ORIGINAL ARTICLE

# Detection of kpc-type Carbapenemases in Clinical Isolates of *Acinetobacter baumannii*

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**Abstract**

*Background:* Multidrug-resistant *Acinetobacter baumannii* is associated with life threatening nosocomial infections and hospital outbreaks. The organism is resistant to most commonly used antibiotics including carbapenems. *Aim and Objectives:* To study carbapenemase production among multidrug-resistant clinical isolates of *A. baumannii*. *Material and Methods:* Fifty multidrug-resistant *A. baumannii* isolates were selected based on PCR identification of the $bla_{OXA-51}$ gene. Minimum inhibitory concentrations were determined to imipenem and meropenem. Carbapenemase production was detected by the Modified Hodge Test (MHT) and the Combined Disc Method (CDM). Presence of the *Klebsiella pneumoniae* carbapenemases (KPC-2) type carbapenemase gene was shown by PCR using specific primers. *Results:* All isolates were resistant to imipenem and meropenem. MHT and CDM results showed carbapenemase production in 78% and 56% of the isolates, respectively. KPC-2 type gene was not observed in any of the isolates. *Conclusion:* No correlation was found between carbapenem susceptibility with phenotypic detection of carbapenemase production or KPC-type gene carriage.

**Keywords:** *Acinetobacter baumannii*, Carbapenemase, Modified Hodge Test, Combined Disc Method, KPC-2

**Introduction:**

*Acinetobacter baumannii* is an important opportunistic pathogen associated with life threatening nosocomial infections and hospital outbreaks. The organism commonly targets critically ill patients in Intensive Care Units (ICU) and is resistant to most commonly used antibiotics including carbapenems [1]. *A. baumannii* is thought to be mostly due to the production of Ambler class A carbapenemases, class B metallo-β-lactamases and class D oxacillinases [2]. *Klebsiella pneumoniae* Carbapenemases (KPC) were initially identified in a *K. pneumoniae* isolate from the United States and were subsequently observed in other members of the *Enterobacteriaceae* worldwide [3]. KPC belong to Ambler class A, Bush subgroup 2f, serine-based beta-lactamases which are active against all beta-lactams, including the carbapenems. Twelve KPC variants (KPC-2 to KPC-11) are currently known. The KPC gene has been found associated with the plasmid-borne transposon Tn*4401* [4].

Modified Hodge test (MHT) has been reported to be a good method for phenotypic detection of KCP in *Enterobacteriaceae* (CLSI, 2014). However, the test does not exclusively detect the KPC-type carbapenemases. Alternatively, a disc diffusion assay using a carbapenem as substrate and 3-aminophenyl boronic acid (combined disc method CDM) has been shown to reliably differentiae isolates producing KPC-type β-

lactamases [5]. It is suggested that carbapenem resistant isolates should be tested by the MHT or boronic acid screening tests for carbapenemase phenotype followed by a confirmatory PCR and sequencing for identification of the KPC-type enzymes [6]. KPC production by *A. baumannii* was first reported from Puerto Rico [3, 7]. Presence of $bla_{KPC}$ gene in *A. baumannii* adds another important element to the organism which already harbors multiple innate and acquired mechanisms of resistance. In addition, the real possibility of horizontal transfer could result in dissemination of this potent carbapenemase among other organisms. We screened for carbapenemase production and detection of $bla_{KPC}$ type gene in Multidrug-Resistant (MDR) clinical isolates of *A. baumannii*.

**Material and Methods:**

**Bacterial Isolates:**

Fifty isolates were collected from Imam Hossein hospital between October 2014 and March 2015. The majority of the isolates were obtained from the intensive care unit (ICU, n=45) and were mostly from sputum (n=34) followed by blood (n=6), catheters (n=3), trachea (n=3), urine (n=2) and wound (n=2). Thirty eight isolates (76%) were from male patients and 12 (24%) were from females. Bacterial identification was carried out by standard biochemical tests including catalase and oxidase tests, sugar fermentation and $H_2S$ production on Triple Sugar Iron (TSI) agar , Oxidation/Fermentation (O/F) test of glucose and growth on MacConkey agar [5-6]. The isolates were stored at -20 °C in brain heart infusion broth containing 8% dimethyl sulfoxide (v/v) until use.

