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EMERGENCE OF *bla*$_{VEB}$ AND *bla*$_{GES}$ AMONG VIM-PRODUCING *PSEUDOMONAS AERUGINOSA* CLINICAL ISOLATES IN ALEXANDRIA, EGYPT

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Thirty-three *Pseudomonas aeruginosa* isolates, resistant to one or more β-lactams, were included in this study. Identification of tested strains was confirmed using MALDI-TOF/MS. Phenotypic and genotypic β-lactamase patterns were investigated. Most of the isolates were resistant to carbapenems (32 out of 33) and to the extended-spectrum cephalosporins (ESC) (30 out of 33). Phenotypically, the production of extended-spectrum beta-lactamase (ESBL), metallo-β-lactamases (MBL), and carbapenemases was detected in 10, 23, and 9 isolates, respectively. However, AmpC hyperproduction was not phenotypically detected among all isolates. Genotypically, ESBL and MBL encoding genes were detected in 23 and 27 isolates, respectively. Altogether 27 strains were detected as *bla*$_{VIM}$ positive and 16 strains carried *bla*$_{OXA-10}$ gene. To the best of our knowledge, this is the first report of *P. aeruginosa* clinical isolates harboring *bla*$_{VEB}$ together with *bla*$_{GES}$ in Egypt, where 5 of our 30 ESC-resistant isolates showed this genotype. Our results confirmed that resistance of *P. aeruginosa* isolates to β-lactam antibiotics is mediated via multiple β-lactamases belonging to different molecular classes. To the best of our knowledge, this is the first report of *bla*$_{VEB}$ among *P. aeruginosa* clinical isolates from Egypt. Ten isolates harbored *bla*$_{VEB}$ and five of them co-harbored *bla*$_{VEB}$ together with *bla*$_{GES}$, *bla*$_{VIM}$, and *bla*$_{OXA-10.}$

Keywords: β-lactamases, carbapenemases, ESBL, *Pseudomonas aeruginosa*, MALDI-TOF/MS

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Introduction

Pseudomonas aeruginosa is a non-fermentative, Gram-negative bacteria that is widely disseminated in nature. It has a remarkable ability to survive on various surfaces in both community and hospital settings. Thus, *P. aeruginosa* has a significant role in nosocomial infections being responsible for a wide variety of infections including wound and burn infections, respiratory tract infections, urinary tract infections, and blood stream infections [1].

P. aeruginosa demonstrates intrinsic resistance to several antimicrobial agents due to the reduced permeability of its outer membrane. This natural resistance evolves against penicillin G, aminopenicillin, and cephalosporins of the first and second generations. Therefore, treatment of infections caused by *P. aeruginosa* is usually limited to only few antimicrobial agents. These agents include extended-spectrum penicillins, such as ticarcillin and piperacillin (PRL), some third-generation cephalosporins such as ceftazidime (CAZ), and all the fourth-generation cephalosporins, carbapenems, and monobactams. Unfortunately, there is a rising resistance against these agents causing a critical challenge to choose the effective antimicrobial therapy for optimized clinical outcome [2, 3].

Among the different mechanisms that mediate β-lactam resistance, antibiotic cleavage by β-lactamase enzymes is considered to be of major importance. There are two classification schemes for β-lactamases that are generally adopted. The first one depends on similarity in the amino acid sequence, whereas the other depends on the functionality [4, 5]. According to the amino acid sequence similarity, Ambler [6, 7] divided β-lactamases into four classes, namely A, B, C, and D. All classes possess serine in their active site except for class B, which requires zinc ion to be active. Therefore, class B is commonly known as metallo-β-lactamases (MBL). The scheme that depends on functionality separates β-lactamases into three groups, namely 1, 2, and 3. Group 1 consists of cephalosporinases, which correspond to class C in Ambler classification. Group 2 consists of serine β-lactamases that include classes A and D of the Ambler classification. Group 3 consists of MBL and they correspond to class B in Ambler classification. Then, each of these three groups is divided into various subgroups [5]. To the best of our knowledge, this is the first study in Egypt to tackle all classes of β-lactamases among *P. aeruginosa* and to report the co-existence of VEB and GES β-lactamases along with other β-lactamases. Here, we determined the phenotypic resistance patterns to β-lactam antibiotics and identified the genetic determinants responsible for β-lactamases-induced resistance among *P. aeruginosa* clinical isolates resistant to one or more of the β-lactams.

