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Pyocin-Based Molecular Typing of Local Isolates of *Pseudomonas Aeruginosa* Isolated from Blood Samples

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Abstract

Background *Pseudomonas aeruginosa* has assumed an increasingly prominent role as the etiological agent in a variety of serious infections in hospitalized patients. *Pseudomonas aeruginosa* strains produce three distinct types of bacteriocins (bactericidal substances). Bacteriocins of *P. aerugonos*a strains can be classified according to their morpology, or according to functions into pore forming pyocin and DNase activity–pyocins.

Objectives To type by a molecular method local isolates of *Pseudomonas aeruginosa* utilizing the genes encoding for the potent bacteriocin (pyocin).

Methods Fifty bacterial isolates of *P. aeruginosa* were re-identified by standard bacteriological methods and were subjected to PCR-amplification for the genes responsible for the production of three bacteriocins (pyocin S1, S2, and S3).

Results Out of the fifty local isolates of *P. aeruginosa* enrolled in this study there were forty five (45) isolates which showed the presence of the genes encoding for the two mentioned bacteriocins (S1 and S2) corresponding to 95% of the isolates and there were forty five (41) isolates showed the presence of the gene encoding for the pyocin S3 corresponding to 82% of the isolates.

Conclusions The present work showed a high genotypic relatedness of the studied clinical isolates of *P. aeruginosa* and it also emphasized the ability of the use of molecular typing of pyocins as more advanced and accurate methods for typing purposes and epidemiological studies.

Key words Pyocins, Bacteremia, bacteriocins, polymerase chain reaction (PCR), electrophoresis

Introduction

Pseudomonas aeruginosa has assumed an increasingly prominent role as the etiological agent in a variety of serious infections in hospitalized patients [(1)]. At particular risk are patients who have suffered major trauma or burns and are exposed to intensive care units [(2)]. Also at risk are normal individuals exposed to a compromising occupational or recreational environment.

Pseudomonas aeruginosa strains produce three distinct types of bacteriocins (bactericidal substances); proteins that have killing activity against strains of the same species. Bacteriocins of *P. aerugonos*a strains can be classified according to their morphology into three distinct types of bacteriocins designated R, F, and S pyocins, or according to functions into pore forming pyocin and DNase activity-pyocins [(3)].

Pyocins are composed of three functional domains, the receptor-binding domain, the translocation domain, and the DNase domain, as reported previously, pyocins S1 and S2 inhibit the lipid synthesis of sensitive cells [(4)].

S-type pyocins (S1, S2, AP41, and S3) cause cell death by DNA breakdown due to an endonuclease activity located at the C-terminal end of the larger component. However these compounds do not express any DNase when it is engaged in the complex [5].

Researchers stated that neither a revised pyocin typing technique nor O-serotyping provides all the requirements of the ideal typing system for P. aeruginosa. O-serotyping provides a rapid indication of antigenic differences when these occur. In an epidemic situation, however, the value of serotyping is limited unless the strains isolated belong to unusual serotypes. Pyocin typing requires a period of 24 h to achieve a result but provides adequate discrimination on which to base more confident epidemiological judgment [6].

Molecular techniques offer a considerable improvement, and can complement phenotypic data to obtain a better understanding of bacterial diversity [8]. PFGE is commonly employed, and has achieved widespread recognition as the 'gold standard' for *P. aeruginosa* DNA typing [9]. However, this method is limited by technical complexity, expense and prolonged turnaround times for results [8]. As an alternative, repetitive-element based PCR (rep-PCR) has shown considerable potential as a DNA typing tool in the laboratory (Lau et al., 1995; Rep-PCR assays utilize primers targeting highly conserved repetitive sequence elements in the bacterial genome. Two such groups of repetitive elements are the enterobacterial repetitive intergenic consensus (ERIC) sequences common to Gram-negative enteric bacteria, and the BOX elements, originally detected in *Streptococcus pneumoniae* [10].

This study was designed to find the frequency of a particular bacterial isolate associated with bacteremia as indicated by new PCR-based typing method for the genes encoding for pyocin S1, S2, and S3 located in the genome of local isolates of *P. aeruginosa*.

Methods

Sampling

This study recruited fifty septicaemic *Pseudomonas aeruginosa* isolates obtained from four sources, eighteen isolates were taken from the department of microbiology, College of Medicine, Al-Nahrain University, thirteen isolates from College of Science, Al-Mustansyria University, nineteen from department of pharmacology, Al-Kindey College of Medicine, Baghdad University, and three isolates from Al-Kadhiymia teaching hospital, Baghdad. These isolates were taken from patients of leukemia, lymphoma, urogenital, gastrointestinal, breast cancers, or from those suffering from burn infections and also from neonates. All of these isolates were collected in a period from February to December 2010. The isolates were subjected to various biochemical tests and standard bacteriological procedure to re-confirm the preliminary identification according to previous work [11].

