African Journal of Microbiology Research Vol. 6(2), pp. 371-378, 16 January, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR11.1068
ISSN 1996-0808 ©2012Academic Journals

Full Length Research Paper

Detection of common resistance genes of Gram-negative bacteria by DNA microarray assay

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Accepted 21 October, 2011

To design a DNA microarray for the detection of common resistance genes of Gram-negative bacteria, we collected 70 strains of Gram-negative bacilli (*Klebsiella pneumoniae*, *Escherichia coli*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*), and the primers and probes were designed and synthesized according to the known resistance genes to prepare the deoxyribonucleic acid (DNA) microarray for the detection of these resistance genes. Then, hybridization and subsequent scanning were performed. The results of DNA microarray assay were compared with results from the polymerase chain reaction (PCR**). Results showed that the consistency rate was 100.00% in the detection of 8 resistance genes (TEM, SHV, CTX-M, DHA, CIT, VIM, KPC and OXA-23). PCR assay revealed the resistance gene IMP in 1 strain of *P. aeruginosa*, which was absent in the DNA microarray assay. The DNA microarray designed in the present study has clinical value in the detection of common resistance genes of Gram-negative bacteria.**

Key words: Gram-negative bacteria, resistance gene, DNA microarray.

INTRODUCTION

In recent years, the statistics of national Bacterial resistance monitoring network (Mohnarin) show that Gram-negative bacilli account for 70% of infection (Xiao et al., 2008). With the wide application of broad-spectrum antibiotics such as third and fourth-generation cephalosporins, carbapenems and fluoroquinolones, bacterial drug resistance has become increasing serious. Traditionally, the detection of pathogenic bacteria depends on bacterial culture, and physiological and biochemical identifications and disk diffusion susceptibility testing are employed to confirm the drug resistance and identify the antibiotics to which bacteria resist. This method has a lot of procedures and is time-consuming. In addition, the sensitivity and specificity of this method are limited by the conditions for culture and the report is often delayed. Thus, in a majority of cases, the physicians estimate the potential pathogens and the drug resistance according to the epidemiology, clinical symptoms and findings on imaging and empirically apply the antibiotics which can cover the potential pathogens. When the results of bacterial culture are obtained, the mediaction is then adjusted. This strategy significantly limits the timely and effective application of antibiotics to which pathogens are sensitive, especially in patients with severe infection.

DNA microarray is also known as DNA chip and is one of biochips developed in the past decade (Yoo and Lee, 2008). In the DNA microarray assay, the known nucleic acid sequences are used as probes which then hybridize the target nucleic acid sequences. Subsequently, the hybridization signals are determined for quantitative and qualitative analysis. The DNA microarray assay has the advantaged in high sensitivity, specificity and ability to detect multiple gene simultaneously which significantly save time and enhance the detection efficiency. In the present study, we designed a DNA microarray for the detection of multiple resistance genes of Gram-negative bacteria. Assay with this DNA microarray is time-saving and efficient and can be used to guide the timely and

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accurate application of antibiotics.

MATERIALS AND METHODS

Materials

According to the national bacterial resistance monitoring report and local information of bacterial resistance, we selected 5 clinically common Gram-negative bacteria bacilli (*Klebsiella pneumoniae*, *Escherichia coli*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*) for the preparation of DNA microarray. All the strains were confirmed by the French Merieux French Merieux VITEK-II microbiology analysis system and the sensitivity analysis. A total of 70 multi-drug resistant strains were selected including imipenem resistant *K. pneumoniae* (n = 17), imipenem resistant *P. aeruginosa* (n = 17), imipenem resistant *A. baumannii* (n = 7), ceftazidime resistant *E. coli* (n=16) and ceftazidime resistant *E. cloacae* (n = 13). The standard strains were *E.coli* ATCC25922 (negative control) and ATCC35218 (positive control). All bacteria were purchased from the agricultural culture collection of China.

Culture of bacteria and DNA extraction

The bacteria were thawed and seeded in plates followed by extraction once. Single colony was used for preparation of bacterial suspension with 1 × PBS at the turbidity of 1.0. Then, 200 µl of suspension were centrifuged and the supernatant was removed. DNA extraction was performed according to DNeasy Tissue Kit (Qiagen, Crawley, UK) and the concentration and quality of DNA were determined using the ND-1000 Spectrophotometer.