Material and Methods

All culture media and antibiotic disks used in this study were purchased from Oxoid (Cambridge, UK).

Collection of clinical isolates

A total of 33 *P. aeruginosa* isolates resistant to one or more β-lactams were collected during the period from September 2014 to June 2015. They were obtained from different clinical samples submitted to the Microbiology Department, Medical Research Institute, Alexandria University. The identification of *P. aeruginosa* isolates was confirmed using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF/MS; Bruker, Billerica, MA, USA).

Antimicrobial susceptibility

Kirby–Bauer method [8] was used for the antimicrobial susceptibility testing of *P. aeruginosa* strains on Mueller–Hinton agar plates. β-lactam antibiotics were chosen according to the CLSI recommendations [9]. The disks used were CAZ, cefepime (CFP), PRL, PRL/tazobactam (TZP), aztreonam (ATM), imipenem (IPM), and meropenem (MEM). The sizes of the inhibition zones were interpreted according to CLSI M100-S27 and the organisms were reported as sensitive, intermediate, or resistant to the agents that have been tested.

β-lactamases phenotypic characterization

Strains that were found to be resistant to CAZ and/or CFP were further screened for the presence of extended-spectrum beta-lactamase (ESBL) and AmpC β-lactamases hyperproduction, whereas those resistant to IPM and/or MEM were further screened for the presence of carbapenemases and MBL.

Screening for ESBL production

P. aeruginosa isolates resistant to CAZ and/or CFP were investigated for ESBL production using combined disk method [10]. Disks containing CAZ (30 μg) alone and in combination with clavulanic acid (30/10 μg) were used. They were placed far apart on a Mueller–Hinton agar plate containing 400 μg of

3-aminophenyl boronic acid (APB; Arcos Organics, New Jersey, USA) and inoculated with 0.5 McFarland suspension of the tested strain. After overnight incubation at 35 °C, ESBL-producing strains demonstrated at least 5-mm increase in the inhibition zone in presence of clavulanic acid [10].

Screening of AmpC hyperproduction

P. aeruginosa isolates resistant to CAZ and/or CFP were investigated for AmpC hyperproduction using combined disk method, as described by Coudron et al. [11]. Briefly, a disk containing 30 μg of CAZ and another one containing 30 μg of CAZ in combination with 600 μg APB were placed far apart on the surface of Mueller–Hinton agar plates inoculated with 0.5 McFarland suspension of the tested strain and then incubated overnight at 35 °C. Strains that showed at least 5-mm increase in the inhibition zone in the presence of APB were considered to be AmpC hyperproducers [11].

Screening for carbapenemase production

P. aeruginosa strains resistant to one or more of the carbapenems were screened for carbapenemase production by modified Hodge test (MHT) using carbapenem-susceptible *Klebsiella pneumoniae* as the indicator organism [12]. Mueller–Hinton agar plate was inoculated with 0.5 McFarland suspension of the indicator strain. Then, two disks containing IPM (10 μg) and MEM (10 μg) were placed on the agar plate away from each other. Heavy inoculum of the test strain was streaked onto the Mueller–Hinton agar plate in a straight line from the edge of one disk to the plate periphery. Carbapenemase production induces a cloverleaf-shaped indentation of growth of the indicator strain after overnight incubation [12].

Screening for MBL production

P. aeruginosa strains resistant to one or more of the carbapenems were screened for MBL production using combined disk method, as described by Yong et al. [13]. One disk containing IPM (10 μg) and one containing IPM and EDTA (10/930 μg) were placed on a Mueller–Hinton agar plate inoculated with 0.5 McFarland suspension of the tested strain and incubated overnight at 35 °C. MBL-producing strains showed 7 mm or greater increase between the inhibition zone around IPM disk alone and that around IPM–EDTA disk [13].

