APPLICATION OF MOLECULAR TECHNIQUES TO THE STUDY OF *Pseudomonas aeruginosa* CLINICAL ISOLATE IN CLUJ-NAPOCA, ROMANIA

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Abstract. The goal 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*. Twenty seven *P. aeruginosa* isolates from the Clinical Hospital for Infectious Diseases in Cluj-Napoca, Romania, were analyzed. The bacteria were isolated over two years period, in 2008 and 2009 and serotyped at the Clinical Hospital of Infectious Disease, Cluj-Napoca. The isolates were recovered from patients with multiple types of infections, mostly respiratory tract, urinary tract and postoperative wound infections. In order to identify the genes implicated in antimicrobial resistance mechanisms, we used a direct PCR technique. DNA extraction was skipped and the bacterial cell wall denaturated in the first step of the reaction. We did not identify the presence of bla_{VIM} or bla_{IMP} family genes at any of the tested isolates of *P. aeruginosa*. We determined instead the PstS gene and from the ESBL group, we detected the presence of OXA type genes, namely OXA-50 and OXA-2. The presence of bla_{OXA-50} is important in order to identify and track the spread of multidrug-resistant *P. aeruginosa* clones since that bla_{OXA-50} may be another potential clonality marker. This study is the first report of presence of PstS and bla_{OXA} type genes in *P. aeruginosa* isolates, in Romania.

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 [25]. *P. aeruginosa* is one of the most important nosocomial pathogens, being responsible for various types of infections with more and more limited therapeutic options [4]. 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 imposes a serious therapeutic problem [5]. 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 [30]. 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 multidrug-resistant (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 [29], and therefore the ability to discriminate between virulent versus non-virulent phenotypes among multi-drug resistant 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 [26]. 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 our 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.

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

MATERIALS AND METHODS

Clinical isolates. We examined 27 clinical isolates of *P. aeruginosa* from the Clinical Hospital for Infectious Diseases in Cluj-Napoca, Romania, a medical center treating patients from all over the western part of Romania. The bacterial isolates were collected between September 2008 and October 2009 from patients with multiple types of infections, mostly respiratory tract, urinary tract, postoperative wound infections and sporadic infections. The isolates were identified with Vitek 2 Compact for Gram-Negative Identification, card 2GN (bioMerieux-Vitek, Inc., Hazelwood, Mo.).

Antimicrobial susceptibility testing. Antimicrobial susceptibility tests were performed by Kirby-Bauer or disk diffusion method and minimum inhibitory concentration (MIC) method with Vitek 2 Compact system for Gram-Negative bacteria, card AST-NO22, according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI).

The MICs of potent antibiotics for *P. aeruginosa*, including, quinolones (ciprofloxacin, pefloxacin), aminoglycosides (gentamicin, amikacin, tobramycin), betalactams: carboxypenicillin (ticarcillin), ureidopenicillin (piperacillin), carboxipenicillin and ureidopenicillin with betalactamases inhibitors (piperacillin/tazobactam, ticarcillin/clavulanic acid), third-generation cephalosporin (ceftazidime), fourth generation cephalosporin (cefepime, cefpirome), monobactams (aztreonam) and polymyxins B (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. We used a direct PCR technique, DNA extraction was skipped and the bacterial cell wall denaturated 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. 3 µl of this suspension was used as the template for amplification by PCR. Using this technique we can skip the expensive DNA extraction and the self contamination of workers is minimized [7]

PCR protocol

A typical 25-µl PCR mixture contained 2.5 µl 10xPCR reaction buffer, 25 pmol of each primer, 200 µM concentrations of each dNTP, 2 µl $MgCl_2$ 25 mM (2 mM final concentration) 0.75 U of *Taq* polymerase, 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 55-60°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 1.5 % agarose gel, stained with ethidium bromide. The optimisation of PCR was made after McPherson and Møller, (2001) [12] and Roux, (2003) [24]. The primers (Table 1) were designed with "Pick Primers" programe (http://biotools.umassmed.edu accessed in September 2010) according to the sequences found at NCBI data base . A primer pair targeting the consensus region of the bacterial 16S rRNA gene was used as a PCR internal-control target

