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Molecular Characterisation of Carbapenemase-Producing *Acinetobacter baumannii* isolates from Hospitalised Patients in Iraq

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Abstract

Carbapenem-resistant *Acinetobacter baumannii* has been considered one of the major threats to patients worldwide. To evaluate carbapenemase in several clinical isolates using phenotypic and genotypic approaches. A total of 49 *A. baumannii* isolates were tested against imipenem and meropenem discs on Muller Hinton agar, then screened phenotypically through the modified Hodge test (MHT), combined disc test (CDT) and modified carbapenem inactivation method (mCIM). The tested isolates have been subjected to polymerase chain reaction (PCR) detection to identify some carbapenemase-encoding genes and one insertion sequence. The carbapenem resistance profile showed 96% and 94% resistance to imipenem and meropenem, respectively. MHT and mCIM were able to produce carbapenemase in 94% and 98% of isolates, respectively, while CDT was able to produce metallo-β-lactamase (MBL) only in 59.2% of isolates. The PCR amplification of *bla*$_{\text{OXA-51}}$ has been observed in all isolates. We found *bla*$_{\text{OXA-23}}$ in 98% of isolates. Insertion sequence IS*Aba1* was present in all positive *bla*OXA-23 strains (98%). A *bla*$_{\text{VIM}}$ gene encoding MBL was present in 71% of isolates, but none of the isolates has been positive for *bla*$_{\text{KPC}}$ and *bla*$_{\text{NDM}}$. The high rate of carbapenem resistance in *A. baumannii* became a serious threat worldwide. Concerning phenotypic tests, mCIM was the most sensitive compared to MHT and CDT. This study established that *bla*$_{\text{OXA-23}}$ and *bla*$_{\text{OXA-51}}$ have been the most prevalent among class D carbapenemase, and *bla*$_{\text{VIM}}$ among class B carbapenemase. The present study suggests that there might be silent carbapenemase genes in carbapenem-sensitive strains.

Keywords: *Acinetobacter baumannii, Carbapenem Resistance, Metallo-beta-lactamase, Oxacillinases, Modified Carbapenem Inactivation Method.*

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I. INTRODUCTION

Acinetobacter baumannii is a gram-negative coccobacillus bacterium which is a very common cause of nosocomial infections (Martin-Aspas *et al.*, 2018). *A. baumannii* is well-known for causing hospital-acquired infections like UTIs, surgical-site infections, secondary meningitis and ventilator-associated pneumonia. Though all hospitalised patients are susceptible to getting such infections, the immunocompromised—particularly those admitted to intensive care units (ICU) or burn units—are more prone to infections (Munoz-Price and Weinstein, 2008; Peleg *et al.*, 2008; Almasaudi, 2018).

Carbapenems are broad-spectrum β-lactam antibiotics which include meropenem, imipenem, doripenem and ertapenem. Carbapenems is regarded as a treatment for infection that is caused by multidrug-resistant *A. baumannii* (Falagas *et al.*, 2006). Carbapenem-resistant *Acinetobacter baumannii* (CRAB) was recently deemed as the most dangerous bacteria for public health, ranked at the top of the worldwide priority list of antimicrobial-resistant pathogens by the WHO (Tacconelli *et al.*, 2017). The most common carbapenem resistance mechanism is the enzymatic hydrolysis of antibiotics. Carbapenemases are classified into three Ambler classes: class A (KPC and GES), class B (VIM, SIM, IMP, and NDM) and class D (OXA or CHDLs) β-lactamases. They have evolved and rapidly spread around the world. Carbapenem-hydrolysing class D β-lactamase (CHDL) or oxacillinases could be intrinsic (OXA-51 like) or acquired (OXA-23 like, OXA-58 like, OXA-24 like, and OXA-143 like) (Evans and Amyes, 2014). The presence of an insertion sequence upstream of oxacillinase leads to its over-expression and confers a high carbapenem resistance level to the *A. baumannii* (Turton *et al.*, 2006).

The presence of carbapenemase must be determined in order to assess problem severity and to guide the use of the anti-microbial stewardship recommendations so as to prevent the spread of

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carbapenem-resistant variations amongst the isolates of the *A. baumannii*. The aim of this study is to detect phenotypic and molecular characterisation of carbapenem-resistance in *A. baumannii* in different isolates in the Iraqi Kurdistan region using phenotypic methods and conventional PCR.

