Disease Markers 20 (2004) 317–323
IOS Press

Evaluation on the use of β-lactamase and Aminoglycoside modifying enzyme gene sequences as markers for the early detection of antibiotic resistance profile of *Pseudomonas aeruginosa*

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Abstract. *Pseudomonas aeruginosa* is one of the major causes of infections including the hospital acquired (Nosocomial) infections. Detection of them and their antibiotic resistance profile by conventional method takes about three days. Recently, DNA based diagnostic methods are being used for the identification of the pathogens. Hence we have tested a rapid and sensitive method using DNA sequences as markers for detecting the presence of three genes coding for the enzymes that inactivate the two most commonly used Anti-pseudomonadal drugs such as β-lactam antibiotics (Penicillin, and its derivatives) and Aminoglycosides such as Gentamicin, Tobramycin, Amikacin, Streptomycin. The internal region of these genes were used for designing and synthesizing primers and these primers were used in Polymerase Chain Reaction (PCR) to screen for the presence of these genes in the clinical isolates and to label them non-radioactively with Biotin. They in turn were used to detect the presence of the antibiotic resistance genes in the clinical isolates by hybridization. The specificity (ratio of positive results obtained in both methods and the sensitivity (the minimum amount of sample DNA and the labeled probe required for the tests) were evaluated.

Keywords: Gene marker, DNA probes, antibiotic resistance, drug-modifying enzymes, non-radioactive labelling, anti-biogram, culture and sensitivity test

1. Introduction

One way of confirming antibiotic resistance by an organism is through the synthesis of enzymes capable of inactivating the natural or semi-synthetic antibiotics, circulating *in vivo*[11]. Anti-pseudomonadal β-lactam antibiotics commonly used are; Penicillin, Carbenicillin, Ticarcillin, Azlocillin, Mezlocillin, Cefotaxime, Cephaloridine, Ceftazidime, Monobactum, Aztreonam, Carbapenem, Imipenem. The mechanism of resistance to these β-lactam antibiotics is due to chromosomally mediated production of β-lactamases encoded by *bla* gene. But few strains elaborate plasmid mediated β-lactamases [4].

The aminoglycosidic antibiotics used against Pseudomonads are Gentamicin, Netilmicin, Tobramycin, Amikacin, Streptomycin, Neomycin, Kanamycin, Isep amicin, etc., *Pseudomonas aeruginosa* modifies these aminoglycosides by acetylation, phosphorylation or adenylation [11]. Gentamicin and Amikacin are modified by Aminoglycoside 3'-acetyltransferase (AAC-3') or 6'- acetyltransferase (AAC-6') produced by

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ISSN 0278-0240/04/$17.00

Pseudomonas aeruginosa. Streptomycin and Spectinomycin are modified by 3'- adenyl transferase (AAD(3')). The internal regions of the gene sequences coding for these enzymes in the organism were used to design suitable primers for setting up Polymerase Chain Reactions (PCR) [8,11].

The results of the PCR experiments and the time needed for carrying out the PCR were compared with those of conventional antibiogram methods. As every clinical lab is not equipped to carry out PCR, we have simplified a membrane based DNA (from clinical isolates) hybridization method using the Biotin labeled PCR amplified products as probes for detecting the antibiotic resistant gene sequences in the clinical isolates. The blots were developed using Streptavidin-Alkaline Phosphatase conjugate and detected by the chromogenic and chemiluminescent reactions. Presence of the these genes detected by these DNA based methods were compared with the actual phenotypic characteristics (resistance to various drugs) shown by the clinical isolates in the culture and sensitivity tests (antibiogram) [3,14].

2. Materials and methods

2.1. Sample collection

53 Clinical isolates from the infected patients at PSG Institute of Medical Sciences and Research Hospital (PSGIMSRH), Coimbatore, India were collected and subjected to Antibiotic Susceptibility tests. All of them were stored as glycerol stocks and out of which 20 samples were selected for the PCR based typing methods. Another set of 50 samples were collected from the PSGIMSR hospital and from another 300 bed hospital(G.K. Naidu Memorial Hospital, Coimbatore, India). Antibiotic susceptibility test for these samples were carried out and the results were recorded.

