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Molecular characterisation of *Pseudomonas* species isolated from diabetic patients with urinary tract infection (UTI) by AFLP.

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Abstract: **Present work is a prospective study on UTI infected diabetic patients from Bangalore region, molecular character and antimicrobial susceptibility of *Pseudomonas* sp. infections. Among seventy seven samples, 10 % were identified as *Pseudomonas* sp. are isolated on C-TAB agar media and confirmed by morphological and biochemical tests. On testing antimicrobial activity by well diffusion methods, all organisms showed highest resistance to Ampicillin (77%) followed by amplified fragment length polymorphism. Dendrogram analysis of ten variables of *Pseudomonas* sp. by using different primer (E+CTT)(M+AAT) and (E+AAG) (M+GGC) gives total diversity among 10 accession showed within 5.4 units of genetic distance.**

Key words: Diabetic, infection, antimicrobial, polymorphism, accession.

I INTRODUCTION

Urinary tract infection represents one of the most common diseases encountered in medical practice today and occurring from the neonate to geriatric age group [1, 2]. Nearly 10 % of human population will experience a UTI during their lifetime [3]. Urinary tract infections are amongst the most common infections described in outpatients setting despite an increasing population of patients with chronic renal insufficiency [4, 5]. Literature on the management of UTI in patients is sparse. Patients with underlying diabetes are a specific population at risk [6, 7]. Urinary tract infections are very often encountered in patients with diabetes mellitus [8, 9]. UTI complications (bacteremia, renal abscesses and renal papillary necrosis) occur more often in diabetic patients, it is important to recognize UTIs in this patient group [10, 11]. Bacteriuria is more common in diabetic than in non-diabetic women because of a combination of host and local risk factors [12].

Bacterial resistance to antimicrobial agents has been increasing over the last few years due to many factors, including overall increase in number of antibiotic prescriptions. Urinary tract infections were the second in frequency to respiratory tract infections [13]. Different investigators reported the prevalence of *Escherichia coli* in urinary tract infections of 70-90 % [14, 15]. *Pseudomonas aeruginosa* colonization reportedly occurs in more than 50 % of humans and is the most common *pseudomonas* species [16]. Number of organism found in urinary tract infection was *Klebsiella pneumonia*, *Proteus mirabilis*, *Proteus morganii Staphylococcus aureus* and *Streptococcus faecalis*. Other Gram-negative bacteria responsible for the infection were *Pseudomonas*, *Enterobacter sp*., *Proteus*, *Serratia sp*. [17]. DNA fingerprinting techniques such as restriction fragment length polymorphism (RFLP) and random primer polymorphism amplification detection (RAPD) have been described as powerful molecular typing methods for microorganisms [18]. One of the newest and most promising methods is amplified-fragment length polymorphism (AFLP) analysis. AFLP overcomes many of the problems of RFLP and RAPD. AFLP has been used to establish genetic linkage maps and to localize disease resistant genes [19]. AFLP technique is a new high-resolution genotypic tool for classification and also emphasize that, this powerful DNA fingerprinting method is important for bacterial taxonomy in general [20]. Several typing methods have been used effectively for epidemiological studies of *P. aeruginosa* infection including macro restriction analysis resolved by pulsed-field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP). Molecular studies were performed to study the clonal transmission and determine the antibiotic resistance mechanisms that could explain this pan resistance [21].

There is not much of the work has been done on *pseudomonas sp*. isolated from UTI, for this reason made small attempt to find the variation by using AFLP. In present study to identify pseudomonas sp. responsible for causing Urinary tract infection and determine the antimicrobial susceptibility of the organisms against the following (Ampicillin, Tetracycline, Gentamycin and Ciprofloxacin) and the diversity among the organisms using markers.

II MATERIAL AND METHODS

Isolation of organism

77 urine samples were collected randomly from patients of the Diabetic centers in Bangalore. Mid-stream urine samples were collected in sterile containers. Patients on antibiotic therapy were excluded from the study. Urine samples were inoculated on Cetrimide agar to isolate *pseudomonas sp*. Identification of isolates was done by colony morphology, gram staining and standard biochemical tests.

Siderophore Detection

Using Kings B medium (pH 7), Chrome Azurels-S (CAS) 60.5 mg dissolved in 50 ml water and mixed with 10 ml of 1mm ferric chloride solution (1 mm $Fecl_3 6H_2o$ in 10 mm HCL). Solution was slowly added to 72.9 mg of hexadecyl trimethyl ammonium bromide (HDTMA) dissolved in 40 ml water. (Dark blue solution autoclaved). Nutrient agar medium containing difco bacto agar was autoclaved. One volume of CAS solution was added to nine volume of the medium (molten agar) is whirled to mix without foaming and immediately poured into sterile petri plates. After agar solidified, another layer of top agar containing only the agar solution was poured over it and incubated at 37 ^{0}C for 24 hours. Change in colour is observed.

