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ERIC-PCR: A Molecular Typing Tool for Genotyping Multi Drug Resistant *Pseudomonas aeruginosa* Isolated from the Pus Samples

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ABSTRACT

Pseudomonas aeruginosa is a typical and major human pathogen, it causes numerous risky opportunistic infections like cystic fibrosis, wound infection, urinary tract infection, ear infections, endocarditis, nosocomial infections and bacteremia, etc. Furthermore, due to their phenotypic variations; identification maybe sometimes harder and consequently which delays their early diagnosis and treatment in infected patients. Hence, a quick and accurate method for the identification of *P. aeruginosa would* be more helpful in their early diagnosis. The present work was aimed to use species-specific primer for the rapid and precise identification of *P. aeruginosa* strains. Multiple drug-resistant strains were selected and their genetic variability was studied using ERIC-PCR. The results showed that out of ten *P. aeruginosa* isolates, eight were found to be unique and genetically diverse.

Keywords: *Pseudomonas aeruginosa*, ERIC-PCR, MDR. Wound infection, Pus sample.

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INTRODUCTION

Pseudomonas aeruginosa is an aerobic non-spore forming Gram-negative rod-shaped with a remarkably adaptable capacity to survive and persist under a broad range of environmental conditions [1] (Dworkin *et al.,* 2006). Asa common opportunistic human pathogen; acquired from both hospital and community settings [2] (Driscoll *et al.,*2007). Also, they found in a variety of aqueous solutions, including disinfectants, soaps, ear drops and eye drops, as well as in sinks, hot tubes, respiratory equipment and showerheads. *P. aeruginosa* is rarely found in the microbial flora of healthy persons but may colonize the gastrointestinal tract of hospitalized patients [3] (Brooks *et al.,*2007). However, the taxonomic complexity, uncertain phylogeny, and paucity of genomic sequence data of the dozens of species within the broad genus Pseudomonas present an obstacle in the genotypic identification assays. In the 1980s, a new standard for identifying bacteria began to develop. In the laboratories, it was shown that the phylogenetic relationship of the bacteria and all life-forms, could be determined by comparing a stable part of the genetic codes [4, 5] (Tortoli, 2003 and Woese,1987). Candidates for this area in bacteria included the genes that code for the 5S, the 16S (small subunit), and the 23S rRNA and the spaces between these genes. The part of the DNA now a day most commonly used for the taxonomic purpose for the bacteria is the gene-specific primer [6] (Bottger, 1989).

Clinical strains of *P. aeruginosa* resistant to many classes of antimicrobial agents, including β-lactams, aminoglycosides and fluoroquinolones, are often isolated [7] (Potron*et al.,*2015). However, an increase in strains resistant to the third- and fourth-generation cephalosporins and carbapenems has become a serious clinical problem worldwide. The primary cause of cephalosporin resistance in *P. aeruginosa* isolates is the overexpression of the chromosomal AmpC enzyme, and the production of the Metallo-β-lactamases [8] (Bae *et al.,* 2014 and Kos *et al.,*2016). However, ESBL-positive *P. aeruginosa* strains that produce extended-spectrum-β-lactamases (ESBLs) are frequently isolated [9] (Poirel*et al.,*2010). The features of these enzymes are utilized in the phenotypic tests used for the detection of ESBL-producing strains.

The clinical importance of *P. aeruginosa* is usually associated with its high resistance against antimicrobial drugs, and additionally to itsintric resistance to antibiotics, it becomes quickly resistant against the antibiotics during the treatment[10] (Tsutsui *et al.,*2011). The prevalence of multidrug-resistance (MDR) isolates has been increasing worldwide and poses a serious problem in hospital settings, with a significant rise in patients' morbidity and mortality[12] (Hocquet*et al.,*2010). One of the widely used repetitive DNA elements in PCR-based genotyping methods is the ERIC PCR common to Gram-negative enteric bacteria [13] (Syrmis *et al.,*2004). The present study was designed to investigate the genetic diversity of a collection of multidrug-resistant *P. aeruginosa* strains isolated from clinical samples by using ERIC-PCR and their species identification. Also, the determination of genetic relationships among the resistant strains allows mapping the dynamics of infection transmission in the region of the study and this will provide a better understanding of the epidemiology of the resistant strains.