II. Materials And Methods

A. Bacterial isolates

In this cross-sectional study, 486 clinical specimens were collected from patients that were admitted to four hospitals in Duhok province over a period of six months (2021-2022). The samples have been obtained from various clinical specimens: urine (198), wounds (95), sputum (112), and blood (81). The bacterial identification was based upon colony morphology on MacConkey agar, Gram staining and biochemical tests (i.e., catalase and oxidase). Isolates have been confirmed by VITEK2 ID-GNB (bioMerieux, France) assay that performed in Golan General Hospital/Akre. Furthermore, 16s rRNA housekeeping gene was amplified using molecular method to identify *A. baumannii* isolates. All of the isolates have been tested for carbapenem resistance by the approach of disc diffusion utilizing the imipenem (10 µg) and meropenem (10 µg) discs on Muller Hinton agar. The results have been interpreted by the determination of minimal inhibitory concentration based on the guidelines of the CLSI (Wayne, 2021).

B. Phenotypic detections of carbapenemase enzyme

Modified Hodge test (MHT): which represents a simple phenotypic test for secerning the presence of carbapenemase enzyme in the bacteria. The test was performed as it has been described by Benmahmod *et al*. (2019). A suspension of McFarland Standard 0.5 of indicator organism (*E. coli* ATCC 25922) has been prepared in the broth or saline, then diluted to 1:10. The suspension was swabbed onto Mueller-Hinton agar (MHA) plates and allowed to dry for several minutes. A meropenem disc (10 µg) has been added to the center of the plate. From the edge of the disc, *A. baumannii* has been streaked in a straight line to the exterior of the plate. The plates have been incubated overnight at a temperature of 37℃. The presence of growth indicated a positive result.

Modified carbapenem inactivation method (mCIM): A novel phenotypic test for the detection of the carbapenemase enzyme, which has been performed along CLSI guidelines (Wayne, 2021). Four to five pure *A. baumannii* colonies isolated from a fresh blood agar plate were added to a tube containing 2ml of tryptone soya broth (TSB). A meropenem disc (10 µg) has been inserted into the suspension and incubated at 37 ºC for 4 hrs. Prior to completing the 4-hour bacterial suspension, a McFarland Standard 0.50 suspension of *E. coli* ATCC 25922 (indicator organism of mCIM that is susceptible strain for the meropenem disc) was prepared, then inoculated on an MHA plate. Plates were allowed to dry. The meropenem disc has been removed from the TSB-meropenem suspension tube by the use of a sterile inoculating loop. The disc was placed on a previously inoculated MHA plate. Plates have been incubated overnight at a temperature of 37°C. Following incubation, the inhibition zone has been measured. When the diameter was 6mm – 15mm or 16mm – 18mm with small colonies in the inhibitory zone, the mCIM has been considered positive.

Combined disc test (CDT): A screening test for detection of the production of Metallo-beta-lactamase enzyme in bacteria. Performed as described by Abouelfetouh *et al*. (2019). A McFarland Standard 0.5 suspension of *A. baumannii* was prepared and then diluted to 1:10. The diluted suspension has been swabbed on an MHA plate. Two imipenem (10 µg) discs were added to the plate 20mm apart, and 10µl of 0.5M EDTA was added to one of the Imipenem discs. The plates were incubated overnight at a temperature of 37ºC. The result was considered positive in the case where the zone of the inhibition around the imipenem/EDTA disc increased by ≥ 7mm in comparison with the other imipenem disc.

Table 1: Primer sequences and their amplicon size.

Genes	Nucleotide sequence (5'-3')	Size (bp)	Reference
16S rRNA	CAGCTCGTGTCGTGAGATGT CGTAAGGGCCATGATGACTT	150	Ghaima *et al*., 2016
bla$_{OXA-51}$	TAATGCTTTGATCGGCCTTG TGGATTGCACTTCATCTTGG	353	Woodford *et al*., 2006
bla$_{OXA-23}$	GATCGGATTGGAGAACCAGA ATTCTTGACCGCATTTCCAT	501	Woodford *et al*., 2006
bla$_{VIM}$	GATGGTGTTTGGTCGCATA CGAATGCGCAGCACCAG	390	Ellington *et al*., 2006
bla$_{NDM}$	CACCTCATGTTTGAATTCGC CTCTGTCACATCGAAATCGC	984	Poirel *et al*., 2010
bla$_{KPC}$	GTATCGCCGTCTAGTTCTGC GGTCGTGTTTCCCTTTAGCC	637	Hong *et al*., 2012
IS*Aba1*	GTGCTTTGCGCTCATCATGC CATGTAAACCAATGCTCACC	430	Poirel and Nordmann, 2006

