



Molecular Characterization of Carbapenem resistant *Escherichia coli* and *Klebsiella pneumoniae* in Erbil, Iraq

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ABSTRACT

Antimicrobial drugs known as carbapenems are used to treat infections caused by bacteria that produce extended-spectrum β -lactamases, such as those found in the family Enterobacteriaceae. Carbapenemases in drug-resistant Gram-negative bacteria must be identified and differentiated at the phenotypic and molecular levels for effective infection management. Resistance genes in *Klebsiella pneumoniae* and *Escherichia coli* were characterized here by analyzing their phenotypic and genotypic profiles. Genotypic confirmation of carbapenemase synthesis in 98 *K. pneumoniae* and *E. coli* isolates was found. PCR was used to look for the metallo- β -lactamase genes OXA-48 and NDM-1 in the collected isolates. The blaOXA-48 and blaNDM-1 genes were found in 22.45 and 12.24% of the isolates, respectively. The genes for carbapenemase resistance, blaOXA-48, and blaNDM-1, were identified in *K. pneumoniae* and *E. coli* isolates from the city of Erbil in the Kurdistan area of Iraq.

Keywords: *Escherichia coli*, *Klebsiella pneumoniae*, OXA-48, NDM-1.

INTRODUCTION

Studying antibiotic resistance, genetic background, mechanisms, and tracking gene transference are the hot trend that scientists are mostly concerned about, as the antibiotic resistance profiles of bacteria are showing increasingly worrying patterns of emerging resistance (1). The consistent development of antibiotic resistance has led to the proliferation of antibiotic-resistant organisms (AROs) such as extended-spectrum β -lactamases (ESBL), methicillin-resistant staphylococcus aureus

(MRSA), carbapenem-resistant enterococci (CRE), and vancomycin-resistant enterococci (VRE), which have become a global threat due to the appearance of multidrug-resistant organisms and extensive resistance organisms (2). The rise of carbapenem-resistant Enterobacteriaceae is one of the ongoing trends that is becoming a significant topic. This strain of bacteria is being reported more frequently all over the world. This resistance is brought on by bacteria that produce an enzyme that hydrolyzes carbapenems into β -lactamases (3).

Another concern is emergence of hidden carbapenem resistance in bacteria, in which a bacterium with resistance genes and positive phenotypic tests would still be responsive to carbapenem antibiotics (4). Also after the covid-19 pandemic that increased the risk of bacterial coinfection and wrong consumption of antibiotics, higher rates of antibiotic resistance is expected (5). *Klebsiella pneumoniae* & *Escherichia coli* are significant members of Enterobacteriaceae family responsible for a variety of infections including pneumonia, UTI, bacteremia, cystitis, wound infections etc. *E. coli* & *K. pneumoniae* respectively are the leading cause for urinary tract infection and bloodstream infection (6). The blaOXA-48 gene was selected due to its endemicity in the neighboring country Turkey, (7), whereas blaNDM-1 which is endemic in India (8), while many cases have been reported from Saudi Arabia, Iran, and Turkey (9-11). This affected Kurdish people who are traveling to these endemic countries for medical treatments, tourism, pilgrimage, and the acquisition of this resistant genes after admission or performing surgical procedures in their hospitals. Carbapenem resistance can arise from different mechanisms by Enterobacteriaceae, including Metallo beta-lactamase alone or together with porin protein loss. Plasmid mediated, carbapenem hydrolyzing MBL have been reported from various countries (12). Thus, this study was carried out to study the molecular characterization of *E. coli* and *K. pneumoniae* isolates physically and genetically using blaOXA-48 and blaNDM-1 genes.

MATERIAL AND METHODS

Study Design

This study was a cross sectional study which was conducted at Rizgari Teaching Hospital and PAR Hospital in Erbil, Kurdistan region of Iraq, from November 2017 to May 2018. This study was approved by Research Board Committee of College of Pharmacy, Hawler Medical University, with ethical approval No. 161206155, in 2017. All the participants signed an information consent form before giving the samples. This study characterizes *E. coli* and *K. pneumoniae* isolates physically and genetically utilizing blaOXA-48 and blaNDM-1 genes. In

total, 98 *E. coli* and *K. pneumoniae* isolates from the participant were examined. They were monitored for resistance genes and genotyped and phenotyped.

Bacterial Isolates

In this study, there were a total of 74 *Klebsiella pneumoniae* and 24 *Escherichia coli* isolates that were identified using the Vitek-2 system. The collected specimens include urine, sputum, blood, stools, swabs, and cerebrospinal fluid (CSF). All the collected specimens were cultured in MacConkey agar plates and were incubated in a 37 °C incubator overnight. Subsequently, pure isolated colonies were selected and were kept in 1.5 mL Eppendorf tubes using thioglycollate broth medium with 20% (v/v) glycerol and were stored at -80 °C for further study.

