Random Amplification of Polymorphic DNA Based Typing of *Pseudomonas Aeruginosa*

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Abstract

Pseudomonas aeruginosa was isolated from various sources during the course of an epidemic outbreak of bacterial endophthalmitis following an eye camp at Sangli, Maharashtra. 15 distinct isolates were obtained from clinical samples. Typing of the 15 isolates was performed by random amplified polymorphic DNA (RAPD) analysis, pyocin typing and antibiogram. RAPD typing was rapid, labour friendly and could be done within six hours. RAPD analysis produced reproducible electrophoretic band patterns on the basis of which three distinct amplification patterns could be visualised. The conventional typing methods were labour intensive and took about 48 hours. However, the results of RAPD typing, pyocin typing and antibiogram did not correlate with each other. This study suggests that RAPD typing could be an additional rapid typing method for studying the epidemiology of infectious disease outbreaks due to *P aeruginosa*.

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Key Words :Antibiogram; *Pseudomonas aeruginosa*; Pyocin

Introduction

Genomic fingerprinting methods are now regarded as the most accurate methods for the typing of micro-organisms for epidemiological purposes [1]. These methods include pulse field gel electrophoresis (PFGE) [2], ribotyping [3], and polymerase chain reaction (PCR) - based fingerprinting methods [4]. Since *P aeruginosa* is one of the most common nosocomial pathogens and is often a major problem in intensive care units (ICUs), many studies have been directed at this micro-organism. However, most studies concern outbreaks or studies between different hospitals for the occurrence of common types [5-8]. In the present study, *P aeruginosa* strains were isolated from the eyes of patients from an outbreak of endophthalmitis following an eye camp at Sangli, Maharashtra, India. The organisms were identified using conventional techniques. This was followed by pyocin typing, an antibiogram, and an analysis of RAPD patterns.

Material and Methods

Strains of *P aeruginosa* isolated from patients admitted to the district hospital at Sangli, were sent for identification to BJ Medical College for conventional typing and Armed Forces Medical College for molecular typing. The isolates were obtained from eye swabs. The colonies were examined by conventional tests for oxidase and pigment production in Kings A and B medium.

Pyocin typing was done by the scrape and streak method. A 1 cm strip across a 9 cm agar plate was plated with each standard and incubated. The growth was carefully removed with a microscope slide. The plate was inverted over a small pad of filter paper soaked with 1-2 ml of chloroform taking

care not to let liquid chloroform touch the plate. Plates were aired in a sterile environment for 30 minutes. A set of 21 indicator strains were used for the test. 8 test strains were inoculated in thin lines across the plate at right angles to the strip. After incubation plates were examined for inhibition across the original streak. The numerical values of the strains inhibiting growth for each isolate were determined by standard techniques [9]. The values were analysed for Pearson's Coefficient of Correlation for relatedness amongst strains using a standard database programme.

Antimicrobial susceptibility (ABST) was done by the Stokes method [10]. Known control strains of *P aeruginosa* (NCTC 11561) were plated on Mueller Hinton agar with lysed horse blood and antimicrobial sensitivity tested against a wide variety of antibiotics so as to obtain an antibiogram against carbenicillin, netilmicin, amikacin, cefpirom, nalidixic acid, gentamicin, ciprofloxacin and polymyxin B.

RAPD analysis was performed on 15 representative isolates. *P aeruginosa* strains were grown on sheep blood agar. Crude bacterial lysates were prepared by suspending a 1 µl loopful of bacteria in 20 µl of 50mM NaOH-0.25% sodium dodecyl sulfate (SDS) and heating for 15 minutes at 95°C. Lysates were diluted with 980 µl of water, and 2.5µl was used for amplification in a 25 µl PCR mixture. PCR tubes further contained 0.5U of DNA polymerase (Bangalore Genie), a 400µM concentration of each deoxynucleoside triphosphate, primer, reaction buffer, and 2.5 mM MgCl₂. Amplification was performed with annealing at 52°C, extension at 72°C and melting at 97°C. The PCR products were mixed with 2.5µl of gel loading buffer and electrophoresed on 2% agarose gel 1.0XTAE buffer (Tris-Acetate-EDTA) at 100 V for 2 hours. Molecular size markers used were a 100bp ladder. The primer used was 5'C AGC

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Table 1

Pyocin typing pattern of the isolates

Pyocin typing pattern	Set 1	Set 2	Set 3	Set 4	Set 5	Set 6	Set 7	Pearsons correlation coefficient
2100300	2	1	0	0	3	0	0	0.736362
7011200	7	0	1	1	2	0	0	0.356614
2111712	2	1	1	1	7	1	2	0.911392
2101732	2	1	0	1	7	3	2	0.920269
3101732	3	1	0	1	7	3	2	0.977319
3111742	3	1	1	1	7	4	2	0.526959
6045710	6	0	4	5	7	1	0	0.628539
7745737	7	7	4	5	7	3	7	1
7745737	7	7	4	5	7	3	7	1
7745737	7	7	4	5	7	3	7	1
7745737	7	7	4	5	7	3	7	1
6015300	6	0	1	5	3	0	0	0.821292
4035710	4	0	3	5	7	1	0	0.738648
6115700	6	1	1	5	7	0	0	0.672119
6111200	6	1	1	1	2	0	0	0.68085