Detection of β-lactamase encoding genes

Genotypic detection of different β-lactamase genes belonging to ESBL, MBL, and OXA classes was performed using polymerase chain reaction (PCR). All primers used in this study are listed in Supplementary Tables I–III. The primers were purchased from Biosearch Technologies (Novato, CA, USA). The PCR Master mix MyTaq HS Red Mix was supplied by BioLine (London, UK). PCR amplification of the extracted DNA was carried out on Veriti Thermal Cycler (Applied Biosystems, CA, USA).

Bacterial DNA was extracted by boiling method; shortly 3–4 colonies were suspended in sterile Tris-EDTA buffer to make a heavy suspension. The suspension was incubated in a boiling water bath for 15 min followed by rapid cooling on ice and centrifugation. The supernatant was used as a DNA template. PCR was performed in a total volume of 25 µl including 12.5 µl 2X MyTaq HS Red Mix, 10 picomoles of each primer, and 0.5 µl DNA extract. A negative control was prepared by the addition of the same contents to the tube without DNA extract.

Results

Identification and antimicrobial susceptibility

The identification of 33 isolates included in this study as *P. aeruginosa* was confirmed using MALDI-TOF/MS. Twelve (36.4%) out of them were obtained from wound swabs, 11 (33.3%) from urine samples, 7 (21.2%) from respiratory tract infections, and 3 (9.1%) from pus syringes.

Antibiotic susceptibility testing using Kirby–Bauer method showed that 32 out of 33 *P. aeruginosa* isolates were resistant to IPM and/or MEM, with only one strain sensitive to both. Thirty out of 33 *P. aeruginosa* isolates were resistant to CAZ and/or CFP. The detailed results for the disk diffusion test are shown in Table I.

Detection of ESBL

Phenotypic detection of ESBL was carried out using combined disk method; only 10 (33.3%) *P. aeruginosa* isolates showed ESBL production.

Detection of ESBL genes

The results of the detection of bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$, $bla_{\text{PSE-1}}$, bla_{GES}, bla_{PER}, and bla_{VEB} genes among 30 *P. aeruginosa* strains, which were resistant to CAZ and/or CFP, are shown in Table II.

Table I. Resistance of the 33 *P. aeruginosa* isolates to different β-lactam antibiotics

	Resistance		Intermediate		Sensitive	
Antibiotic	*N*	%	*N*	%	*N*	%
CAZ	29	87.88	1	3.03	3	9.09
CFP	30	90.91	2	6.06	1	3.03
PRL	14	42.42	17	51.52	2	6.06
TZP	14	42.42	15	45.46	4	12.12
ATM	7	21.21	22	66.67	4	12.12
IPM	31	93.94	1	3.03	1	3.03
MEM	31	93.94	0	0	2	6.06

Note: CAZ: ceftazidime; CFP: cefepime; PRL: piperacillin; TZP: piperacillin/tazobactam; ATM: aztreonam; IPM: imipenem; MEM: meropenem.

Table II. ESBL gene detection among 30 *P. aeruginosa* isolates

ESBL genes	bla_{TEM}	bla_{SHV}	$bla_{CTX\text{-}M}$	bla_{PER}	bla_{VEB}	$bla_{PSE\text{-}1}$	bla_{GES}
Positive	9 (30%)	1 (3.3%)	4 (13.3%)	3 (10%)	10 (33.3%)	2 (6.6%)	15 (50%)
Negative	21 (70%)	29 (96.7%)	26 (86.7%)	27 (90%)	20 (66.7%)	28 (93.3%)	15 (50%)

Note: ESBL: extended-spectrum beta-lactamase.

