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Laboratory Detection of Carbapenemases in Gram-Negative Bacteria

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Abstract

The nosocomial infections, caused by multi-drug resistant bacteria, are the most important cause of mortality throughout the world. One important mechanism against beta-lactam antibiotics is the production of beta-lactamases. *Enterobacteriaceae* and non-fermentative bacteria, like *Pseudomonas aeruginosa* and *Acinetobacter baumannnii* may produce these enzymes. Currently, there is no vaccine to prevent the infections caused by β-lactamase-producing bacteria. Consequently, it is necessary to identify β-lactamase-producing bacteria by phenotypic and molecular methods.

Keywords: Beta-Lactamases, Gram-Negative Bacteria, Drug Resistance, Carbapenemases, Antibiotics

1. Context

Antibiotic therapies are widely used for treating infectious diseases (1). Nowadays, antibiotic-resistant bacteria are a great concern of worldwide public health (2). The nosocomial infections, caused by multidrug-resistant (MDR) bacteria, are one of the most important causes of mortality throughout the world (3). Therefore, multidrug-resistant Gram-negative rods will be a serious problem if no adequate action is taken (4, 5). Unfortunately, the issue of antibiotic resistant bacteria in Iran is increasing, following the rising trend of its global counterpart (6). One of the most common reasons for this trend is represented by the widespread overuse and incorrect prescribing practices. Resistant bacteria possess resistance gene cassettes, which protect antibiotic-producing bacteria against these biological active molecules (7-9). The most important resistance mechanism against beta-lactam antibiotics is producing beta-lactamases, especially by Gram-negative bacteria (10, 11). The β-lactamases are classified into four molecular classes, according to Bush-Jacoby classification system (12). During the last years, the distribution of carbapenemase-producing *Enterobacteriaceae* has emerged almost globally. There is no general protocol for treating severe infections that are caused by carbapenemase-producing bacteria. Fosfomycin, tigecycline and polymyxins such as polymyxin B or colistin are offered for these cases. In serious circumstances, combination therapy is recommended, rather than monotherapy. Several newly discovered antibiotics have been approved to aid treatment options. Avibactam, as a non-beta-lactam beta-lactamase inhibitor, acts against OXA-48 carbapenemases and almost all A and B beta-lactamase classes (including strains expressing class A carbapenemases and/or derepressed AmpC enzymes). Ceftazidime or aztreonam, in combination with avibactam, are also effective (13, 14). *Enterobacteriaceae* and non-fermentative bacteria, like *Pseudomonas aeruginosa* (*P. aerugionsa*) and *Acinetobacter baumannnii* (*A. baumanii*) are able of producing carbapenemases. The classification of these enzymes is as follows: group A (KPC, SME, IMI and NMC), group B (VIM, IMP, SPM, GIM, NDM, SIM, DIM and AIM) and group D (OXA-23, OXA-24, OXA-48, OXA-58 and OXA-143). Since the 1990s, metallo-β-lactamase (MBL) producing bacteria have been reported from many regions of the world (12, 15). An important percentage of Gram-negative pathogenic bacteria are resistant to β-lactam antibiotics, through MBLs production. Verona integron-encoded MBLs (VIM) (12, 16) and imipenemase (IMP) (12) are the main MBLs family members.

2. Imipenemase-Type Beta-Lactamases

For the first time, IMP-type enzymes were detected in Japan, in the late 1980s. Thereafter, these enzymes have been detected in *Enterobacteriaceae* and Gram-negative non-fermenters (mainly in *P. aeruginosa* and *Acinetobacter*

spp.), throughout the world. The bla_{IMP-1}, as an allele for IMP is within a class 3 integron, adjacent to an *aac(6′)Ib*-like gene and its position is on a large plasmid (120 kb) (17). More than 53 IMP allotypes have been known so far. However, the IMP-type enzymes have little activity against temocillin (a 6 α-methoxy penicillin), while possessing a wide spectrum substrate and affinity for cephalosporins and carbapenems (18).

