Genotyping of multidrug-resistant strains of *Pseudomonas aeruginosa* isolated from burn and wound infections by ERIC-PCR[1]

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DOI: http://dx.doi.org/10.1590/S0102-865020160030000009

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ABSTRACT

PURPOSE: To determine the genetic diversity of MDR *P. aeruginosa* strains isolated from burn and wound infections in Ahvaz, Iran, by ERIC-PCR.

METHODS: From total 99 strains of *P. aeruginosa* defined as MDR by using drug susceptibility testing, 66 were subjected to ERIC-PCR analysis, comprises 53 strains isolated from burn infection, and 13 randomly selected strains from wound infection with higher resistance to combinations of more numbers of drugs.

RESULTS: Eight clusters (I to VIII), and 50 single clones were generated for tested MDR isolates analyzed by ERIC-PCR. The high heterogeneity was observed among the isolates from burn infections including 16 isolates which were categorized in eight clusters and 37 single clones. The isolates in clusters II, III, VI, VIII showed 100% similarity.

CONCLUSIONS: The high level of genotypic heterogeneity in *P. aeruginosa* strains demonstrated no genetic correlation between them. Extremely high drug resistance in isolates from burn, suggests that efficient control measures and proper antibiotic policy should be observed.

Key words: Drug Resistance, Microbial. Polymerase Chain Reaction. Genotyping Techniques. Pseudomonas aeruginosa.

Introduction

Pseudomonas aeruginosa is one of the most common causes of nosocomial infections which affects mainly immunocompromised hospitalized patients, and in particular, is the leading cause of life threatening infections in patients in burn units[1-2]. The clinical importance of *P. aeruginosa* is mostly related to its high resistance against antimicrobial drugs, and in addition to its intrinsic resistance to antibiotics, becomes quickly resistant against the drugs during the treatment[3]. The prevalence of multidrug-resistant (MDR) isolates has been increasing worldwide and poses a serious problem in hospital settings, with significant rise in patients' morbidity and mortality[4]. Previous studies show that the prevalence of MDR is dramatically increasing among *P. aeruginosa* isolates from burn and other hospitalized patients in Iran[5-7].

Nowadays, different typing methods such as biotyping, serotyping, pyocin typing have been proposed to determine the common clones of *P. aeruginosa*[8]. These clones and correlation between them are of importance in epidemiologic studies of *P. aeruginosa* nosocomial infections. However the discrimination power of these methods is lower compared to molecular typing system[4]. So, in order to differentiate between the isolates and clonal groups of *P. aeruginosa,* several molecular typing schemes have been described[9]. These methods include pulsed field gel electrophoresis (PFGE), ribotyping, enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) and random amplification of polymorphic DNA (RAPD). Among them, the main typing method with wider application is PFGE known as gold standard genotypic technique, though being high specific and sensitive, is limited by technical complexity, expense and prolonged turnaround times for results[10-11].

One of the widely used repetitive DNA elements in PCR-based genotyping methods, are the ERIC sequences common to Gram-negative enteric bacteria[10]. ERIC sequences may offer greater potential as an example for the study of the evolution of bacterial interspersed repetitive sequences because they are longer and thus more informative in comparative analyses and are found in a wider range of species[12]. The ERIC -based PCR genotyping (ERIC-PCR) method has already been used to study the epidemiology of *P. aeruginosa* in Australia and Brazil[8,13].

Since the rapid and discriminative subtyping methods are useful for determining the clonality of the strains in nosocomial outbreaks, the present study was designed to investigate the genetic diversity in a collection of the MDR strains of *P. aeruginosa* isolated from burn and wound infections by ERIC-PCR. The determination of genetic relationships among the MDR strains, allows mapping the dynamics of infection transmission in the region of the study and will also provides a better understanding the epidemiology of the resistant strains.

Methods

This study has been approved by the Infectious and Tropical Diseases Research Center.

Bacterial strains

From total 99 MDR strains, 66 were subjected to genotyping. These were came from 150 *Pseudomonas aeruginosa* isolates which were originated from wound and burn infections in Ahvaz, Iran, over a period of six months from January to November 2013, and the details are under publication[14]. The MDR determination was according to the criteria described by other investigators[15].

DNA extraction

DNA extracted from colonies of all isolates by simple boiling method as described elsewhere[16]. In brief, a few colonies were dissolved in TE (Tris-EDTA) buffer and boiled at 100°C for 10 minutes with subsequent precipitation in a 14000 x g refrigerated centrifuge at 4°C for 3 min. The supernatant containing DNA was used as template for PCR amplification. The concentration of extracted DNA was measured by biophotometer (Eppendorf, Germany) at 260-280 nanometer and were kept at -20°C until use.

