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Research Article

# Detection of Plasmid-Mediated *blaNDM1* and *blaNDM2* Genes in Clinical Isolates of *Acinetobacter baumannii* from Iraqi Patients

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## ABSTRACT

The widespread of *Acinetobacter baumanniiis* concerning. The horizontal gene's transfer of this bacteria is crucial in acquiring unique traits like antibiotic resistance, which has been related to a significant increase in infection and fatality rates in patients. The New Delhi carbapenemase enzyme, which hydrolyzes all-lactam antibiotics (except aztreonam), including the broad-spectrum carbapenem antibiotic, is one of the most therapeutically significant carbapenemes. NDM (New Delhi metallo-beta lactamase) is a carbapenem enzyme that efficiently hydrolyzes lactams; as a result, NDM-producing bacteria only have a few treatment alternatives. VITEK-identified multidrug-resistant (MDR) *A. baumannii* isolates from Iraqi pneumonia patients were included in the investigation. Using 0.7 percent agarose gel electrophoresis, each isolate was checked for the presence of plasmid(s). Using the polymerase chain reaction technique, positive isolates were submitted to genetic identification of plasmid containing blaNDM1 and blaNMD2. The presence of the plasmid was discovered in all the isolates tested. NDM1-R1 enzyme was found to be positive in 61.5 percent of isolates, NDM1-R2 enzyme in 61.5 percent of isolates, and the NDM-2 enzyme in 61.5 percent of isolates. In addition, 38.4 percent of the participants tested negative for both enzymes. The importance of plasmid-mediated horizontal gene transfer in the acquisition of multiple drug resistance in MDR *A. baumannii* is highlighted by these findings. It is the first time to detect plasmid-mediated *blaNDM1* and *blaNDM2* genes in MDR *A. baumannii* in Iraq. The purpose of this study was to determine the incidence of NDM variants, metallo-β-lactamases, among *A. baumannii* isolates from diverse clinical samples in Iraq

**Keywords:** *Acinetobacter baumannii*, Plasmid, *NDM-1, NDM-2*

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## INTRODUCTION

*Acinetobacter baumanniiis* a Gram-negative, non-fermenting, aerobic, and nonmotile coccobacillus (Siroy A, *et al*., 2005). Currently, this bacterium is a typical occurrence in intensive care units and quickly became resistant to most antibiotics. Numerous outbreaks caused by this organism have been reported globally, (Huang YC, *et al*., 2002; Chan PC, *et al*., 2007; Hammerum AM, *et al*., 2015; Perez F, *et al*., 2007) including India, Egypt, and China (Chen Y, *et al*., 2011; Kaase M, *et al*., 2011; Karthikeyan K, *et al*., 2010).

*Acinetobacter* can cause Infections of the urinary tract, wounds, and burns (Fishbain J and Peleg AY, 2010). 3%-5% of nosocomial pneumonia cases are caused by it and are most common among patients in intensive care units (Dijkshoorn L, *et al*., 1993).

Due to *A. baumannii*'s proclivity to develop genes for resistance (Corbella X, *et al*., 2000), these infections are difficult to treat which yielded a multidrug-resistant (MDR) strain.1 This resistance developed as a result of the overuse of antibiotics (Peleg AY, et l., 2008). It is suggested that carbapenems can be used in treating patients and saving their lives (Gao L, *et al*., 2017). Carbapenems are the chosen medication to treat this pathogen (Costa LMD, *et al*., 2013).

The New Delhi metallo-β-lactamase 1 (bla*NDM-1*), also known as the carbapenem resistance gene, (Boulanger A, *et al*., 2012) was reported from a 59-year-old Swedish male in India. The man was hospitalized first in Punjab and then in New Delhi (Johnson AP and Woodford N, 2013). In January 2008, he was repatriated to Sweden, where an isolate of *Klebsiella pneumoniae*, which showed resistance to multiple antibiotics such as carbapenems, was obtained from his urine culture on the day after admission (Yong D, *et al*., 2009).