3. Verona Integron-Encoded-Type Beta-Lactamases

The VIM-type enzymes, as an acquired MBL, consist the second major group. In 1997, VIM-1 was detected for the first time in a *P. aeruginosa* strain isolated in Verona, Italy. This clinical isolate was resistant to piperacillin, ceftazidime, imipenem, and aztreonam (β-lactam antibiotics). The bla_{VIM-1} gene, as a gene cassette, was integrated into a class 1 integron that carries an integrase gene, typical of class 1 integrons. The bla_{VIM-1} gene cassette and an *aacA4* gene cassette encoding resistance to aminoglycosides can be carried on this integron. The bla_{VIM-1} containing integron, in this *P. aeruginosa* isolate, might be on the chromosome (18).

4. New-Delhi Metallo-Beta-Lactamases

New Delhi metallo-β-lactamase (NDM) outbreak, among isolates of *Enterobacteriaceae*, especially *Klebsiella pneumoniae* (*K. pneumoniae*) and *Escherichia coli* (*E. coli*), is a matter of great concern, because NDM can hydrolyze almost all β-lactam antibiotics. In 2008, this enzyme was isolated from a *K. pneumoniae* strain, isolated from a Swedish patient, hospitalized in India for the first time. The 16 variants of NDM are different in one or two amino acid substitutions (http://www.lahey.org/studies/) (12, 19). The NDM-1 is a transferable molecular class B beta-lactamase that has been recently discovered. It has zinc ions in its active site and can hydrolyze all beta-lactam antibiotics, except for monobactam. Most NDM-1-positive bacteria carry additional resistance mechanisms, like for aminoglycosides, fluoroquinolones, macrolides and sulfonamides against other antimicrobial classes. The bla_{NDM-1} gene may be found from a chromosome of plant pathogens, such as *Pseudoxanthomonas*, and related bacteria that outspread in the environment (20). The NDM-1 producing enterobacterial strains that are sporadic hospital outbreaks were reported from multiple countries in the Mediterranean area: France, Italy, Lebanon, Morocco, Spain, Tunisia and Turkey. Very recently, *E. coli* with NDM-5 was found in Algeria. Other cases have been reported from Australia, Greece, Canada, Singapore, USA, China, Japan, Kenya, Oman and China's-Taiwan region. Therefore, sensitive and accurate identification testing represents a very important strategy for rapid diagnosis and control of NDM-1 (12, 21). Patients screening and controlling infections in NDM-1 high-risk areas might be better approaches than the limitation of international medical tourism. To blame a region for spreading a new bug, because of its identification in that region in the first place is inadequate and unprofessional. Also, the results of research performed by pharmaceutical companies and the European Union health politics are incongruent and result in biased conclusions (18, 22).

5. Carbapenem-Hydrolysing Oxacillinase-48-Type Carbapenemase

The Carbapenem-Hydrolysing Oxacillinase-48 (OXA-48) was first identified in *K. pneumoniae*, in Turkey, in 2001. Since then, other countries, such as Croatia, Egypt, France, Greece, Italy, Lebanon, Libya, Slovenia, Spain, Tunisia (the Mediterranean countries) have been widely reporting it between the sources of nosocomial outbreaks. The OXA-48, as the most common carbapenemase type, circulates in these regions, especially in Spain and France. The OXA-48 producers are more prevalent in The middle east and north Africa. In the last few years, OXA-48-producing *Enterobacteriaceae* have been disseminated as a nosocomial agent in certain Moroccan hospitals. The OXA-48 problem occurred as an endemic outbreak with the isolation of this enzyme in the community members and also in environment. Recently, the OXA-48 gene existing in a *K. pneumoniae* isolate has been reported in Algeria (12, 23). The accurate and rapid carbapenem-resistant isolates identification is a major prevention stage against such MDR strains and reduces treatment failure. As carbapenemases are encoded on mobile genetic elements that carry other antibiotic resistant genes, therapeutic protocol selection is often limited. In this case, the only option for treatment is colistin (24).

6. *Klebsiella pneumoniae* Carbapenemase

The *K. pneumoniae* carbapenemase (KPC) was first detected in a *K. pneumoniae* strain isolated in north Carolina, in 1996. Since then, it has spread as a common cause of MDR and pandrug resistance among *Enterobacteriaceae* throughout the world (22, 25). The KPC-producing bacteria are also causes of nosocomial and systemic infections; the most common agents are members of *Enterobacteriaceae* and the least common agents are *Pseudomonas aeruginosa* isolates. The most efficient members of carbapenems

(imipenem, meropenem and ertapenem) fail to treat enterobacterial infections by KPC β-lactamases (KPC-1 to KPC-7) producers, which are also resistant to multiple other non-β-lactam molecules. Routine antibiotic susceptibility tests are not sufficient to detect KPC-producing bacteria. Therefore, infection control policies for controlling the spread of these pathogens usually fail (26).

