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16S rDNA sequence for Identification and Discriminating *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa **is emerging, as one of the leading causes of nosocomial infection. It's ubiquitous nature and pathogenicity is attributed to their enormous genome that encrypts for virulence, antibiotic resistance and other regulatory genes which are fundamental for the survival and growth of this bacterium. A comparative analysis of Random Amplified Polymorphism across genomic DNA (RAPD) and ribosomal DNA (RArDNA) with Amplified Ribosomal DNA Restriction Analysis (ADRDA) targeting 16S rDNA region, were attempted in typing *Pseudomonas aeruginosa* isolates obtained from clinical (UTI) and diverse environmental sources. RAPD proved to be a better typing technique than RArDNA and ADRDA, though none of these techniques gave conclusive patterns for clinical samples. RArDNA gave unique patterns for only 3 environmental samples. ADRDA gave restriction pattern with BamH1 only. An amplicon of 956 base pairs by 16S rDNA-PCR for Oxidase and Gelatin negative *Pseudomonas aeruginosa* strains established the superiority of molecular assay in bacterial identification**

Key words: Random Amplified Polymorphism across genomic DNA, Amplified Ribosomal DNA Restriction Analysis, *Pseudomonas aeruginosa*

Abbreviations: ADRDA: Amplified Ribosomal DNA Restriction Analysis; bp: base pair; PCR: Polymerase Chain Reaction; *Ps.aeruginosa: Pseudomonas aeruginosa*; RAPD: Random amplified Polymorphic DNA; RArDNA: RandomAmplification of ribosomal DNA; rDNA: ribosomal DNA; UTI: Urinary Tract Infection

Nosocomial or Hospital Acquired Infections (HAIs) are looming global health concern, causing 1.5 to 3 million deaths per year[1]. HAI are accessory infections that are procured from hospitals, within the first 48 hrs or 4 days of hospital admission, and those acquired within 30 days after the hospital disacharge. According to the Nosocomial Infection Control Consortium (NICC) surveillance study of 2002-2007[2], the top three pathogen in Intensive Care Unit's (ICUs) were *Ps.aeruginosa, Klebsiella pneumoniae* and *Acinetobacter baumannii* and that of non-ICU were *Escherichia coli, Ps.aeruginosa and Klebseilla pneumoniae.* Healthcare-associated infection (HCAI) studies by International- NICC from 2004-2007 on seven Indian cities have shown 28.6% of the *Ps.aeruginos*a strains were resistant to ciprofloxacin, 64.9% to ceftazidime and 42.0% to imipenem[3]. These data are indicative of the growing emergence of *Ps. aeruginosa* as one of the most dreaded pathogen.

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Ps.aeruginosa, a versatile denizen of intestinal microbial flora of humans, is a non-sporulating, rod shaped Gram negative bacterium with polar flagella. Apart from being a gut bacterium the main habitat of *Ps.aeruginosa* is controversial as these microbes are ubiquitously found in soil, water and in man-made environment without any geographical preference[4]. This bacterium is addressed as an epitome of opportunistic pathogen because it owns a huge genome with a size that ranges from 5.2 to 7Mbp which includes a repertoire of genes ranging from virulence determinants to antibiotic resistance[5]. A study about the genetic diversity of these bacteria among various sources (both clinical and environmental) can provide insights about their genetic circuit, epidemiology, adaptability, pathogenicity and further facilitate in discriminating strains. The success of the particular cram relies on sample identification and molecular tools with typeability. In past, the identity of *Ps.aeruginosa* was established by selection on cetrimide agar with positive results in Oxidase and Gelatin test[6], but with rapid developement of molecular assays, ribosomal region of 16S rDNA that are specific for *Ps.aeruginosa* were targeted to confirm the identity[7]. A complete complementary within these "microdiverse clusters" of different strains have shown upto 30% genetic difference on sequencing of their complete genome[8]. Based on these fundamentals this particular study explores 16S rDNA region, as a target site for identification and discrimination of the candidate bacterium.