Carbapenem resistance was linked to bacteria's ability to propagate *via* mobile genetic elements, according to the phenotypic examination of isolates (Miriagou V, *et al*., 2010). The ISAba125 element, which is found upstream of the *NDM-1* gene, may enhance the rapid dispersion of the *NDM-1* gene; it is one of several factors that can influence NDM transmission (Poirel L, *et al*., 2012).

Carbapenem resistance is caused by a variety of causes. First, it is through the formation of hydrolyzing carbapenem-β-lactamases by *A. baumannii* (Hornsey M, *et al*., 2011). β-lactamases are enzymes produced by some of these bacteria that can make them resistant to various classes of β-lactam antibiotics, the main treatment for these infections. In the mid-1980s, a new group of these enzymes was detected, the extended-spectrum β-lactamases (ESBLs), which confer resistance to expanded-spectrum cephalosporins but not to carbapenems. Carbapenems are primarily used for treating infections due to ESBL-producing Enterobacteriaceae. These particular groups of β-lactamases are classified as class B (metallo-β-lactamases (MBLs)-like) and class D (oxacillinases), including *OXA-23*-like, *OXA-24/40*-like, and *OXA-58* (Ambler RP, *et al*., 1991). Second, it is through alterations in porin permeability in the outer membrane of the microorganism, in addition to efflux pumps and modifications in penicillin-binding protein affinity (Abbott I, *et al*., 2013). The bla*NDM-1* gene encodes an *NDM-1* enzyme that is resistant to all-lactam antibiotics, including carbapenems, which have been discovered in Enterobacteriaceae. (*Klebsiella pneumoniae* and *Escherichia coli*) (Yong D, *et al*., 2009; Kumarasamy KK, *et al*., 2010) as well as in *A. baumannii*, where the bla*NDM-1* and bla*NDM-2* genes were recently discovered (Espinal P, *et al*., 2013). The rapid evolution of bla*NDM-1* has been linked to a mobile plasmid that may be

passed from one bacteria to the next, from one person to the next, and even from one country to the next (Fallah F, *et al*., 2011).

The first *NDM-2*-producing *A. baumannii* isolate was discovered in the urine of an Egyptian woman brought to Tawam Hospital (Al Ain, UAE) in May 2009, after she had been hospitalized for cancer treatment in Cairo and Beirut several times the previous year. The second group of isolates was discovered in July 2009 during a thorough surveillance investigation conducted on patients admitted to two wards of the Tel Aviv-based TA-Sourasky-MA Rehabilitation Hospital (the Sourasky Medical Center) (Espinal P, *et al*., 2013).

Kaase M, *et al.* (Kaase M, *et al*., 2011) recently described an *NDM-2* variation (proline to alanine substitution at position 28). This allele was originally discovered in an MDR *A. baumannii* strain isolated from a German patient who had previously been hospitalized in Egypt (Kaase M, *et al*., 2011), followed by another isolate from Israel.

These new findings inspired us to be the first to explore and investigate the transferable plasmid, which is thought to play a role in the dissemination of *A. baumannii* since many Iraqi patients have traveled to India and other nations for medical treatment in recent years, which may have aided in the acquisition of this gene.

## MATERIALS AND METHODS

### *Bacteria collection and identification*

*A. baumannii* sample was collected from 39 sputum and bronchioalveolar lavage samples taken from patients suffering from pneumonia, admitted to Al-Yarmuk hospital from September 2019 until February 2020. Bacteria identification was achieved using the VITEK system. All confirmed specimens were cultured on Mueller-Hinton agar plates to test their susceptibility using the Kirby-Bauer disk diffusion method according to the described guidelines, which were suggested by the Clinical and Laboratory Standards Institute (CLSI).