Table 1 Primers used for PCR amplification

Target gene	Function	Sequence (5'→3')	Amplicon size (pb)	Accesion no.
IMP-7	β-lactamase	AAGGCAGTATCTCCTCTCATTTTC/ ACTCTATGTTCAGGTAGCCAAACC	243	EF606914
IMP-10	β-lactamase	AATGCTGAGGCTTACCTAATTGAC/ CCAAGCTTCTATATTTGCGTCAC	388	DQ288156
IMP-13	β-lactamase	AGACGCCTATCTAATTGACACTCC/ CCACTAGGTTATCTTGAGTGTGACC	311	AM931299
IMP-25	β-lactamase	GCAGTATTTCCTCACATTTCCATAG/ TCACCCAAATTACCTAGACCGTAG	295	EU352796
VIM-7	β-lactamase	TATCCGACAGTAGATGACATACCG/ CTAGAGGAGAGCGCTTTTAGAGAG	386	NC009739
PstS	multidrug resistance	GGCTTTCGAGCAGAAGTACG/ ATGTAGCCGTCCTTGACCAC	606	EF601159
bla_{OXA-50}	ESBL	GAAAGGCACCTTCGTCCTCTAC/ CAGAAAGTGGGTCTGTTCCATC	400	AM117128
bla_{OXA-2}	ESBL	ATACACTTTTTGCACTTGATGCAG/ TGAAAAGATCATCCATTCTGTTTG	510	AJ620678
ARNr 16S	consensus region	AGAGTTTGATCCTGGCTCAG/ ACGGCTACCTTGTTACGACTT	1499	HM045838

RESULTS

Phenotypic traits. All tested strains were sensitive to colistin, three strain were susceptible only to imipenem and meropenem, one was susceptible only to piperacillin-tazobactam and seven of them were sensitive to aztreonam and resistant to all others antibiotics. Two isolates were susceptible to all the antibiotic that we tested.

PCR failed to detect VIM-7 and IMP family tests whereas controls with bla_{IMP} and bla_{VIM} genes yielded PCR products of the expected sizes. Amplicons of the expected sizes were obtained for PstS, $bla_{OXA\ 50}$ and bla_{OXA-2} genes at the isolates that were resistant to antimicrobials (Fig. 1, 2 and 3). At the sensitive isolates amplicons were not obtained.

The results of the amplification using the primer pair targeting the consensus region of the bacterial 16S rRNA gene are presented in fig. 4.

DISCUSSION

P. aeruginosa is one of the most important nosocomial pathogens, being responsible for various types of infections with more and more limited therapeutic options. One of the most alarming characteristic of *P. aeruginosa* is its low antibiotic susceptibility. This situation is due in large part to the rapid accumulation of antimicrobial resistance mechanisms. The four main mechanisms by witch microorganisms exhibit resistance to antimicrobials are: drug inactivation through the production of β-lactamases, alteration of target site (e.g. alteration of PBP-the target site of penicillins), alteration of metabolic pathway and reduced drug accumulation by decreasing drug permeability and/or increasing active efflux (pumping out) of the drug across the cell surface.

Development of multidrug resistance by *P. aeruginosa* isolates requires several different genetic

events including acquisition of different mutations and/or horizontal transfer of antibiotic resistance genes.

In this study we used PCR method in order to identify the genes implicated in antimicrobial resistance mechanisms (MDR included) in *P. aeruginosa* isolates collected in an important medical center from the western part of our country.

Since the first report of acquired metallo-β-lactamase (MBL) in Japan in 1994 [19], genes encoding IMP- and VIM type enzymes have spread rapidly among *Pseudomonas* spp. [13, 24, 29]. Acquired metallo-β-lactamases (MBLs) are mostly encoded by integron-borne genes and confer resistance against all β-lactams except for the monobactams. VIM-type MBLs were reported from several European countries and also from countries outside Europe such as Korea and the United States [10, 18, 21, 27]. The prevalence of MβL-producing gram-negative bacilli has increased in some hospitals, particularly among clinical isolates of *P. aeruginosa* [23]. Since MBL production may confer phenotypic resistance to virtually all clinically available β-lactams, the continued spread of MBL is a major clinical concern [28].

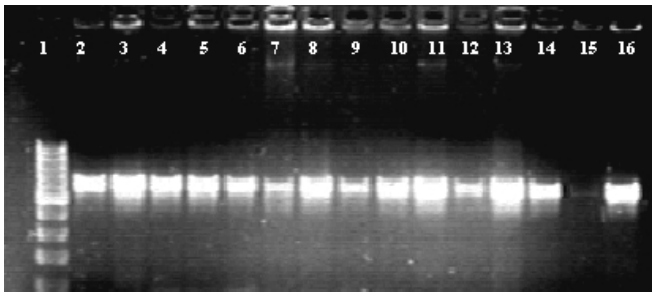

Figure 1. Agarose gel electrophoresis of PCR products after amplification of *PstS* gene. Lanes: 1 – molecular weight marker (O'Range Ruler 100 DNA Ladder, SM1143-Fermentas); 2-16– different strains of *P. aeruginosa* (*PstS* gene products).