Different types of metallo-β-lactamases dissemination in Iran are presented in Table 1.

7. How Can Metallo-Beta-Lactamases and Carbapenemases Producing Bacteria be Identified?

7.1. Phenotype Tests for Carbapenemase

7.1.1. Broth Microdilution Screening for Metallo-Beta-Lactamases

Determination of minimum inhibitory concentration (MIC) values in the absence and presence of 0.2 mmol/L EDTA, along with 0.02 mmol/L 1, 10-phenanthroline, has been described by screening wells containing IMP at concentrations of 0.25 - 1.024 μg/mL. It is considered that a ratio $\geq$ 4 in the MIC for IMP, compared to the MIC value for IMP, in the presence of chelators (IMP + EP), is a positive result in MBL production (37).

7.1.2. Modified Hodge Test

The recommended technique by clinical and laboratory standards institute (CLSI) to confirm carbapenemase production is the modified hodge test (MHT). The *K. pneumoniae* is the most common carbapenemase producer among *Enterobacteriaceae* family members in the United States. Also, the MHT is positive for other carbapenemases, such as the MBL and SME-1 in *Serratia marcescens*. These enzymes are commonly being detected in the USA (38).

7.1.3. E-Test for Metallo-Beta-Lactamases Detection

The E-test MBL strips, containing IMP (IP) and IMP + EDTA (IPI) is recommended to detect MBLs. The strips are used to detect MBLs according to the instruction of the manufacturers. A reduction in MIC value, in the presence of EDTA, of greater than or equal to eight-fold (IP/IPI $\geq$ 8) is interpreted as MBL activity (39-41).

7.1.4. CHROMagar Medium

CHROMagar KPC® (CHROMagar, Paris, France) is another screening medium, which contains a carbapenem, as the selector for resistance. It is also precisely designed for KPC producers screening. By means of the CHROMagar medium, carbapenemase-producing isolates with MIC values < 4 μg/mL are detected, with much higher detection limits (42).

7.1.5. Combined Disk Diffusion Test

The other test for identification of MBLs is the combined disk diffusion test (CDDT). This test is based on imipenem and meropenem alone and in combination with EDTA. If the difference of inhibition zone between antibiotic alone discs and antibiotic + EDTA disks is $\geq$ 7 mm, the result of MBL production is positive (43, 44).

7.1.6. Carbapenemase Nordmann-Poirel Test

The carbapenemase nordmann-poirel (Carba NP) test is a rapid test to identify carbapenemase production in *Enterobacteriaceae*. This test is based on hydrolysis of a carbapenem like imipenem. The sensitivity of the Carba NP test is 100% and is more specific, compared with molecular methods. The advantages of this rapid test, which requires < 2 hours to be performed, are its inexpensiveness and easiness of performance in laboratories (45).

7.1.7. Blue-Carba

The Blue-Carba test is a rapid test to detect all carbapenemase producers with 100% sensitivity and 100% specificity. All noncarbapenemase producers (including extended-spectrum β-lactamase- and/or AmpC-producing isolates), give negative results. Different times are required to observe a positive result for different carbapenemases types (e.g., KPC or MBL at the first 30 minutes, versus most OXA-type enzymes at 1 hour 30 minutes to 2 hour). Additionally, a higher inoculum results in stronger color changes for OXA types from *Acinetobacter* spp. Blue-Carba is proved to have specificity and sensitivity (100%) similar to those of Carba NP test and presents additional advantages, as follows: (1) increased protocol simplicity due to the direct use of colonies (instead of bacterial extracts); (2) significantly reduced cost per reaction (over 200 $\times$), taking into account the use of Tienam (about 10 $\times$ cheaper than an imipenem monohydrate formula) and the dispensability of the extraction buffer (B-PER II), which is used to obtain bacterial extracts; (3) the validation of the test for the detection of OXA-type carbapenemases commonly identified in *Acinetobacter* spp (46).