MATERIAL AND METHODS

Sample collection

Seventy clinical isolates were collected from the Department of Microbiology, Rajah Muthiah Medical College (RMMCH), Chidambaram, Cuddalore District, Tamil Nadu, identity of isolates were confirmed using a series of microbiological tests such as Gram staining, motility, catalase test, oxidase test and standard biochemical tests such as indole, methyl red, Voges–Proskauer, citrate, urease, nitrate reduction and triple sugar ion tests (Edition, 2013).

Antibiotic susceptibility test

The following Antibiotics were used to screen the resistance of the isolates. Amikacin (30μg), Aztreonam (30μg), Cefepime (30μg), Ceftazidime (30μg), Ciprofloxin (5μg), Gentamicin (10μg), Imipenem (10μg), Levofloxacin (5μg), Meropenem (10μg), Piperacillin (100 μg), Tobramycin (10μg) Norfloxacin (50μg), Ofloxacin (30μg), Cefixime(30μg), Tetracycline (10μg).(Hi media, Mumbai). Drug-resistant patterns of the bacterial isolates were determined by using the Kirby-Bauer disc diffusion method according to CLSI guidelines and followed by (Hudzicki, 2009). Pure isolate culture was swabbed on the Muller Hinton Agar plate (Himedia, Mumbai). Antibiotic discs were dispensed on the surface of the seeded agar plates and the plates were incubated at 37°C for 18-24 hrs. After that agar plates were examined for inhibition of growth and the zone of inhibition was measured.

Among the 70 *P. aeruginosa* isolates tested for antibiotic resistance, 10 strains showed a higher degree of resistance to many antibiotics (i.e.) multidrug resistance (MDR), so these 10 strains alone were selected for further species-specific identification and ERIC-PCR based genotyping studies.

DNA Extraction

1.5 ml of overnight grown bacterial isolates maintained in nutrient broth were transferred to 2 ml of microcentrifuge tube and centrifuged at 10,000 x g for 2 min and the pellet was collected. The same was repeated for another 1.5 ml of culture to harvest enough quantity of cells (100 mg). The pellet was washed with 0.9 % saline and was suspended in 1 ml of CTAB solution (Cetyltrimethyl

ammonium bromide buffer (100 mMTris, 1.4 M NaCl, 1.11% EDTA and 2% CTAB) was incubated at 60 °C with occasional shaking in tightly capped microcentrifuge tubes for 60 min. The sample was then extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and centrifuged for 10 min at 10,000 x g. The top aqueous layer was transferred to a new tube. To it, 0.5 volumes of 7.5 M ammonium acetate was mixed gently and 2 volumes of 100 % ice-cold ethanol were added. It was centrifuged for 5 min at 5000 x g. The DNA pellet was washed with 70 % ethanol. The pellet was air-dried and re-suspended in 25μl of TE buffer (pH 8.0) and stored at -20 °C.

Gene-specific amplification of *Pseudomonas aeruginosa*

The gene-specific primers (PA-SS-F 5'-GGGGGATCTTCGGACCTCA -3' and PA-SS-R 5'-TCCTTAGAGTGCCCACCCG -3') were used for the amplification of the 16S rDNA gene-specific fragment. The PCR cycling conditions were as follows: an initial denaturation for 5 min at 95 ºC, followed by 30 cycles of denaturation at 95ºC for 20 secs, annealing at 58ºC for 20 sec and extension at 72 ºC for 40 secs and then a final extension for 5 min at 72ºC. The 16s rDNA amplification reaction mixture (25μl) consists of 2X Amplicon Red master mixes (amplicon®) with 10 ng of total genome of each isolate, 10 pmol of each forward and reverse primer. The amplified PCR products were electrophoresed on 1.5% agarose gel. The gel was stained in ethidium bromide and photographed with a gel documentation system.

Enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) analysis of the MDR *P. aeruginosa*

ERIC-PCR was performed for a total of 10 MDR *P. aeruginosa* isolates which were resistant to more than one or two antibiotics were selected for molecular typing. This technique was carried out in a Thermocycler (Agilent SureCycler 8800. USA) using the primer ERIC (F): 5'-CAGCCATGAACAACTGGTGGCG -3' and ERIC (R): 5'-TGCTTTGCGCAGGGAAGATTCC 3' following cycle condition: initial denaturation 95 °C for 7 min) followed by 30 cycles of denaturation (95 °C for 30 sec), annealing (52°C for 1min), extension (72 °C for 5 min), and a final cycle of extension at 72 °C for 15 min. The PCR products were loaded on a 1.5% agarose gel (Sigma-Aldrich) at 70 V for 1 hr, and the banding patterns were visualized on an ultraviolet illumination. ERIC-PCR results analysis Te ERIC patterns were analyzed by online data analysis service (inslico.ehu.es). ERIC profiles were compared using the Dice coefficient method and dendrogram based cluster analysis using by unweighted pair group method using arithmetic averages (UPGMA) program.

RESULTS AND DISCUSSION

The major public health wellbeing is to find a suitable treatment for *P. aeruginosa* infection considering the disturbing patterns of developing drug-resistant to multiple antibiotics [21] (Perez *et al.,*2007). In the present study, *Pseudomonas aeruginosa* isolates collected from clinical samples of RMMCH were identified using standard biochemical tests. The results showed that all the 70 isolates have belonged to *Pseudomonas aeruginosa*. The 70 strains were subjected to antibiotic susceptibility test using the disk diffusion method and the results showed that 10 out of 70 (i.e.) 14.3% of them were found to be resistant to multiple antibiotics.
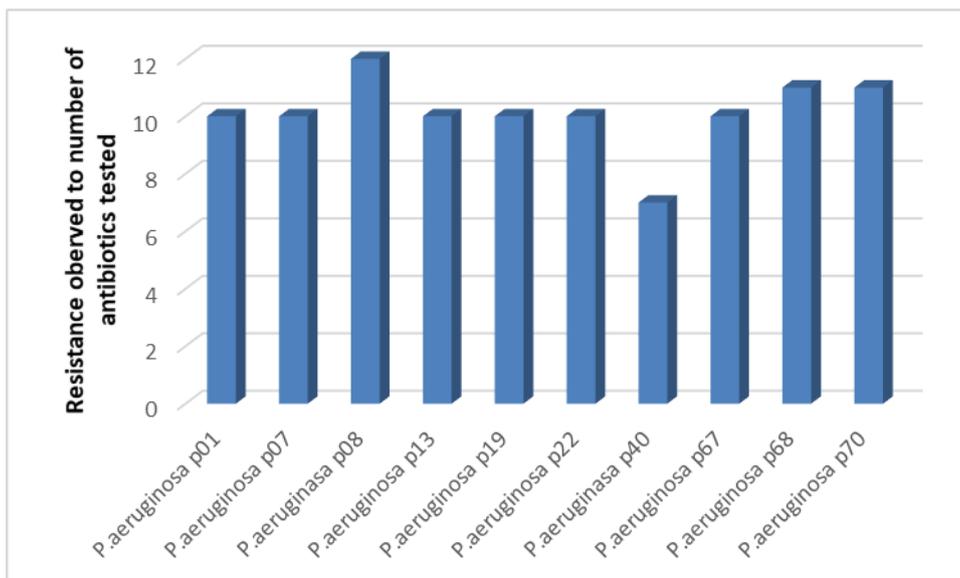
Figure1: Multidrug resistance profile of selected *P. aeruginosa* isolates to different antibiotics

Figure 1 shows the multidrug resistance (MDR) profiles of selected strains tested against 15 different antibiotics. The isolate *P. aeruginosa* 08 was found to be the most resistant among the other 9 MDR isolates i.e. it showed resistance to 12 antibiotics and sensitivity was observed only to 3 antibiotics namely Meropenam, Cefexime and Tobramycin. Nearly 9 out of 10 *P. aeruginosa* isolates were almost resistant to 9 to 10 antibiotics tested. The isolate *P. aeruginosa* 40 isolate showed least resistant among the other isolates tested i.e. it was resistant to only 7 antibiotics.

