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MOLECULAR CHARACTERIZATION OF
METALLO- β -LACTAMASE PRODUCING
PSEUDOMONAS AERUGINOSA STRAINS

MOLEKULARNA KARAKTERIZACIJA
SOJEVA *PSEUDOMONAS AERUGINOSA* KOJI
PRODUKUJU METALO- β -LAKTAMAZE

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Ključne reči

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Abstract

Metallo- β -lactamases (MBLs) are emerging resistance determinants detected in multidrug-resistant nosocomial isolates of *Pseudomonas aeruginosa* and other Gram-negative pathogens. In MBL-producing *P. aeruginosa* isolates from Military Medical Academy (MMA) patients in the period of January 2008 to December 2009, the *bla*_{VIM} genes were found in 16 isolates and they were all carried by class 1 integron. PCR with primers specific for *bla*_{VIM-1} was positive in 4 isolates, and with primers specific for *bla*_{VIM-2} in 12 isolates. Differences in the position of the MBL genes on integron indicated multiple introduction of MBL-producing *P. aeruginosa* strains in MMA.

INTRODUCTION

Pseudomonas aeruginosa is a leading cause of nosocomial infections worldwide [1]. These infections are often difficult to treat because of the natural resistance of the species due to low permeability of outer membrane, constitutive expression of efflux pumps and naturally occurring chromosomal AmpC β -lactamase [2]. Besides that, *P. aeruginosa* easily acquires additional resistance to multiple groups of antimicrobial agents. Different mechanisms of resistance often exist simultaneously, thus conferring combined resistance [3].

Carbapenem antibiotics have potent antibacterial activity and are among the drugs of choice for the treatment of infections caused by multiresistant nosocomial pathogens, including *P. aeruginosa*. However, the

development of carbapenem resistance severely compromises effective therapeutic options. The mechanism leading to carbapenem resistance in *P. aeruginosa* is usually multifactorial, and can comprise alteration of outer membrane permeability, activation of efflux systems or acquisition of carbapenem-hydrolysing β -lactamases via horizontal gene transfer. The production of acquired metallo- β -lactamases (MBL) is the major mechanism of resistance to carbapenems both in terms of its effectiveness and epidemiological significance. The first reported MBL was IMP, and was found in Japan [4]. In contrast, the VIM type MBLs were first reported in Europe [5]. These two main types of MBL, together with some new variants of enzyme, can now be found in various Gram-negative bacteria worldwide. In Serbia, the first MBL-producing *P. aerugi-*

nosa was isolated in the Military Medical Academy in Belgrade [6]. Sequencing of the gene revealed that enzyme was identical with VIM-2.

Most of metallo- β -lactamase genes are carried by integrons, genetic structures capable of capturing gene cassettes. Integrons possess two conserved segments, 5'-CS and 3'-CS, located on either side of integron, and variable region between them [7]. Variable region consists of gene cassettes comprising an antibiotic resistance genes. Downstream of inserted genes are located recombination sites, known as 59-base elements, that are recognized by the integrase [7]. Genes encoded integrases are located at the 5' conserved segment. Integrons can be located on transposons and plasmids which can serve as vehicles for the transmission of antibiotic-resistance genes.

The aim of this study was to determine types of MBLs produced by *P. aeruginosa* strains isolated from patients in Military Medical Academy, and to perform molecular characterization of genome region encoding MBLs.

MATERIALS AND METHODS

Bacterial strains. Imipenem non-susceptible *P. aeruginosa* isolates were cultured from clinical specimens from patients hospitalized on various departments of Military Medical Academy in Belgrade, in the period of January 2008 to December 2009. Control strains were PA396, VIM-1 producing *P. aeruginosa* characterized earlier [8], and 722, VIM-2 producing *P. aeruginosa* also characterized earlier [6].

Antibiotic susceptibility. Resistance to carbapenems was detected by disk diffusion test on Mueller-Hinton agar (Bio-Rad Laboratories, Marnes-la-Coquette, France), using breakpoints recommended by the Clinical and Laboratory Standards Institute [9]. To screen for MBL production, the imipenem-EDTA, ceftazidime-EDTA, and cefepime-EDTA combined disk tests were used [10].