Design and synthesis of primers for resistance genes

The clinically common resistance genes including TEM, SHV, CTX-M, DHA, CIT, IMP, VIM, KPC and OXA-23 were used as target genes. The primers and probes were designed by National Engineering Research Center of Biochip in Shanghai and synthesized in Invitrogen, Shanghai. The primers sequences are shown in Table 1.

PCR assay

Single-plex PCR

The mixture (15 µl) for PCR assay included 1.5 µl of 10 × PCR Buffer, 0.6 µl of $MgCl_2$ (25 mM), 0.2 µl of 10 mM dNTP , 0.2 µl of 20 pm each primer, 0.2 µl of Taq DNA Polymerase (5 U/µl), 1.0 µl of template DNA and sterilized distilled water. The conditions for PCR assay were as follows: pre-denaturation at 95℃ for 5 min, 30 cycles of denaturation at 95℃ for 30 s, annealing at 56℃ for 40 s and extension at 72℃ for 50 s and a final extension at 72℃ for 10 min. The products were then subjected to 2% agarose gel electrophoresis followed by EB staining. The bands were observed by using the GIS2010 gel image system and the presence of corresponding bands was defined as positivity. The primers and anticipated size of products are shown in Table 1.

Multiplex PCR

Multiplex PCR assay was performed in two groups (Table 1). The mixture (15 µl) for PCR included 1.5 µl of 10 × Titanium Taq PCR Buffer, 0.2 µl of dNTP Mixture (10 mM), 1.0 µl of primer mixture (1:1), 0.1 µl of Titanium Taq DNA Polymerase, 1.0 µl of template DNA and sterilized distilled water. The conditions for PCR assay were as follows: pre-denaturation at 95℃ for 5 min, 30 cycles of 95℃ for 30 s and 68℃ for 40 s and a final extensio n at 68℃ for 3 min. Agarose gel electrophoresis and analysis of results were similar to those earlier.

Preparation of DNA microarray and hybridization

Design and synthesis of probes for hybridization

Probes were designed by the National Engineering Research Center of Biochip in Shanghai and synthesized in Invitrogen, Shanghai. The probes sequences are shown in Table 1.

Microarray spotting

The Optical Grade Aldehyde Base Film was used in the preparation of DNA microarray. The probe solution was prepared at 100 pmol/µl. On the basis of designed DNA microarray (Table 2), 5 µl of probe solution were mixed with hybridization solution of equal volume and the mixture was placed in 384-well plate for sample spotting. The OmniGrid™ 100 microarrayer (USA) was used for sample spotting. There were 10 reaction regions in each microarray and detection was performed at least 3 times for each region. The distribution of target sequences in the microarray is shown in Table 2.

Pretreatment of microarray

Preparation of pretreatment solution was done with 312 µl of water, 40 µl of 20 × SSC, 40 µl of 100 × BSA, 8 µl of 10% SDS and water with a final volume of 400 µl. The pretreatment solution was evenly added to the hybridization region which was allowed to keep at room temperature for 30 min.

Purification of PCR products and SBE labeling

SAP and ExoI were used for the purification of products from Multiplex PCR. In brief, 4 µl of DNA were mixed in 1 µl of SAP and 1 µl of ExoI followed by incubation at 37℃ for 30 min and than at 85℃ for 10 min. A total of 15 µl of solution were used for SBE labeling and included 3 µl of purified products, 1.5 µl of 10 × ThermoPol Reaction Buffer, 0.2 µl of Thermo Sequenase DNA polymerase (enzyme for sequencing), 0.2 µl of marker and 0.2 µl of ddATP-Cy3 (1/10). The conditions for SBE labeling were as follows: pre-denaturation at 95℃ for 5 min, 40 cycles of denatu ration at 95℃ for 30 s, annealing at 60℃ for 30 s and exten sion at 72℃ for 20 s and a final extension at 72℃ for 5 min.

Hybridization on the microarray

The products following Single-base extension (SBE) labeling were mixed with positive control and hybridization solution, and this mixture was added to the microarray carrying resistance genes for hybridization at 48℃ for 2 h. Then, the microarray wa s washed.

Microarray scanning and result analysis

Microarray scanner (GenePix 4000B) was used to scan the microarray and the intensity of fluorescence signal was analyzed by using the GenePix Pro 6.0 software.

Table 1. Primers and probe for PCR assay.