C. Genotypic detection of carbapenemase genes and insertion sequence

All isolates have been stored at deep freezing in 50% glycerol in brain heart infusion broth for genetic stability. DNA extraction was done by the boiling approach (Dashti *et al*., 2009; Adzitey *et al*., 2013). Several colonies from fresh and pure culture were added to 300 µl of deionised water. The suspension was incubated for 20 min at 100℃ then frozen at a temperature of -20℃ for 5min. samples were centrifuged at 14,000rpm for 10min. The supernatant was stored at -20℃. DNA purity and concentration have

been checked by Nanodrop (Thermofisher, UK). PCR was performed for the identification of class A (*bla*$_{KPC}$), class B (*bla*$_{NDM}$ and *bla*$_{VIM}$) and class D carbapenemase-encoding genes (*bla*$_{OXA51}$ and *bla*$_{OXA-23}$), and insertion sequence (ISAba1). Primers that have been utilized in the present study are described in Table1. An amount of 20µl of the PCR reaction was prepared to contain 10 µl of *Taq* master 2x, 2µl DNA template, 1 µl of every forward and reverse primer and 6µl deionised water. The amplification condition is explained in Table2. PCR products have been subjected to electrophoresis in 1.5% agarose gel containing SYBR green dye. The amplicons were visualised under UV light after running at 80 volts for 45 minutes.

D. Ethical considerations

The Duhok Health Directory and the research ethical committee of Duhok Polytechnic University approved this project (Reference number: 18082021-8-19).

E. Statistical analysis

Data has been analyzed with statistical packaging for asocial science program (IBM SPSS Statistics Data Editor, v. 26). Frequency and percentage were used to indicate the results.

III. RESULTS

A. Bacterial isolates

Throughout this study, a total of 49 (10.1%) samples have been recovered from hospitalised patients. The frequency of samples obtained from urine was 12 (24.4%), sputum 15 (30.6%), blood 9 (18.4%) and wounds 13 (26.5%). All of them were confirmed by VITEK 2 Systems. The initial test for carbapenem resistance showed 96% and 94% resistance against imipenem and meropenem according to CLSI criteria.

Table 2: PCR amplification conditions.

Genes	Initial denaturation	Denaturation	Annealing	Extension	Final extension
	1 cycle	30 cycles			1 cycle
16s rRNA	95°C/ 3min	95°C/ 1 min	55°C/1 min	72°C/1 min	72°C/ 5min
bla$_{OXA-51}$ *bla*$_{OXA-23}$	95°C/ 1min	95°C/ 15 s	52°C/15 s	72°C/10s	72°C/ 10min
bla$_{VIM}$ IS*Aba1*	94°C/ 5min	94°C/ 40 s	54°C/1 min	72°C/2min	72°C/ 5min
bla$_{kpc}$	94°C/ 5min	94°C/ 40 s	58°C/1 min	72°C/2min	72°C/ 5min
bla$_{NDM}$	94°C/ 5min	94°C/ 40 s	54°C/1 min	72°C/2 min	72°C/ 5min

B. Phenotypic detection

Production of carbapenemase enzyme was evaluated through three different phenotypic screening tests (MHT, mCIM, and CDT). Out of 49 *A. baumannii* isolates, the highest frequency was detected via mCIM test (48; 98%), followed by MHT test (46; 94%), while only 29 isolates (59.2%) were positive for CDT.

C. Molecular detection

A total of 49 (100%) isolates have been confirmed as *A. baumannii* using the PCR approach relying on the presence of the species-specific gene of 16s rRNA gene for *A. baumannii* with a 150-bp amplicon size (Fig. 1). The positive rate of carbapenemases *bla*$_{OXA-51}$ were 100%, *bla*$_{OXA-23}$ and IS*Aba1*, were 98%, *bla*$_{VIM}$ were 71%, and *bla*$_{NDM}$ and *bla*$_{KPC}$ were 0%. Out of the six genes examined, two genes (*bla*$_{NDM}$ and *bla*$_{KPC}$) have not been identified in any of the investigated isolates.

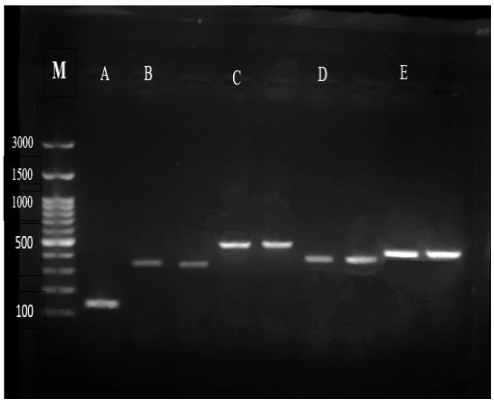

Figure 1. Molecular detection of genes encoding carbapenemase and the IS*Aba1* insertion sequence among *A. baumannii*. M: DNA ladder; lane A: 16s rRNA (150bp); B: *bla*$_{OXA-51}$ (353bp); C: *bla*$_{OXA-23}$ (501bp); D: *bla*$_{VIM}$ (390bp); E1 and E2: IS*Aba1* (430bp).