PCR amplification. DNA of *P. aeruginosa* was extracted with QIAamp DNA Mini Kit (Quiagen, GmbH, D-40724 Hilden) and used as a template in PCR experiments. The presence of genes encoding MBLs and integrase was detected by PCR. Specific primers for *bla*_{VIM-1}, *bla*_{VIM-2}, *bla*_{IMP}, *bla*_{SPM}, *int1*, and *int3* genes were used for amplification and their sequences were previously described [11, 12]. To identify the location of the MBL gene on integron, PCR reaction was performed with reverse primer for integrase gene and reverse primer for *bla*_{VIM-1} or *bla*_{VIM-2}. The composition of the reaction mixture and conditions for all PCR amplifications were the same, and were reported by Senda et al. [13].

RESULTS

Detection of metallo- β -lactamase and integrase genes. In the period from January 2008 to December 2009 altogether 214 imipenem non-susceptible clinical isolates were screened by phenotypic test for MBL production. For 22 of them, the imipenem-EDTA, ceftazidime-EDTA, and cefepime-EDTA combined disk tests all demonstrated a ≥ 7 mm increase of the inhibitory zones around the antibiotic disk in the presence of EDTA. These isolates were further analysed for the presence of genes encoding MBL. PCR experiments demonstrated the presence of *bla*_{VIM} genes in 16/214 isolates (7.5%), which were all class 1 integron-borne. In 4 isolates we found *bla*_{VIM-1} gene, and *bla*_{VIM-2} gene in 12. Isolates that proved negative for the presence of *bla*_{VIM-1} or *bla*_{VIM-2} genes were examined with primers specific for the genes coding other types of metallo- β -lactamase. Genes *bla*_{IMP} or *bla*_{SPM} were not detected in any one of these isolates.

The position of the metallo- β -lactamase genes on the integron. In all 16 isolates positive in PCR for *bla*_{VIM-1} or *bla*_{VIM-2} genes, the position of these genes on the integron class 1 was established. PCR with a reverse primer for integron class 1, and a reverse primer for *bla*_{VIM-1} revealed that in two of four isolates that possessed *bla*_{VIM-1} gene, this cassette was located

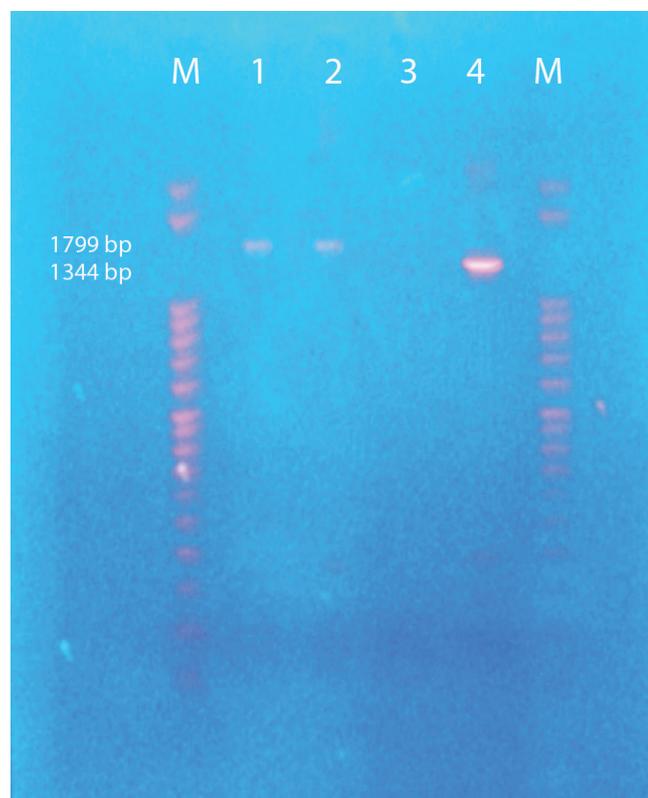


Fig. 1. Determination of the position of *bla*_{VIM} genes on the integron. Lanes: M, molecular weight marker, 50 bp DNA ladder; 1, control strain 722, and 2, strain 8885, amplified with reverse primers for integron class 1 and *bla*_{VIM-2}; 3, control strain 396, and 4, strain 727/I, amplified with reverse primers for integron class 1 and *bla*_{VIM-1}.