DNA Extraction

DNA extraction was carried out by using genetic DNA purification pp-206, by (Jena Bioscience-Germany). Briefly, a colony selected from MacConkey agar using a sterile loop and is suspended in 300µl of cell lysis solution. 1.5µl of RNase was added and mixed by inverting. Then the solution was incubated at 37 for 15-30 minutes and cooled on ice for 1 minute, Vortexing at high speeds for 30 seconds after adding 100 µl of protein precipitation solution. After that, the mixture was centrifuged for 5 minutes at 15000 rpm. The supernatant was placed into a microtube with 300 µl of isopropanol > 99% that had already been sterilized. The DNA pellet was then washed with 500 µl of washing buffer, and the tube was inverted numerous times. The mix was centrifuged again at 15000rpm for 1 minute. The ethanol was discarded after that. The tubes were air dried at room temperature for 15 minutes. 100µl of DNA hydration solution was added to the air-dried DNA pellet. The DNA was then incubated at 65°C for one hour. For future use the DNA was stored at -40°C.

Detection of Carbapenemase genes by PCR

The presence of genes responsible for carbapenem resistance, specifically the blaNDM-1 and blaOXA-48, were examined using PCR

testing. PCR was then done using specific oligonucleotide primers listed in Table 1. The PCR reaction for amplifying the genes of interest was composed of a mixture of 20µl of pre-mixed PCR reagents, 1µM of each primer, and 2ng of DNA template. This was performed in a total volume of 20µl. The DNA samples from both K. pneumoniae and E. coli were amplified using specific primers for the blaOXA-48 and blaNDM-1 genes, using both forward and reverse primers for each. The PCR process was carried out with the following parameters: first, the

sample was incubated at 95 degrees Celsius for 5 minutes, then it underwent 30 cycles of heating at 95 degrees Celsius for 30 seconds, cooling at 52 degrees Celsius for 30 seconds, and extending at 72 degrees Celsius for 1 minute. Afterwards, there was a final extension step at 72 degrees Celsius for 7 minutes. The resulting PCR product was then examined using a 1.5% agarose gel, which was stained with ethidium bromide. The size of the product was determined by comparing it to a 100 bp DNA ladder.

TABLE 1: Primers used for amplification of blaOXA-48 and blaNDM-1 genes

Name	Sequence 5'→3'	Gene Name	Tm °C	Size (bp)
OXA-48-F	CCAAGCATTTTACC CGCATCKACC	BlaOXA-48	55°C	438
OXA-48-R	GYTTGACCATACGCT GRCTGCG			
NDM-F	GGTTTGGCGATCTGG TTTTC	BlaNDM	52°C	621
NDM-R	CGGAATGGCTCATCA CGATC			

NDM, New Delhi metallo-β-lactamase; OXA, oxacillinase

RESULTS

In our study, using the PCR techniques, we found that 22.9% of E. coli and 20.8% of K. pneumoniae isolates tested positive for the OXA-48 gene. Additionally, we also found that 12.2%

of E. coli and 12.5% of K. pneumoniae isolates tested positive for the NDM-1 gene. The data on the frequency and percentage of OXA-48 and NDM-1 positive isolates is represented in Figure 1.

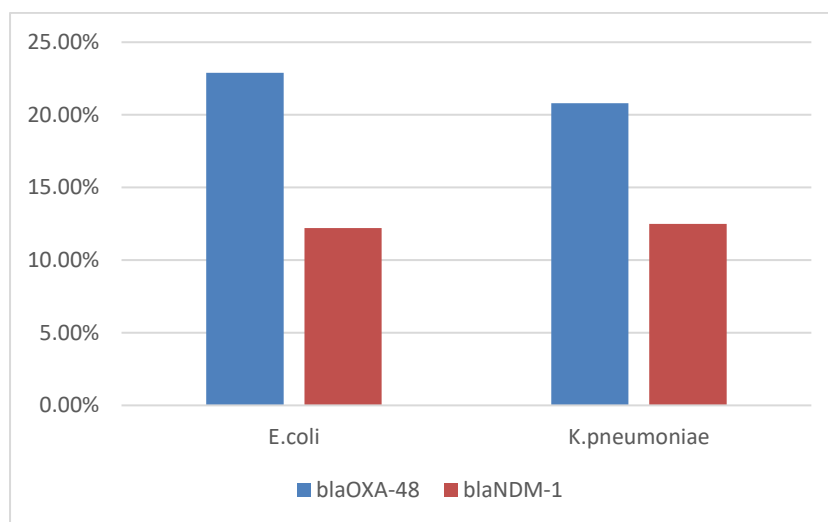


FIGURE 1: Frequency of bla-OXA48 gene and blaNDM-1 gene among isolates

The most common source for the detected genes was urine as (53%) of the isolates came from urine specimen (32.4%) of the detected genes from E. coli and (25%) of the detected genes from

