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Detection of GES-2, a Class A β-Lactamase Produced by *Pseudomonas aeruginosa* in a Teaching Hospital in Iran

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Background: Guiana Extended-Spectrum (GES-2) as one of the Extended-spectrum β-lactamases (ESBLs), produced by *Pseudomonas aeruginosa,* is able to compromise the effectiveness of imipenem and tends to be geographically limited.
Objectives: The aim of this study was to detect the spread of GES-2 β-lactamase gene among ESBL- producing *P. aeruginosa* isolates in a teaching hospital in Kashan, Iran.
Materials and Methods: Detection of β-lactamase gene blaGES-2 in eight ESBL producing isolates of *P. aeruginosa* collected from 100 clinical and environmental specimens was performed by PCR method. The susceptibility of the isolates to eight antibacterial agents was determined by disk diffusion method. ESBL production of the isolates was detected by double disk synergy test. DNA sequencing and aligning with the reference sequence were performed using BLAST and CLUSTAL W for selected PCR products of the blaGES-2 gene.
Results: A total of 100 environmental and clinical isolates of *P. aeruginosa* were collected. Eight of the isolates were found to be ESBL positive. The highest resistance rate was detected for piperacillin (75%), while the lowest resistance rate was for ciprofloxacin (12.5%). Fifty percent of the isolates (four out of eight) were imipenem-susceptible and MDR. The blaGES-2 gene was detected in all ESBL-producing isolates. Sequencing of both forward and reverse strands of the blaGES-2 gene were identified by BLASTn search as the ATP-binding cassette (ABC) transporter permease, partial cds, clone G-1 *P. aeruginosa* (Accession no. AB591379.1).
Conclusions: According to the present data, this is the first report on the presence of blaGES-2 gene of ESBL-producing *P. aeruginosa* from teaching hospitals of Iran. Fifty percent of the blaGES-2 positive *P. aeruginosa* isolates were multi-drug resistance (MDR).

Keywords: Pseudomonas aeruginosa; beta-Lactamases; Multi-Drug-Resistant

1. Background

Extended-spectrum β-lactamases (ESBLs) are a group of enzymes that mediate resistance to extended-spectrum cephalosporins, such as ceftriaxone, cefotaxime, ceftazidime, and the monobactam aztreonam. Clavulanic acid-inhibited extended-spectrum β-lactamases (ESBLs) have been described primarily in *Enterobacteriaceae* then in *Pseudomonas aeruginosa* (1, 2). Beta lactamase TEM (Temoneria) and SHV (refering to sulfhydral variable) have been known as parental ESBL enzymes. Other types of ESBL enzymes such as PER (*Pseudomonas* extended resistance), GES (Guiana Extended-Spectrum), certain OXA type enzymes (preferentially hydrolysis oxacillin and cloxacillin), have also been explained in some of countries (1).

GES, the new class of ESBLs, was reported in 2000 in a clinical isolate of *Klebsiella pneumoniae* from French Guiana (2). Identification of GES-2 is an interesting research progress on ESBLs in *P. aeruginosa* (3). The GES-2 β-lactamase is a class A carbapenemase, the emergence of which is an alarming advance in clinically considerable bacterial pathogens as the enzyme confers resistance to carbapenem antibiotics (4). The integron genetic framework of GES-2 is the essential factor that emerges resistance to broad-spectrum β-lactam antibiotics and other dissimilar classes of antimicrobials (3).

Outcome of introducing such characteristics brings out a huge challenge around effective treatment and control of these isolates on a health system (5). The blaGES-2 is located on a gene cassette in class 1 integron. For the first time, GES-2 was reported in MDR clinical isolates of *P. aeruginosa,* originating from a university hospital in South Africa (6). Poirel et al. reported that GES-2 was associated with an outbreak occurring in the same hospital in 2000 (7). Rapid recognition of resistant strains and detection of resistance genes is necessary to succeed in antimicro-

Implication for health policy/practice/research/medical education:
GES-2 β-lactamase is a class A carbapenemase, the emergence of which is an alarming advance in clinically considerable bacterial pathogens as the enzyme confers resistance to carbapenem antibiotics. To establish the appropriate antimicrobial therapy and infection control measures, rapid detection of resistant strains and identification of resistance genes are important.

bial therapy and infection control policy. Although, epidemiological data indicate that ESBL-producing *P. aeruginosa* has sparse prevalence in Iran.

2. Objectives

This study was performed to investigate the presence of β-lactamase blaGES-2 gene in ESBL-producing isolated *P. aeruginosa* from a tertiary care teaching hospital in Kashan, Iran, DNA sequencing for selected PCR products of the blaGES-2 gene, sequence comparing and aligning with reference sequence and evaluate their resistance to eight commonly-used antibiotics.

3. Materials and Methods

A total of 100 clinical and environmental isolates of *P. aeruginosa* were non-repetitively and consecutively obtained from clinical specimens and wet environment of Beheshti hospital in Kashan, Iran, from February 2010 through September 2011. Species identifications were done by standard methods. Eight ESBL-producing isolates of *P. aeruginosa* were detected. The strains were isolated from blood [1], trachea [2], urine [2], gastrointestinal fluid [1], bronchoscopy fluid samples [1] and wet environment of the hospital [1].