The *modus operandi* for differentiating/ typing the variant strains of the particular microbe was based on PCR-Fingerprinting decree. Three molecular approaches comprising of Random Amplified Polymorphic DNA (RAPD)[9], Random amplification of ribosomal DNA (RArDNA) and Amplified Ribosomal DNA Restriction Analysis (ADRDA)[10] were employed to accomplish the objective. The analysis of amplified pattern by the arbitrary primers across genomic DNA is termed as RAPD and those within ribosomal region is named as RArDNA to prevent any confusion about the region under study even though the methodology is same. ADRDA that employs restriction enzymes to interpret the presence/ absence of respective recognition sites within the amplified locations of 16S ribosomal DNA based on the digestive patterns obtained.

Both these methods are preliminary discriminatory technique that can be used prior to sequencing, the ultimate tool in authenticating any degree of difference among different strains.

MATERIAL AND METHODS

Collection, Processing of samples and Screening of *Ps.aeruginosa*

Twenty one samples comprising of thirteen environmental and eight clinical samples (Table: 1) were collected. All the clinical samples were from Urinary Tract Infection (UTI), whereas three and ten of each environmental isolates was collated from soil and water samples respectively. Atmost priority was given for sample collection wherein environmental samples were collected from locations far away from any hospital to avoid cross contamination of non-clinical with clinical isolates. Soil samples were weighed, mixed with equal volume of water and left for sedimentation followed by decantation, serial dilution and plating. Water samples were filtered to remove any solid contaminants and the filtrate was plated after serial dilution. Two reference strains of *Ps. aeruginosa*, a pyocin producer: PS4 (NCIM 5029) and a non-pyocin producer: PS3 (NCIM 2948) from National Collection of Industrial Microorganisms (NCIM) along with *E.coli* DH5α (Banglore Genei) as a negative control were used. The respective samples were plated on cetrimide agar and well spread individual colonies were selected for further experiments after overnight incubation at 43^0C.

Identification of *Ps.aeruginosa*

Biochemical and Microbial method

For all the isolates microbial tests comprising of Gram's staining, Flagella staining along with biochemical characterization using Oxidase, Gelatin, Citrate (Table 2) and IMViC test (result not shown) were performed in accordance with Bergey's Manual for the Determinative Bacteriology Identification[11]

Molecular method (16srDNA-PCR)

Molecular confirmation was achieved by bacterial isolation followed by symmetrical amplification of conserved region of 16S rDNA with designed primers. Chromosomal DNA

extraction was carried out with certain modification of Jones and Bartlet (1990). The extracted DNA was resuspended in 50µl of TE buffer (10 mM Tris, 1 mM EDTA pH 8.0) with 20µg/ml DNase-free RNase A. The bacterial DNA with a value of 1.8 at A_{260nm}/A_{280nm} was used for amplification.

For selective amplification of 16S ribosomal DNA, primers were designed against a strain prototype, PAO1 (wound isolate of *Ps.aeruginosa).* The specific forward and revere primers (Ferguson *et al*, 2007) were scanned across the operon regions of 16S rDNA as well as the whole genome to validate an amplicon size within the candidate region. For the 16 rDNA-PCR reaction, 100ng of the isolated DNA was mixed with 0.1mM each of *FP: 5'GGG GGA TCT TCG GAC CTC A 3'* and *RP: 5' TCC TTA GAG TGC CCA CCC G 3*' (Ferguson et al, 2007), 1U of Taq polymerase, 1X reaction buffer, 800mM dNTP and nuclease free water to make up a reaction volume of 25µl. The reaction soup of the respective samples were amplified in a thermocycler (*Bioer*) with a programmed cyclic reaction comprising of initial denaturation at 94°C for 2 min followed by 25 cycles of 94°C for 1min, 58^0C for 30 sec, 72°C of 1 min that is culminated by a final extension of 72°C at 2 min. 5µl of the PCR products were subjected to electrophoresis in 1.5% agarose gel. The PCR products were purified by gel extraction and purification kit (Chromous Biotech) for further study.