Antibiotic susceptibility

Antibiotic susceptibility test was done by disc diffusion method using Muller-Hinton agar(pH 7.4) media. The media was poured into all sterile petri plates and allowed to solidify. 100 µl cultures spread on the media by sterile spreader and one plate was taken as control (*E. coli*), disc was placed in the centre of agar surface in all the inoculated plates. They were incubated at 37 °C for 24 hrs.

Calculation for percentage of anti-microbial activity is

$$\frac{\text{Control (cm)} - \text{Sensitivity (cm)}}{\text{Control (cm)}} \times 100$$

Genomic DNA extraction from Bacteria

DNA extraction was carried out by SDS method. Ten colonies of *Pseudomonas sp.* was inoculated into 25 ml nutrient broth and incubated at 28±2 °C overnight. 5 ml of the overnight culture was transferred into a 10 ml eppendorf tube and centrifuge for 10 min at 6000 rpm. Bacterial cells were collected by discarding the supernatant and resuspended in 6 ml lesion buffer (25 mM Tris-HCL, pH 7.4, 10 mM EDTA and 1 % SDS), mix well by vertex and incubate for 45 min in water bath at 65 °C. Allow to cool for room temperature and then add 3 ml of (5m) sodium acetate incubate at 4 °C for 10 min. Centrifuge at 10000 rpm for 10 min. Take supernatant and add double volume of ice cold ethanol, keep it for ½ hr at 4 °C for 10 min, then take the pellet dry, dissolve it in 25 µl TE buffer. Quantity and quality was determined by spectrophotometric and gel electrophoresis analysis.

Molecular Characterizations of organisms by AFLP

AFLP screening was performed using different primers (E+CTT) (M+AAT) and (E+AAG) (M+GGC). Reaction mixtures (10.2 µl) were prepared as follows: [Template DNA (Pre-amp dilution) 3 µl, EcoRI + N*N*N*- 2 µl, Mse I + N*N*N* 2 µl, 1 mM dNTP mix 2 µl, 10X Taq buffer 1 µl, IU Taq Poly (5 U/µl) 0.2 µl]. Amplifications were performed in a CG 1-96 thermo cycler. Amplified samples run in Urease PAGE and visualize by silver staining.

Data analysis

Results were analyzed using static software. In the statistical matrix, only 2 characteristics of the bands were used, 0 (no band present) and 1 (band present). Clustering was carried out in Statistica 7.0 for Windows (Stat Soft Inc. USA) using algorithm "unweighted pair-group average linkage analysis". Distances between the clusters were performed using "Percent of disagreement".

III RESULTS AND DISCUSSION

Out of 77 samples collected in this study and grew on cetrimide agar selective media to isolate *Pseudomonas sp.* from contaminated specimens, 10 % were confirmed as *Pseudomonas sp.* using different morphological and biochemical tests [22, 23]. Siderophore detection was done by using CAS method and colour changes were observed after 48 hr. Relationship between siderophore production and bacterial growth rates has led to belief that, siderophore production enhances bacterial virulence [24, 25]. All 10 organisms were highly resistant to ampicillin (77 %), ciprofloxacin (10-15 %), tetracycline (20-25 %) and Gentamycin (Table.1), but *Pseudomonas aeruginosa* is naturally resistant to β-lactams, including broad-spectrum of cephalosporins, chloramphenicol, quinolones and tetracyclines, mainly because of very low permeability of their cell wall [26].

On the contrary, range of sensitivity of *pseudomonas aeruginosa* to ciprofloxacin was to found be 85-96 % and 65-70 % for tetracycline [27]. Sensitivity of *P. aeruginosa* to ciprofloxacin in our isolates was 80.4 %, compared to 73.2 % in Latin America [28] and 10 %-32 % in Europe [29].

Table.1. Sensitivity percentage against different antibiotics

Isolates	Age/Gender	Tetracycline	Gentamycin	Ampicillin	Ciprofloxacin
E1	29/M	23.53%	25%	80.77%	6%
E2	31/F	5.89%	18.75%	57.70%	4%
E3	37/F	26.48%	12.5%	61.54%	10%
E4	47/F	32.36%	32.5%	65.39%	8%
E5	27/F	23.53%	31.25%	65.39%	2%
E6	65/M	11.77%	18.75%	84.62%	16%
E7	60/M	32.36%	25%	80.77%	20%
E8	43/F	26.48%	37.5%	88.47%	16%
E9	47/M	29.46%	37.5%	80.77%	6%
E10	49/F	14.7%	43.75%	73.08%	20%

P. aeruginosa isolates were susceptible to the fluoroquinolones. Resistance has now developed with 10 % strains being resistant to ciprofloxacin. A similar level of resistance has also been recorded in other areas of the world including USA [30, 31]. Ciprofloxacin and gentamicin were both weakly effective against these resistant isolates like *Klebsiella pneumonia* (36 %) and *Pseudomonas aeruginosa* (24 %). Graft survival at 2 years was similar in UTI (87.2 %) and control group (81.2 %, p = 0.32) [32, 33]. Selection of antibiotics based on the above references showing comparatively more resistance for the ciprofloxacin.