The used antibiotics were ampicillin (MIC ≥ 32 mg/l), aztreonam and ceftriaxone (MIC ≥ 64 mg/l), gentamicin (MIC ≥ 16 mg/l), ciprofloxacin (MIC ≥ 4 mg/l), imipenem (MIC ≥ 16 mg/l), levofloxacin (MIC ≥ 8 mg/l), tigecycline (MIC ≥ 1 mg/l), and colistin (MIC ≥ 1 mg/l).

Identified bacteria were then stored at -80°C in nutrient broth with 20% glycerol. Isolates were cultured in Luria-Bertani broth for plasmid extraction.

### *Plasmid extraction and molecular detection of* NDM-1 *and* NDM-2 *genes*

Extraction of plasmid from the isolates was done according to the manufacturer's instructions (Wizard plus SV MInipreps DNA purification system, Promega, USA) with modification so that the elution buffer was minimized in the last step of plasmid DNA extraction to 35 µl. For plasmid detection, 5 µl of plasmid extract was mixed with 2 µl of loading dye (Promega, USA) and resolved on 0.7% gel electrophoresis, which was run at 70 V for 2 h. Then, the plasmid molecular size was specified according to a ladder, ranging 10,200-500 bp. (Bioneer, Republic of Korea).

For genetic detection of *NDM-1* and *NDM-2* genes, the Polymerase Chain Reaction (PCR) technique (PCR Express, Hybaid, USA) was applied. The primers used are listed in *Table 1*. Master Mix (ambGood, Canada) was applied according to the manufacturer's instructions, and the total volume was 50 µl. Thermocycler conditions applied for both genes are listed in Table 2. PCR products were later resolved by 1.5% gel electrophoresis at 70 V for 1.5 h using a ladder ranging from 2000 to 100 bp (Bioneer, Republic of Korea).

## RESULTS

Confirmed positive bacteria were cultured on Mueller-Hinton agar plates to test their susceptibility using the Kirby-Bauer disk diffusion method; the rate of the resistance was different from one antibiotic to another. For ampicillin, the rate of resistance was 92%; aztreonam, 90%; cefotaxime, 87.2%; gentamicin, 72.5%; imipenem, 46%; levofloxacin, 45.1%; tigecycline, 41.4; and colistin, 27.2%.

The plasmid was detected in all 39 isolates, and the molecular size was more than 10,200 bp (*Figure 1*). PCR was later conducted to detect the presence of bla*NDM1* and bla*NDM2* genes in the plasmid of 39 isolates. The PCR product sizes for both NDM1-R1 and NDM1-R2 were 476 bp and 391 bp, respectively (*Figure 2*). For *NDM-2*, the PCR product size was 946 bp (Figure 3). The results revealed that 24 out of 39 (61.5%) isolates demonstrated positivity for NDM1-R1 and NDM1-R2, while 15 out of 39 (38.4%) isolates showed negative results for these genes. For NDM2-R, 24 out of 39 (61.5%) isolates appeared to have this gene, whereas 15 out of 39 (38.4%) isolates showed negative results (*Table 3*). R1 and R2 (reverse primers): Referring to the used primer, which is a short sequence of single-stranded DNA that marks both ends of the target sequence; the reverse primer attaches to the stop codon of the complementary strand of DNA.

**Table 1: List of primers used to amplify the genes encoding carbapenemases**

| Gene | Primer sequence | Product size (bp) |
|---|---|---|
| *NDM1-R1* | F:5-GGGCAGTCGCTTCCAACGGT-3 | 476 |
| | R:5-GTAGTGCTCAGTGTCGGCAT-3 | |
| *NDM1-R2* | F:5-GGGCAGTCGCTTCCAACGGT-3 | 391 |
| | R:5-GTGCCGTAGCTCCCAACGGT-3 | |
| *NDM2* | F:5-GTCGCAAAGCCCAGCTTCGCA-3 | 945 |
| | R:5-GCCTCGCATTTGCGGGGTTTTTA-3 | |