**Molecular Identification of *A. baumannii:***

Identification of *A. baumannii* was confirmed by detection of $bla_{OXA-51}$ gene using primers listed in Table 1 [8]. DNA extraction was carried out by boiling (REF Zanganeh) [9]. Briefly, a loopful of an overnight grown culture of each test isolate was suspended in 500 µL of sterile double-distilled water, boiled at 100°C for 10 min, and centrifuged at 13,000 × *g* for 10 minutes. The supernatant was then used as the DNA template for PCR amplifications. PCR reaction mixtures (25 µL) contained 1 µL of DNA template, 1.4 mM $MgCl_2$, 0.24 mM of each dNTP, 4 pmol primer, and 2.5 U of Taq DNA polymerase in the 1X buffer provided by the manufacturer (CinnaGen, Iran). The amplifications were performed in a thermocycler (Techne TC-3000G; UK) with the following program: initial denaturation at 94°C for 5 min, 30 cycles of 94°C for 25 sec, 52°C for 40 sec, and 72°C for 50 sec, followed by a final elongation step at 72°C for 6 min [8]. PCR products were separated on 1% agarose gels and observed after staining with RedSafe (iNtRON Biotechnology, Korea) using an image analysis system (UVLtec; St John's Innovation Centre, UK).

**Table 1: PCR Primers for the Detection of OXA-51 Genes**

| Primer | Sequence | Product size. bp |
|---|---|---|
| **Forward** | 5'-TAATGCTTTGATCGGCCTTG-3 | 353 |
| **Reverse** | 5'-TGGATTGCACTTCATCTTGG-3 | 353 |

**Carbapenem Susceptibility Testing:**

Minimum Inhibitory Concentrations (MIC) for imipenem and meropenem (Loghman Pharmaceuticals, Tehran, Iran) were determined using broth microdilution according to the guidelines recommended by the Clinical and Laboratory Standards Institute (CLSI 2014).

**Screening of Carbapenemase Production by Modified Hodge Test:**

Carbapenemase phenotype was initially detected using the modified Hodge test (MHT) (CLSI, 2014). Briefly, A 1:10 dilution of an overnight grown culture of *Escherichia coli* ATCC 25922 in Muller Hinton Broth (MHB, Liofilchem), previously adjusted to the turbidity of 0.5 McFarland standard, was streaked as lawn on a Mueller Hinton agar plate (MHA, Liofilchem) followed by placing a 10 µg meropenem disc (Mast, UK) in the center of the plate. Colonies from the test organisms from overnight culture plates were streaked from meropenem disc to the edge of the plate before incubation at 37°C for 16-24 hours. The test was considered positive if a distorted inhibition zone was observed around the meropenem disc.

**Screening of Carbapenemase Production by Combined Disc Method:**

The combined disc method (CDM) was also employed for detection of carbapenemases using phenyl-boronic acid as an inhibitor for KPC enzymes. Briefly, an overnight grown culture of test isolates adjusted to the turbidity of 0.5 McFarland standard in Muller Hinton Broth (MHB, Liofilchem), was streaked as lawn on a Muller Hinton agar (MHA, Liofilchem), followed by placing a 10 µg imipenem disc (Mast, UK) and 10 µg imipenem disc containing 400 µg of phenylboronic acid and 10 µg imipenem disc containing 600 µg of phenylboronic acid on the plate followed by incubation at 37˚C for 18 hours. The test was considered positive if the difference in the inhibition zone diameters was ⩾ 5 mm between the zone produced by combination discs (imipenem+phenylboronic acid), and the zone produced by imipenem disc alone [10].