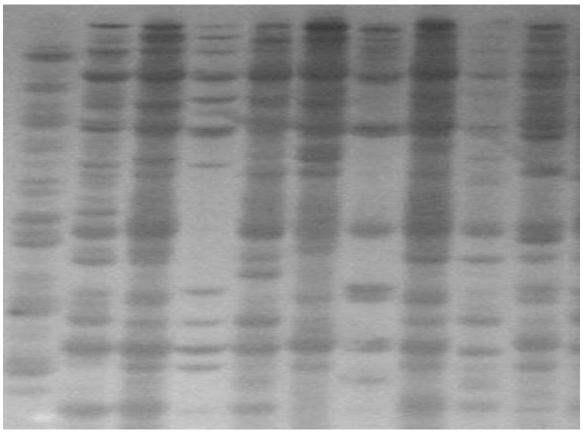

Fig.1. Amplification of DNA using primer (E+CTT) (M+AAT)

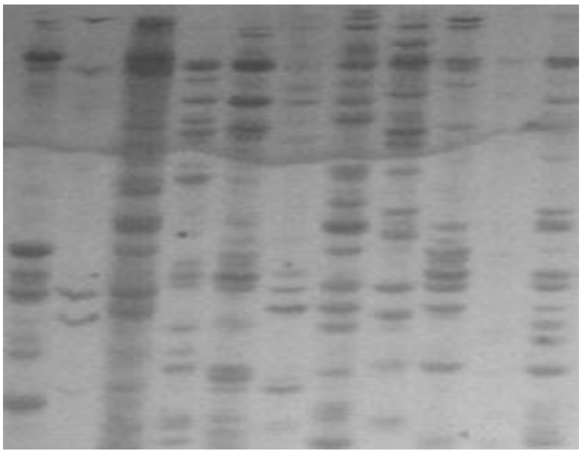

Fig.2. Amplification of DNA using primer (E+AAG) (M+GGC)

Genetic diversity

A total of 61 bands were amplified from two primers combinations, out of that 15 % were monomorphic and 26 % on average polymorphic (Fig.1 and 2). Genetic relationship among all AFLP patterns of *Pseudomonas sp*. according to dendogram (Fig. 3) analysis of 10 variables of *Pseudomonas sp.* by unweighted pair group average, Euclidean distances by using both the primers **(E+CTT) (M+AAT)** and **(E+AAG) (M+GGC),** total linkage distance among 10 variables within 5.4 units. 10 organisms can be classified into two groups like group I and group II. Group I having only PAU 1 and group II having PAU 2, PAU 3, PAU 4, PAU 5, PAU 6, PAU 7, PAU 8, PAU 9 and PAU 10. Lowest linkage distance is showing PAU 2 and PAU 3 within 4.2 units. For a wide range of taxa, including plants, fungi and bacteria, AFLP markers have been used to uncover cryptic genetic variation of strains or closely related species, that have been impossible to resolve with morphological or other molecular systematic characters. Therefore, AFLP have broad taxonomic applicability and have been used effectively in a variety of taxa including bacteria [34] and fungi [35].

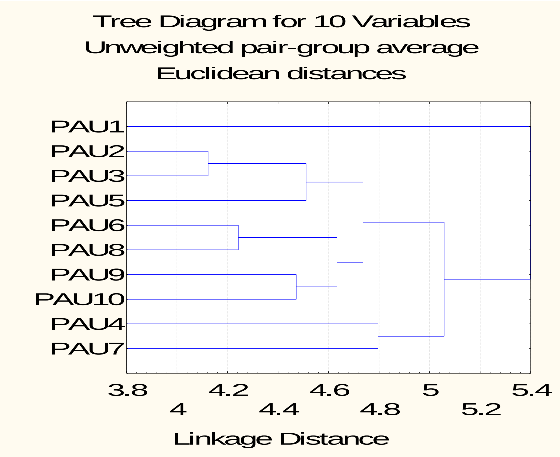

Fig.3. Dendrogram of 10 variables

In view of the results in the present study, complex AFLP patterns were obtained using two different primers pairs and genomic similarity analyses derived from qualitative data enabled us to identify 10 isolates of *pseudomonas sp*. whose taxa had been uncertain based on morphological criteria. We have demonstrated that, AFLP markers useful in the study of genetic variation of *pseudomonas sp.* Using two primer combinations with EcoR1 (E)+1 and MseI (M)+1 at the 3' end of the primer of 10 isolates, a total of 61 bands were amplified.

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