Detection of AmpC hyperproduction

Phenotypic detection of AmpC hyperproduction was carried out on 30 *P. aeruginosa* strains, which were resistant to CAZ and/or CFP, using APB combined disk method. However, none of our 30 strains was an AmpC hyperproducer.

Detection of carbapenemases

Thirty-two *P. aeruginosa* strains resistant to IPM and/or MEM were phenotypically tested for the presence of carbapenemases using MHT. Only 9 out of 32 strains were positive.

Detection of MBL

Thirty-two *P. aeruginosa* strains resistant to IPM and/or MEM were phenotypically tested for the presence of MBL using combined disk method. Twenty-three out of 32 strains were positive determined using IPM–EDTA combined disk method.

Detection of MBL genes

Thirty-two carbapenem-resistant *P. aeruginosa* were investigated for the presence of the following genes: bla_{VIM}, bla_{IMP}, bla_{SPM-1}, bla_{NDM-1}, and bla_{GIM}. bla_{VIM} was the only detected gene in 27 (84.4%) isolates (Table III).

Detection of OXA genes

Thirty-three *P. aeruginosa* strains were screened for the presence of bla_{OXA-1}, bla_{OXA-2}, and bla_{OXA-10} genes. Only bla_{OXA-10} gene was detected among 16 (48.5%) strains, as shown in Table IV.

Distribution of β-lactamases genes among our clinical isolates

In this study, 11 (33.3%) out of 33 *P. aeruginosa* strains harbored 3–5 genes belonging to 3 β-lactamase classes: ESBL (class A), MBL (class B), and OXA β-lactamases (class D). Whereas, 8 (24.2%) harbored 2–3 genes belonging to 2 β-lactamase classes A and B. Five (15.2%) isolates harbored one gene belonging to the MBL class, as shown in Table V. Interestingly, two isolates did not harbor any of the tested β-lactamase encoding genes; one of them was resistant to carbapenems, whereas the other was resistant to all tested β-lactams.

Table III. Detection of MBL genes among 32 carbapenem-resistant *P. aeruginosa*

MBL genes	bla_{IMP}	bla_{VIM}	bla_{NDM-1}	bla_{SPM-1}	bla_{GIM-1}
Positive	0 (0%)	27 (84.4%)	0 (0%)	0 (0%)	0 (0%)
Negative	32 (100%)	5 (15.6%)	32 (100%)	32 (100%)	32 (100%)

Note: MBL: metallo-β-lactamase.

Table IV. Detection of OXA genes among 33 *P. aeruginosa* isolates

OXA genes	bla_{OXA-1}	bla_{OXA-2}	bla_{OXA-10}
Positive	0 (0%)	0 (0%)	16 (48.5%)
Negative	33 (100%)	33 (100%)	17 (51.5%)

Table V. Distribution of β-lactamase genes and classes among 33 *P. aeruginosa* strains

No. of isolates	β-lactamase genes	Number of genes	β-lactamase molecular class
3	bla_{VEB}, bla_{TEM}, bla_{GES}, bla_{VIM}, and bla_{OXA-10}	5	A, B, and D
1	bla_{PER}, bla_{VEB}, bla_{PSE-1}, bla_{VIM}, and bla_{OXA-10}	5	A, B, and D
1	bla_{VEB}, bla_{PSE-1}, bla_{GES}, bla_{VIM}, and bla_{OXA-10}	5	A, B, and D
1	bla_{PER}, bla_{VEB}, bla_{CTX-M}, bla_{VIM}, and bla_{OXA-10}	5	A, B, and D
1	bla_{TEM}, bla_{VEB}, bla_{CTX-M}, bla_{VIM}, and bla_{OXA-10}	5	A, B, and D
1	bla_{TEM}, bla_{GES}, bla_{VIM}, and bla_{OXA-10}	4	A, B, and D
1	bla_{VEB}, bla_{GES}, bla_{VIM}, and bla_{OXA-10}	4	A, B, and D
1	bla_{VEB}, bla_{CTX-M}, bla_{VIM}, and bla_{OXA-10}	4	A, B, and D
1	bla_{VEB}, bla_{VIM}, and bla_{OXA-10}	3	A, B, and D
1	bla_{PER}, bla_{TEM}, and bla_{VIM}	3	A and B
1	bla_{TEM}, bla_{GES}, and bla_{VIM}	3	A and B
1	bla_{SHV} and bla_{VIM}	2	A and B
1	bla_{TEM} and bla_{VIM}	2	A and B
2	bla_{GES} and bla_{OXA-10}	2	A and D
4	bla_{GES} and bla_{VIM}	2	A and B
2	bla_{VIM} and bla_{OXA-10}	2	B and D
2	bla_{TEM} and bla_{CTX-M}	2	A
1	bla_{GES}	1	A
5	bla_{VIM}	1	B
2	No genes	0	–