K. pneumoniae were from urine. Only two (2.7%) gene positive E. coli came from swab specimen.

TABLE 2: Distribution of gene positive-Escherichia coli & Klebsiella pneumoniae

Organism	Source	Gene-positives	Percentage
Escherichia Coli	Urine	24	32.4
Escherichia Coli	swabs	2	2.7
Total		26	35.1
Klebsiella pneumoniae	Urine	6	25
Klebsiella pneumoniae	Sputum	2	8.3
Klebsiella pneumoniae	Blood	1	4.1
Total		9	37.5

Out of the 35 genes that were identified, only 52.2% of them showed positive results in the tests for detecting MBL genes (CDT and MHT).

Additionally, 47.8% of the MBL positive isolates did not contain either the OXA-48 or NDM-1 genes.

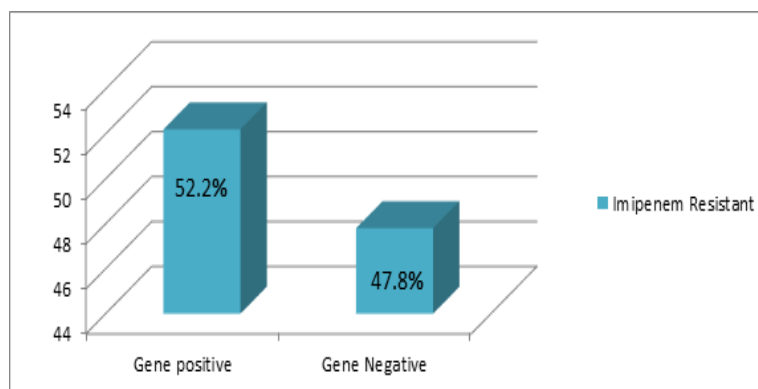


FIGURE 2: Percentage of MBL and non-MBL producing Klebsiella pneumoniae & Escherichia coli with gene detection

In the total of 12 positive blaNDM-1 only (3) (8.82%) were from IMP sensitive isolates and (9) (26.5%) from IMP resistant isolates. In 22 positive blaOXA-48 (18) were from IMP

sensitive isolates and (4) (11.8%) were IMP resistant isolates. Details The proportion of MBL gene among the isolates that are sensitive and resistant to IMP. are shown in Table (3).

TABLE 3: The proportion of MBL gene among the isolates that are sensitive and resistant to IMP.

MBL genes	IMP-Sensitive No. (%)	IMP-Resistant No. (%)	Total No. (%)
NDM-1	3 (8.82%)	9 (26.5%)	12(35.3%)
OXA-48	18 (52.9%)	4 (11.8%)	22 (64.7%)
Total	21 (61.8%)	13 (38.2%)	100%

DISCUSSION

Carbapenemase production could be mediated by various genes including NDM, IMP, VIM, KPC and OXA-48 encoded by blaNDM, blaIMP, blaVIM, blaKPC and blaOXA-48 respectively. These genes could be found among

Enterobacteriaceae family coding for resistance traits, and the rapid distribution of Enterobacteriaceae clinical isolates exhibiting resistance to carbapenems and most or all other available antibiotics become a major concern (12). The blaNDM-1 and blaOXA-48 genes are