	Gene	Primer	Primer sequence (5'- 3')	PCR length (bp)	Probe sequence (5'- 3')
1	OXA-23	OXA-L OXA-R	ATGGAAGGGCGAGAAAAGG TTGCATGAGATCAAGACCGATA	127	AGTGGATCTTGTACGTGGACCGCAAGTTCCTGATAGACTGGGACTGC
	IMP	IMP-L IMP-R	GCAGAGCCTTTGCCAGATTT CCGCCCGTGCTGTCACTA	254	AGACTCTCACTGCAAGCTGTAGCCACGTTCCACAAACCAAGTGACTA
	VIM	VIM-L VIM-R	CCAGATTGCCGATGGTGTT AATCTCGCTCCCCTCTACCTC	310	TTGACGCTACAGGTGACGATACAATGAGACCATTGGACGGGTAG
	CTX-M	CTX-M-L CTX-M-R	CGGGAGGCAGACTGGGTGT TCGGCTCGGTACGGTCGA	381	CCTGACTGCAATAGATCCTGACGGCCATCACTTTACTGGTGCTGC
	KPC	KPC-L KPC-R	CGCTGGTTCCGTGGTCACCC GAGCGCGAGTCTAGCCGCAG	557	CACTGAACAGCTGACATACG CGGGCCGCCCAACTCCTTCAGCAAC
2	CIT	CIT-L CIT-R	AGAGGCAATGACCAGACGC AGAGGCAATGACCAGACGC	174	GCTACGATACTGCAGAACCTCTTCGGCGTCAAGTTGTCCCGGAGA
	TEM	TEM-L TEM-R	GTCGCCGCATACACTATTCTCA CGCTCGTCGTTTGGTATGG	258	GTCAGCGAGAACATGTGTACGCGGTTAGCTCCTTCGGTCCTCCG
	SHV	SHV-L SHV-R	GCCTTGACCGCTGGGAAAC GGCGTATCCCGCAGATAAAT	319	CGAATCAGTCTTGCTCATCGTGTCGCCCTGCTTGGCCCGGATAAC
	DHA	DHA-L DHA-R	AACTTTCACAGGTGTGCTGGGT CGGGTCTGGGCGAGATACA	573	GTACACGATTCAGAGAGAGGCCGGGACGGCTGCCACTGCTGATAG

Table 2. Distribution of target sequences in the microarray.

P	P	P	P	P
P	OXA-23	…	IMP	VIM
P	CTX	KPC	CIT	…
P	TEM	SHV	…	DHA
P	…	…	…	…
P	…	…	…	…
P	…	…	…	…
P	…	…	…	…
P	…	…	…	…
P	…	…	…	…
P	…	…	…	…
P	…	…	…	…
P	N	N	B	B

P: positive probe; N: negative probe; B: blank probe; …: reserved.

Table 3. Resistance of different Gram-negative bacilli to antibiotics.

Antibiotics	*K. pneumoniae* (17 strains)		*E. coli* (16 strains)		*E. cloacae* (13 strains)		*P. aeruginosa* (17 strains)		*A.baumannii* (7 strains)	
	strains	Resistance rate (%)	strains	Resistance rate (%)	strains	Resistance rate (%)	strains	Resistance rate (%)	strains	Resistance rate (%)
Ampicillin	17	100.00	16	100.00	13	100.00	17	100.00	7	100.00
Ampicillin / sulbactam	17	100.00	15	93.75	13	100.00	17	100.00	7	100.00
Cefepime	16	94.12	16	100.00	7	53.85	15	88.24	7	100.00
Cefatriaxone	17	100.00	16	100.00	13	100.00	17	100.00	7	100.00
Ceftazidime	17	100.00	16	100.00	13	100.00	17	100.00	7	100.00
Cefotetan	17	100.00	3	18.75	13	100.00	17	100.00	7	100.00
Cefazolin	17	100.00	16	100.00	13	100.00	17	100.00	7	100.00
Aztreonam	17	100.00	16	100.00	13	100.00	17	100.00	7	100.00
Piperacillin / tazobactam	17	100.00	3	18.75	5	38.46	17	100.00	7	100.00
Cotrimoxazole	1	5.88	11	68.75	11	84.62	2	11.76	5	71.43
Ciprofloxacin	16	94.12	15	93.75	9	69.23	16	94.12	7	100.00
Gentamicin	14	82.35	14	87.50	5	38.46	13	76.47	5	71.43
Tobramycin	14	82.35	11	68.75	8	61.54	14	82.35	5	71.43
Imipenem	17	100.00	3	18.75	2	15.38	17	100.00	7	100.00
Levofloxacin	16	94.12	15	93.75	8	61.54	16	94.12	7	100.00
Kanamycin	14	82.35	2	12.50	3	23.08	15	88.24	5	71.43
Nitrofurantoin	15	88.24	1	6.25	5	38.46	15	88.24	7	100.00