2.2. Antibiogram

Uniform spreading of the clinical sample on the nutrient agar plates were done and 17 antibiotic discs (of which 5 were aminoglycosides and 12 were penicillin derivatives) were placed at least 24 mm apart. Plates were incubated at 37°C overnight and the zone of inhibition around the discs were noted [2]. Based on the diameter of the zone of inhibition, the resistant or sensitive characteristics were determined using the recommendations of National Committee for Clinical Laboratory Standards (NCCLS), USA.

Table 1
List of antibiotics used

Abbreviations used	Name of the Antibiotic
A	Ampicillin
Ac	Amoxyclav
Ak	Amikacin
Am	Amoxycillin
Cb	Carbenicillin
Cs	Cefoperazone
Ca	Ceftazidime
Ci	Ceftriaxone
Cu	Cefuroxime sodium
Ce	Cephotaxime
Cf	Ciprofloxacin
Do	Doxycyclin
G	Gentamicin
Na	Nalidixic acid
Nt	Netillin (netilmicin sulphate)
Nx	Norfloxacin
Of	Ofloxacin
Pc	Piperacillin
Sc	Sparfloxacin
Ti	Ticarcillin
Tb	Tobramycin
Co	Co-trimoxazole
Nf	Nitrofurantoin

2.3. DNA extraction

A simplified method for preparation of DNA [6] based on the alkaline lysis procedure was used to extract the bacterial DNA and the concentration was measured spectrophotometrically (at λ_{260}) [1]. The quality of the DNA was analyzed by agarose gel electrophoresis [10].

2.4. Primer designing

From the GenBank sequences of *bla*, *aac*(6') and *aad* (3') (Accession numbers X56809, L06163, X02340 respectively) three sets of primers were designed (for the internal regions of these genes) using the software package downloaded from the URL http://www.genome.wi.mit.edu/cgi-bin/ and from http://128.84.203.244. The oligonucleotides used in this study are listed in Table 2.

2.5. PCR amplification

100 ng of template DNA prepared as above was used in the polymerase chain reaction (PCR) which was set up with the following parameters; one minute initial denaturation followed by 35 cycles of one minute denaturation at 92°C, one minute annealing at 55°C for *bla* gene primers (at 52°C for *aac*(6') gene primers) and one and half minute synthesis at 72°C. Final extension was done for 10 minutes at 74°C.

Table 2
Oligonucleotides used for amplification and hybridization

Primer	Nucleotide sequence (5'-3')
bla(F)*	c(94) gtgagtccgatttccctatg(115)
bla(R)*	c(970)gctgttcagtgcggcaacgat(948)
aac(F)	a(222)accttgaccgaacgcagcg(342)
aac(R)	g(481)tgaagcataccgatgtcg(460)
aad(F)	a(203)accttgaccgaacgcagcg(223)
aad(R)	g(845)gacctaccaaggcaacgcta(824)
bla(int-F)	g(735)actccctcggtgggttggtatg(758)
bla(int-R)	t(855)ttggctatctgcgttcgtag(834)
aac(int-F)	a(498)aacaaagttaggcagcac(517)
aac(int-R)	g(590)tgaagcataccgatgtc(571)g

*(F) and (R) denote Forward and Reverse primers

2.6. Agarose gel-electrophoresis

Electrophoretic separation of the PCR amplified products were carried out using 1 to 1.5% agarose gels in 1X TAE buffer (0.04 M Tris-Acetate/0.001 M EDTA, pH 8.3). The sizes of the gels were 10 * 7 or 10 * 14 and the voltage used was 3 V/cm.