Table 2

Antibiogram pattern of the isolates

Carbenicillin	Netilmicin	Amikacin	Cefpirom	Nalidixic acid	Gentamicin	Ciprofloxacin	Polymyxin B
R	S	S	S	MS	R	MS	S
R	R	R	R	R	R	R	S
R	R	S	MS	R	R	R	S
R	R	S	R	R	R	R	S
S	S	S	S	MS	R	MS	S
R	S	S	S	MS	R	MS	S
R	R	S	R	R	R	R	S
R	R	S	R	R	R	R	S
S	S	S	S	MS	R	MS	S
R	R	R	S	R	R	R	S
R	S	R	S	R	R	R	S
S	S	S	S	MS	R	MS	S
R	R	S	S	S	R	R	S
R	MS	R	S	R	R	R	S
S	S	S	S	MS	R	MS	S

CAG C 3'. Isolates from each patient typed by RAPD analysis in a single PCR run were analysed by photographing the gels and marking the position of the bands in order to facilitate comparison of strains between patients. Strain differentiation was done by observing readily discernible band patterns.

Results

Pyocin typing revealed identical patterns in four strains (strain 8,9,10 and 11). None of the other strains showed any similarity in pyocin typing patterns. Results are shown in Table 1. The relatedness amongst strains was calculated by the Pearson's correlation coefficient.

All the strains were sensitive to polymyxin B and resistant to gentamicin. One isolate was resistant to all the antibiotics except polymyxin B. 8 of the 15 isolates showed resistance to 5 or more antibiotics. 11 isolates were resistant to carbenicillin. Three distinct antibiogram patterns were

observed amongst the isolates. Only 8 of the 15 isolates had identical antibiograms in clusters of 3 and 2 each (strains 4,7,8; resistant to carbenicillin, netilmicin, cefpirom, nalidixic acid, gentamicin and ciprofloxacin), (strains 5,9,12 were resistant to gentamicin and moderately sensitive to ciprofloxacin) and (strain 1,6 were resistant to carbenicillin, gentamicin and moderately sensitive to nalidixic acid and cipro-floxacin). The results are presented in Table 2. No correlation could be demonstrated between pyocin typing and antibiogram patterns.

Electrophoretic patterns of RAPD typing are shown in Fig. 1. Analysis of the RAPD band patterns shows that three distinct amplification patterns could be visualized. Strains having identical band patterns were distinctly discerned proving their genotypic similarity. Strain 1,5,6,9,12,15 showed identical band patterns. Strain 3,4,7,8,13 had similar band patterns. Other strains (10,11,14) demonstrated independent band patterns and were not similar strains.

Fig. 1 :

Strain 2 was nontypeable. Results of RAPD typing were rapid and reproducible in triplicate runs. However, in this study there was no correlation observed between the three different typing methods.

Discussion

This study emphasizes the role of RAPD as an additional, rapid and reproducible method in epidemiological typing of *P. aeruginosa*. While pyocin typing and antibiogram highlight the phenotypic characteristics of the strains, RAPD is a genotypic method giving reproducible results and rapidly differentiates apparently unrelated strains. This study showed no correlation between pyocin typing, antibiogram and RAPD analysis. Other studies have recommended RAPD typing as a useful method. Campbell et al in their study of 200 isolates, blinded in triplicate and evaluated by RAPD found all 600 samples to be typeable; 197 of 200 isolates giving identical results on three occasions, and 131 distinct RAPD types were identified [11]. In another study comparing three typing methods for *P. aeruginosa* by Chen CH et al, RAPD typing performed on 57 isolates classified them into 15 RAPD types, pyocin typing method into 10 types and ribotyping into 22 types. According to the results obtained in this study, the ribotyping had a discriminatory index of 0.865, RAPD 0.785, and pyocin typing 0.676, respectively [12]. RAPD typing has been used successfully as a reliable, reproducible, accurate and sensitive discriminatory method in epidemiological typing of various other organisms [13-16]. Conventional methods like pyocin typing are time consuming and inconsistent [17]. Antibiogram patterns are largely determined by the antibiotic usage in the hospital. RAPD being a genotypic technique, can serve as an additional reliable, reproducible and rapid epidemiological tool. Other molecular methods like PFGE, ribotyping, and

restriction fragment length polymorphism (RFLP) are also valuable methods for studying clonal relationship of *P. aeruginosa* isolates [2-4]. RAPD analysis in this study, demonstrated three distinct amplification patterns showing clonal relatedness of the strains of *P. aeruginosa*, however, conventional methods used showed no correlation with the genotypic method. Interestingly isolate 2 was not only resistant to all antibiotics (except polymyxin) but was also untypable by RAPD.

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