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Molecular Characterization and Sensitivity Pattern of *Pseudomona aeruginosa* Among Patients With Different Diseases in Khartoum, Sudan

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ABSTRACT

The aim of this study was to determine the presence of the genes implicated in resistance to agents used for chemotherapy of infectious diseases caused by *Pseudomonas aeruginosa*. Seventy four *P. aeruginosa* strains were isolated from patients hospitalised in the Khartoum Teaching Hospital, Sudan. The strains were isolated in the period from February to June 2011 and serotyped studied at the research laboratory in Sudan University of Science and Theology. The isolates were recovered from patients with multiple types of infections, mostly respiratory tract, urinary tract and postoperative wound infection. Direct PCR technique was used to identify the genes implicated in antimicrobial resistance mechanisms. DNA extraction was skipped and the bacterial cell wall was denaturized in the first step of the reaction. The presence of IMP family genes, in the tested isolates of *P. aeruginosa* was identified, namely IMP-7 and IMP-10. This was the first report of the presence of IMP-7 and IMP-10 type genes in *P. aeruginosa* isolates in Khartoum State, Sudan.

المستخلص

هدفت هذه الدراسة إلى دراسة الزائفة الزنجارية من بين المرضى مع مختلف الأمراض في ولاية الخرطوم، السودان وتم كشف الزائفة الزنجارية بواسطة الكشف الجزئي ونمط الحساسية. وقد أجريت هذه الدراسة خلال الفترة من فبراير إلي يونيو 2011م، وشملت (74) عينة تحمل مختلف الأمراض من الزائفة الزنجارية تم جمعها من الجنسين ومختلف الأعمار والغالبية كانت من عينات مسحات التهاب الجروح والبول. هذه العينات تم جمعها واختبارها في مستشفي الخرطوم التعليمي، وأجريت دراستها في معمل البحوث بجامعة السودان للعلوم والتكنولوجيا، واستخدمت الاختبارات البيوكيميائية للتعرف علي البكتيريا المعزولة بواسطة استخدام اختبار (بي سي آر) باستخلاص الـ (دي ان. آى). وقد أجري تفاعل البلمرة المتسلسل لعائلة أي أم بي للجين (آي أم بي – سفن) والجين (آي أم بي – تن) للزائفة الزنجارية المعزولة. خلصت الدراسة إلى أن هذا يعد أول تقرير لوجود أنواع الجينين (أي أم بي – سفن) و(أي أم بي – تن) بالنسبة للزائفة الزنجارية في ولاية الخرطوم – السودان.

KEYWORDS: *Pseudomonas aeruginosa*; molecular techniques; PCR.

INTRODUCTION

Nosocomial infections are an important source of morbidity and mortality in many hospitals affecting millions of patients each year [(1)]. *P. aeruginosa* is one of the most important nosocomial pathogens, being responsible for various types of infections with more and more limited therapeutic options [(2)]. Infection due to *P. aeruginosa* continues to be a major cause of mortality among critically ill and immuno-compromised patients despite the development of newer and more powerful antibiotics. *P. aeruginosa* is characterized by inherent resistances to a wide variety of

antimicrobials. Its intrinsic resistance to many antimicrobial agents and its ability to develop multidrug resistance impose a serious therapeutic problem [3]. Multi-drug-resistant (MDR) strains of *P. aeruginosa*, defined as resistant to at least three of the following antibiotics: ceftazidime, imipenem, gentamicin or ciprofloxacin, are often isolated from patients exposed to prolonged intensive care-type therapies [4]. Its resistance to anti-pseudomonal β-lactams, advanced generation of cephalosporins, monobactams and carbapenems is also an increasing clinical problem. Carbapenems, mainly imipenem and meropenem, are potent agents for the treatment of infections due to MDR *P. aeruginosa*. The immunoevasive nature of *P. aeruginosa,* as well as its acquisition of multi-drug resistance makes elimination of this organism a particular challenge. Yet antibiotic resistance itself does not confer enhanced virulence [5], and therefore the ability to discriminate between virulent versus non- virulent phenotypes among MDR isolates would be a major step in predicting the particular threat of a colonizing strain of *P. aeruginosa.*

Historically, the analysis of nosocomial pathogens has relied on a comparison of phenotypic characteristics such as biotypes, serotypes and antimicrobial susceptibility profiles [6]. This approach has begun to change over the past 2 decades, with the development and implementation of new technologies based on DNA or molecular analysis. Studies of microbial pathogenicity at the molecular level have made substantial contributions to understanding of the epidemiology, clinical manifestations, diagnosis, treatment, and immunoprophylaxis of infectious diseases. One of the most exciting and profound technical advances in the past years has been the development of nucleic acid amplification techniques and their application to the study of microbial pathogenesis and the diagnosis of infectious diseases. Comparing with traditional methods, molecular analysis has a higher accuracy, and results are obtained much faster and much cheaper.

The goal of this study was to identify the presence of bacterial genes involved in multiple resistances to antimicrobials in *P. aeruginosa* using the PCR method.