Typing of *Ps.aeruginosa*

RAPD and ADRDA were performed for typing of the strains of *Ps. aeruginosa*. 25µl of reaction mix for the random amplification included 100ng of the DNA (genomic DNA for RAPD and PCR product for RArDNA), 0.5mM of PRT2 *5' AC GCG CAA C 3'*, 1U of Taq pol, 800mM dNTP and nuclease free water. The amplifying condition were similar to 16S rDNA-PCR with annealing at 35°C for 30 sec . The amplicons were examined on 1.5% agarose gel. 5U/µl each of common cutter (*BamH* I) and rare cutter *(EcoR*V) were employed to perform ADRDA on 100ng/µl of the PCR product that was left for overnight incubation at 37°C to facilitate complete digestion. The restriction activity of *Bam*HI and *Eco*RV was checked on pUC 18 and pACYC184. The digestion patterns were visualized on 4% agarose gel.

Table 1. List of sample collected

Nature		Isolates	Origin
Environmental	Soil samples	Ws	Waterfall soil
		Gs	Garden soil
		Px	Palm beach soil
	Water samples	Tw	Turtle water
		Wwt	Treated waste water
		Wwu	Untreated waste water
		Aw	Aquarium water
		Sw	Sea water
		Cw	Cooler water
		TapW	Tap water
		M	Milk
		Cp	CBD Pond water
		Pw	Palm Bach water
Clinical	Urinary Tract Infection(UTI samples)	Sa,Da,Sh3,S1, S2,S3,S4,S5	Urine sample
Controls	Reference strain, Pyocin producer *(Ps.aeruginosa)*	*PS3*	NCIM 5029
	Reference strain, Non- Pyocin producer *(Ps.aeruginosa)*	*PS4*	NCIM 2948
	Negative control*(E. coli)*	*E*	Banglore Genei

Table 2. Biochemical, Microbial and Molecular assays of sample

Tests	Water samples										Soil samples			UTI								*Ps.aeruginosa*		*E.coli*	Positive	Negative
	Cw	Wat.	Wwu	Tap W	M	Tw	Pw	Aw	Sw	Cp	Ws	Px	Gs	S1	S2	S3	S4	S5	Sa	Da	Sh3	PS3	PS4	*E.coli*		
CITRATE	-	-	+	-	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-		
LYOCIS	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	+	-		
OXIDASE	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	+	+	-		
CATALASE	-	-	+	-	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-		
GELATIS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-		
SELECTIVE CITRATE AGAR WITH ACETAMIDE			+		+	+	-	-	-	+	+	-	+	+	+	+	+	+	-	-	-	+	+	-		
GENOMIC DNA	1 cm	1 cm	1 cm	1 cm	0.5 cm	0.7cm	1 cm	1 cm	1 cm	0.4cm	0.5cm	1 cm	0.7cm	0.5 cm	0.7cm	1 cm	0.7cm	0.4cm	1 cm	1 cm	1 cm	0.7cm	0.4cm	1 cm	*S. coli* genome is 4.6Mbp Whereas *Ps.aeruginosa* genome ranges from 5.2-7 Mbp	
PCR**	X	X	—	X	—	—	X	X	X	—	—	X	—	—	—	—	—	—	X	X	X	—	—	X	The PCR product is 956bp	
REMARKS	R	R	S FN	R	S FN	S FN	R	R	R	S FN	S	R FP	S	S FN	S FN	S FN	S FN	S FN	R FN	R FN	R	S	S	S	5 clinical and 7 Environmental samples were selected for the study	

R=Rejected, S=Selected, FN=False Negative, FP=False Positive, Cw=Coolant water, Wwu=treated waste water, Wwu=untreated waste water, TapW=Tap water, M=Milk water, Tw=Turtle water, Pw=Palm Beach water,Sw= sea water, Cp=CBD pond water,Ws=Waterfall soil, Px=Palm Beach soil, Gs=Garden soil,S1-S5 UTI samples from Shiv Pathology laboratory Sa,Da,Sh3=Shree hospital * Variation in the genomic size of the *Ps.aeruginosa* isolates are indicated by the distance migrated by the DNA from respective wells