2.7. Biotin labeling

Biotin-14 dATP (Gibco-BRL) was used to label the PCR amplified target sequences (internal gene probes) by nick translation using 0.5 μg of the DNA, 10 mM dNTP mix without dATP and 20 mM Biotin-14-dATP in a total volume of 45 μl of the nick-translation buffer (50 mM Tris.Cl, 10 mM $MgSO_4$, 0.1 mM dithiothreitol, 50 μg/ml BSA (Fraction V), pH 7.5) [13]. 5 μl of Pol I/DNase I was used and incubated for 90 minutes at 15°C. The reaction was stopped with 1 μl of 0.5 M EDTA (pH 8.0) and the labeled probe was precipitated by ethanol precipitation followed by 70% ethanol wash and the labeled DNA was dissolved in 50 μl of sterile distilled water [10].

2.8. Dot-Blot

The DNA samples of the clinical isolates to be tested were blotted onto the nylon membrane [1] and fixed by UV-crosslinking by exposing the membrane over the UV-transilluminator for 3 minutes [9].

2.9. Hybridization

Prehybridization washing of the membrane was done for one hour followed by hybridization at 42°C for 3 hours in 2.5 ml of hybridization buffer containing 50% formamide, 5x SSC, 25 mM sodium phosphate pH 7.5, 0.5 mg/ml sheared calf thymus DNA, 5x Denhard's solution and freshly prepared denatured probe (50 ng/ml) [13].

2.10. Chromogenic detection

After washing of the blots in wash buffer (2x SSC, 0.1% SDS and 0.1% SSC, 0.1% SDS) at 42°C, membrane was incubated for 30 minutes in blocking solution (3% BSA in wash buffer (0.1 Tris-HCl, 0.15 M NaCl, pH 7.5)) followed by 10 minutes incubation with Streptavidin-alkaline phosphatase conjugate (10 μg/10 ml). Rinsed with the wash buffer and the color was developed using the substrate Bromo Chloro-Indolyl Phosphate (BCIP, 0.3%) and Nitroblue Tetrozollium (NBT, 0.1%) in development buffer (0.1 Tris, 0.1 M NaCl and 50 mM $MgCl_2$), pH 9.5 [13].

2.11. Chemiluminescent detection

For development of the blots, chemiluminescent reagent CDP-Star (1.5% aqueous solution of Disodium 2- chloro -5- (4 methoxyspiro [1,2-dioxetane-3,2'-(5'-chloro) -tricyclo [3,3,1,1] decan]- 4yl) phenyl phosphate), (Amersham Pharmacia, UK) was pipetted over the membrane, left for 5 minutes, excess reagent was drained off and exposed to the x-ray film in the dark for 30 minutes followed by developing and fixing of the same using the standard procedures.

3. Results and discussion

3.1. Antibiogram

When all the 53 clinical isolates obtained from the patients were subjected to antimicrobial susceptibility tests (Table 1), these isolates showed broad spectrum resistant profile (data not shown). Another 50 samples were collected from two sources, sensitivity tests and hybridization analysis were carried out for all the samples. The results obtained in both methods were compared and subjected to statistical methods. Besides, the intensity of the dots obtained in the blots were subjected to spot densitometric analysis and compared against the amount of DNA samples blotted on each dot. A titration curve (dot intensity as a function of DNA concentration) was drawn.

Table 3
The recorded Antibiogram Pattern and the PCR results

Sample No.	Antibiogram Results*		PCR results**	
	Betalactam	Aminogiycosides	bla	aac
1	−	−	+	+
2	−	+	+	+
3	+	−	+	+
4	+	+	+	+
25	+	+	−	+
26	+	+	+	+
28	+	+	+	+
29	+	+	+	+
30	+	+	+	+
34	+	+	+	+
35	+	+	+	+
36	+	+	+	+
38	−	+	+	+
39	+	+	+	+
41	+	+	+	+
44	+	+	+	+
46	+	+	+	+
47	+	+	+	+
48	+	+	+	+
49	+	+	+	+

*(+),(−) Shows resistant and sensitive phenotypes respectively

** (+),(−) Shows resence and absence of resistant genes respectively

3.2. PCR based diagnosis versus susceptibility test

3.2.1. bla gene marker

Of the 20 samples that were subjected to PCR analysis, 19 were found to be positive harboring β-lactam resistant gene as the expected amplification product of about 870 bp (corresponding to the nucleotide 94 to 970 in the gene sequence) was obtained in each 19 cases. Whereas only 17 samples showed β-lactam resistant phenotype in the antibacterial susceptibility test (Table 3, Fig. 1, partial gel picture).