MATERIALS and METHODS

Clinical isolates: Seventy four clinical isolates of *P. aeruginosa* were obtained from the Clinical Hospital for Infectious Diseases in Khartoum Teaching Hospital, Sudan medical center. The bacterial isolates were collected between February to June 2011from patients with multiple types of infections, mostly respiratory tract, urinary tract, postoperative wound (Septicaemia, Skin and soft tissue infection, Ecthyma gangrenosum), Eye, External ear, Respiratory tract (pulmonary infection), Gastrointestinal (Diarrheal infection), Chronic otitis media and external and Melioidosis infections. The isolates were identified with Compact for Gram-Negative Identification, card 2GN (bioMerieux-Vitek, Inc., Hazelwood, Mo.).

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by Kirby-Bauer disk diffusion method. The minimum inhibitory concentration (MIC) was done with Vitek 2 Compact system for Gram-Negative bacteria, according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI).

The MICs of: ciproflox- acin, pefloxacin, gentamicin, amikacin, tobramycin, ticarcillin, piperacillin, carboxipenicillin and ureidopenicillin with betalactamases, piperacillin, ticarcillin, ceftazidime, cefepime, cefpirome, aztreo- nam and colisin were determined. *P. aeruginosa* ATCC 27853, and *Escherichia coli* ATCC 25922 were used as reference strains in susceptibility testing.

Preparation of samples and DNA amplification

Direct PCR technique was used, DNA extraction was skipped and the bacterial cell wall was denaturized in the first step of the reaction. One or two bacterial colonies from a plate that was incubated overnight were suspended in 100 ml of sterile water and then diluted to a concentration of approximately 10^6 CFU/ml. an eliquot of 3 μl of the suspension was used as the template for amplification by PCR. Using this technique skips the expensive DNA extraction and minimizes self contamination of workers [7].

PCR protocol

A typical 20 μl PCR mixture with 4 μl master mix which contained 0.5 μl 5 x PCR reaction buffer, 0.2 μl of each primer, 200 μ M concentrations of each dNTPs, 2.5 μl $MgC1_2$, 12.5 mM (2 mM final concentration), 0.75 U of *Taq* polymerase, blue dye (Migration equivalent to 3.5 - 4.5 kb DNA fragment), yellow dye (Migration rate in excess of primers in 1% agarose gel < 35 -45 bp and 3 μl bacterial suspension. PCR was performed in a Thermocycler, (Gradient Palm-Cycler™, Corbett Life Science). The parameters for amplification were as follows: initial denaturation at 94°C for 4 min, 30, cycles of: 1 min each at 94°C, 1 min. at 57-62°C (depending on the primer), 1 min. at 72°C and a final extension step at 72°C for 10 min. Amplicons have been separated on 1% agarose gel, stained with ethidium bromide. The optimization of PCR was made after McPherson and Møller [8], and Roux [9]. The primers (Table 1) were designed with "Pick Primers" programe according to the seque1nces found at NCBI data base.

Table (1) Primer used for PCR amplification of Pseudomonas aeruginosa ATCC 27853

Target gene	Function	Sequence ($\overline{5}-\overline{3}$)	Amplification size (pb)	Accession number
IMP- 7	β -lactamase	AAGGCAGTATCTCCTCTCATTTC/ ACTCTATGTTCAGGTAGCCAAACC	243	EF606914
IMP-10	β -lactamase	AATGCTGAGGCTTACCTAATTGAC/ CCAAGCTTCTATATTTGCGTCAC	388	DQ288156

IMP- 7:

IMP-7 Outer - F: AAGGCAGTATCTCCTCTCATTTC/

IMP-8 Outer - R: ACTCTATGTTCAGGTAGCCAAACC

Outer - R1: GGTTTGGCTACCTGAACATAGAGT

IMP-10 :

IMP-10 Outer-F: AATGCTGAGGCTTACCTAATTGAC/

IMP-11 Outer – R: CCAAGCTTCTATATTTGCGTCAC

Outer- R1: GTGACGCAAATATAGAAGCTTGG

RESULTS

Phenotypic traits: Out of one hundred and fifty samples, seventy four isolates were *P. aeruginosa*. Most of these tested strains were highly sensitive to antibiotic drugs. Seventy strains were susceptible to imipenem, sixty to ceftazidim and sixty of them were sensitive to ciprofloxacin and highly resistant to others antibiotics, fifty strains were resistant to carbenicillin and fourty strains were resistant to piperacillin. Fifty seven isolates were recognized to have IMP-7 and IMP-10, only twenty six isolates produce IMP-7 and the remainder thirty one isolates had IMP-10. Five isolates of them were giving same reactives in tow genes IMP-7 & IMP-10.