The ten MDR *P. aeruginosa* isolates were further confirmed by PCR using a species-specific primer. PCR amplification yielded a 956bp product for all the MDR isolates tested (Figure 2). Similarly, a study was done by Spilker *et al.,*2004 on *Pseudomonas* sp., found 14 out of 14 were *Pseudomonas aeruginosa*[24].
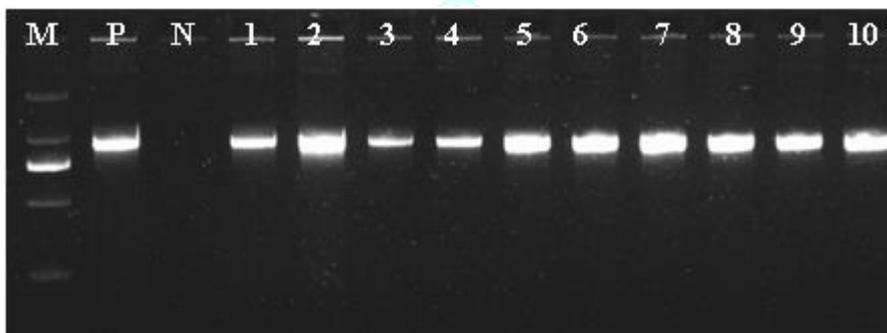

Figure 2: Gene specific amplification of *Pseudomonas aeruginosa* showing amplified PCR product of 956bp in1.5 % agarose gel. Lane M: 250- 1.5kb bp DNA Ladder, Lanes P: Positive control. Lanes N: Negative control, Lanes 1-10: Species specific amplification profile of *P. aeruginosa* isolates PA01, 2-PA07, PA08, PA13, PA19, PA22, PA40, PA67, PA68 and PA70.
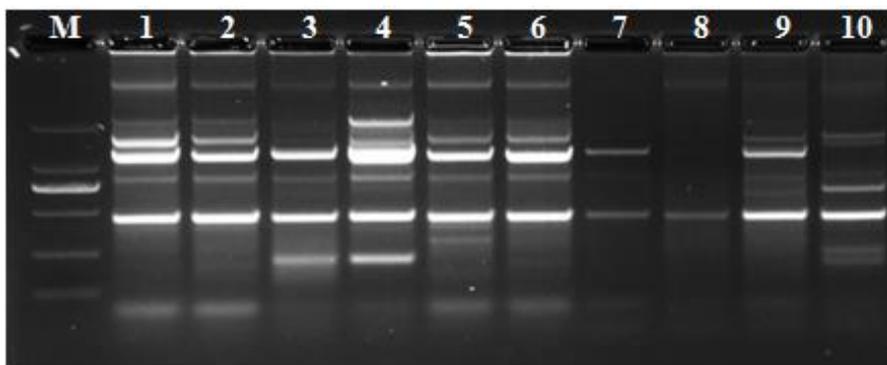

Figure3: ERIC-PCR analysis of *P. aeruginosa*; Lane M: 250- 1.5kb bp DNA Ladder, Lanes 1-10: ERIC profile isolates PA01, 2-PA07, PA08, PA13, PA19, PA22, PA40, PA67, PA68 and PA70.
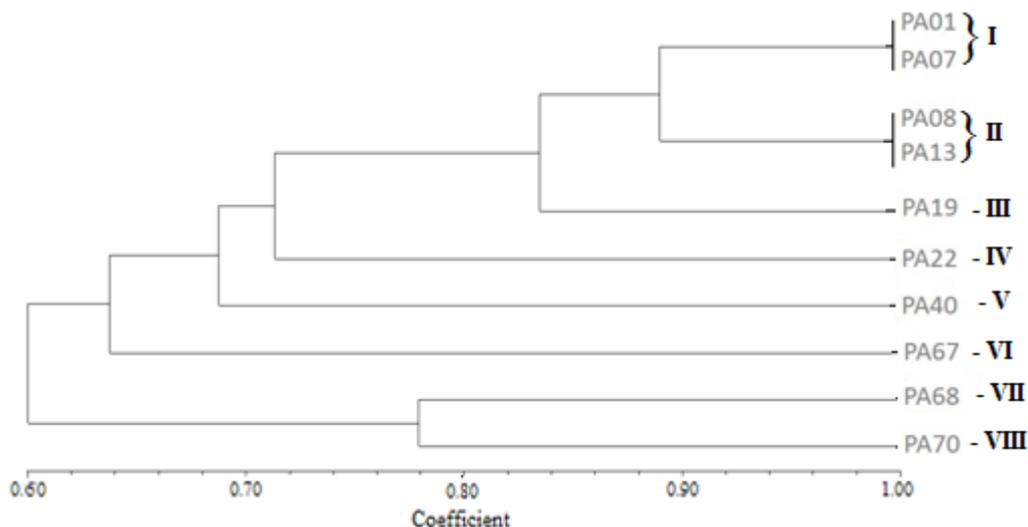
Figure 4: ERIC-PCR based dendrogram analysis of MDR *P. aeruginosa* isolates