**Detection of $bla_{KPC}$ Gene by PCR:**

PCR amplification of $bla_{KPC}$ gene was carried out using the DNA template prepared by boiling and two sets of primers listed in Table 2 [11-13]. The PCR reaction mixtures were prepared as described above. The amplifications were performed in a thermocycler (Techne TC-3000G; UK) with the following program: initial denaturation at 94°C for 3 min, 30 cycles of 94°C for 1 min, 52°C for 1 min and 72°C for 1 min, followed by a final

**Table 2: PCR Primers for the Detection of KPC genes**

| Primer | Sequence | Product size. bp |
|---|---|---|
| **KPCa-Forward** | 5'-GTATCGCCGTCTAGTTCTGC-3 | 636 |
| **KPCa-Reverse** | 5'-GGTCGTGTTTCCCTTTAGCC-3 | 636 |
| **KPCb-Forward** | 5'-ATGTCACTGTATCGCCGTCT-3 | 880 |
| **KPCb-Reverse** | 5'-TTACTGCCCGTTGACGCCCA-3 | 880 |

elongation step at 72°C for 10 min [10-12]. Positive DNA control for $bla_{KPC}$ was obtained from the Pasteur Institute in Tehran and was included in PCR experiments. PCR products were separated on 1% agarose gels and were visualized as mentioned above.

**Results:**

Biochemical test results as well as presence of $bla_{OXA-51}$ gene (Fig. 1) in all test isolates confirmed their identity as *A. baumannii.* All isolates (100%) were resistant to imipenem and meropenem with MIC values between 32 and 512 µg/ml. . Carbapenemase production shown by MHT was detected in 39 isolates (78%). Of these, 79.48% (31 isolates) were from sputum samples of male patients in the intensive care unit, 12% (6 isolates) showed negative carbapenemase phenotype, the majority of which (66.7%, 4 isolates) were also obtained from males in ICU. Finally, 10% (5 isolates) showed indeterminate carbapenemase production (60%, 3 isolates) from males in ICU and 40% (2 isolates) from males in infectious diseases ward). The CMT showed positive results for 28 isolates (56%) and negative results for 22 isolates (44%). The isolates which had shown indeterminate carbapenemase production using MHT, were distributed equally among the CDM positive and negative isolates. Carbapenemase negative isolates by the MHT were also negative for carbapenemase production in CDM. Higher inhibition zone diameters for the combination imipenem disc with 600 µg phenylboronic acid compared to that of the imipenem disc with 400 µg phenylboronic acid was noticeable.

**Detection of $bla_{KPC}$ gene:**

Despite the high percentage of carbapenemase production by the isolates, none carried the $bla_{KPC}$ gene.

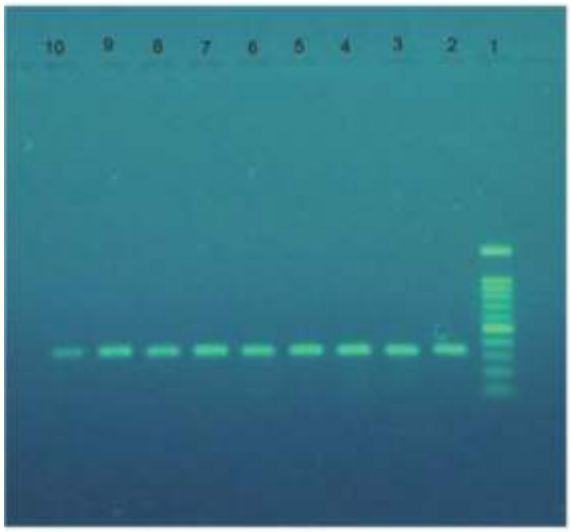


**Fig.1: Distribution of OXA-51 Gene in Isolates of *Acinetobacter baumannii***

Profile 1. 100 Kb DNA molecular weight marker, Profiles 2 to 10. Show the clinical isolates (353 bp)

