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* _OXA-23_ has been observed in all isolates. We found *bla* _OXA-23_ in 98% of isolates. Insertion sequence IS _Ab1_ was present in all positive *bla* _OXA-23_ strains (98%). A *bla* _VIM_ gene encoding MBL was present in 71% of isolates, but none of the isolates has been positive for *bla* _KPC_ and *bla* _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* _OXA-23_ and *bla* _OXA-51_ have been the most prevalent among class D carbapenemase, and *bla* _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.*

**I. INTRODUCTION**

*Acinetobacter baumannii* is a gram-negative cocccobacillus 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...
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-GBN (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 discerning 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. The plates have been incubated overnight at a temperature of 37°C. The presence of growth 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 37°C. The result was considered positive in the case where the zone of the inhibition around the imipenem/EDTA disc increased by ≥ 7 mm in comparison with the other imipenem disc.

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 (Dushiti 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°C then frozen at a temperature of -20°C for 5min. samples were centrifuged at 14,000rpm for 10min. The supernatant was stored at -20°C. DNA purity and concentration have

Table 1: Primer sequences and their amplicon size.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Nucleotide sequence (5'-3')</th>
<th>Size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA</td>
<td>CAGCTCGTGTCTGTAGATGTGTAAAGGCGCATGATGCTT</td>
<td>150</td>
<td>Ghaima et al., 2016</td>
</tr>
<tr>
<td>bladec</td>
<td>TAATGGCTTGAATGCGCCTTGGTGGATTGCACTTACCTG</td>
<td>353</td>
<td>Woodford et al., 2006</td>
</tr>
<tr>
<td>bladec</td>
<td>GATTCGAGTTGAGGAAACGAATTCTTAAGGAGCATTTCATC</td>
<td>501</td>
<td>Woodford et al., 2006</td>
</tr>
<tr>
<td>bladec</td>
<td>GATGGGTATTGCTAGCATACTTGTAGCCGACACCAG</td>
<td>390</td>
<td>Ellington et al., 2006</td>
</tr>
<tr>
<td>bladec</td>
<td>CACTCAATGTTTGAACCTCGCTCTGTCACTGACAATGACCC</td>
<td>984</td>
<td>Poirel et al., 2010</td>
</tr>
<tr>
<td>bladec</td>
<td>GTATCCGGCGCTAGTTAGCGGCTGTTCTTCCTTGAGGCC</td>
<td>637</td>
<td>Hong et al., 2012</td>
</tr>
<tr>
<td>ISAba1</td>
<td>GTGGTTTGCGCTCATGCTGACCATATGTCATGC</td>
<td>430</td>
<td>Poirel and Nordmann, 2006</td>
</tr>
</tbody>
</table>
been checked by Nanodrop (Thermofisher, UK). PCR was performed for the identification of class A (blaKPC), class B (blaNDM and blaVIM) and class D carbapenemase-encoding genes (blaOXA51 and blaOXA23), and insertion sequence (ISAba1). Primers that have been utilized in the present study are described in Table 1. An amount of 20µl of 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 Table 2. 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.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Initial denaturation</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Extension</th>
<th>Final extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>16s rRNA</td>
<td>95°C/1 min</td>
<td>95°C/1 min</td>
<td>55°C/1 min</td>
<td>72°C/1 min</td>
<td>72°C/5 min</td>
</tr>
<tr>
<td>blaOXA51</td>
<td>95°C/15 s</td>
<td>95°C/15 s</td>
<td>52°C/15 s</td>
<td>72°C/10 s</td>
<td>72°C/10 min</td>
</tr>
<tr>
<td>blaOXA23</td>
<td>95°C/1 min</td>
<td>95°C/1 min</td>
<td>55°C/1 min</td>
<td>72°C/2 min</td>
<td>72°C/5 min</td>
</tr>
<tr>
<td>blaVIM</td>
<td>94°C/40 s</td>
<td>94°C/40 s</td>
<td>54°C/1 min</td>
<td>72°C/2 min</td>
<td>72°C/5 min</td>
</tr>
<tr>
<td>ISAba1</td>
<td>94°C/40 s</td>
<td>94°C/40 s</td>
<td>58°C/1 min</td>
<td>72°C/2 min</td>
<td>72°C/5 min</td>
</tr>
<tr>
<td>blaKPC</td>
<td>94°C/40 s</td>
<td>94°C/40 s</td>
<td>54°C/1 min</td>
<td>72°C/2 min</td>
<td>72°C/5 min</td>
</tr>
<tr>
<td>blaNDM</td>
<td>94°C/40 s</td>
<td>94°C/40 s</td>
<td>54°C/1 min</td>
<td>72°C/2 min</td>
<td>72°C/5 min</td>
</tr>
</tbody>
</table>

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 blaOXA51 were 100%, blaOXA23 and ISAba1, were 98%, blaVIM were 71%, and blaNDM and blaKPC were 0%. Out of the six genes examined, two genes (blaNDM and blaKPC) have not been identified in any of the investigated isolates.

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

The present study established that the \( \text{bla}_{\text{OXA-23}} \) 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 \( \text{bla}_{\text{OXA-51}} \) and \( \text{bla}_{\text{OXA-23}} \) 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.

\( \text{ISAba}1 \) plays a significant role in providing strong promoter activity when its located upstream of \( \text{bla}_{\text{OXA-23}}, \) promoting carbapenem resistance (Segal et al., 2007). In this study, \( \text{ISAba}1 \) was positive in all \( \text{bla}_{\text{OXA-23}} \)-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 \( \text{bla}_{\text{VIM}} \) 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 \( \text{bla}_{\text{VIM}} \) 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 \( \text{bla}_{\text{VIM}} \) (Ali et al., 2019). \( \text{bla}_{\text{NDM}} \) 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 \( \text{bla}_{\text{KPC}}, \) 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 \( \text{bla}_{\text{VIM}} \) 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 \( \text{bla}_{\text{SIM}}, \) \( \text{bla}_{\text{IMP}} \) and \( \text{bla}_{\text{SHV}}. \) Additionally, the CDT test failed to detect \( \text{bla}_{\text{VIM}} \) 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 \( \text{bla}_{\text{OXA-51}}, \text{bla}_{\text{OXA-23}}, \) and \( \text{bla}_{\text{VIKM}} \). 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 \( \text{bla}_{\text{OXA-51}}, \text{bla}_{\text{OXA-23}} \) and \( \text{bla}_{\text{VIKM}} \) genes among isolated A. baumannii, which is a serious concern. This study did not detect any \( \text{bla}_{\text{OXM}} \) and \( \text{bla}_{\text{KPC}} \) genes among isolates; more research is required for larger screening. The present study suggests that some carbapenem-sensitive strains may have unexpressed carbapenemase genes.

REFERENCES