3.2.2. aac (6') gene marker

All the twenty samples tested were positive in the PCR for the presence of the acetyltransferase gene (amplifying the expected 260 bp product) but two among them (sample 1 and 3) showed aminoglycoside-resistant phenotype in the susceptibility tests (Table 3, Fig. 2, partial gel picture). The variation of 10% seen between the two methods in case of both the commonly used antibiotic markers can be due to the differences between the actual diameter of the zone of inhibition recorded, the variation of the control values (NCCLS, USA) used as standards for the susceptibility or resistant phenotypes etc., Or it may be due to the genetic factors such as repression, mutation, and low specific activity of the β-lactamase gene products present. In contrast, sample number 25 which was negative in PCR

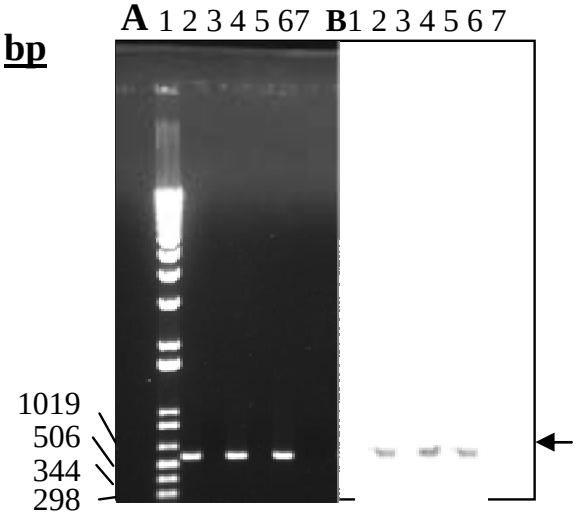

Fig. 1. PCR amplification and Southern Blot analysis of bla gene in the clinical isolates of Pseudomonas aeruginosa. A) 1in 1X TAE buffer containing 0.5 μg/ml ethidium bromide. Lane 1 is 1 kb step ladder (2 μg/lane). Lane 2,4, and 6 correspond to sample isolates 1, 2 and 38 (Beta lactam positive phenotype). Lane 3,5 correspond to sample isolate 25, 51 (Beta lactam negative) and lane 7 was loaded with the reagent control (mix without any template DNA). Arrow head showing the expected (870 bp) amplification product. B) Southern hybridization of the samples in gel A after transferring to Nylon membrane.

showed resistance to β-lactam antibiotics in the susceptibility test. This may be due to activation of multidrug efflux pumps operating on the membrane of the pathogenic bacteria [12] in spite of the absence of the gene.

3.2.3. aad (3') gene

There was no amplification product obtained for the aad(3') gene. As the aad(3') gene codes for the Streptomycin and Spectinomycin resistance and these drugs are not used in the common susceptibility tests, the phenotypic characteristics of all the isolates have to be tested with respect to these two drugs before correlating the PCR results obtained.

3.3. Hybridization tests versus PCR based detection

Specificity of the amplified sequences were checked out by southern hybridization analysis using the Biotin-labeled probes specific to the internal sequences of β-lactam and aminoglycoside drug modifying genes (Table 2, Figs 1 and 2).

When the blots (dot-blots) of 50 samples (second set of collections) were used for hybridization with the labeled probes, the following results were obtained;