Among the 10 MCR *P. aeruginosa* isolates tested for genetic diversity and heterogeneity analysis using the ERIC-PCR and were further sub-typed into 8 types (I-VIII) based on ERIC profiles. The similar ERIC-PCR-type (EPT)-1 was observed for the isolates PA01, PA07 Cluster-I whereas EPT-2 was observed for the isolates PA08, PA13 Cluster-II. So, they might have originated from the same source or possible cross-infection and also eight of the isolates were found to be heterogeneous. The remaining genotypes of 3-8 were observed as single clones (i.e.) PA19, PA22, PA40, PA67, PA68 and PA70 respectively. The isolates in the cluster I and Cluster II shared 100% similarity. In each cluster, there were two identical isolates found and each of them has formed a single clone (Figure 3 and Table 1).

The results showed that though the gene-specific primers specifically identify the *Pseudomonas* strains, the ERIC-PCR genotyping method differentiates the isolates more accurately. Hence, the combination of both the techniques i.e. species-specific primers with ERIC-PCR would be more useful in the precious identification of the Pseudomonas species.

Table 1: Genetic similarity coefficient of isolates strain

Isolate	PA01	PA07	PA08	PA13	PA19	PA22	PA40	PA67	PA68	P70
PA01	1.000									
PA07	1.000	1.000								
PA08	0.711	0.711	1.000							
PA13	0.685	0.685	0.685	1.000						
PA19	0.889	0.889	0.711	0.685	1.000					
PA22	0.889	0.889	0.711	0.685	1.000	1.000				
PA40	0.597	0.597	0.597	0.597	0.597	0.597	1.000			
PA67	0.597	0.597	0.597	0.597	0.597	0.597	0.778	1.000		
PA68	0.833	0.833	0.711	0.685	0.833	0.833	0.597	0.597	1.000	
PA70	0.635	0.635	0.635	0.635	0.635	0.635	0.597	0.597	0.635	1.000

When dealing with MDR pathogens, earlier identification and confirmation of the source will be helpful in treating the infections easily. Genotypic identification methods would be expected to circumvent the problems associated with the identification process[18] (De-Vos, *et al.,*1997). In the present work, we used a simple molecular typing method ERIC-PCR to find the hereditary relatedness among the extremely MDR pathogenic isolates of *P. aeruginosa* isolates and also compared each other using ERIC-PCR. The dendrogram analysis of the ERIC-PCR profiles showed phylogenetic relationships among the MDR *P. aeruginosa* isolates (Figure 4).

ERIC-PCR a PCR based technique utilized for studying *P. aeruginosa* endemicity and being a rapid and cost-effective approach tends to be utilized and can give some clues for the possible transmission pattern of multidrug-resistant pathogens[22, 23] (Aljindan *et al.,* 2018 and Ranjbar *et al.,* 2017). Lim *et al* 2009 suggest ERIC-PCR and PCR amplification with species-specific primer as a good method

to determine the diversity of *P. aeruginosa*. Several other reports have shown that ERIC-PCR can be an excellent tool for the genotypic screening of *P. aeruginosa* isolates[25, 26, 27,] (Goudarzi *et al*., 2011 , Dawson *et al.,* 2002, and Inacio *et al.,* 2014). The present result is in agreement with Khosravi *et al.,* 2016 as they observed genetic diversity and heterogeneity nature of the MDR *P. aeruginosa* isolates of clinical origin[28].

CONCLUSION

The ever-increasing antibiotic resistance among *P. aeruginosa* starting from different sources has been proved incontestably true in the present study. The high level of resistance to multiple drugs tested against the clinical isolates of *P. aeruginosa* suggests that efficient and persistent control measures and strict antibiotic policies have to be made to combat drug resistance. ERIC-PCR as a powerful discriminating tool can be used to analyze the diversity and sub-type the *P. aeruginosa* strains and also

their epidemiological surveillance and transmission in the hospital environments. The heterogeneity in the ERIC genotype results suggests a genetically diverse nature of the MDR *P. aeruginosa* isolates.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest in the study.

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