on the first position of the integron. In isolates with VIM-2 type of MBL, PCR with a reverse primer for integron class 1, and a reverse primer for *bla*_{VIM-2} revealed that this cassette was located on the first position of the integron in 8 out of 12 isolates. (Fig.1)

DISCUSSION

The frequency of isolation of MBL-producing *P. aeruginosa* strains from clinical isolates is increasing worldwide over the past years. After the first isolation in Italy [5], *bla*_{VIM}-type genes have been spreading among *P. aeruginosa* and other gram-negative pathogens, being the most prevalent in Europe [14, 15, 16]. Strains producing MBLs have been responsible for serious infections and prolonged nosocomial outbreaks, and are considered as one of the major emerging challenges to antimicrobial chemotherapy [17]. Patients infected with MBL-producing *P. aeruginosa* were more likely to receive multiple antibiotics and carbapenems [18]. But Cornaglia G et al [19] have analysed the antimicrobial chemotherapy received by the patients before isolation of the MBL-positive *P. aeruginosa* and revealed that only (15%) of the patients had received therapy with carbapenems. Authors suggested that MBL-producing *P. aeruginosa* can spread as hospital infections without the use of antibiotics.

In one study in Hungary, 758 carbapenem-resistant *P. aeruginosa* isolates were screened for MBL production [14]. PCR with VIM- and integron-specific primers gave positive results for 50 isolates (6.6%). MBL genes were all identified as *bla*_{VIM-4}, a variant of

*bla*_{VIM-1} type, and integron was identified as class 1. Differences regarding position of the *bla*_{VIM-4} gene in integron existed. This study, and studies in hospitals in other countries, indicated dissemination of *bla*_{VIM} through clonal spread, but other mechanisms, such as horizontal transfer, were also involved [14, 20, 21].

VIM-1-like and VIM-2-like are two main clusters of MBLs with amino acid identity of 90% [22]. Other subtypes are determined by sequencing of *bla*_{VIM} genes. The 59-base elements of *bla*_{VIM-1} and *bla*_{VIM-2} gene cassettes differed in size and structure suggesting a separate origin in each cassette [22]. The first isolate of metallo-β-lactamase-producing *P. aeruginosa* in Serbia produced VIM-2 type MBL and harboured a novel class 1 integron with a *bla*_{VIM-2}-like cassette in the first position [6]. In this study, both VIM-1 and VIM-2 metallo-β-lactamase-producing *P. aeruginosa* isolates were detected, and position of *bla*_{VIM} genes in class 1 integron was different, indicating multiple introduction of metallo-β-lactamase-producing *P. aeruginosa* strains in MMA. After the introduction, *bla*_{VIM} genes were further disseminated through clonal spread and horizontal transfer.

The occurrence of MBL-producing bacterial pathogens in a hospital setting possess a therapeutic problem. Once introduced, MBL resistance genes disseminate rapidly and may lead to treatment failure and increased morbidity and mortality. Therefore, the early detection of MBL-producing *P. aeruginosa* and the use of infection control measures are of primary importance in avoiding the spread of these multidrug-resistant isolates.

Apstrakt

Metalo-β-laktamaze (MBL) su determinante rezistencije koje se posljednjih godina sve češće detektuju kod multirezistentnih izolata *Pseudomonas aeruginosa* i drugih Gram-negativnih patogena. Molekularnom karakterizacijom *P. aeruginosa* koji proizvode MBL, izolovanih iz pacijenata sa Vojnomedicinske akademije (VMA) u periodu od januara 2008. do decembra 2009. godine, kod 16 izolata su nađeni *bla*_{VIM} geni, koji su se svi nalazili na integronu klase 1. PCR sa prajmerima specifičnim za *bla*_{VIM-1} gen bio je pozitivan kod 4 izolata, a sa prajmerima specifičnim za *bla*_{VIM-2} gen kod 12 izolata. Razlike u položaju ovih gena na integronu ukazuju na višestruko unošenje sojeva *P. aeruginosa* koji proizvode MBL u VMA.

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