D. Sensitivity of phenotypic tests

The sensitivity of phenotypic tests was evaluated. mCIM was the most sensitive method (98%) to detect carbapenemase production but could not differentiate between the kinds of carbapenemase, followed by MHT (91.8%) and CDT (55.1%).

IV. DISCUSSION

Carbapenems were medications of choice for the treatment of infections that are caused by the β-lactam-resistant *A. baumannii*, but their previous use has led to improved incidence of carbapenem resistance throughout the last years (Nordmann and Poirel, 2019). The presence of carbapenem-resistant bacterial pathogens is mostly accompanied by antibiotic failure, leading to significant morbidity and mortality (Makharita *et al.*, 2020).

Carbapenem resistance acquisition in *A. baumannii* is usually associated with the production of carbapenemases, the most common of which are CHDLs, followed by MBLs like VIM, IMP and NDM (Walsh *et al.,* 2005; Walther-Rasmussen and Høiby, 2006).

The present study used different phenotypic tests—which are economical and easy to perform—to evaluate carbapenemase production in *A. baumanmii* in 49 isolates obtained from different clinical specimens. In addition, PCR has been utilized to detect some carbapenemase-encoding genes and one insertion sequence and was compared to phenotypic tests to evaluate their sensitivity.

This study found resistance to imipenem (96%) and meropenem (94%) by the disc diffusion approach. Clos to this study's findings, a previous study in Dohuk by Qader (2021) reported that 80% and 90% of isolates exhibited resistance to imipenem and meropenem, respectively. However, Smail and AL-otrachi (2020) found a higher rate of resistance (100%) to the above-mentioned antibiotics in a study performed in Erbil.

Class D carbapenemase gene *bla*$_{\text{OXA-51}}$ has been detected in all *A. baumannii* isolates, while 98% of isolates harboured *bla*$_{\text{OXA-23.}}$ *bla*$_{\text{OXA-51}}$ is intrinsic to *A. baumannii* and naturally present in chromosomal DNA of all strains (Turton *et al.,* 2006), while the *bla*$_{\text{OXA-23}}$ gene is transferable and its production by *A. baumannii* is enough to promote carbapenem resistance (Héritier *et al.,* 2005). *bla*$_{\text{OXA-51}}$ and *bla*$_{\text{OXA-23}}$ were previously reported as the main mechanisms of resistance to imipenem in *A. baumannii* (Mohajeri *et al.,* 2013).

The present study established that the *bla*$_{\text{OXA-23}}$ gene is the most prevalent of the carbapenemase genes in *A. baumannii*, occurring in 98% of isolates, in comparison with other screened genes as reported in earlier studies in Erbil (92%) (Ganjo *et al.,* 2016), Iran (90.8%) (Rezaei *et al.,* 2018), Turkey (94.2%) (Asgin *et al.*, 2019) and Baghdad (90.74%) (Tawfeeq *et al.,* 2020). In a previous study, Hou and Yang reported that only (48.38%) and (46.31%) of isolated strains harbored *bla*$_{\text{OXA-51}}$ and *bla*$_{\text{OXA-23}}$ respectively which were disagreement with study findings (Hou and Yang, 2015). The inconsistency between studies may be due to differences in samples selection and gene variations in different ethnicities as well as sample size.

IS*Aba1* plays a significant role in providing strong promoter activity when its located upstream of *bla*$_{\text{OXA-23}}$, promoting carbapenem resistance (Segal *et al.,* 2007). In this study, IS*Aba1* was positive in all *bla*$_{\text{OXA-23}}$-positive isolates. This finding was similar to previous studies from Iran (Tafreshi *et al.,* 2019), and Baghdad (Ridha *et al.,* 2019). While the finding was disagreement with study by Avila-Novoa *et al.* (2019) which reported rate of 33.3%.