1. DNA Extraction

The genomic DNA was extracted from bacterial cells using Wizard genomic DNA purification kits (Promega®, USA) and according to the manufacturer's instructions. Agrose gel (1.5%) electrophoresis was adopted to confirm the presence and integrity of the extracted DNA [12].

2. Primer

Primers were purchased from Bioneer® (South Korea) with melting temperatures and PCR product size of 58 ^{o}C; 278 bp and 53 ^{o}C; 541 bp for pyocin S1,S2 and pyocin S3, respectively.

To determine the type of S pyocin produced by different *P. aeruginosa* isolates, certain primers were used to detect the presence of the genes encoding for pyocin S1 and S2 which cannot be detected separately as they share the same antigenic properties, the3 used primers were as follows:

Because pyocins S1 and S2 share the same immunity protein, primers corresponding to the region encoding this protein were chosen [4].

For pyocin S1 and S2 immunity proteins, the following primers were selected:

a. S1S2imm-Fw (5-CACAAGGGAGGGAAGTGA-3).

b. S1S2imm-Rv (5-CGGCCTTAAAGCCAGGAA-3).

1. For pyocin S3 the following primers were selected:

a. RB-fw (5'-CGTATCACGAGACAGGCA-3').

b. RB-rv (5'-TGCCGCTTCTTCCGCTTT-3').

3. PCR Program

The preparatory step for PCR included the addition of 5 µl of the template bacterial DNA onto preloaded master mix eppendorff tubes followed by the addition of 1.5 µl (10 picomol\µl) of the specific primers, the final volume was completed to 20 µl by the addition of distilled water; finally the PCR program for pyocin S1 and S2 was run using the conditions mentioned in table 1.

Table1. Cycling protocol for the PCR amplification of pyocin S1 and S2 gene

Step		Temperature (°C)	Time	No. of cycles
Initial denaturation		94	30 seconds	1
First loop	Denaturation	94	30 seconds	30
	Annealing	528	30 seconds	
	Extension	72	30 seconds	
	Final extension	72	5 minutes	

For amplifying pyocin S3 gene, the primer concentration was 10 picomol\µl and the PCR mixture was prepared as for pyocin S1 and S2 while the cycling conditions run as mentioned in table 2.

Note: Cycling conditions were adopted as trial and error approach relying upon previous study [(4)].

Table2. Cycling protocol for the PCR amplification of pyocin S3 gene

Step		Temperature (°C)	Time	No. of cycles
Initial denaturation		94	30 seconds	1
First loop	Denaturation	94	30 seconds	30
	Annealing	56	30 seconds	
	Extension	72	30 seconds	
	Final extension	72	5 minutes	

4. Agarose Gel Electrophoresis

The preparation of reagents, buffers, stains, and the method of electrophoresing PCR products were prepared and done according to previous work. Agarose at 1.5, 1 grams was dissolve in 100 ml of 1X tris-borate EDTA buffer (or TAE) for PCR product and genomic DNA, respectively. The mixtures were then solubilized by heating with stirring, then they were left to cool at 60°C; ethidium bromide was then added to the mixtures and finally they were poured into the taped plate [(12)].

Results

The results of the current study revealed that all the fifty bacterial isolates (pre-identified as local isolates of *P. aeuginosa*) were re-identified as *P. aeruginosa* because they appeared as gram negative rods, capable of growth at 42°C, oxidase positive, sweet musty odor was produced, and were confirmed to be oxidative but not fermentative when applying oxidation/fermentation test.

The results of this study confirmed that all the 50 isolates enrolled in the study were viable and produced visible growth when activated and

sub-cultured; meanwhile the presence and integrity of their chromosomal DNA were also confirmed using agarose gel electrophoresis (Figure 1).