RESULTS

In vitro antimicrobial susceptibility testing

The Gram-negative bacilli in the present study were multidrug resistant. All the bacilli were resistant to all the beta-lactam antibiotics including ampicillin, cefazolin, ceftazidime and ceftriaxone and the rate of resistance against quinolones, aminoglycosides, and carbapenems was more than 50%. Only the rate of resistance to sulfonamides was relatively low. Moreover, the rate of resistance against all antibiotics except for cotrimoxazole was higher than 80% in the imipenem resistant *K. pneumoniae* and *P. aeruginosa*; the 7 strains of *A. baumannii* were resistant against 17 antibiotics; the ceftazidime resistant *E. coli* were sensitive to cefotetan, piperacillin/tazobactam, imipenem, kanamycin and nitrofurantoin; Ceftazidime resistant *E. cloacae* were relatively sensitive to cefepime and imipenem when compared with other bacteria. The results of susceptibility testing are shown in Table 3.

Single-plex PCR

Among these bacteria, the detection rate of β-lactamase resistance genes was high and the TEM resistance gene was common in all bacteria. The detection of rate of TEM was 100 and 94.12% in imipenem resistant *A. baumannii* and *K. pneumoniae*, respectively. The SHV resistance gene was mainly found in imipenem resistant *K. pneumoniae* and ceftazidime resistant *E. cloacae*, but absent in the ceftazidime resistant *E. coli* and imipenem resistant *A. baumannii*. The DHA and CIT resistance genes were only identified in the imipenem resistant *K. pneumoniae* (88.24 and 94.12%, respectively). The OXA-23 resistance gene was predominantly identified in all the 7 strains of imipenem resistant *A. baumannii*. The IMP and VIM resistance genes were only noted in the *P. aeruginosa*.

Table 4. Resistance genes in different bacteria determined by PCR.

Resistance gene	*K. pneumoniae* (17 strains)		*E. coli* (16 strains)		*E. cloacae* (13 strains)		*P. aeruginosa* (17 strains)		*A. baumannii* (7 strains)	
	Strains	Detection rate (%)	Strains	Detection rate (%)	Strains	Detection rate (%)	Strains	Detection rate (%)	Strains	Detection rate (%)
TEM	16	94.12	9	56.25	8	61.54	5	29.41	7	100.00
SHV	16	94.12	0	0.00	9	69.23	1	5.88	0	0.00
CTX-M	17	100.00	8	50.00	4	30.77	0	0.00	0	0.00
DHA	15	88.24	0	0.00	1	7.69	0	0.00	0	0.00
CIT	16	94.12	3	18.75	2	15.38	0	0.00	0	0.00
KPC	17	100.00	6	37.50	1	7.69	0	0.00	0	0.00
OXA-23	2	11.76	0	0.00	7	53.85	0	0.00	7	100.00
IMP	0	0.00	0	0.00	0	0.00	1	5.88	0	0.00
VIM	0	0.00	0	0.00	0	0.00	5	29.41	0	0.00

Multiplex PCR

Most of the imipenem resistant *K. pneumoniae* had multiple resistance genes: 15 of 17 strains of imipenem resistant *K. pneumoniae* had 6 or more resistance genes; ceftazidime resistant *E. coli* had 2 or 3 resistance genes, but only 1 strain of *E. coli* had 1 resistance gene (TEM or CTX-M). Ceftazidime resistant *E. cloacae* also had 2 or more β-lactamase resistance genes. In 17 stains of imipenem resistant *P. aeruginosa*, only 1 had TEM, SHV and VIM resistance genes, 8 had TEM or VIM resistance gene, and the remaining 8 had no resistance genes detected in the present study. In addition, 7 strains of imipenem resistant *A. baumannii* had both the TEM and OXA-23 resistance genes Table 4.

Microarray scanning

In the present study, the IMP resistance gene was not detected among these bacteria using the present microarray and the results from the detection of resistance genes by using microarray were consistent with those from PCR. Results from detection with microarray are shown in Table 5 and those from microarray scanning in Figure 1 A, B, C and D.