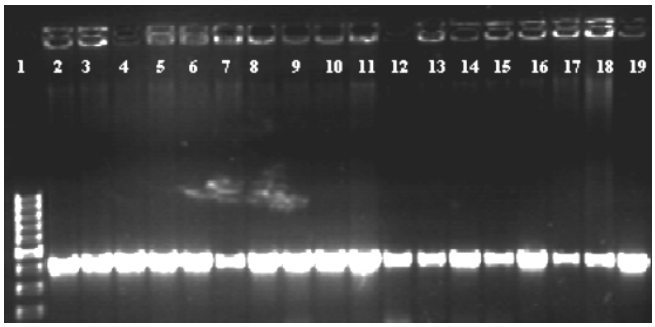

Figure 2. Agarose gel electrophoresis of PCR products after amplification of bla_{OXA-50} gene. Lanes: 1–molecular weight marker (O'Range Ruler 100 DNA Ladder, SM1143-Fermentas); 2-19–different strains of *P. aeruginosa* (bla_{OXA-50} gene products).

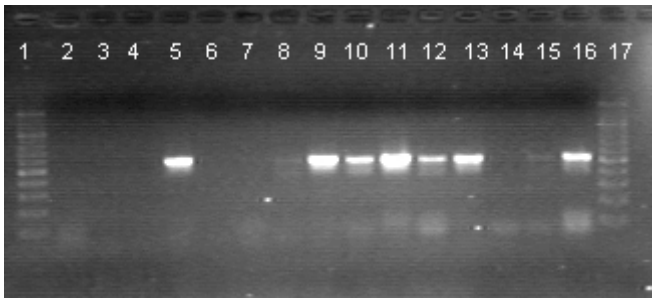

Figure 3. Agarose gel electrophoresis of PCR products after amplification of bla_{OXA-2} gene. Lanes: 1 and 17-molecular weight marker (O'Range Ruler 100 DNA Ladder, SM1143-Fermentas); 2-16–different strains of *P. aeruginosa* (bla_{OXA-2} gene products).

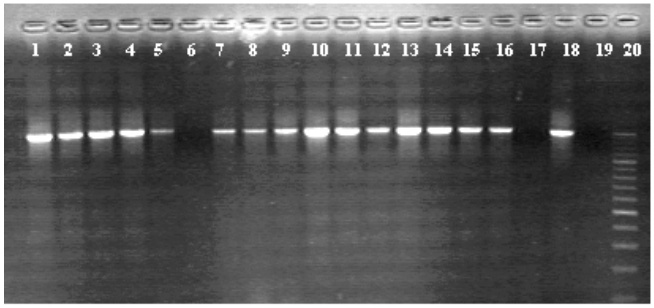

Figure 4. Agarose gel electrophoresis of PCR products after amplification of the consensus region of the bacterial 16S rRNA. Lanes: 20-molecular weight marker (O'Range Ruler 100 DNA Ladder, SM1143-Fermentas); 1-5, 7-16 and 18–different strains of *P. aeruginosa*; 6, 17, 19-negative control (no template).

With the recent detection of IMP and VIM-producing strains in several Eastern-European countries [21, 25], the appearance of MBL-producing clinical isolates of *P. aeruginosa* can be anticipated in Romania. Our results suggest the lack of these bla_{VIM} and bla_{IMP} genes at 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. PER (*Pseudomonas e*xtended *r*esistant) β-lactamases are one of the rarer ESBL families; however, their prevalence may be increasing [4]. Since 1995, PER producing organisms have been disseminating in Italy [20, 22] and, more recently, in Belgium [16], France [3], Spain [14], Romania [17], Hungary and Serbia [11], Korea [8], Japan [29], and China [6].

From the ESBL group, we detected only the presence of OXA type β-lactamases, namely OXA-50 and OXA-2 at the tested isolates. These genes were found mainly in *P. aeruginosa* isolates from Turkey [1, 9] and France [3]. This aspect is important in order to identify and track the spread of multidrug-resistant *P. aeruginosa* clones since that bla_{OXA-50} may be another potential clonality marker for *P. aeruginosa* [4].

PstS is another gene that we identified in almost all tested isolates. PstS proteins are the cell-bound phosphate-binding elements of the ubiquitous bacterial ABC phosphate uptake mechanisms. Primary and tertiary structures, characteristic of pstS proteins, are conserved in proteins, which are expressed in secretory operons and induced by phosphate deprivation, in *Pseudomonas species* [2, 15]. The presence of this periplasmic phosphate binding protein (PstS) confers a highly virulent phenotype of MDR isolates of *P. aeruginosa* [31].

Our study revealed that PstS, OXA-50 and OXA-2 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 our country.

This study is the first report of presence of PstS and blaOXA type genes in *P. aeruginosa* isolates, in Romania. 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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