**Table 2: PCR thermocycling conditions**

| Gene | Initial denaturation | Denaturation | Annealing | Extension | Final extension | Number of cycles |
|---|---|---|---|---|---|---|
| *NDM-1* | 95°C/10 min | 94°C/30 s | 60°C/30 s | 72°C/1 min | 72°C/7 min | 30 cycles |
| *NDM-2* | 95°C/10 min | 94°C/30 s | 62°C/30 s | 72°C/1 min | 72°C/7 min | 30 cycles |

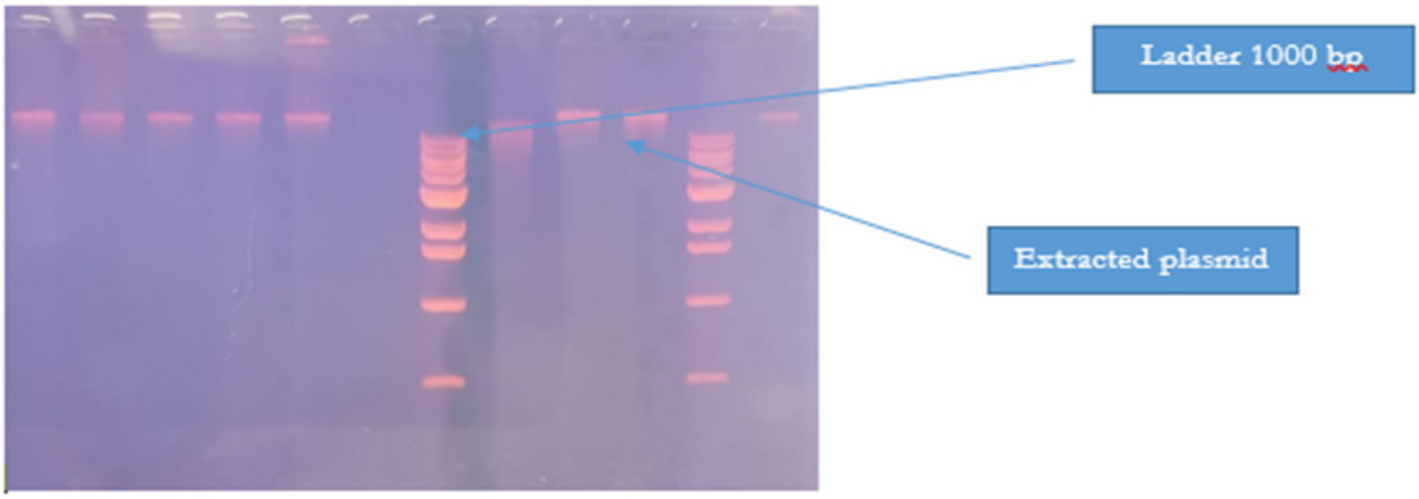


**Figure 1: Gel electrophoresis of plasmids extracted from *A. baumannii* isolates with 10,200 bp size**

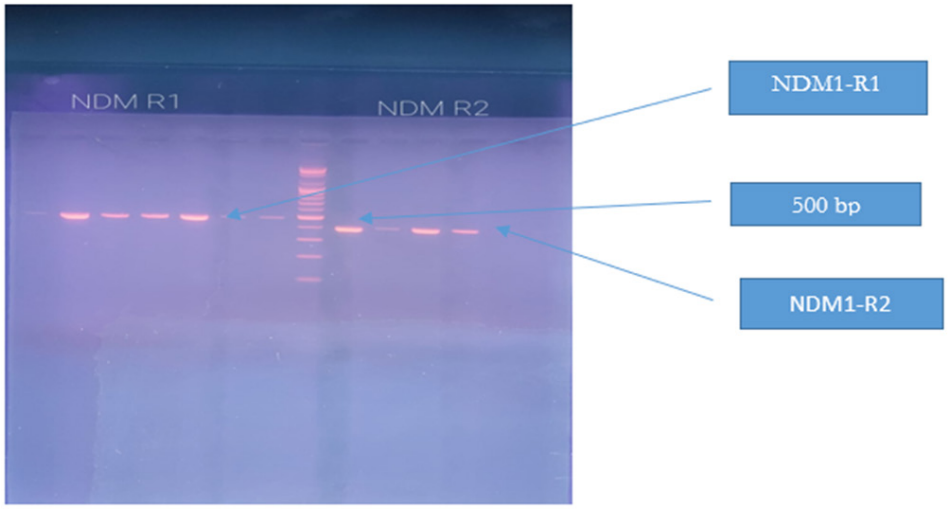


**Figure 2: PCR result of *NDM-1* on 1.5% agarose gel electrophoresis**

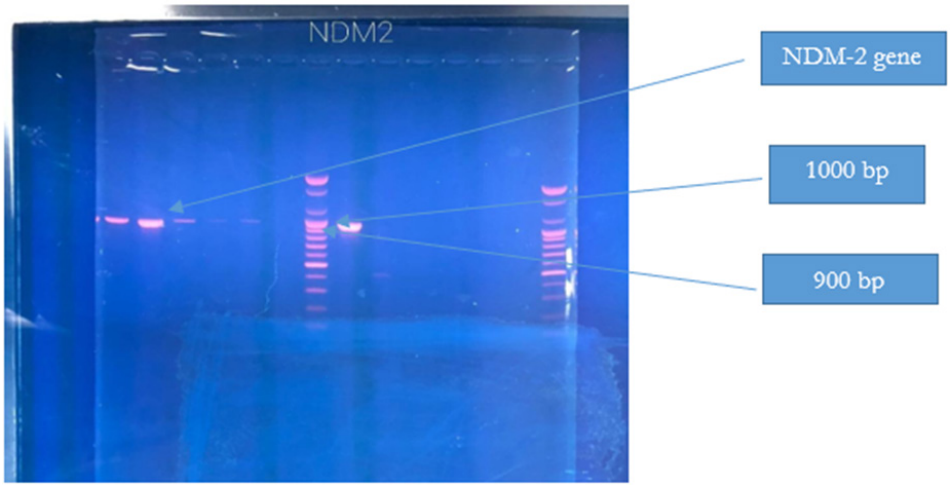


**Figure 3: PCR result of *NDM-2* on 1.5% agarose gel electrophoresis**

**Table 3: Number of positive and negative isolates harboring NDM-1 and NDM-2 genes**

| Gene | No. of positive samples | Positive percentage | No. of negative samples | Negative percentage |
|---|---|---|---|---|
| *NDM1-R1* | 24/39 | 61.50% | 15/39 | 38.40% |
| *NDM1-R2* | 24/39 | 61.50% | 15/39 | 38.40% |
| *NDM-2* | 24/39 | 61.50% | 15/39 | 38.40% |

## DISCUSSION

*A. baumannii*, a prominent nosocomial pathogen, is a serious public health concern, especially in intensive care units. Lactams, aminoglycosides, carbapenems, and fluoroquinolones are among the antimicrobials to which some strains of this bacterium are resistant to (Abbo A, *et al*., 2005). The growing multidrug resistance, which includes colistin and carbapenem, causes concern about this pathogen.

Even though these plasmids play a significant role and crucial part in *A. baumannii* resistance towards the antibiotic, due to their ability to transmit virulence and antimicrobial genes and despite their significant role in the pathogenicity of the bacteria, there has been no research into the role of plasmids in the transmission of *NDM-1* and *NDM-2* genes in Iraq.