Antimicrobial susceptibility testing was performed on all eight isolates according to the standard method established by Clinical and Laboratory Standards Institute (CLSI) (8). Imipenem (10 μg), ciprofloxacin (5 μg), ceftazidime (30 μg), ceftriaxone (30 μg), cefotaxime (30μg), aztreonam (30 μg), piperacillin (100 μg) and gentamicin (10 μg) [Mast Group Ltd., Merseyside, UK] were determined by disk diffusion method. Multi-drug resistance was defined as resistance to three or more classes of antibiotics. The quality control of antibiotic susceptibility was determined by *P. aeruginosa* ATCC 27853. Double disk synergy test was used for the assessment of ESBLs production.

Detection of β-lactamase blaGES-2 gene was performed under standard PCR conditions using the published set of primers (Table 1). The primers used for these tests were obtained from Cinagene Company, Iran. To perform PCR, a 1:10 dilution of a 24-hour culture was boiled for 10 minutes. Then amplification was performed with 1:10 of this dilution as the DNA template. PCR settings involved 30 cycles of amplification under the following conditions: Denaturation at 95°C for 30 seconds, annealing at 50°C for 60 seconds and then at 72°C for 60 seconds. Then, cycling was followed by a final extension at 72°C for 5 minutes. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel. After electrophoresis, the gel was stained using ethidium bromide and photographed under a UV trans-illuminator (Ingenius, Syngene).

A 100-base pair (bp) DNA (Bioneer, Korea) ladder was used as the molecular size marker. DNA sequencing for selected PCR products of the blaGES-2 gene was performed for identification of detected bla gene using primers as shown in Table 1. For this matter, the PCR products of the above-mentioned gene were subjected to direct sequencing of both strands using an automated sequencer (ABI system, 3730XL) of Macrogen Company, Korea. The nucleotide sequences were analyzed by Chromas LITE software, version 2.01. Sequences were compared and aligned with the reference sequence using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and CLUSTAL W (http://www.ebi.ac.uk/clustalw).

Table 1. Primers Used for Detection of GES-2, a Class A β-Lactamase Produced by*P. aeruginosa*

Gene Type	Primer	Sequences (5´ to 3´)	Product Size, bp	Reference
GES-2	GES-2	GTTTTGCAATGTGCTCAACG	371	(9)
	AGES-2 B	TGCCATAGCAATAGGCGTAG		

The nucleotide sequences of blaGES-2 gene and the partial gene encoding ABC transporter permease (G-1) were deposited in Gene Bank through DNA Data Bank of Japan (DDBJ), and the accession numbers assigned were AF326355.1 and AB591379.1, respectively.

4. Results

The prevalence of ESBLs in *P. aeruginosa* isolates was 8%. The resistance pattern of eight ESBL-producing *P. aeruginosa* isolates in our study showed an extra high resistance to piperacillin (75%) and all extended-spectrum cephalosporins (62.5%). The isolated ESBL-producing *P. aeruginosa* samples were more sensitive to imipenem, gentamicin and ciprofloxacin compared to other tested antibiotics (Table 2). Fifty percent of the isolates, (four out of eight) were resistant to at least three classes of antibiotics and classified as multi-drug resistant (MDR). By using the blaGES-2primer pair, visible bands were observed near the location of the expected size in eight isolates. In order to increase the accuracy of the results, sequencing of both forward and reverse strands of the blaGES-2 gene were carried out twice (Figure 1). Sequence alignment with the reference sequence of blaGES-2 (Accession no. AF326355.1) by BLASTn search and CLUSTAL W revealed no similarity and BLASTn search identified them as the ATP-binding cassette (ABC) transporter permease, partial cds, clone G-1 *P. aeruginosa* (Accession no. AB591379.1), with 84% coverage and 100% maximum identity.

Table 2. Antibiotic Sensitivity Pattern of Eight Strains of ESBL-Producing*P. aeruginosa*

Antibiotics	Sensitive, No. (%)	Intermediate, No. (%)	Resistant, No. (%)
Piperacillin (100 g)	2 (25)	0 (0)	6 (75)
Imipenem (10 g)	4 (50)	0 (0)	4 (50)
Gentamicin (10 g)	4 (50)	0 (0)	4 (50)
Cefotaxime (30 g)	1 (12.5)	2 (25)	5 (62.5)
Ceftriaxone (30 g)	2 (25)	1 (12.5)	5 (62.5)
Ceftazidime (30 g)	2 (25)	1 (12.5)	5 (62.5)
Aztreonam (30 g)	3 (37.5)	1 (12.5)	4 (50)
Ciprofloxacin (5 g)	4 (50)	3 (37.5)	1 (12.5)

Figure 1. The nucleotide sequences of blaGES-2 gene of *P. aeruginosa* Strains Isolated From Clinical and Environmental Specimens of Beheshti Hospital in Kashan