7.1.8. Boronic Acid Disk Test for the Phenotypic Detection of Klebsiella pneumoniae Carbapenemase-Producing Isolates

The stock solution is prepared by dissolving phenylboronic acid in dimethyl sulfoxide, at a concentration of 20 mg/mL. From this solution, 20 μL (containing 400 μg of boronic acid) are dispensed onto a meropenem disk. The disk is then dried and used within 60 minutes. The test is performed by inoculating Mueller-Hinton agar by the standard diffusion method and placing disc with or without boronic acid onto the agar. After an overnight incubation at 37°C, the diameter of the growth-inhibitory

Table 1. Dissemination of Different Types of Metallo-Beta-Lactamases in Iran

City	MBL Type, No. (%)				Year	Organism	Reference
	bla_{IMP}	bla_{VIM}	bla_{AIM}	bla_{NDM-1}			
Tabriz	19 (61)	9 (29)	-	-	2011	*A. baumannii*	(27)
Northwest of Iran	6 (5.77)	18 (17.31)	-	-	2010	*P. aeruginosa*	(28)
Tehran	-	23	-	-	2010	*P. aeruginosa*	(29)
Ahvaz	-	8 (19.51)	-	-	2008	*P. aeruginosa*	(30)
Tehran	-	-	-	1	2013	*K. pneumoniae*	(31)
Kurdistan	-	8	-	-	2012	*P. aeruginosa*	(32)
Zanjan	10 (24.3)	23 (56)	-	-	2013	*P. aeruginosa*	(33)
Tehran	3 (3.48)	15 (17.44)	-	-	2014	*A. baumannii*	(34)
Tehran	3	55	1	-	2014	*P. aeruginosa*	(35)
Mashhad	-	3	-	-	2013	*A. baumannii*	(36)

zone around a meropenem disc with boronic acid is compared with that around the corresponding meropenem disc without boronic acid. The test is considered positive for KPC enzyme production when the diameter of the growth-inhibitory zone around a meropenem disc with boronic acid is $\geq$ 5 mm larger than that around a disc containing the meropenem substrate alone (47).

7.1.9. Double-Disk Synergy Test

The double-disk synergy test (DDST) is performed according to Arakawa et al. and Lee et al. (48). The test strains are being adjusted to a turbidity equivalent to that of a 0.5 McFarland standard and are used to inoculate Mueller-Hinton agar plates. Depending on the test, a 10 μg imipenem disc or a 30 μg ceftazidime disc is placed on the plate, and a blank filter paper disc (6 mm in diameter, Whatman filter paper no. 2) is placed at a distance of 15 mm (edge to edge). Ten microliters of a 0.5 M EDTA solution are added to the blank disc. After overnight incubation, the presence of any synergistic inhibition zone is interpreted as positive.

7.2. Molecular Techniques for Carbapenemase Genes Detection

7.2.1. Loop-Mediated Isothermal Amplification

The MHT is neither sensitive, nor specific to detect carbapenemase. Therefore, carbapenemase presence confirmation needs molecular methods to confirm a positive MHT result. The carbapenemases (including KPC- and NDM-mediated resistance) can be detected by molecular methods, including sequencing of PCR products, target-specific probes in real-time assays, microarray and loop-mediated isothermal amplification (LAMP) detection. The LAMP technique results have a high rate of false-positive results; however, it holds multiple advantages (e.g. simplicity of performance, high efficiency under isothermal conditions and specificity) (49).

7.2.2. Matrix Assisted Laser Desorption Ionization-Time of Flight

Recently, matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) has been introduced in clinical microbiology for species identification. During the last 2 years, a number of studies have shown the proof of concept concerning the detection of β-lactamases using this technology, which is a rapid method to detect KPC carbapenemase (in 45 minutes) or MBL (in 150 minutes). It is based on analysis of the degradation of carbapenem molecule. These studies have either investigated a small set of strains or focused on the detection of hydrolysis, rather than the verification of specific enzymes. All studies have used different protocols and different sets of species/enzyme combinations (24, 50).