Discussion

β-lactams that are usually used for treatment of *P. aeruginosa* infections include extended-spectrum penicillins, monobactams extended-spectrum cephalosporins (ESC), and carbapenems [3].

In this study, most of the *P. aeruginosa* isolates were resistant to both carbapenems (93.94%) and ESC (90.91%); on the other hand, a much lower resistance was observed against ATM, PRL, and TZP (21.21%, 42.42%, and 42.42%, respectively).

Detection of ESBL genes was carried out for 30 *P. aeruginosa* isolates resistant to the ESC. bla_{GES} was detected in 15 (50%) of the isolates, followed by bla_{VEB} in 10 (33%) and bla_{TEM} in 9 (30%). To the best of our knowledge, this is the first study to report *P. aeruginosa* clinical isolates co-harboring bla_{GES} and bla_{VEB} in Egypt. Lower detection rate was observed for bla_{PER}, which was detected in 3 (10%), bla_{PSE-1} in 2 (6.6%), and bla_{SHV} in 1 (3.3%). Seven isolates had three ESBL genes and six isolates had two genes. bla_{GES} was detected alone in eight isolates, whereas no ESBL genes were detected in six isolates.

ESBL genes were detected in 24 (80%) out of 30 ESC-resistant *P. aeruginosa* isolates. However, according to the phenotypic method used in

this study, ESBL production was detected in 10 (33.3%) out of 30 P. aeruginosa strains resistant to the third- and fourth-generation cephalosporins. This confirms that the phenotypic detection of ESBL, which is based on the use of clavulanic acid as an inhibitor, is unreliable with a low sensitivity (37.5%).

Jiang et al. [14], Zafer et al. [10], and Poulou et al. [15] supported previous implication that the ongoing ESBL detection methods are uncertain in *P. aeruginosa*, as there is no standardized method for the detection of ESBLs in *P. aeruginosa*.

In this study, only bla_{VIM} of the MBL genes was detected among our isolates. It was detected in 27 (84.4%) out of 32 carbapenem-resistant *P. aeruginosa* isolates. On the other hand, the rest of the tested genes such as bla_{IMP}, $bla_{\text{SPM-1}}$, $bla_{\text{GIM-1}}$, and $bla_{\text{NDM-1}}$ genes were not detected among our isolates.

In this study, 23 (71.9%) out of 32 carbapenem-resistant *P. aeruginosa* isolates were phenotypically MBL-positive. Four (17.4%) out of 23 phenotypically MBL-positive isolates did not harbor any of the tested MBL genes. Genotypic screening of 32 *P. aeruginosa* isolates showed that 27 (84.4%) of them harbored bla_{VIM} gene of which 8 (29.6%) were phenotypically MBL-negative. On the other hand, four *P. aeruginosa* isolates showed enlarged zone in absence of genes encoding for MBL. False-positive and false-negative phenotypic detection of MBL has been observed with a sensitivity and specificity of 70.37% and 20.0%, respectively.

Similar findings were reported by Gerges and Amin [16] who found that eight out of their isolates, which were phenotypically MBL-negative, carried one or more MBL genes.

Aghamiri et al. [17] reported similar results as 11 isolates that were phenotypically MBL-negative carried MBL genes as detected by PCR.