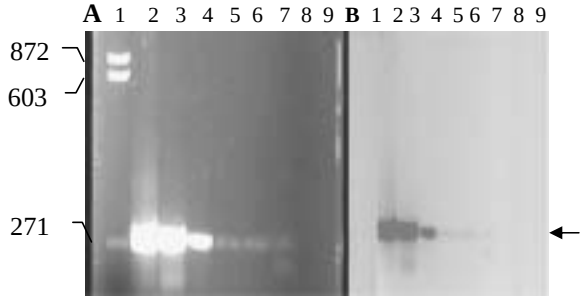

Fig. 2. PCR amplification and Southern Blot analysis of *aac*(6') gene in the clinical isolates of *Pseudomonas aeruginosa*. A) 1.5% agarose gel in 1X TAE buffer containing 0.5 μg/ml ethidium bromide. Lane 1 DNA Ml. wt. Marker (∅ X 174 DNA- *Hae* III digest DNA). Lane 2 and 3 correspond to aminoglycoside positive samples 2 and 4 (1 μg of DNA). Lane 4 to 7 correspond to sample number 28 (aminoglycoside positive) and was loaded with 500, 250, 50 and 20 ng of PCR products respectively. Lane 8 was loaded with sample number 61 (aminoglycoside negative) Lane 9 was loaded with the reagent control (without template DNA). Arrow head showing the expected (260 bp) amplification product. B) Southern hybridization of the samples in gel A after transferring to Nylon membrane.

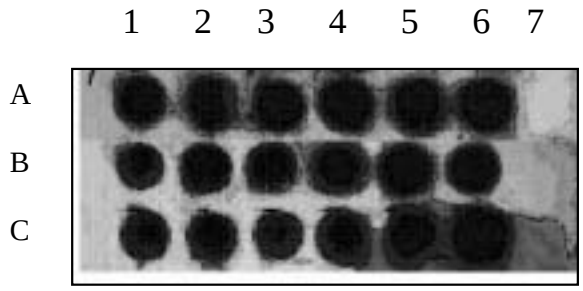

Fig. 3. Chemiluminescent dot-blot of the *bla* and *aac*(6') gene positive and negative isolates (20 numbers). Spots A1 to A6 were that of positive (bla^r) samples (54, 55, 58, 60, 64 and 65). Spots B1 to B6 were that of (bla^r)positive samples 66, 67, 68, 73, 78 and C1 to C6 correspond to $(aac(6'))^r$ samples 79, 80, 82, 84, 95 and 96. Negative samples 51 bla^s and 61$(aac(6'))^s$ were spotted on to Spots A7 and B7.

All the 45 samples with β-lactam resistant phenotype showed positive results both in the chemiluminescent and chromogenic hybridization reactions with bla probes(Partial results shown in Fig. 3). Sample number 73 with a sensitive phenotype also showed positive result in the hybridization analysis. Likewise 48 samples were both aminoglycoside-resistant and were positive for the *aac*(6') gene except the sample number 96 which was aminoglycoside sensitive but was positive for *aac*(6') gene in the hybridization tests.

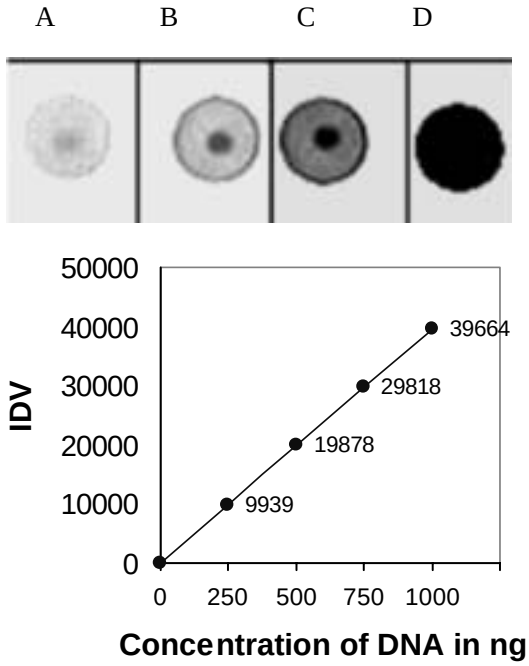

Fig. 4. Chromogenic Detection of *bla* gene. A, B, C and D correspond to 250, 500, and 750, 1000 ng of DNA blotted onto the membrane. Probe was used at the concentration of 50 ng/ml and was detected with the BCIP/NBT substrate as given in the materials and methods. The dots were scanned and the IDV, Integrated Density value (sum of all pixels) of each dot was measured automatically by the instrument after the background correction. Titration graph (dot intensity as IDV versus the DNA concentration spotted) was drawn and was found to be linear (intensity directly proportional to the amount of DNA used).

