 regions, which were considered as the best sites for differential purposes (13,14). Digested products coming from these two pairs of primers were all two fragments. Adoption of incomplete digestion was based on the consideration that it can provide information for the whole digestible sites. Except products, digested by *Hae III*, from ER10-ER11G of *P. mirabilis* and *P. vulgaris*, whose fragments were different in lengths, the other isolates' digested fragments of 16S rRNA genes were only of family specificities. For example, products of ER10 and ER11G of *Staphylococcus* only had a 214-bp fragment (the other one was too short to be detected); strains of nonfermenting genera only gave 92-bp and 163-bp fragments. It was obvious that RFLP-CE-ID data of the selected 16S rRNA genes might be too limited to provide sufficient information for the utilization of bacterial differentiation.

To overcome the shortcomings of 16S rRNA gene data, we introduced the 16S-23S rRNA spacer region genes to improve the differentiation. In prokaryotes, the rRNA genetic loci contain the genes for all three rRNA species: 16S, 23S, and 5S genes. These genes are separated by spacer regions, which exhibit a large

degree of sequence and length variation at the levels of genera and species (Fig. 1) (15). Within a single genome these loci also show a significant degree of variations of the insertion numbers and types of tRNA sequences (16). From Fig. 2, most of the products of the studied strains showed different patterns with length and/or number of polymorphisms. But some patterns, for example, *P. vulgaris* and *P. mirabilis*, took a similar appearance. We ascribed this trait to the conservative sequences amid the bacteria (17). In some cases weak and variable secondary amplification products in the size range over 800 bp were generated. These products were observed most frequently in the genera of *Proteus*, *Salmonella*, and *Staphylococcus*, and to a much lesser degree in the genus of *Escherichia*. The yield of such products was significantly lower than the yield of products in lengths from 280 to 850 bp. Although the source and nature of these larger fragments were not well understood, their primary utility, we thought, might provide additional information for identification. To eliminate the effects of the secondary products with lower quantities and to efficiently make use of the relatively stable fragments, the detection range for RFLP-CE-ID was restricted in 50–500 bp, and the digestion samples were properly diluted before analysis.

Unlike the RFLP-CE-ID analysis, CE-SSCP analysis of the whole strains generated more regular results among the isolates, although some similar patterns still could be discerned among strains belonging to the same genera. Because every pair of primers of the 16S rRNA genes were labeled with different fluorescent dyes, the four fragments could be distinguished easily in their electropherograms even if the PCR products were separated together. Also owing to the remarkable length differences and migration velocity diversities, ER11G and P11P were labeled with the same fluorescent dye without resulting in overlapping peaks. SSCP analysis for fragments beyond the range of 200–400 bp were

usually not very satisfying (18), so PCR products of 16S-23S spacer region genes were not analyzed by CE-SSCP.

It seemed to be reasonable that the data from RFLP-CE-ID and CE-SSCP analysis of rRNA genes could be used to distinguish microbes potentially. But the manual comparison of those data was labor-intensive and time-consuming. An ANN is an information processing paradigm that is inspired by the way biological nervous systems, such as the brain, process information. It is composed of a large number of highly interconnected processing elements (neurons) working in unison to solve specific problems. ANNs, like people, can learn by training. This technology has been used as an efficient pattern recognition method in many arenas (19,20). Based on the consideration of reliabilities of the constructed networks, species with no more than nine isolates were discarded. Data of the remaining 241 bacteria, 60% as training set, 20% as validating set, and 20% as prediction set, were fed to EasyNN. Under the applied conditions in this research, the maximum variance of detected fragments less than 500 bp was about 0.2%, which meant that fragments with 1-bp differences could be differentiated correctly. According to that criterion, RFLP-CE-ID data from 16S-23S spacer region genes could be classified to 36 different fragment types (variables). Those data were then analyzed by ANN with the following settings; if certain fragment presented in certain strain, the corresponding input variable of that strain was "1," otherwise the value was "0." For 16S rRNA gene SSCP data, the retention time of each fragment was directly used as an input variable. Accuracy of ANN prediction based on 16S-23S rRNA spacer region gene RFLP-CE-ID data was about 98.0% (48/49). It was much higher than the accuracy of ANN based on 16S rRNA SSCP data (79.6%, 39/49). Statistical analysis proved the significance ($P < 0.01$) was remarkable. Most of the wrongly predicted strains by ANN based on 16S rRNA gene data were involved in *Enterobacteriaceae* (Table 3). This problem was perfectly solved by ANN based on 16S-23S rRNA spacer region gene data, although one isolate of *P. aeruginosa* was still incorrectly predicted.

Bacteremia is a kind of relatively dangerous infection and usually needs rapid pathogenic reports. PCR is undoubtedly the suitable choice because in most circumstances bacteremia is caused by one certain kind of bacteria. Many kits extracting bacterial DNA from blood samples have been developed. PCR can be performed when positive blood culture is acquired. Although gene sequencing can give definite results for bacterial identification, the performance is not economic. From this research, we concluded that ANN was a powerful tool to facilitate rapid identification of bacteria. Data from 16S-23S rRNA spacer region gene RFLP

analysis was more reliable than data from 16S rRNA gene SSCP analysis when ANN assay was employed as a pattern recognition method. This strategy could prove valuable in clinical bacterial identification if similar levels of accuracy can be demonstrated through direct examination of clinical samples (blood, plasma, etc.).

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