ERIC PCR fingerprinting

PCR amplification was performed on extracted DNA by using previously reported primers of ERIC1 (5'-ATGTAAGCTCCTGGGGATTCAC-3') and ERIC2 (5'-AAGTAAGTACTGGGGTGAGCG-3')[17], in an Eppendorf thermocycler (Roche Co, Germany). PCR mixture was prepared in a final volume of 50 µl containing 10 µl of 10x PCR buffer, 50 mM $MgCl_2$, 2.5 mM dNTP mix, 10 µM of each primer,1U of *Taq* DNA polymerase and 50 ng of template DNA. The amplification parameters were initial denaturation at 94 for 3 min, followed by 35 cycles of PCR consisting denaturation at 94 for 1min, annealing at 55 for 7min, and extension at 72 for 2 min and a final extension at 72°C for 5 min. The amplified products were subjected to electrophoresis on 1.5% agarose gel, stained with 0.5 µg/µl ethidium bromide (Qiagen, Germany) and analyzed under UV light in a gel documentation system (Proteinsimp, USA). *Pseudomonas aeruginosa* ATCC 27853 was used as positive

control. Each ERIC-PCR test was performed in duplicate to ensure conformity of each fingerprint.

ERIC Fingerprint analyses and dendrogram construction

A TIFF image of gel was created by photographed with UV gel Doc (BIO-RAD, USA) and the DNA banding patterns entered into a database in BioNumerics 7.1 software (Applied Maths, Sint-Martens-Latem, Belgium). The ERIC-PCR patterns obtained were interpreted and compared as described by Ghazi *et al*.[18]. Similarity analysis was calculated using the Dice coefficient and the unweighted pair group average (UPGMA) for cluster analyses. The criterion for related clones or classified as the same type when the pattern similarity was 80% or more similar bands.

Results

According to criteria determined for MDR *P. aeruginosa*[15], 99 strains (66%) were identified as MDR, of which 53 (70.66%) were belonged to burn and 46 (61.33%) were originated from wound infections. These isolates showed more than 90% resistance to the most of tested antibiotics (Table 1). From total 99 MDR isolates, 66 were subjected to ERIC-PCR analysis. These were included all 53 isolates from burn patients, which were showed resistance to at least nine of antibiotics, and 13 randomly selected from wound infections with the resistance patterns similar to those isolated from burn infections.

TABLE 1 - Disk diffusion antimicrobial resistance patterns of 150 *P. aeruginosa* isolates. The isolates designated as MDR are among the most resistant isolates.

Antibiotic	Disk content (µg)	Resistant N. (%)
ofloxacin	5	110(73.33)
ciprofloxacin	5	106(70.67)
gentamicin	10	103(68.66)
imipenem	10	93(62)
meropenem	10	93(62)
ceftazidime	30	102(68)
amikacin	30	83(55.33)
aztreonam	30	100(66.67)
ceftriaxone	30	105(70)
cefotaxime	30	109(72.67)
cefepime	30	119(79.33)
ticarcillin	75	106(70.67)
piperacillin	100	68(45.33)
carbenicillin	100	107(71.34)

Figure 1 represents the ERIC-PCR profiles of some *P. aeruginosa* strains isolated from burn infection by gel electrophoresis. Eight clusters were generated for analyzed MDR isolates, which were designated as: I, II, III, IV, V, VI, VII, VIII, and 50 single clones. In clusters II, III, VI and VIII; the isolates showed 100% similarity, and each of them has formed one real clone. All MDR isolates from wound infections showed heterogeneity as single clone.

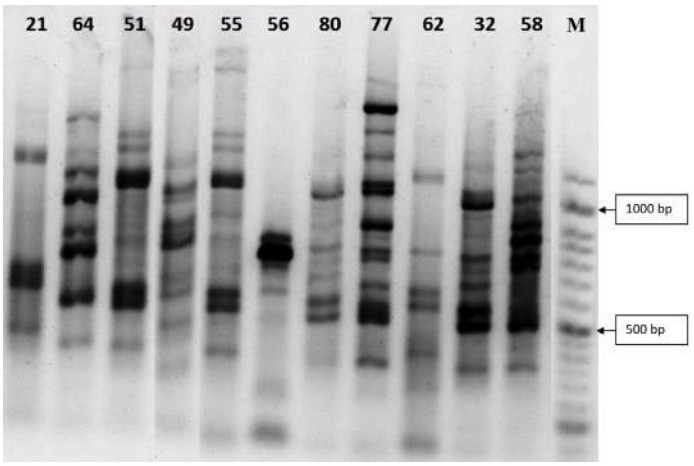

FIGURE 1 - DNA-banding profiles of some *P. aeruginosa* strains isolated from burn infection by gel electrophoresis following ERIC-PCR.