4. Chromogenic and chemiluminescent detection

To make it feasible for any small laboratory to do DNA based diagnostic testing, a simple method of growing the isolates obtained from patients in suitable broth for 4 hours followed by boiling for 10 minutes and blotting the lysate containing DNA onto the nylon membrane were carried out. Hybridizing the same with the biotin labeled probes for each of the antibiotic resistant gene was done and the blots were developed using Streptavidin – Alkaline Phosphatase Conjugate and the BCIP – NBT or CDP – Star as the substrates [7,13]. Our results showed that the sensitivity of chromogenic and chemiluminescent methods in detecting the target genes is 250 ng and 25 ng of the sample DNA respectively when the probe was used at the concentration of 50 ng/ml (Figs 4 and 5).

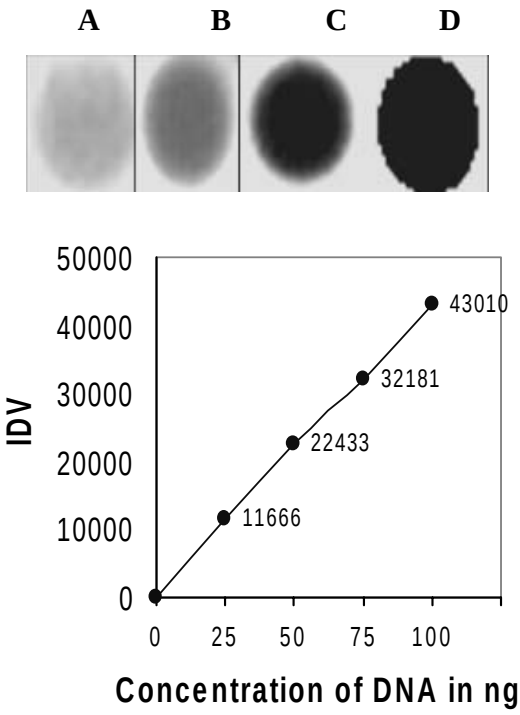

Fig. 5. Chemiluminescent Detection of *aac*(6) gene. A, B, C and D correspond to 25, 50, 75 and 100 ng of DNA blotted onto the membrane. Probe was used at the concentration of 50 ng/ml and was detected with the BCIP/NBT substrate as given in the materials and methods. The dots were scanned and the IDV, Integrated Density value (sum of all pixels) of each dot was measured automatically by the instrument after the background correction. Titration graph (dot intensity as IDV versus the DNA concentration spotted) was drawn and was found to be linear (intensity directly proportional to the amount of DNA used).

5. Specificity of each assay methods

Since the number of samples used in this study were more than hundred, the population t test was used to find out the level of significance (at 95% level) between each methods. When the results of Antibiogram method was analyzed against the results of the PCR method, the Z value was 1.05 and 0 for the *bla* and *aac* genes respectively which is within the limits of 1.96 (5% significance level). Likewise the level of significance between the Antbiogram method and the DNA-Hybridization based method was 0.333 and 0.58 for the *bla* gene and *aac* genes respectively which is well below the limits of 1.96 (at 5% significance level).

5.1. Summary

The results obtained by using DNA sequence of the pathogen as a marker for detecting antibiotic resistance were statistically significant with those obtained by the traditional Culture and sensitivity methods. Whether the small discrepancy between these two methods is due to the differences in the specificity and sensitivity of the methods involved or due to the mutation or inactivation of the genes present and thereby showing sensitive phenotype needs to be analyzed.

The time taken for the DNA marker based detection method is only about 62% of what is required for the traditional method (antibiotic susceptibility test) and with above 95% level of significance this method may enable early detection of the resistant traits of the pathogen in acute and life threatening infections leading to timely and appropriate antibiotic regimen.

Acknowledgments

This study was supported by the project grant of the Indian Council of Medical Research, New Delhi, India (ECD- I, 5/3/3/7/98). Technical help offered by Mr. V. Murali Krishnan is acknowledged.

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