Due to the quick disruption of the gene between Enterobacterales and *Acinetobacter* spp., NDM carbapenems have been identified in most parts of the world (Antunes LCS, *et al*., 2014). Only a few studies on *NDM-1*-producing *A. baumannii* have been published in Africa, and those that have been published are largely from northern or southern African countries, as well as Benin. Another study was done in Iraq by Sarah and Suhad in 2018 on *Pseudomonas aeruginosa* to detect these genes (Ismail SJ and Mahmoud SS, 2018). This is the first study on the dissemination of bla*NDM-1* and bla*NDM-2* genes in Al-Yarmuk hospitals among *A. baumannii* isolates (Ismail SJ and Mahmoud SS, 2018).

The results of antibiotic susceptibility exhibited different patterns of resistance to different types of antibiotics. Results showed resistance to at the very least, three types of antibiotics; As a result, the isolates were classified as MDR.

The isolates demonstrated the highest resistance to ampicillin (92%); these results are a little lower than another study conducted by Al-Harmoosh and Jarallah, (Al-Harmoosh RA and Jarallah EM, 2015) where they found that their resistance was 100% due to the wide range use of ampicillin in Yarmuk hospital. The present study also showed their high resistance to aztreonam (90%), and this result is close to another survey done by Alubaidi, *et al.* (Alubaidi GT, *et al*., 2021) where their result was 90.8. Also, the result was 80% in Al-Hilla hospital in a study by Al-Harmoosh and Jarallah, (Al-Harmoosh RA and Jarallah EM, 2015) and there was a moderate resistance to cefotaxime (87.2), which is in accordance with the result of Alubaidi, *et al.* (Alubaidi GT, *et al*., 2021) who noted that the resistance was 87.3, while the result of Al-Harmoosh and Jarallah (Al-Harmoosh RA and Jarallah EM, 2015) was (60%). The development of porin-deficient mutants may be a cause of resistance to cephalosporin antibiotics (Manchanda V and Singh NP, 2013). Furthermore, an increasing number of bacterial strains exhibit various forms of β-lactamases, including inducible and/or plasmid-mediated AmpC enzymes, which may raise the risk of cephalosporin resistance (Al-Harmoosh RA and Jarallah EM, 2015).

The resistance to gentamicin was 72.5, which is lower than the result of Alsehlawi and his colleagues, who found the resistance to be 83.3% (Alsehlawi ZS, *et al*., 2014). Other studies that were done in Thailand by Leepethacharat and Oberdorfer (Leepethacharat K and Oberdorfer P, 2007) showed a result of 60%; their result was in parallel with that of a study conducted in Turkey conducted by Özdemir and his colleagues (Özdemir H, *et al*., 2011). In *A. baumannii*, resistance to gentamicin is frequently caused by the production of modifying enzymes such as aminoglycoside-modifying enzymes (AMEs), acetylases, phosphorlyases, and adenylases, all of which can reduce antibiotic efficacy. Changes in bacterial membrane permeability and ribosomal protein alteration are two further resistance mechanisms (Barros JCS, *et al*., 1999). In the current study, the impedance against ciprofloxacin (quinolone antibiotic) was 68.7, while in a study by Al-Harmoosh and Jarallah, (Al-Harmoosh RA and Jarallah EM, 2015) it was 40%. In China, Zhou and his colleagues discovered that clinical isolates were resistant to ciprofloxacin in a significant percentage of cases (>95%) (Zhou H, *et al*., 2007). Alsehlawi and his colleagues discovered 91.6 percent resistance to ciprofloxacin in another investigation in Najaf (Alsehlawi ZS, *et al*., 2014). Quinolone resistance is caused by mutations in type II topoisomerases that are chromosomally encoded, as well as overexpression of efflux pumps or point-related genes (Drlica K and Zhao X, 1997; Tran JH, *et al*., 2005). The plasmid *qnr* genes are playing an increasingly important role in the spread of fluoroquinolone resistance (Firoozeh F, *et al*., 2014).