Comparison

Evaluation of reliability

When the results from PCR were used as standards, the consistency rate of IMP resistance gene was 98.57% and that of remaining resistance genes was 100%. The adjusted consistency rate was higher than 75%. The Kappa of IMP resistance gene was 0 and that of remaining resistance genes was 1. These findings suggest that PCR and microarray assay have high consistency rate except in the detection of IMP resistance gene.

Evaluation of truthfulness

When the results from PCR were used as standards, the results of microarray assay were consistent with those from PCR. Except for IMP (sensitivity of 0), the sensitivity of detection of the remaining resistant genes was 100% by using microarray. These results indicate that the detection of resistant genes with the present microarray has good clinical application value.

DISCUSSION

Currently, the sensitivity of bacteria to antibiotics is determined based on the phenotype. The pathogenesis of drug resistance is diverse and some resistance genes might be silenced in the bacteria. Thus, the potential resistance might exist. Under the proper conditions, the bacteria with potential resistance may transform into resistant bacteria or transfer the resistance gene into other bacteria. PCR hybridization is a common method to detect the resistance gene. However, to screen one or severe resistance genes in numerous resistance genes is usually time consuming and requires repeated detection.

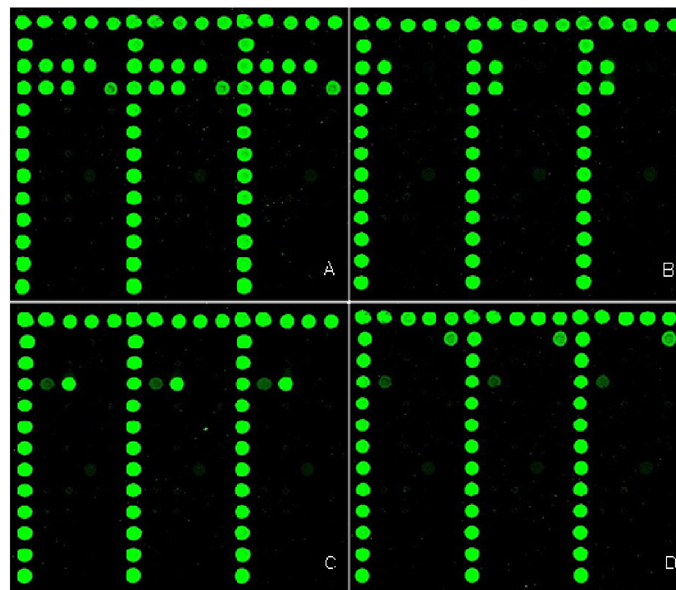

Figure 1 Results of microarray scanning. A, *Klebsiella pneumoniae*; B, *Escherichia coli*; C, *Enterobacter cloacae*; D, *Pseudomonas aeruginosa*

Table 5. Resistance genes in different bacteria in the microarray assay.

Resistance gene	*k. pneumoniae* (17 strains)		*E. coli* (16 strains)		*E. cloacae* (13 strains)		*p. aeruginosa* (17 strains)		*A. baumannii* (7 strains)	
	Strains	Detection rate (%)	Strains	Detection rate (%)	Strains	Detection rate (%)	Strains	Detection rate (%)	Strains	Detection rate (%)
TEM	16	94.12	9	56.25	8	61.54	5	29.41	7	100.00
SHV	16	94.12	0	0.00	9	69.23	1	5.88	0	0.00
CTX-M	17	100.00	8	50.00	4	30.77	0	0.00	0	0.00
DHA	15	88.24	0	0.00	1	7.69	0	0.00	0	0.00
CIT	16	94.12	3	18.75	2	15.38	0	0.00	0	0.00
KPC	17	100.00	6	37.50	1	7.69	0	0.00	0	0.00
OXA-23	2	11.76	0	0.00	7	53.85	0	0.00	7	100.00
IMP	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00
VIM	0	0.00	0	0.00	0	0.00	5	29.41	0	0.00

Microarray assay has high throughput and can detect multiple resistance gene simultaneously, which significantly increases the detection efficiency.