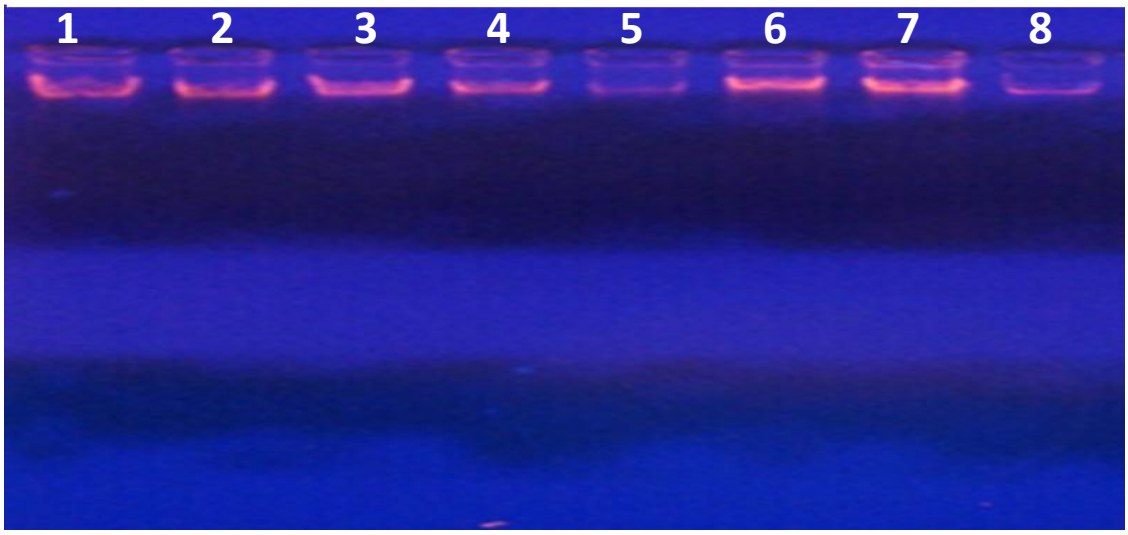

Figure 1. Chromosomal DNA bands on 1% agarose gel at 4V/cm for one hour

The results of PCR amplification for the genes encoding pyocin S1&S2 indicated that out of the fifty local isolates of *P. aeruginosa* enrolled in this study, there were forty five (45) isolates which showed the presence of the genes encoding for the two mentioned bacteriocins (S1 and S2) corresponding to 90% of the isolates (287 bp PCR products) while only five (5) isolates corresponding to 10% of the total fifty (50) isolates were recorded to be negative. The presence of the bands reflecting successful PCR amplifications and the absence of these bands indicated positive and negative results, respectively (figure 2).

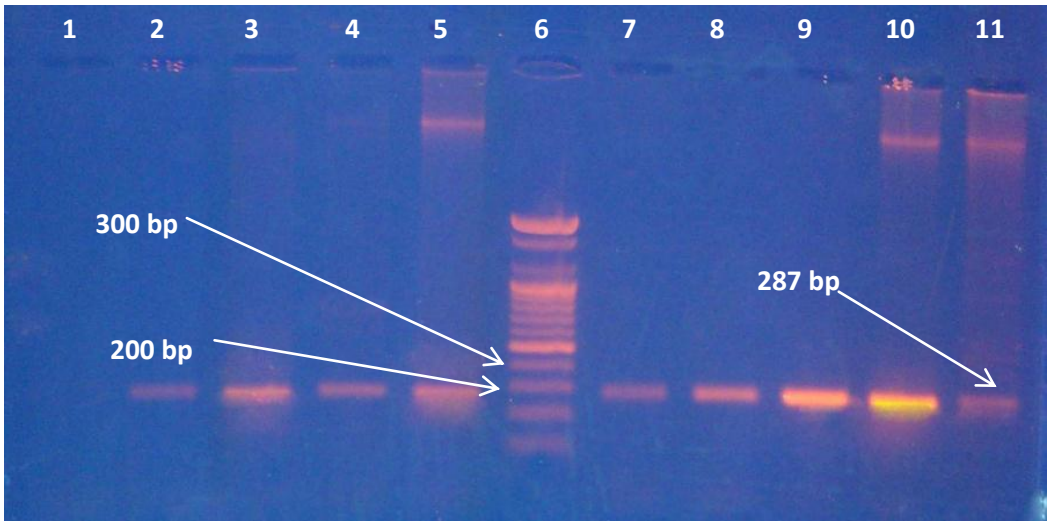

Figure2. Electrophoresis profile of PCR products of pyocin types 1 and 2 found in *P. aeruginosa*. From left to right: lane 2-5 represent the bands of PCR products belonged to four bacterial isolates, lane 6 represents the molecular size marker (100 bp) and lane 7-11 represent the bands of PCR products belonged to five bacterial isolates. Bands run on 1.5% agarose gel.

The results of PCR amplification for the gene encoding pyocin S3 revealed that out of the fifty local isolates of *P. aeruginosa* enrolled in the current study, there were forty one (41) isolates showed the presence of the gene encoding for the pyocin S3 corresponding to 82% of the isolates (415bp PCR products) while only nine (9) isolates corresponding to 18 of the total fifty (50) isolates were recorded to be negative; the positivity and negativity of PCR amplification was evidenced by the presence of the bands and the absence of these bands when amplification failed, respectively (Figure 3).