Additionally, Class B carbapenemase-encoding gene *bla*$_{VIM}$ was detected in 71% of isolates, making it the most prevalent MBL. The rapid spread of this gene in the isolates of *A. baumannii* is probably the result of the location of this gene on the plasmid, resulting in transfer through the plasmid to other susceptible bacteria and becoming resistant strains (Hujer *et al.,* 2006). Similar results have been reported in a study in Thi-Qar-Iraq which reported *bla*$_{\text{VIM}}$ in 65% of *A. baumannii* isolates (Kadom and Abid, 2020). Furthermore, another study in Baghdad reported 87.9% (Abbas-Al-Khafaji *et al.,* 2021), which is compatible with this study's results. However, this finding is in disagreement with research that has been carried out in Sulaymaniyah, which reported that 11.4% of isolates contained *bla*$_{\text{VIM}}$ (Ali *et al.,* 2019). *bla*$_{\text{NDM}}$ has not been detected in any of our isolates; this result was in agreement with research in Sulaymaniyah (Ali *et al.,* 2019). While a study in India was in disagreement with this finding, reported NDM in 18.8% of isolates (Rahman *et al.,* 2018).

Genes encoding class A carbapenemases, like *bla*$_{\text{KPC}}$, have also not been found in any of our isolates. These findings were consistent with previous research in Kufa-Iraq (Tuwaij, 2016) and Baghdad (Ghaima, 2018), but disagreed with a study in Erbil (Haji *et al.,* 2021) that recorded KPC in its isolates.

This is likely the first report of carbapenemase genes in clinical isolates of *A. baumannii* in Duhok province, Iraq. It is important to highlight that this study found carbapenemase genes in meropenem- and imipenem-sensitive strains. This may hold different explanations: first, this study tested imipenem and meropenem only to detect the carbapenem susceptibility against isolated *A. baumannii*. The isolates may be resistant to other carbapenem generations like ertapenem and doripenem as has been described by Ali *et. al*. (2019). Second, the presence of silenced genes that could not be screened by the disc diffusion approach. Third, the sensitive strains may have had false positive results that were examined by the disc diffusion approach.

A comparison of PCR results to phenotypic test results showed that the sensitivity of mCIM, MHT and CDT were 98%, 91.8% and 55.1%, respectively. The ability of MHT to detect carbapenemase-producing *A. baumannii* isolates was 94%. This finding is comparable with a study conducted in Baghdad that recorded a high rate (91.1%) (Ridha *et al.,* 2019). A study conducted in Iraq disagreed with this study's findings, recording 40% (Al Meani *et al.,* 2020). The ability of CDT to diagnose MBL isolates was only 59.2% in the current study. Our findings concur with a study conducted in Sulaymaniyah in which CDT was able to detect MBL in 26 isolates (59%) (Ali *et al*., 2019). Another study in Egypt (El-Hady *et al*., 2021) reported 44.4%. In the current study, the *bla*$_{VIM}$ gene has not been detected in seven isolates that showed a positive result for CDT, which might be a result of the presence of other MBL genes such as *bla*$_{\text{SIM,}}$ *bla*$_{\text{IMP}}$ and *bla*$_{\text{SPM}}$. Additionally, the CDT test failed to detect *bla*$_{\text{VIM}}$ in 14 strains, possibly due to the low level of carbapenemase

activity. Only 25 (n = 49) of the strains displayed positive for all phenotypic tests. The carbapenemase enzyme was detected in 98% of the tested isolates using the mCIM test. Only one isolate was recorded as a false negative result that carried *bla*$_{\text{OXA-51}}$, *bla*$_{\text{OXA-23}}$, and *bla*$_{\text{VIM}}$. This indicate that this method is more sensitive than other phenotypic approaches. Our finding was in agreement with previous studies in Iraq (100%) (Al Meani *et al.*, 2020), and Kuria (100%) (Vu *et al.*, 2020).

V. Conclusion

An increase in carbapenem-resistant *A. baumannii* is a serious challenge worldwide. The present study ascertained high rates of carbapenem resistance in *A. baumannii*, which was mostly caused by carbapenemase-encoding genes on plasmids. Furthermore, proper infection control practices and anti-microbial programs are needed to minimize the spread of these bacteria. In this paper, mCIM showed excellent sensitivity for the detection of carbapenemase compared to MHT and CDT. Our results recommend the use of this method—which is economical, precise, and easy to perform—for early detection of carbapenemase enzymes. This study reported a high prevalence of *bla* $_{\text{OXA-51}}$, *bla* $_{\text{OXA-23}}$ and *bla* $_{\text{VIM}}$ genes among isolated *A. baumannii*, which is a serious concern. This study did not detect any *bla*$_{\text{NDM}}$ and *bla*$_{\text{KPC}}$ genes among isolates; more research is required for larger screening. The present study suggests that some carbapenem-sensitive strains may have unexpressed carbapenemase genes.

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