Table 3. RAPD, RArDNA, ADRA profiles of the samples

samples	Clinical					Environmental					Control		
Test	S1	S2	S3	S4	S5	Tw	Ws	Gs	Wwu	Cp	M	Ps3	Ps 4
Random Amplified Polymorphic DNA													
RAPD profile *	In	In	In	In	In	4	6	1	1	6	6	4	3
RArDNA profile*	x	-	-	-	-	-	-	2	3	-	2	2	2
Amplified Ribosomal DNA Restriction Analysis													
*Eco*RV pattern	-	-	-	-	-	-	-	-	-	-	x	-	-
*Bam*HI pattern	2	2	2	2	-	-	2	2	2	2	x	2	2

In=inconclusive pattern, x= no PCR product,-= no profiles/restriction pattern
* the bands were unique to respective isolates even though some isolates contained the same number profiles

The respective agarose gels contained 0.5mg/ml ethidium bromide for visualization of bands under UV illuminator (Thermo Systems). The gel pictures were captured with Syngene software in Geldoc (Genius Bioimaging System, Syngene). 100bp ladder (Banglore Genei) was used as a marker for all the electrophoresis assays.

RESULTS AND DISCUSSION

Wwu, M, Tw and Cp water isolates (4 out of 10) Ws, Ps and Gs of soil samples and eight clinical samples (S1, S2, S3, S4, S5, Sa and Da) showed growth on cetrimide agar. While none of the selected isolates were Gelatinase positive, only soil isolates showed Oxidase activity. Five of the clinical samples (S1-S5) showed pyocin pigmentation. These results couldn't be used for confirming the identity of the bacterium, as *Ps.aeruginosa* is Oxidase and Gelatin positive. To differentiate between false positive (positive test results given by the subject that does not possess the attribute for which the test is being conducted) and false negative (negative test results given by the subject even though they possess attribute for which the test is being conducted) isolates, molecular assay exploiting the conserved region of 16S rDNA was used. The region of 16S rDNA was located by FASTA search in NCBI genome database of NC_002516 the reference sequence of PAO1, (http://www.ncbi.nlm.nih.gov/nuccore/NC_002516) that consists of 4 operon region (with a single regular (location 722096…723631) region and three complementary regions (4792196…4793731// 267724…5269259// 6043208…6044743). On 16S rDNA-PCR, a band of 956 bp was seen for 4, 2 and 5 of water (Wwu, M, Tw, and Cp), soil (Ws, Gs) and clinical samples (S1, S2, S3, S4 and S5) respectively (Table 2). Eleven samples that gave PCR product and showed selective growth on cetrimide were chosen for further study. All the selected samples showed a great degree of variance in their genome size (Table 2). The specificity of molecular assays over biochemical assays were established with False positive (Px with growth on cetrimide, Oxidase positive and no PCR product) and False negatives (Wwu, M, Tw, S1, S2, S3, S4 and S5 with negative Oxidase test but positive bands) of the test isolates.

Random amplification with PRT2 against genomic DNA gave different profiles with number of bands ranging from 1-6, that were unique and specific to each respective environmental sample whereas inconclusive and blurred bands was obtained for clinical samples (Fig 2a). Reference strains PS3 and PS4 also gave diverse profiles. The RAPD pattern of *Ps.aeruginosa* isolates were different from that of *E.coli* supporting the fact that RAPD can be indeed used as discriminatory tool among bacterial species.

RArDNA showed absence of random amplification within the 16S rDNA region for clinical samples and reference strains PS3 and PS4 (Fig 2b). No detectable amplicons were displayed by sample S1 indicating either multiple amplification, generating low base pair fragment or an experimental error that have prevented the amplification to take place. 100ng of PCR product

was taken for the RArDNA assay and a minimum of 2ng to 10ng of DNA can be detected by ethidium bromide staining[12] which strongly supports the former hypothesis. RArDNA of environmental isolates showed unique profiles in the range of 2-3 bands for M, Gs and Wwu samples.

The patterns obtained by RArDNA is a reason of disconcert, as no complementary sites were found for PRT2 random primer within the 956bp amplicon obtained by analysis of the genome of PAO1. This indicates that the organisms screened and studied for the present study are different in their make up at least within the ribosomal region in comparison to PAO1.