Investigators have attempted to detect the resistance gene with different microarrays and results show the clinical application value of these microarrays to a certain extent. Shen et al. (2007) designed a microarray for the detection of ESBLs and AmpC both of which are produced by the *E. coli*, *K. pneumoniae* and Acid-producing *Klebsiella*, and they performed detection in 225 strains of bacteria with above microarray. Results showed all the bacteria positive for ESBLs were identified by this technique except for a fraction of *E. coli* and *K. pneumoniae* carrying the phenotype AmpC. At the same time, they categorized the CTX-M gene. Weile et al. (2007) designed a microarray for the detection of anti-biotic resistance and virulence factors of *P. aeruginosa*. The detection using microarray included DNA extraction, amplification of target gene, fluorescence labeling and hybridization, which could be completed within 5 h. The sensitivity and specificity were 89 and 83%, respectively. Batchelor et al. (2008) also designed a microarray for the detection of resistance genes of *E. coli* and *Salmonella*. This microarray could be used to identify 47 resistance genes including those of aminoglycoside, trimethoprim, sulfonamides, tetracycline, β-lactamase and extended spectrum β-lactamase. The results from the detection with microarray were similar to those from PCR assay. (Naas et al., 2010) prepared a microarray for the detection of β-lactamase resistance genes of *Enterobacteriaceae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* including TEM, SHV, CTX-M and KPC. Their results showed high consistency between microarray assay and PCR assay. Moreover, the microarray assay has high high-throughput and can be used for the rapid detection of antibiotic sensitivity which is helpful to guide the clinical treatment. (Cassone et al., 2006) designed a microarray for the detection of macrolide resistance genes in 8 types of bacteria. In China, some investigators also applied microarray to detect the resistance genes in clinical practice. (Cassone et al., 2006) designed a microarray to detect the extended-spectrum β-Thalidomide enzyme resistance genes including SHV and CTX-M. The whole procedures from the sample processing to the bacterial identification and detection of resistance spectrum was completed within 6~8 h, which is especially applicable for the patients with severe infection (Mao et al., 2006; Wang et al., 2007) designed a microarray to simultaneously identify Gram-positive bacteria and detect the drug resistance. Their results showed high sensitivity and specificity and this miccroarray could be used to help the clinicians to diagnose and treat disease. Various gene detecting chips were designed by domestic and foreign researchers currently, which have certain positive meaning in their respective areas, but due to the limits of the chip detection range, their directive guidance is not of so significance in clinical selection of the sensitive antibiotics. Our research, which is based on clinical needs, with the resistance genes from the most common clinical Gram-negative bacteria as detection range, has great guiding significance in choosing antibacterial drugs rapidly and accurately.

In the present study, classic molecular biology method served as a control and was used to evaluate the application value of present microarray in the detection of resistance genes. The bacteria in the present study were confirmed by the French Merieux French Merieux VITEK-II Microbiology Analysis System and the sensitivity analysis. The bacteria were found to be resistant or multiresistant. Our results showed the detection rate of extended-spectrum β-lactamase resistance genes (TEM, SHV and CTX-M) was the highest (>90%) in the *K. pneumoniae* which also had AmpC resistance gene. In the present study, the detection rate of carbapenem resistance gene (KPC) was 100, 37.50 and 7.69% in the *K. pneumoniae*, *E. coli* and *E. cloacae*, respectively. However, carbapenem resistance gene was not present in the *A. baumannii* and *P. aeruginosa*. The detection rate of resistance genes was low in the *P. aeruginosa* and the metal dependent β-lactamase resistance genes (IMP and VIM) were only found in the *P. aeruginosa* with low detection rate. These findings suggest that, in the *P. aeruginosa*, β-lactam antibiotics can be used as the preferred antibiotics in the treatment of *P. aeruginosa* induced infection.

In the present study, our microarray can be used to detect common resistance genes of Gram-positive bacteria. Except for the detection of IMP resistance gene, the results from microarray assay showed favorable consistency with those from PCR. In the PCR assay, the IMP band was clear and had no confounding band. Therefore, there might be error in the probe design. Now, we are optimizing the conditions for the detection. In addition, the detection with microarray is being performed with difference DNA concentrations and also in the bacteria outside of the present study. These experiments may be helpful to understand the sensitivity and specificity of detection with present microarray.

In the future studies, we will increase the number of resistance genes in the microarray and attempt to simultaneously identify bacteria and detect the resistance gene. This may broaden the range of detection with microarray and guide the diagnosis and treatment of infectious diseases.

ACKNOWLEDGEMENT

This study was supported by Ningbo Agricultural and Social Develop Major Project (2010C50038).

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