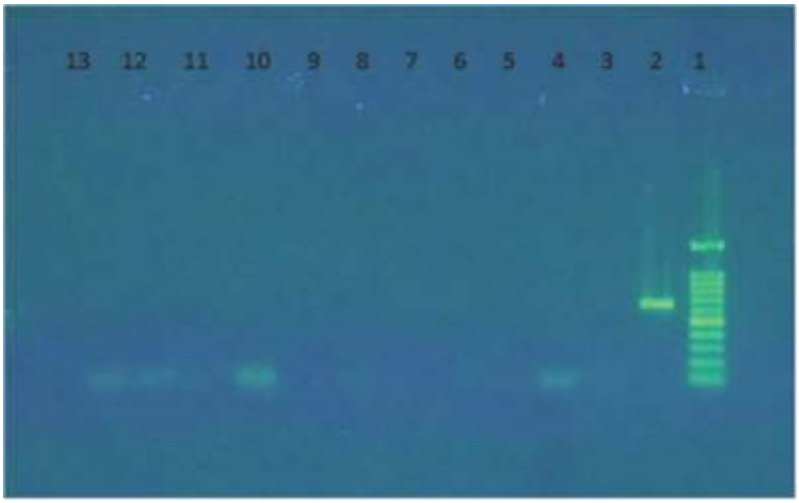


**Fig. 2: Distribution of KPC-2 Gene in Isolates of *Acinetobacter baumannii***
Profile 1. 100 Kb DNA molecular weight marker
Profile 2. Show the positive control DNA (636 bp)
Profiles 3 to 13. Show the clinical isolates that was not obsereved KPC

**Discussion:**

Phenotypic detection of KPC-type carbapenemases is often difficult and not conclusive. In this study,using the MHT, 78% of carbapenem resistant *A.baumannii* isolates showed carbapenemase activity. On the other hand, the CDM showed carbapenemase production by 56% of the isolates. The combined disc method is frequently used for KPC detection in *K. pneumoniae* and not as widely used for *A. baumannii*. In addition, Phenyl-boronic acid which was originally reported as an inhibitor for KPC-type β lactamases was later shown to also inhibit other class A and C β-lactamases [14]. Global emergence of carbapenem-resistant MDR isolates of *A. baumannii* has become a major problem in antibiotic treatment of infections caused by this opportunistic pathogen. We found 100% resistance to imipenem and meropenem among our clinical *A. baumannii* isolates but could not detect KPC-2 type carbapenemases. The first report on KPC production by *A. baumannii* showed that 4.3% of their isolates carried KPC-type genes which were further confirmed by sequencing as KPC 2, KPC 3, KPC 4 and KPC10 [7]. Another study reported that 5% of *A. baumannii* clinical isolates harbored KPC-type genes [3]. Among the Iranian studies, Hakemi-Vala found 100% resistance to imipenem and meropenem in burn isolates of *A. baumannii* but KPC encoding genes were not detected [15] and Mohammadi found 100% resistance to meropenem and 98% resistance to imipenem [16]. Azimi *et. al.* showed 9.23% KPC gene carriage in *A. baumannii* burn isolates [4] . Since KPC was not observed in any of our isolates, it is likely that the high rates of carbapenemase resistance may be due to other mechanisms such as oxacillinase production. Perez *et al*. showed 85% resistance to imipenem and meropenem in *A. baumannii* clinical isolates, mostly due to the presence of

OXA-23 and OXA-24 carbapenemases and did not detect KPC-2 type carbapenemases [17]. Mosqueda et al. reported that the 100% resistance to imipenem and meropenem in nosocomial isolates of *A. baumannii* corelated with the presence of OXA-23 and OXA-24 genes [18]. Our results did not show a correlation between carbapenem susceptibility with phenotypic detection of carbapenemase production or KPC-type gene carriage. Despite the fact that MHT and/or CDM tests are conveniant for detecting carbapenemases, neither can be used as the method of choice for detection of KPC-type carbapenemases in clinical laboratories.

According to Clinical Laboratory Standards Institute (CLSI) and Centers for Disease Control and Prevention (CDC) guideline, MHT is being used for detection of carbapenemases in *Enterobacteriaceae* family and not KPC phenotype. Other carbapenemases such as Metallo-beta-lactamases (NDM, VIM and IMP), class A of carbapenemases (SME-1) and OXA type of carbapenemases can also produce a positive results in MHT method. The MHT test does not have a high sensitivity for $bla_{KPC}$ identification unless it is confirmed by PCR followed by sequencing the PCR product [19].

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