ORIGINAL ARTICLE

Genomic Diversity and Virulence Genes among Clinical Isolates of *Pseudomonas aeruginosa*

HASAN VALADBEIGI [1], ROBAB RAFIEI TABATABAEI [2], ABBAS MALEK [1],
ZAMBERI SEKAWI [3], MOHAMMAD RAFTARI [4], KOLSOOM PARVANEH [1, 5],
SOBHAN GHAFOURIAN [1, 3], NOURKHODA SADEGHIFARD [1]

[1] Clinical Microbiology Research Center, Ilam University of Medical Sciences, Ilam, Iran
[2] Dept of Microbiology, Faculty of Basic Sciences, Islamic Azad University of North Tehran, Tehran, Iran
[3] Department of Medical Microbiology and Parasitology, Faculty of Medicine and Health Sciences, University Putra Malaysia, Malaysia
[4] Faculty of Food Science and Technology, Universiti Putra Malaysia, UPM 43400, Serdang, Selangor, Malaysia
[5] Department of Nutrition and Dietetics, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

SUMMARY

Background: Typing of nosocomial pathogens is necessary to determine the source of an outbreak. The aim was to determine the genomic variability among *Pseudomonas aeruginosa* (*P. aeruginosa*) by random amplification of polymorphic DNA (RAPD) and enterobacterial repetitive intergenic consensus (ERIC) methods.
Methods: Fifty *P. aeruginosa* isolates were obtained from the hospitals. The source of these isolates were burn wound and urinary tract infections. After detection of *P. aeruginosa* by biochemical methods, chromosomal deoxyribonucleic acid (DNA) was extracted by a DNA extraction kit. ERIC-PCR and RAPD-PCR was done by standard methods. The polymerase chain reaction (PCR) products were run and visualized in 1.5% agarose gels stained with ethidium bromide.
Results: Fifty *P. aeruginosa* isolates were analyzed by ERIC-PCR and RAPD-PCR methods. Multiple PCR fragment sizes generated by two PCR methods and PCR product size were between 200 - 3500 bp, and 10 and 7 different PCR patterns were detected by ERIC-PCR and RAPD-PCR, respectively. Eleven isolates were not detected by ERIC-PCR method. Fifteen isolates were typed to a single genotype by the RAPD-PCR method.
Conclusions: We suggested that ERIC and RAPD PCR are equally suitable, inexpensive, fast, reproducible, and discriminatory as rapid DNA typing tools for effective epidemiological surveillance of *P. aeruginosa* isolates. Our results suggest that these DNA typing tools could be used in routine epidemiological surveillance, outbreak surveillance, and in the identification of the source of transmission of *P. aeruginosa*.
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KEY WORDS

Pseudomonas aeruginosa genotyping techniques, polymerase chain reaction, random amplified polymorphic DNA technique, urinary tract infections

LIST OF ABBRIVATIONS

P. aeruginosa - *Pseudomonas aeruginosa*
PCR - polymerase chain reaction
RAPD - random amplified polymorphic DNA
ERIC - enterobacterial repetitive intergenic consensus
DNA - deoxyribonucleic acid

UTI - urinary tract infections
PFGE - pulsed-field gel electrophoresis
LB - lurian brothB

INTRODUCTION

The ability of *P. aeruginosa* to adapt and thrive in a wide variety of environments is due in part to its extensive genetic versatility, which contributes significantly to its potential as a pathogen. *P. aeruginosa* is an opportunistic pathogen that is a common cause of hospital-

acquired infections [1,2]. This ubiquitous bacterium is able to survive under many conditions, on inanimate objects such as respiratory ventilators, patient care equipment, toilets, hospital room sinks and showers, so prevention of exposure to it is impossible [2].