GCCTGAAGGAGGGTGGCGTATCCGCGACAAGCAGCACGTATGCGCAACACCAGCAGCAAC
AGGGCCAGCAGGCCGGCCAGGAGGAACAGGCCGACACCGATTTCCAGGGTGCGGGTTTGC
ATTCAGAAATCTCCAAACATCAAAGCAGTCAGAATGAAGTCGAGCCCCAGCACCGCCAGG
GAGGCATAGACCACGGT CCGGGT CGT CGCCCGGCT GAT TCCT T CCGAGGT CGGCT CGCAG
TCGTAGCCTT GGTAGACCGCGAT CCAGGTCACCAC GAAGGCGAATACGATACT T TT GAT C
ACGCCGTTGAGCACATTGCAAAAC

The nucleotide sequences were analyzed using Chromas LITE software, version 2.01

5. Discussion

In our study, blaGES-2 gene was detected in all ESBL-producing isolates. GES β-lactamase genes which are placed on the broad-host-range conjugative plasmids, are the gene cassettes elements of class 1 integrons found in gram-negative isolates and have been spread worldwide (4). GES-2 β-lactamase is the fourth example of a non-TEM/ non-SHV-type ESBL in *P. aeruginosa* after PER-1, VEB-1 (Vietnamase extended spectrum) and OXA-18 (6). GES-type β-lactamase consists of 15 members (GES-1 to GES-15), isolated from various Gram-negative bacteria in Europe, Asia, Africa, and America (4). Although some GES β-lactamases produce antibiotic resistance profiles like those of classical extended spectrum enzymes, some GES variants (GES-2, 4, 5, and 6) are conferred with reduced susceptibility to imipenem (4).

GES β-lactamases are found in French Guiana, Greece, and South Africa (10). blaGES-1 has also been found in a *P. aeruginosa* strain isolated in a French medical center and from a 63-year-old female who had a hysterectomy (Sao Paulo, Brazil) (9). GES-2 was identified from a *P. aeruginosa* isolate in South Africa and later from eight more strains involved in the outbreak. GES-2 β-lactamase may supply partly to the decreased susceptibility of *P. aeruginosa* to imipenem (6, 7, 11). GES-2 β-lactamase belongs to the class A carbapenemase, the emergence of which is an alarm in *P. aeruginosa* as the enzyme confers resistance to carbapenem antibiotics. Tazobactam is a clinically used inhibitor of class A β-lactamases, which inhibits the GES-2 enzyme efficiently, retrieving the susceptibility to β-lactam antibiotics.

In our study, BLASTn search showed that the blaGES-2 gene sequencing matched as the ATP-binding cassette (ABC) transporter permease, partial cds, clone G-1 *P. aeruginosa* (Accession no. AB591379.1). Same results were obtained by Hirakawa et al. (12). Resistance in *P. aeruginosa* may be mediated via several distinct mechanisms including the production of β- lactamases, efflux pumps, and target-site or outer membrane modifications (13). Efflux pump systems have been revealed as the extremely important causes of multi-drug resistance in *P. aeruginosa*. ABC transporters are the major efflux pump protein families that mediate resistance to antibiotics and are believed to play a crucial role in the development of MDR (14, 15). In general, ABC transporters have been found in both prokaryotic and eukaryotic systems and are responsible for the import and export of various proteins, peptides, polysaccharides, and drugs that utilize ATP hydrolysis to drive the export of substrates (15).

Detection of the ESBL GES-2 from *P. aeruginosa* in seven hospitalized patients and one environmental sample clarified that these isolates had been established in our teaching hospital. GES-2-producing *P. aeruginosa* tends to colonize and mostly infects debilitated patients, considerably increasing both their lengths of hospitalization and costs of treatment. The integron genetic structures that support GES-2, present not only resistance to broad-spectrum β-lactam antibiotics but also to dissimilar classes of antimicrobials, making these isolates very difficult to treat and control successfully. In our study, prevalence of MDR *P. aeruginosa* isolates, was 50% which was lower than the value reported in the study conducted by Aggarwal et al. (100%) (16).

Carbapenems were subsequently introduced into the clinic as the last resorts of antibiotics due to their high potency and exceptional broad spectrum of antimicrobial activity that includes both gram-negative and gram-positive aerobic and anaerobic bacteria. New types of class A β-lactamases, the carbapenemases, are capable of causing resistance to a wide variety of β-lactam antibiotics, including carbapenems (4). In this study, notable resistance (50%) of *P. aeruginosa* was observed against imipenem, while other studies reported that more than 80% of ESBL producing isolates were sensitive to imipenem (17, 18). Our study reports the presence of blaGES-2 in *P.*

aeruginosa for the first time in Iran. ESBLs are uncommon but significant problems in our hospital. GES-2 was found in all of ESBL-positive isolates, of which 50% appeared to be MDR.

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Authors' Contribution

All authors had equal role in design, work, statistical analysis and manuscript writing.

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