PCR used to detect IMP family tests (IMP-7 and IMP-10) genes yielded PCR products of the expected sizes. Amplicons of the expected sizes were obtained for IMP-7 and IMP-10 genes in the isolates that were resistant to antimicrobials (Fig. 1 to 3). At the sensitive isolates amplicons were not obtained

DISCUSSION

Pseudomonas aeruginosa is one of the most important nosocomial pathogens, being responsible for various types of infections with more limited therapeutic options. One of the most alarming characteristic of *P. aeruginosa* is its low antibiotic susceptibility. Since the first report of acquired metallo-β-lactamases (MBL) in Japan in 1994 [10] genes encoding IMP-types (IMP-7 and IMP-10) enzymes have spread rapidly among *Pseudomonas spp.* [5,9,11]. Acquired metallo-β-lactamases (MBLs) are mostly encoded by integron-borne genes and confer resistance against all β-lactams except for the monobactams. IMP-types MBLs were reported from several countries [12]. The prevalence of MBL- producing Gram-negative bacilli has increased in some hospitals, particularly among clinical isolates of *P. aeruginosa* [13]. Since MBL production may confer phenotypic resistance to virtually all clinically available β-lactams, the continued spread of MBL is a major clinical concern [14].

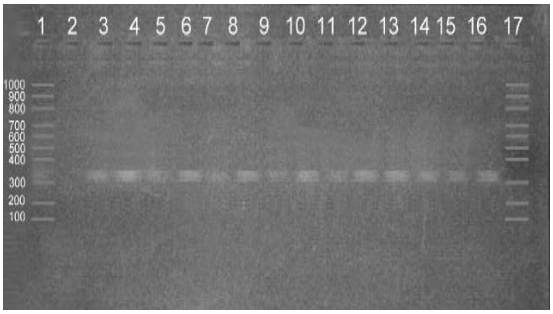

Figure (1) Agrose gel electrophoresis of PCR products after amplification of (IMP-7) gene. Lanes: 1 and 17 – molecular weight marker (O'Range Ruler 100bp DNA ladder); 2 – Negative control; 3 – 16 different strains

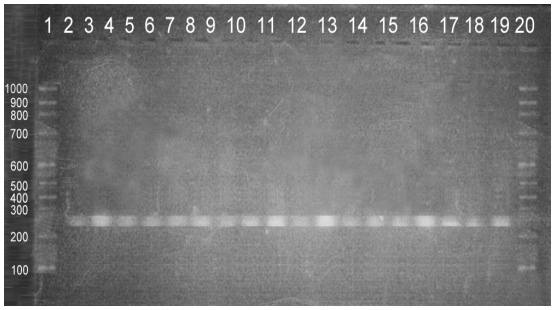

Figure (2) Agrose gel electrophoresis of PCR products after amplification of (IMP-10) gene. Lanes: 1 and 20 – molecular weight marker (O'Range Ruler 100bp DNA ladder); 2 – Positive control; 3 – 19 different strains of P. aeruginosa (IMP-10 gene products)

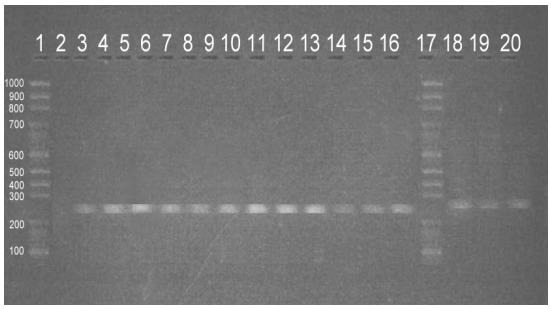

Figure (3) Agrose gel electrophoresis of PCR products after amplification of (IMP-7 and IMP-10) genes. Lanes: 1 and 17 – molecular weight marker (O'Range Ruler 100bp DNA ladder); 2 Negative control; 3 and 16 different strains of P. aeruginosa IMP-7 gene products; 18 – 20 – different strains of P. aeruginosa IMP-10 gene products

With the recent detection of IMP-7 and IMP-10 producing strains in several Eastern-European countries [1], the appearance of MBL-producing clinical isolates of *P. aeruginosa* can be anticipated in Romania. Our results suggest the lack of these IMP-7 and IMP-10 genes in the tested isolates. Further studies are necessary to conclude that this genes family is not present in the *P. aeruginosa* isolates circulating in this area of the country.

Extended-spectrum β-lactamases (ESBLs) that confer resistance to oxyimino-β-lactams are frequently plasmid encoded. *Pseudomonas* extended resistant (PER). β-lactamases are one of the rarer ESBL families; however, their prevalence may be increasing [2]. Since 1995, PER-producing organisms were disseminating in Italy [15, 16] and, more recently, in Belgium [17], France [18], Spain [19], Romania [20], Hungary and Serbia [21], Korea [22], Japan [5], and China [23].

The present study revealed that IMP-7 and IMP-10 genes are implicated in antimicrobial resistance mechanisms in MDR tested isolates of *P.aeruginosa*. Further studies are necessary to conclude if other genes contribute to the antimicrobial resistance in *P.aeruginosa* strains in Sudan.

This study is the first report of presence of IMP type genes in *P. aeruginosa* isolates, in Khartoum State, Sudan. A regular screening and monitoring system should be set up to prevent the spread of the resistance determinants in the country.

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