7.2.3. Ultraviolet Spectrophotometer

The ultraviolet (UV) spectrophotometer is the other instrument that can be used to detect carbapenemase activity. The steps of this method are as follows: 1) bacterial incubation for 18 h (in certain cases it can be shorter, to up to 8 hour); 2) protein extraction; 3) imipenem hydrolysis measurement by a UV spectrophotometer. For every carbapenemase activity, the sensitivity and specificity of it is 100% and 98.5%, respectively. In addition, this method has the accuracy to differentiate carbapenemase producers from non-carbapenemase producers, among carbapenem-non-susceptible isolates [extended spectrum beta-lactamases (ESBLs), cephalosporinase producers and fault in outer

membrane permeability]. Nevertheless, this is a time consuming technique. Recently, mass spectrometry, with its basic rules based on the analysis of degradation of a carbapenem molecule, has been introduced to detect carbapenemase activity. However, it has been assessed that the MALDI-TOF equipment is preferred in bacteriology laboratories (50).

7.2.4. Multiplex Real-Time Polymerase Chain Reaction

The basis of multiplex real-time polymerase chain reaction (RT-PCR) is to detect bla_{KPC} and bla_{NDM-1} in a single reaction among Gram-negative bacteria. The 16S rRNA is an indicator and control for DNA extraction and amplification in any reaction. The KPC or NDM-1 assessment can be done independently by separate primers and probes (51).

7.2.5. Check-Direct Cytopathogenic Effect Assay

A new multiplex RT-PCR assay is check-direct cytopathogenic effect (CPE) assay (Check-Points, Wageningen, the Netherlands). It can detect and differentiate carbapenemase genes in *Enterobacteriaceae* (bla_{KPC}, bla_{OXA-48}, bla_{VIM}, and bla_{NDM}), only by rectal swabs (52).

7.3. Genetic Relatedness by Several Methodologies for MBLs

7.3.1. Random Amplified of Polymorphic DNA Polymerase Chain Reaction (RAPD PCR) and Rep-PCR

The basis of some molecular genetics methods is PCR, for instance, random amplified of polymorphic DNA (RAPD) and repetitive element palindromic PCR (rep-PCR). The RAPD markers are DNA fragments from PCR amplification of random segments of genomic DNA, with single primer of arbitrary nucleotide sequence. Unlike traditional PCR analysis, RAPD does not require any specific knowledge of the DNA sequence of the target organism. No fragment is produced if primers annealed too far apart or 3' ends of the primers are not facing each other. Consequently, if a mutation has occurred in the template DNA, at the site that was previously complementary to the primer, a PCR product will not be produced, resulting in a different pattern of amplified DNA segments on the gel. In rep-PCR DNA fingerprinting, PCR amplification of the DNA between adjacent repetitive extragenic elements is used to obtain strain-specific DNA fingerprints, which can be easily analyzed with pattern recognition computer software. The rep-PCR technique was chosen because of its simplicity, ability to differentiate between closely related strains of bacteria, and, also, its capability to be used for high-throughput studies (53, 54).

7.3.2. Pulsed-Field Gel Electrophoresis

Pulsed-field gel electrophoresis (PFGE) is a highly discriminative molecular typing method that is used in epidemiological studies. It is based on the variable migration of large DNA restriction fragments in a field of alternate polarity. Comparing the fingerprints of two isolates shows whether they belong to the same clone or if they are genetically unrelated. The PFGE is a 'gold standard' for typing the bacterial species. Restriction endonuclease analysis (REA) is the basis of other techniques, like PFGE or fragmentation of the genome, such as ribotyping. Amplified fragments-length polymorphism (AFLP) has been offered as a more powerful and rapid procedure than PFGE. However, it has several limitations, like absence of a standardization of the test (37, 54).

7.3.3. Multilocus Sequence Typing

The multilocus sequence typing (MLST) technique is a method for measuring variation in house-keeping genes. It has been standardized for multiple bacterial species. The phylogenetic information on genetic variations in conserved genes can be plotted by this method. Therefore, MLST can differentiate between different strains. In epidemiological, geographical and/or evolutionary studies, MLST is a preferable procedure (37, 54).

7.3.4. Multilocus Variable Number Tandem Repeat Analysis

In epidemiological studies, multilocus variable number tandem repeat analysis (MLVA), as an almost novel technique, has been applied for several species. This procedure has been used to detect outbreaks and source of bacteria in European countries. Considerably, it has been reported that an MLVA scheme containing several VNTR loci can display diversity, if phylogeny is done in an accurate manner (54).

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