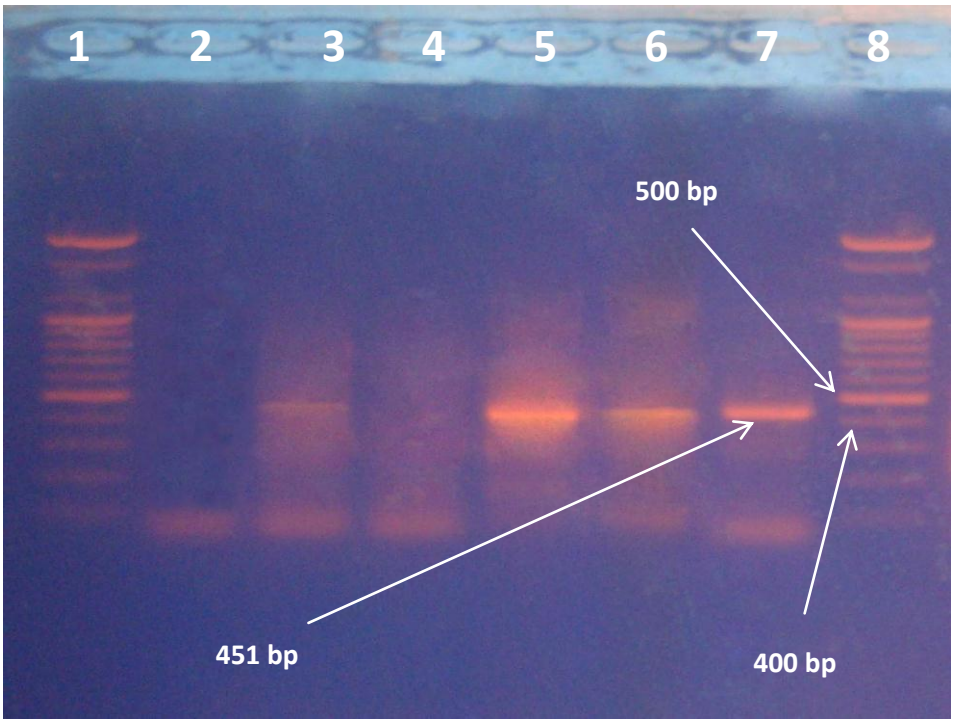

Figure3. Electrophoresis profile of PCR products of pyocin types 3 found in *P. aeruginosa.* From left to right: lane 1 and 8 represent the molecular size marker (100 bp), lanes2-7 represent the bands of PCR products belonged to six bacterial isolates. Bands run on 1.5% agarose gel.

Discussion

P. aeruginosa is an opportunistic pathogen that is able to cause severe invasive diseases in critically ill and immunocompromised patients [13]. It is a common pathogen in hospitals and particularly in intensive care units (ICU) that shows a high phenotypic diversity [14]. Moreover, high rates of resistance to antibiotics and frequent multi-drug resistance (MDR) are associated with nosocomial *P. aeruginosa* strains [15].

Molecular typing demonstrated that most *P. aeruginosa* isolates belonged to distinct genotype, demonstrating again that these methods had a higher discriminatory power than the phenotypic methods (antibiotyping and biotyping) [16].

Apart from discriminatory power, a suitable DNA typing assay must also have a high level of reproducibility, typeability and stability, low complexity and cost, as well as fast result turnaround times [17,18].

In a comparison among different molecular methods for typing bacterial strains, it was agreed that pulse field gel electrophoresis is the golden standard for typing purposes but the cost of PCR- based techniques is less and the shorter hands-on time for the PCR assays means that labour costs were significantly less, and training of personnel in this technology was simpler and more generic, compared to PFGE. The result

turnaround time for the rep-PCR assays was less than 10 h, which was considerably faster than PFGE (4-5 days). Also, as with all PCR-based techniques, the chance of generating artifact rather than detecting true genetic variation is greater if low-stringency PCR conditions are used such as those used in arbitrarily primed-PCR [19]. Rep-PCR assays use highly stringent conditions and therefore are more easily standardized [20]. However, optimization of all parameters of any DNA typing assay is essential to ensure optimal inter- and intra-laboratory standardization [18]. Overall, therefore, rep-PCR assays combine maximum discriminatory power, reproducibility, typeability and stability with cost-effective use of reagents and operator time.

In conclusion, the present work showed a high genotypic relatedness of the studied clinical isolates of *P. aeruginosa*. This finding might lead the way toward better approach for understanding the pathogenicity of the bacterial spp. Isolated from single particular clinical specimens and it may also provide a tool for more accurate typing method for this important nosocomial and opportunistic pathogen as compared to the conventional typing method; the presence of higher percentages of isolates producing pyocin S1,S2, and S3 may explain some the competing mechanisms exhibited by *P. aeruginosa* and this in turn can explain the dominance of a particular isolate in a particular clinical specimens.

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