A low resistance rate to imipenem was recorded (46.5%), which is convergent with another study done by Talib, *et al.* (Talib ST, *et al*., 2018) while for levofloxacin, it was 45.1%, which is close to the result of Alubaidi, *et al.* (Alubaidi GT, *et al*., 2021) but contrast to the result obtained in a study done in Brazil, (Tognim MCB, *et al*., 1999) where it was 20%, as well as the outcome of Patwardhan's and his colleagues' efforts in India (Patwardhan RB, *et al*., 2008). This could be related to the *A. baumannii*-carrying multi-resistant plasmid (Patwardhan RB, *et al*., 2008). The lowest resistance was to colistin, with a rate of 27.2%, which corresponds with another study in which 97% of the bacteria were sensitive to colistin (Tunyapanit W, *et al*., 2014).

The current study's high levels of antibiotic resistance could be due to both innate and acquired mechanisms. Resistance is widespread, posing a significant clinical risk (Mathur P, *et al*., 2002). Antibiotics are also subjected to selection pressure in the hospital setting, resulting in diverse resistances to these medications. According to El-Astal (El-Astal Z, 2005), the increasing resistance rate of *A. baumannii* to routinely used antimicrobial medications could be due to inadequate and erroneous antimicrobial agent administration, as well as a lack of suitable infection management methods (El-Astal Z, 2005).

Recently, we have witnessed an increase in resistance to imipenem and other antibiotics in our hospitals; this resistance is because of this organism's ability to horizontally acquire resistance deterrents. Resistance manifests itself in a variety of ways, including the production of enzymes such as β-lactamases, which hydrolyze the β-lactam group and carbapenem. AMEs are enzymes that modify aminoglycosides.

In the present study, plasmid with a size of more than 10,200 bp was detected in *A. baumannii*. These results coincide with another study in Baghdad conducted by Alubaidi, *et al.* (Alubaidi GT, *et al*., 2021) in Al-Khadmiya Hospital.

In 2012, in China, isolates from a clinical *Acinetobacter lwoffii* strain were studied (Hu H, *et al*., 2012). According to the researchers, the plasmid had

a complete *Tn125* transposon and had a strong horizontal transferability, where they found one large plasmid with a size of 78,125 bp and three smaller ones with sizes of 4797, 1634, and 7865 bp, respectively (Galata V, *et al*., 2015).

Since then, several bla*NDM-1* positive plasmids have been discovered in *Acinetobacter* spp in China. In *A. baumannii*, bla*NDM-1* was discovered on plasmids ranging in size from 30 to 50 kb in a Chinese investigation. *NDM-1* was discovered predominantly on plasmids in *Acinetobacter* spp in china. China, while it was found on the bacterial chromosomes in Europe and North Africa (Hu H, *et al*., 2012; Zhou Z, *et al*., 2012) Jones *et al.* discovered *NDM-1* on numerous bands ranging in size from 45 to 300 kb in an Indian investigation (Jones LS, *et al*., 2014).

Other studies reported that *A. baumannii* possesses plasmids ranging from as small as 2 kb to more than 100 kb in size (Gallagher LA, *et al*., 2015; Lean SS and Yeo CC, 2017). Due to the inclusion of several antibiotic resistance genes and their self-transmissible merit, *A. baumannii* big plasmids are frequently the focus of the investigation (Hamidian M and Hall RM, 2018). Small plasmids, particularly those harboring antibiotic resistance genes, were emphasized in other studies (Gallagher LA, *et al*., 2015; Lean SS and Yeo CC, 2017).

*A. baumannii* that produces *NDM-1* was first discovered in India (Karthikeyan K, *et al*., 2010) and then in China (Chen Y, *et al*., 2011). Non-baumannii species that produce *NDM-1 Acinetobacter genomospecies* have been documented from nosocomial settings and environmental sources in China, including *Acinetobacter lwoffii, Acinetobacter junii, Acinetobacter pittii, Acinetobacter haemolyticus,* and *Acinetobacter haemolyticus.*

Experiments and molecular research confirmed that the *NDM-1* gene is localized on transferable plasmids of 180 and 140 kb belonging to the pNDM-BJ01-like family, in contrast to *NDM-1*-producing *A. baumannii*, the bla*NDM-2*'s reservoir (Hu H, *et al*., 2012). Plasmids in *K. pneumoniae* and *E. coli* isolates are frequently conjugative, which aids in the transmission of genes across strains of different genera (Johnson AP and Woodford N, 2013; Espinal P, *et al*., 2011).