P. aeruginosa is an important cause of both community-acquired and hospital-acquired infections. It is a common cause of hospital-acquired infections, particularly infecting patients with predisposing factors, such as burn victims, immunocompromised hosts, or those with metabolic disorders [3,4]. Hospitalised patients may be infected with this bacterium on admission or may acquire it during their stay in hospital. Nosocomial infections caused by *P. aeruginosa* include UTI, bloodstream infections, surgical and burn site infections, pneumonias, and skin infections.

This pathogen produces multiple pathogenic elements during the infection, therefore, in most situations the immune response can not eliminate the bacteria. It is likely that as part of colonization, invasion, and survival in the human host, *P. aeruginosa* utilizes a unique range of genes; however, the precise repertoire of virulence genes required is unclear.

Typing of this nosocomial pathogen is very important for detecting the source of nosocomial outbreaks and to implement effective control methods for the prevention the spread of the pathogen. A good typing technique should be highly discriminatory, easy to use, inexpensive, reproducible, and fast. Phenotypic and biochemical methods based on antimicrobial susceptibility, serotyping, and bacteriophage typing lack discriminatory power and stability and were replaced with molecular based techniques. Many molecular typing methods have been developed for typing *P. aeruginosa*. Among these methods, PFGE is commonly employed to type closely related isolates of *P. aeruginosa* and is recognized as the gold standard for typing this bacterium. However, this method is expensive, time-consuming (2 or 3 days), and needs specialized equipment and expert operators. To avoid the drawbacks of PFGE, rapid and inexpensive PCR-based typing methods, such as ERIC-PCR and RAPD-PCR, can be used to screen, discriminate, and to determine genetic relatedness among the strains with the same accuracy of PFGE. These two techniques are faster and less expensive than PFGE [2,5]. ERIC-PCR assays utilize primers targeting highly conserved sequences common to Gram-negative enteric bacteria [6,7].

Random amplification of polymorphic DNA-polymerase chain reaction does not require prior knowledge of the genome of the organisms to be investigated. A random primer is used, which is not targeted to amplify any specific sequences on DNA. Each primer gives different PCR products which allows the differentiation of even closely related strains in the same species [8-10]. These two methods have not previously been used to compare *P. aeruginosa* (PA) strains isolated from clinical isolates in Iran. The goals of the present study were to analyze of PA by ERIC- and RAPD-PCR, to deter-

mine the genomic variability among the isolates, and to determine the frequency of alginate and exotoxin A genes among the isolates.

MATERIALS AND METHODS

Bacterial isolates
Fifty isolates of *P. aeruginosa* were obtained during the period from October 2009 to December 2010 from Shafa Hospital in Kerman and Milad Hospital in Tehran. The isolates were from burn patients in Shafa Hospital and urinary tract infections in Milad Hospital. *P. aeruginosa* was identified by biochemical methods [11]. After inoculation of a colony on LB medium and incubation for 18 hours in 37°C, chromosomal DNA was extracted by DNA extraction kit (Fermentas).

PCR for detection of virulence genes
With special primers for alginate, PCR was done using forward primer V1C1:
5'-TTCCCTCGCAGAGAAAACAT-3' and reverse primer V1C2: 5'-CCTGGTTGATCAGGTCGATCT-3' and for exotoxin A, forward primer ETA1:
5'-GACAACGCCCTCAGCATCACCAGC-3' and reverse primer ETA2:
5'-CGCTGGCCCATTCGCTCCAGCGCT-3'.
DNA templates of each isolate were amplified in a total reaction volume of 50 µL containing 2.5 U of AmpliTaq Gold thermostable polymerase (Roche, Switzerland), 50 pmol of primer, 200 µM of each deoxynucleotide, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), and 50 mM KCl (Promega, USA). Amplification for the exotoxin A gene was carried out with denaturation at 94°C for 4 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, 68°C for one minute, and 72°C for one minute, plus a final extension of 7 minutes at 72°C to complete partial polymerizations. Amplification for the alginate gene was performed as for the exotoxin A except for annealing which was done at 62°C for 1 minute. The amplified products were subjected to electrophoresis in 1.2% agarose gels, stained with ethidium bromide, and visualized in a Gel Doc XR molecular imager (Bio-Rad Laboratories).

Enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR)
DNA of each isolate underwent ERIC-PCR typing [12]. ERIC-PCR was performed using 0.2 mL PCR tubes in a Bio-Rad thermal cycler. In each assay, the reaction mix contained $MgCl_2$, DNA template, Taq polymerase, primers, and deoxynucleoside triphosphate [4,12]. PCR amplification was started using an initial denaturation step at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, primer annealing at 56°C for 1 minute and extension at 72°C for 2 minutes and one cycle of further extension at 72°C for 10 minutes. The amplified products were subjected to electrophoresis in 1.2% agarose gels, stained with ethidium

bromide, and visualized in a Gel Doc XR molecular imager (Bio-Rad Laboratories).

Random amplification of polymorphic DNA-polymerase chain reaction (RAPD-PCR)

The RAPD-PCR fingerprinting was performed using AP-ARB11 primer (5'-CTAGGACCGC-3').

DNA templates of each isolate were amplified in a total reaction volume of 50 µL containing 2.5 U of Ampli-Taq Gold thermostable polymerase (Roche, Switzerland), 50 pmol of primer, 200 µM of each deoxynucleotide, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), and 50 mM KCl (Promega, USA). Amplification was carried out with denaturation at 94°C for 7 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, 40°C for one minute, and 72°C for 1 minute, plus a final extension of 10 minutes at 72°C to complete partial polymerizations. The amplified products were subjected to electrophoresis in 1.2% agarose gels, stained with ethidium bromide, and visualized in a Gel Doc XR molecular imager (Bio-Rad Laboratories).

RESULTS

Fifty *P. aeruginosa* isolates were analyzed by ERIC-PCR and RAPD-PCR methods. Multiple PCR fragment sizes were generated by the two PCR methods, and PCR product sizes were between 200 - 3500 bp, 10 and 7 different PCR patterns were detected by ERIC-PCR and RAPD-PCR, respectively. We could not find 11 PA by ERIC-PCR. Some of the isolates between the two hospitals had similar genotypes by two PCR methods which are shown in Table 1 and Table 2.

Table 1. Frequency of ERIC-PCR patterns among *P. aeruginosa* isolates.

Groups	Number of isolates
1	10
2	2
3	4
4	3
5	3
6	2
7	4
8	5
9	2
10	4

Table 2. Frequency of RAPD-PCR patterns among *P. aeruginosa* isolates.

Groups	Number of isolates
1	2
2	7
3	2
4	17
5	10
6	7
7	5

The results of ERIC-PCR typing showed that most of the isolates with similar patterns were found in genotype 1 as shown in Table 1. This genotype circulated in both hospitals. Interestingly, genotypes 2, 6, 7, and 9 were observed just in Milad Hospital and genotypes 3, 4, 5, 8, and 10 were identified in Shafa Hospital., All of the isolates were identified in seven genotypes by RAPD-PCR typing. The most common patterns by RAPD-PCR were observed among 17 (34%) PA isolates and they were found in genotype 4. The findings revealed genotype 4 in both hospitals. Our results showed that genotypes 2 and 6 were found in Milad Hospital, while genotypes 3, 4, 5, and 6 were observed in Shafa Hospital.

According to PCR results, 37 (74%) and 46 (92%) isolates had exotoxin A and alginate genes, respectively. Among *P. aeruginosa* strains from 30 isolates of urinary tract infections, exotoxin A and alginate genes were detected in 21 (70%) and 26 (86.6%) isolates, respectively. In addition, in the remaining strains which included 20 isolates from burn wound sites, exotoxin A and alginate genes were identified in 16 (80%) and 20 (100%) isolates, respectively.