For ADRDA, the preference of rare and common cutter is decisive with respect to GC percentage of the organism under study. The frequency of a particular restriction endonuclease site depends on the G+C content and particular nucleotide sequence recognized along the DNA molecule[13]. On considering the GC content only, in a GC rich organism, higher is the proposition of restriction sites for a GC rich restriction cutter (common cutter) and vice versa for that of AT rich restriction enzyme (rare cutter). *Ps. aeruginosa* has a GC content ranging from 65-67%[14] and for the ADRDA study, *EcoR*V (5'GAT*ATC 3' with GC content: 33%) was chosen as rare cutter and BamH1 (5'G*GATCC3' with GC content: 67%) as the common cutter. The analysis of restriction site within the amplicon indicated a single restriction site for *Bam*H1 (at 637 position) and no restriction site for *EcoR*V.

ADRDA with *EcoR*V (Fig 3a) gave no digestion pattern for the samples (both environmental and clinical) as indicated by the absence of it's restriction site within the amplified region. An exception was seen for sample M that showed no PCR product or any visible restriction pattern. This may be attributed to the presence of numerous restriction sites that would have fragmented the amplicon into smaller bits which were unable to detect on agarose gel.

ADRDA with *BamH*1(Fig 3b) showed partial digestion but gave 2 restriction bands of 607bp and 349bp respectively for 4 of the clinical

Fig. 1. Sequence of 956 bp amplicons of 16S rDNA region (722096-723631) PAO1 with forward (Location: 722288) and reverse (Location: 723244) primer. The sequence showed a single recognition site for *Bam*H1 (G*GATCC) at a location of 637 but showed no site for *Eco*RV (GAT*ATC).The RAPD primer PRT2 had no complementary site within the specified region

I. Random Amplified Polymorphic DNA (RAPD)

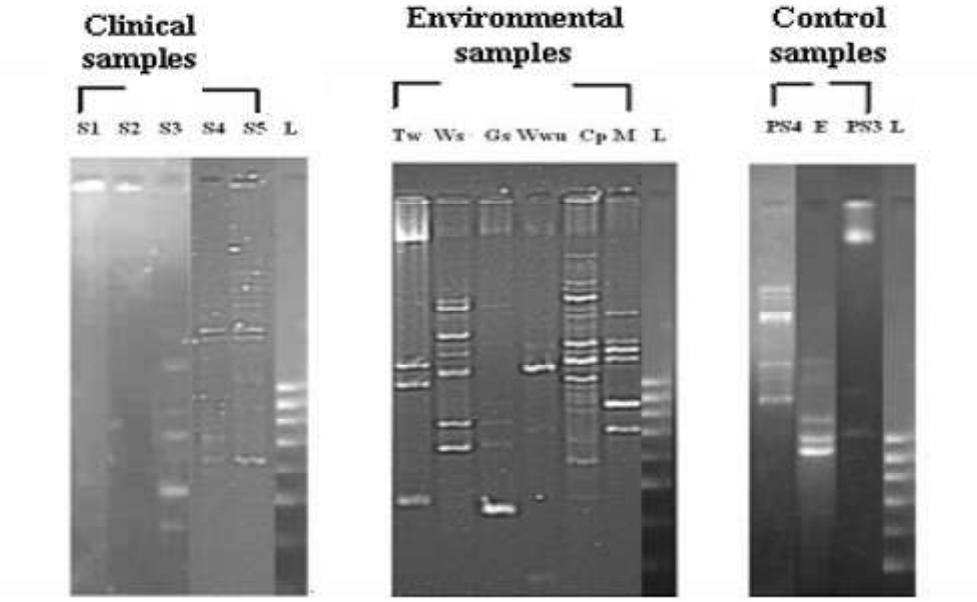

Fig. 2a: RAPD profile of genomic DNA of Clinical, Environmental and control samples on 1.5% agarose gel with 0.5mg/ml ethidium bromide. Clinical samples (UTI): S1-S5, Environment samples: Tw=Turtle water, Ws=Waterfall soil Gs=Garden soil, Wwu=untreated waste water, Cp=CBD pond water, M=Milk water, L= 100bp ladder, PS4=pyocin producing *Ps.aeruginosa*, E=DH5a *E.coli* strain, PS 3= pyocin non-producing *Ps.aeruginosa*