In Figure 2, the dendrogram generated on the basis of the isolates antimicrobial resistance against 14 main antibiotics are presented. The high unusual heterogeneity was observed among the isolates from burn infections, representing 16 isolates which were categorized in eight clusters (each cluster comprised 2 identical isolates), and 37 single clones. The members classified within the clusters, demonstrated highest resistance to more antibiotic categories in present study.

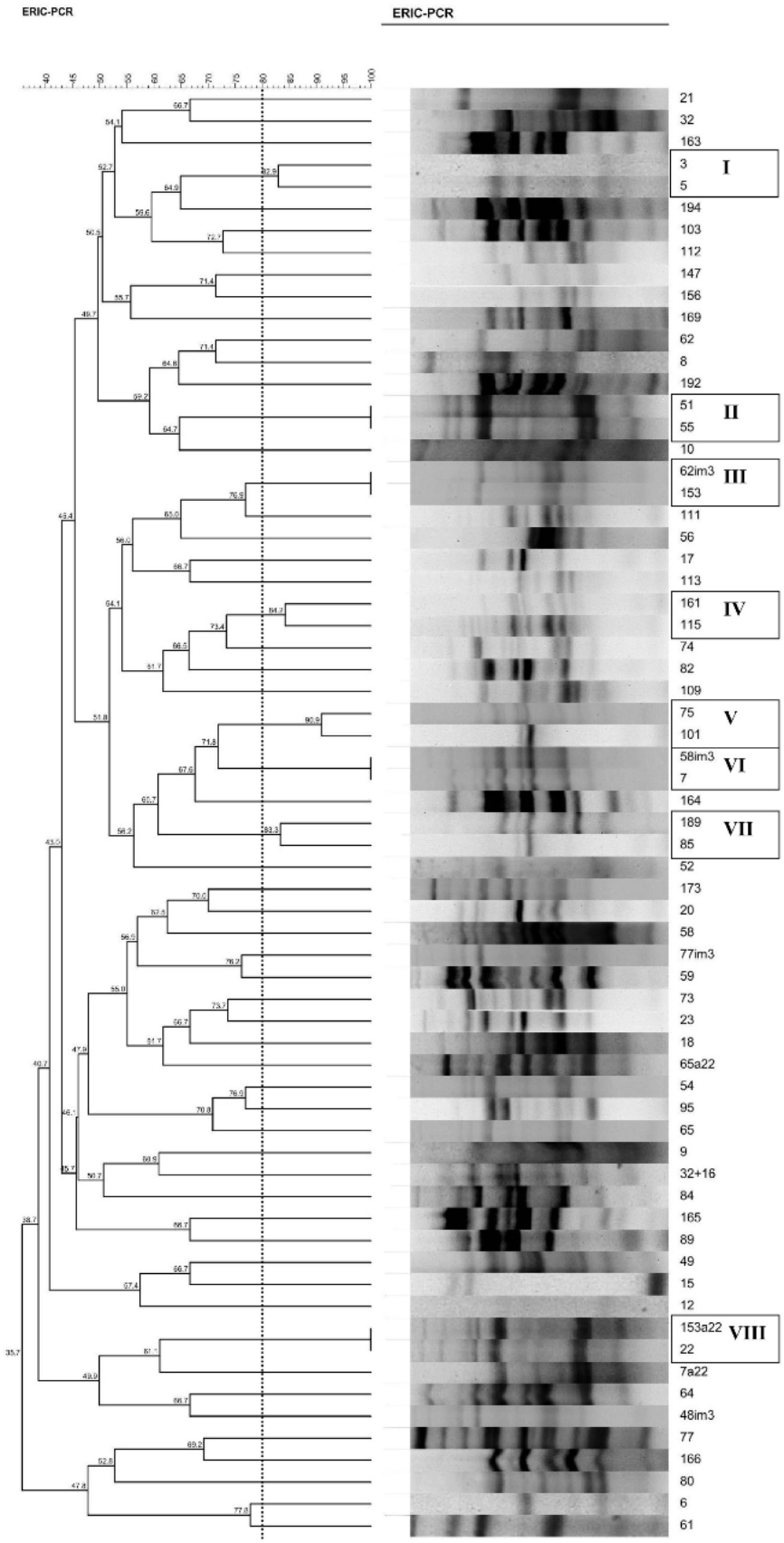

FIGURE 2 - Dendrogram of *Pseudomonas aeruginosa* based on ERIC-PCR. The isolates from both burn and wound infections. The scale at the top represents the genetic distance between the isolates.