DISCUSSION

Many reports around the world supported that nosocomial infections caused by multi resistance organisms like *P. aeruginosa* are very dangerous for hospitalized patients, so detection of the source of transmission of these multi drug resistant bacteria, especially *P. aeruginosa*, by molecular typing methods is very useful and important for controlling hospital outbreaks.

According to PCR results, 37 (74%) and 46 (92%) isolates had exotoxin A and alginate genes, respectively. Results showed that all *P. aeruginosa* strains from the 20 isolates of burn wound sites carried the alginate gene. Maybe this gene is very important in strains which cause burn wound infection.

In this study, we used two PCR based DNA typing methods, ERIC and RAPD PCR. Both techniques provided measures of genetic diversity, but they were not equivalent. Although 11 isolates were not typed by ERIC-PCR, the diversity of this method was signifi-

cantly higher than RAPD-PCR. The ERIC-PCR primers and the single RAPD primer generated banding patterns containing 5 - 16 bands ranging in size from 150 to 3500 bp and 350 to 2800 bp, respectively. Some of the isolates had identical patterns among both hospitals using the ERIC or RAPD PCR method. On the other hand, some of the genotypes were observed just in Milad or Shafa Hospital. For example by ERIC-PCR Genotypes 2, 6, 7, and 9 were observed just in Milad Hospital and genotypes 3, 4, 5, 8, and 10 were identified only in Shafa Hospital.

With ERIC-PCR analysis, most of the isolates with similar patterns were identified in genotype 1, so that 10 (20%) isolates were grouped in this type. All of the isolates were typed in 7 genotypes by RAPD-PCR typing, and the most common pattern by RAPD-PCR method was observed among 17 (34%) isolates and they were typed in genotype 4. Results from this study supported that ERIC and RAPD PCR are suitable, inexpensive, fast, reproducible, and discriminatory as rapid PCR based DNA typing tools for effective epidemiological surveillance of *P. aeruginosa* isolates and are valuable and useful as first screening genotyping methods for typing of *P. aeruginosa*. As previously reported, the ability of ERIC-PCR and RAPD-PCR assays to discriminate types proved to be excellent [12].

The RAPD-PCR method has been successfully utilized for typing of *P. aeruginosa* [13,14]. Kersulyte et al. reported that the RAPD method with 10-nucleotide primers lacked reproducibility; however, our RAPD typing data suggest that 10-nucleotide primers can be used successfully for typing *P. aeruginosa* [14]. All 50 isolates described in this study were typeable by RAPD analysis, and no contamination problems were detected. ERIC-PCR has been successfully applied to *P. aeruginosa* as well as to *Serratia marcescens*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Enterococcus faecalis*, *Staphylococcus epidermidis*, *Acinetobacter baumannii*, and *Escherichia coli* [15-18].

ERIC-PCR has performed well and appears to be a more reliable typing method for *P. aeruginosa* than RAPD-PCR, while some of isolates were not typeable by this method. It is suggested that these two methods be utilized with an additional, more reliable typing method such as PFGE or MLST to confirm relationships between isolates.

In comparing these two PCR base typing methods with PFGE, which is gold standard for typing *P. aeruginosa*, the cost of reagents and our lab costs for ERIC and RAPD PCR are significantly less than PFGE. Training of personnel in these two methods is simpler and more generic, compared to PFGE.

CONCLUSION

Our results demonstrated that these DNA typing tools could be used in routine epidemiological surveillance, outbreak surveillance, and in detection of the source of transmission of *P. aeruginosa*. Results of this study showed that some of the strains have similar patterns by RAPD-PCR and ERIC-PCR and it is assumed that these strains have the same origin.

Declaration of Interest:
The authors have no conflicts of interest to declare.

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Correspondence:
Nourkhoda Sadeghifard
Email: Sadeghifard@gmail.com

Sobhan Ghafourian
Email: sobhan.ghafurian@gmail.com