II. Random Amplified Polymorphism of ribosomal DNA DNA (RArDNA)

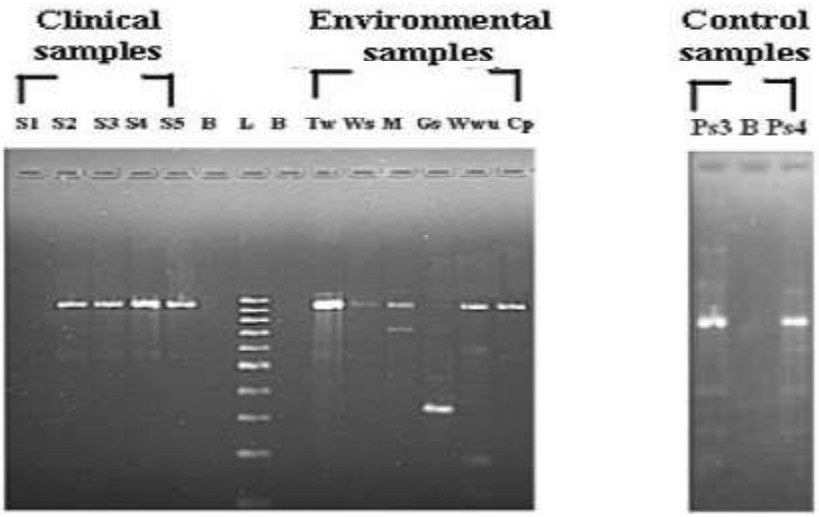

Fig 2b: RAPD profile of 16srDNA-PCR of Clinical, Environmental and control samples on 1.5% agarose gel with 0.5mg/ml ethidium bromide. Clinical samples (UTI): S1-S5, L= 100bp ladder, B= blank, Environment samples: Tw=Turtle water, Ws=Waterfall soil M=Milk water, Gs=Garden soil, Wwu=untreated waste water, Cp=CBD pond water, PS 3= pyocin non-producing *Ps.aeruginosa* E=DH5a *E.coli* strain, PS4=pyocin producing *Ps.aeruginosa*

samples (S1-S4) and 4 of environmental samples (Ws, Gs, Wwu and Cp) which is a definite result with respect to the restriction site on PAO1. However no restriction pattern was observed for S1 and Tw indicating the absence of restriction site for *BamH*1. The environmental sample M gave no visible pattern suggesting excessive digestion which is unlike the restriction patterns obtained from other isolates and PAO1. PS 3 and PS 4 gave no restriction bands with *EcoR*V but