Discussion

Pseudomonas aeruginosa has a remarkable ability to acquire and harbor diverse resistance determinants[19]. Due to its intrinsic and acquired antimicrobial resistance, only limited classes of antibiotics are effective for the treatment of *P. aeruginosa* infection, so outbreaks due to MDR *P. aeruginosa* infection in hospitals despite efficient infection control policies, occurs worldwide[20-21]. The Taleghani burn hospital in Ahvaz, the center of Khuzestan province, is a referral center in Southwestern Iran, serves patients from neighboring provinces. Therefore genotyping of *P. aeruginosa* isolates seems to be essential to clarify the epidemiology of this organism in this hospital. In order to characterize the diversity of MDR strains of *P. aeruginosa* isolated from burn patients, and comparison with the heterogeneity of isolates from wound infections, 66 MDR strains were subjected to ERIC-PCR analysis. These isolates which were comprises all MDR strains isolated from burn infection (n=53) and 13 randomly selected strains from wound infections, were more resistant to combinations of very high numbers of drugs. With ERIC-PCR, the 66 isolates were sub-typed into eight different cluster profiles and 50 single clones. The clusters consisted only 16 isolates from burn infection and none of the wound isolates were included in the clusters. The majority of burn isolates and all those from wound infections, showed the diversity as single clones, which shows a high genotypic variation among the tested isolates. In a study conducted on 75 *P. aeruginosa* isolates from various sources, weak clonality was observed with 80% of the clinical strains belonging to only 3 clones[21], which was not in agreement with our study. Other investigators reported genetically diversity and heterogeneity among their 48 tested *P. aeruginosa* clinical isolates and concluded that both used analyses methods of ERIC-PCR and REP-PCR have been described as equally effective in characterizing clinical isolates of *P. aeruginosa*[10]. Although the study of Lim *et al*.[22], was against the mentioned work, as they found that ERIC-PCR was more discriminatory than REP-PCR. In their report, ERIC-PCR, was able to differentiate isolates that were not differentiated by REP-PCR. Moreover the molecular typing of the MDR *P. aeruginosa* isolates revealed 21 genetic profiles with predominance of three clones in another study[23]. In our study, high level of genetic diversity was observed among the isolates, demonstrating no genetic correlation between them. We could not find any significant differences between genotypic diversity of isolates from burn and wound infections, due to high rate of heterogeneity in both isolates. In agreement to our study, ERIC-PCR produced 53 different ERIC fingerprints for *P. aeruginosa* isolates in the study of Goudarzi *et al*.[24], from Iran, 49 of which contained only 1 strain. Eight of the isolates had 100% similarity, forming four real clones. Besides, Inacio et al.[9], demonstrated a high level of genetic heterogeneity among their tested isolates using ERIC-PCR analysis.

This *P. aeruginosa* heterogeneity differences in several studies from different parts of the world, demonstrate the impact of environmental factors and the level of hospital hygiene on the distribution and genetic clonal formation variation. As the results demonstrated, our studied MDR *P. aeruginosa* clinical isolates were genetically diverse and heterogeneous, suggesting that multiple subtypes of the species are involved in infection. Moreover the findings of present work suggest that genotyping by ERIC-PCR, may play an important role in routine epidemiological surveillance, and in the identification of the source of transmission of *P. aeruginosa* in the hospitals. As previously reported[25], the ability of ERIC-PCR assay to discriminate types, proved to be excellent, and can be used as first screening genotyping methods for typing of *P. aeruginosa*.

Conclusions

Despite the strain diversity among clinical isolates both from burn and wound infections in present study, extremely high level of drug resistance especially in isolates from burn, suggests that efficient and sustained control measures and proper antibiotic policy should be observed. Moreover the high level of genotypic heterogeneity in *P. aeruginosa* strains demonstrated by ERIC-PCR, showed no genetic correlation between them.

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Received: Nov 21, 2015
Review: Jan 18, 2016
Accepted: Feb 16, 2016
Conflict of interest: none
Financial source: Ahvaz Jundishapur University of Medical Sciences (Grant No. 91132)

[1]Research performed at Health Research Institute, Infectious and Tropical Diseases Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.