Chu et al. [18] reported that methods using EDTA are highly sensitive but not specific when analyzing MBL production, as this method may lead to false-positive results. This was confirmed by Marra et al. [19] who reported a false-positive detection rate (69.6%) with EDTA.

Franco et al. [20] stated that EDTA has a bactericidal activity, which causes a synergistic effect with carbapenems resulting in increased inhibition zones. Chu et al. [18] reported that methods using EDTA are highly sensitive but not specific suggesting that caution must be taken when analyzing MBL production, as this method may lead to false-positive results. In addition, EDTA may affect the membrane permeability, which causes increased susceptibility to carbapenems and other antimicrobial agents. Thereby, it causes false reading of the MBL tests involving EDTA [21]. It is noteworthy that phenotypic tests for MBL in *P. aeruginosa* have not yet been standardized [20, 22].

CLSI recommended that *Enterobacteriaceae* with increased inhibitory concentrations (MICs) or decreased inhibition zones should be screened for carbapenemases production using MHT. It should be noted that *P. aeruginosa* is not included in that recommendation in spite of the rising carbapenem resistance among its isolates [9].

Pasteran et al. [12] reported that, with the MHT, a high proportion of indeterminate results were observed in 22% and 43% of carbapenemase producers and non-producers, respectively, with a sensitivity and specificity of 78% and 57%, respectively.

In *P. aeruginosa*, AmpC β-lactamases are an inducible chromosomally encoded enzyme. Normally, *P. aeruginosa* strains produce low levels of AmpC β-lactamases when an inducing β-lactam is lacking. In absence of any other resistance mechanism, these strains may be susceptible to broad spectrum penicillins, penicillin-inhibitor combinations, cephalosporins, and carbapenems. However, when *ampC* is derepressed, it results in high levels of *ampC* expression, thereby resulting in resistance to practically all β-lactams [1].

Screening for AmpC induction relies on the disk approximation (D-test) assay using cefoxitin/ceftazidime (FOX/CAZ) [23]. In this study, induction of AmpC using D-test assay was not possible since all of our 30 *P. aeruginosa* isolates were resistant to CAZ.

In this study, a combined disk test using CAZ/APB was used instead. Accordingly, none of our 30 ESC-resistant isolates was considered as AmpC hyperproducer, since the addition of APB did not result in the enlargement of the inhibitory zone diameter around the disk (CAZ/APB) by ≥5 mm.

Class D β-lactamases are also called OXA enzymes (oxacillinases) as they favorably hydrolyze oxacillin and cloxacillin rather than benzylpenicillin. These enzymes are generally narrow spectrum, and induce resistance to narrow spectrum cephalosporins, aminopenicillins, as well as carboxypenicillins [4].

In this study, $bla_{\text{OXA-10}}$ gene was detected among 16 (48.5%) of 33 *P. aeruginosa* strains. Our results agree with Zafer et al. [10] who reported that the prevalence of $bla_{\text{OXA-10}}$ was 41.7% out of their 122 *P. aeruginosa* Egyptian isolates.

To the best of our knowledge, this is the first time to detect bla_{VEB} in *P. aeruginosa* clinical isolates from Egypt. bla_{VEB} was found in 10 of our isolates together with other β-lactamase genes. Five of the isolates harbored bla_{VEB} with bla_{GES}, bla_{VIM}, and $bla_{\text{OXA-10}}$, whereas the other five isolates harbored bla_{VEB}, bla_{VIM}, and $bla_{\text{OXA-10}}$.

According to our results, resistance of *P. aeruginosa* isolates to β-lactam antibiotics was found to be mainly mediated via multiple β-lactamases belonging to different molecular classes. Twenty-three isolates (69.7%) harbored 2–5 genes

belonging to more than one β-lactamase class (2–3 classes). Only six isolates harbored a single gene belonging to a single β-lactamase class (A or B).

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Conflict of Interest

The authors declare no conflict of interest.

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