among class B and D beta-lactamase Ambler classification that shows resistant to carbapenem antibiotics (13). These two genes were selected due to their endemicity in neighboring countries like Turkey with OXA-48 (7), and NDM-1 in India (14) which is attributed from Kurdish patient's transportation to India for medical treatments and acquiring this resistant gene after admission or performing surgical procedures in their hospitals. There are many ways for controlling and stopping spread of resistance, and genes that are easily transmitted between microorganisms in healthcare institutes (15), one of them being phenotypic assays for carbapenem resistance and molecular assays to identify resistant genes (16). The most predominant gene in 98 isolates was OXA-48 gene (38.2%), which reflective of the high prevalence of OXA-48 which is considered the most common carbapenemases in the Middle Eastern countries. Our study results shows that total of (22), (22.4%) of the isolates were possessing blaOXA-48 gene in which only (5) were from *K. pneumoniae* isolates and (17) OXA-48 positive *E. coli* isolates. Since the first report of this gene in Turkey, the Middle East and North Africa had become reservoirs expanding from India to Saudi Arabia (17, 18). A study from Saudi Arabia (9) showed that major carbapenemases in 519 clinical isolates was OXA-48 with 71.2% (n=292). Similar studies showed closer results to ours, like a study that was conducted in Turkey, where 346 *E. coli* and *K. pneumoniae* isolates, (96.8%) of the *E. coli* isolates were harboring OXA-48 and (70.9%) of *K. pneumoniae* that tested positive for OXA-48 (19). Our results detected (12) (12.2%) which comparatively with neighboring countries it seems that we reached endemic levels. A similar results from Saudi Arabia was reported by (20) they detected blaNDM-1 gene in 12.69% in total of 189 isolates. However, only 7.9% OXA-48 in total of 189 isolates were reported (20). On the other hand, a study from Iran detected blaNDM-1 in (94%) of 114 CRE isolates (21), another study from India reported that from 353 endotracheal aspirate bacterial isolates 21.5% of them showed the presence of blaNDM-1 gene (22). Our results are in agreement with a study carried out by (23) in Diyala, another city in Iraq, they performed

(PCR) assay for detection of blaNDM-1 gene on 250 clinical samples of *Klebsiella pneumoniae*, (13) of *Klebsiella pneumoniae* isolates were positive for blaNDM-1 gene. Likewise, in a study in Iran (24), a total of 29 carbapenem resistant isolates were studied and 27 of the were carriers of bla NDM-1. On the contrary to our results, in Baghdad a study tested 20 carbapenem resistant isolates and reported all 20 isolates to be carrying blaNDM-1 100% (25). Apart from that, a study conducted in Turkey tested the presence of blaNDM-1 gene among their isolates showed 20.4% positives (26). In our previous study, we investigated the prevalence of MBL enzymes in the isolates (27), after running them for genetic observation. Our result showed that half of our MBL positive isolates confirmed the absence of both OXA-48 and NDM-1 which indicated the presence of other carbapenem resistant genes.

Studying and surveilling NDM-1 carriers is very crucial to slow down the spread of other resistance genes as well, because usually NDM-1 gene can be carried on plasmids that are carrying other resistance genes as well resulting in MDR strains or even XDR or PDR. These results in comparison with other studies from neighboring cities and countries shows an alarming pattern of emergence of resistance genes. NDM-1 is considered by some studies to be a significant public health threat, on par with HIV, tuberculosis, and malaria. One way that the spread of NDM-1 producing bacteria could be prevented is by closely monitoring and screening individuals who are traveling to regions where NDM-1 is prevalent, particularly if they are coming from or returning to these areas. Moreover, some studies suggested that specific blood groups might be susceptible to have specific bacterial infection (28, 29, 30, 31). In fact, some factors might also have role in inducing the bacterial infection such as stress and diet as they might affect individuals' immunity and put them at risk of those bacterial infection (32). Thus, those factors should be also investigated to shed light about their association with these bacterial infections. Apart from that, it is necessary to limit the access of antibiotics to the general public without a prescription from a doctor in order to reduce the risk of antibiotic resistance, and to closely track the spread of

multidrug resistant bacteria, particularly CRE which is linked to high death rates (as reported by the CDC). Therefore, based on the findings of this study, it can be seen that more frequent reporting of antibiotic resistance in Enterobacteriaceae are needed and particularly in the Kurdistan region and Iraq. Antibiotic overuse must be monitored, and managed, and effective timely infection control measures must be put into place. We recommend restricted carbapenem, early prevention of infection, detection, and proper infection control policies to help in limiting the spread of these resistance genes. Moreover, there is an urgent need to develop new antimicrobials for carbapenem-resistant Enterobacteriaceae, together with strict hand-hygiene to control the Enterobacteriaceae infections also determination of the clonal relatedness of the isolates could help with.

CONCLUSION

The development and dissemination of antibiotic resistance among Enterobacteriaceae must be halted, and phenotypic testing is an essential tool in doing so. Isolates harboring genes for carbapenemase resistance were found using conventional phenotyping methods and PCR tests. Through our investigation, we have found that two important carbapenemase genes, NDM-1 and OXA-48, are present in hospitals in Erbil, Iraq. This sheds light on the emergence of resistance genes in the Ambler Class B group, specifically one MBL-type (NDM-1) and one non-MBL-type (OXA-48) in the Class D group. It has been observed in the current study that OXA-48 gene is more prevalent among IMP-sensitive isolates compared to NDM-1 gene. Among the isolates 47.8% of MBL producers didn't show presence of neither OXA-48 nor NDM-1 genes which suggests presence of other MBL genes. Further molecular studies are recommended to include other carbapenemase producing genes.

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