Following the sequencing of the *NDM-1*-bearing plasmid, a fragment of ISAba125 was discovered just upstream of the *NDM-1* gene (Poirel L, *et al*., 2011). According to several researches, the NDM gene is located between two copies of the ISAba125 element, generating the *Tn125* composite transposon. These transposons can mobilize a wide range of resistance genes, aiding in the spread of antimicrobial resistance (Mahillon J and Chandler M, 1998). These transpositions are one of the most common causes of bacterial DNA rearrangements, which can result in gene expression alterations (Sinha MH, 2004). Transposable elements (ISAba125 element) give the 35 sequences of the hybrid promoter in *A. baumannii*, which is responsible for *NDM* gene production (Poirel L, *et al*., 2011). In *A. baumannii*, the genes *ISAba1, ISAba2, ISAba3, ISAba4*, and IS18 are frequently related with carbapenemase gene expression (Villalón P, *et al*., 2013).

Only one *NDM-1* variant (*NDM-2*) was detected in *A. baumannii* isolates, and it was described in 2011 by Kaase, *et al.* It differed by a single amino acid (Kaase M, *et al*., 2011). Following that, Espinal, *et al.* (Espinal P, *et al*., 2011) sequenced the *NDM* gene, finding a twofold nucleotide alteration from cytosine to guanine at position 82 and alanine to guanine at position 468 from the start codon. Only the first modification, which was earlier published and called *NDM-2*, resulted in an amino acid substitution from P (proline) to A (alanine) at position 28, and the other was a quiet mutation. This variation has also been found in a German patient (who had previously traveled to Egypt) and in a patient from the United Arab Emirates (Ghazawi A, *et al*., 2012). In 2010, *NDM-1* and *NDM-2* were found in *A. baumannii*, and in 2011, NDM-5 was found in *E. coli* (Hornsey M, *et al*., 2011). In 2018, Sarah and Suhad detected *NDM-1* and *NDM-2* among *Pseudomonas aeruginosa* (Ismail SJ and Mahmoud SS, 2018).

In the current study, *NDM-1* and *NDM-2* genes were harbored by a plasmid with a size ranging from approximately 10,200 bp, which can be conjugated and transmitted to *E. coli* Although these investigations were conducted *in vitro*, we could predict that the *NDM* genes were spread from *Acinetobacter* species to Enterobacteriaceae because of this plasmid's interspecies transfer (Bonnin RA, *et al*., 2014).

The first case of an MDR *A. baumannii* strain generating *NDM-1* and *NDM-2* in Iraq is described here. The possibility of variations developing will increase if this bacterium containing the *NDM-1* and *NDM-2* genes continues to spread. This is a crucial factor to consider when developing genetic tools to target carbapenem resistance genes, especially because many Iraqi patients seek treatment in India, which could make getting these genes easier.

We were inspired by these new results to be the first to research and explore the transferable plasmid, which is assumed to play a role in the spread of *A. baumannii*. In recent years, many Iraqi patients have sought medical treatment in India and other countries, which may have contributed to the acquisition of this gene.

## CONCLUSION

As in the present study, isolates with the NDM-positive *A. baumannii* gene that produces New Delhi metallo-β-lactamase exhibit resistance to most antimicrobials recommended by CLSI (Clinical and Laboratory Standards Institute, 2012) and appear to be MDR, especially against ampicillin, where the resistance rate was 92%, and this may create a serious problem in choosing therapy. This New Delhi metallo-β-lactamase, which is produced by the *NDM-1* gene, can be a target for a new therapy.

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