III. Amplified Ribosomal DNA Restriction Analysis

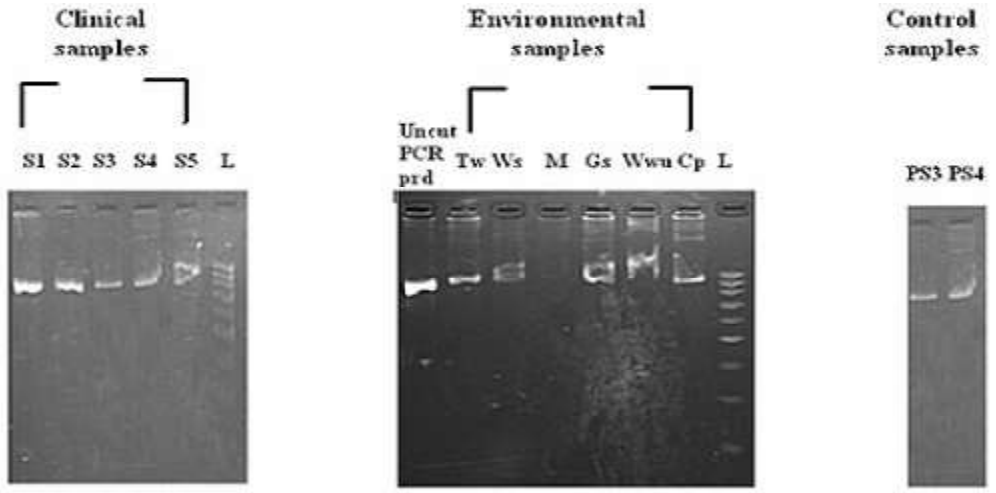

Fig 3a: Digestive pattern of 16srDNA-PCR product of Clinical, Environmental and control samples by rare cutter, *Eco*RV on 4% agarose gel containing 0.5mg/ml ethidium bromide. Clinical samples (UTI): S1-S5, Environment samples: Tw=Turtle water, Ws=Waterfall soil, M=Milk water, Gs=Garden soil, Wwu=untreated waste water, Wwu=treated waste water, Cp=CBD pond water, L= 100bp ladder, PS 3= pyocin non-producing *Ps.aeruginosa*, PS4=pyocin producing *Ps.aeruginosa*

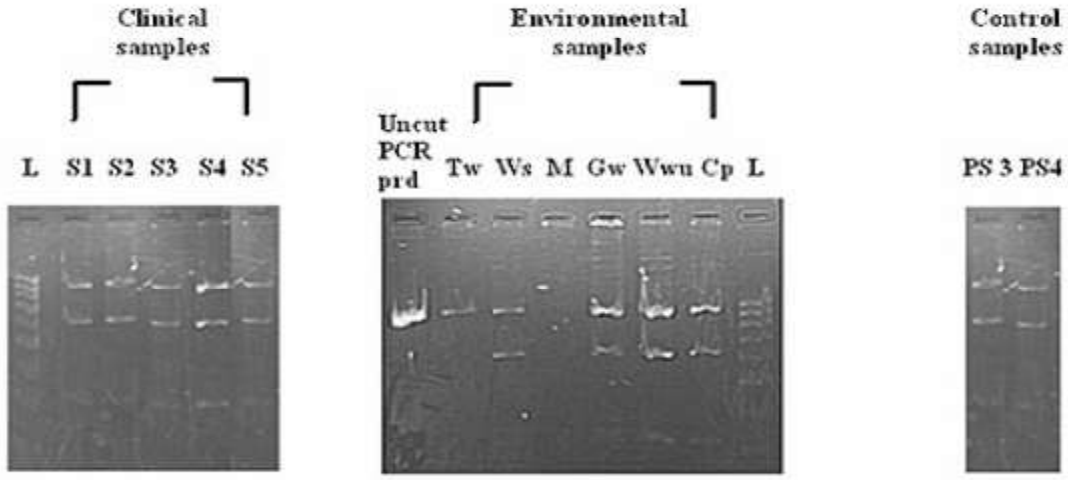

Fig. 3b: Digestive pattern of 16srDNA-PCR product of Clinical, Environmental and control samples by common cutter, *Bam*H1 on 4% agarose gel containing 0.5µg/ml ethidium bromide. Clinical samples (UTI): S1-S5, Environment samples: Tw=Turtle water ,Ws=Waterfall soil Gs=Garden soil, Wwu=untreated waste water Cw=Coolant water, Wwu=treated waste water, Cp=CBD pond water, M=Milk water, L= 100bp ladder,PS4=pyocin producing *Ps.aeruginosa*,PS 3= pyocin non-producing *Ps.aeruginosa*

with *BamH*1 gave 2 bands as observed in other samples (Fig 3a, 3b).

CONCLUSION

Molecular assay of 16S rDNA-PCR established authentication of the identity of *Ps.aeruginosa* over microbial and biochemical assays. RAPD showed 55% typeability (6 out of 11) whereas RArDNA displayed only 27% typeability (3 out of 11) indicating that RAPD is a better typing tool than RArDNA. ADRDA with *Bam*H1 showed restriction pattern than *Eco*RV even though a clear distinction between isolates were not obtained. Of the three molecular tools RAPD proved to be the most discriminative and ADRDA proved to be least favored for differentiating purposes. Sequencing of the samples that have provided definite type able pattern can confirm the degree of variance within these strains.

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