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Carbapenem Resistant *Klebsiella Pneumonia* (CRKP) Harbor NDM-1 Gene Isolated from Wastewaters in Basrah, Iraq

Ismaal Jmia Abas* and Nooralhuda waleed abdulredha
Department of Biology, College of Education, Qurna University of Basrah, Basrah, IRAQ
*Corresponding author ismaaljmia@gmail.com

ABSTRACT
Resistance to carbapenems is developing around the world *Klebsiella pneumonia* resistance to carbapenems is associated with production of carbapenem-hydrolyzing class B metallo-β-lactamases (MBL).New Delhi metallo-β-lactamase 1 (NDM-1) is one of the most recently discovered MBL among E.coli .The present work was aimed to study the prevalence of bla NDM-1 gene among carbapenem-resistanti isolates from *Klebsiella pneumonia* five s Wastewaters in Basrah, Iraq Isolates identified and antibiotics susceptibility were assayed by using VITEK2Compact.Phenotypic detection of carbapenemase was performed using combined-disc synergy test (CDST), Chrom ID Carba agar and using RAPIDEC CARBA NP test, isolates were also subjected to the polymerase chain reaction detection of bla NDM-1 gene.
Keywords: Carbapenem resistant, *Klebsiella pneumonia*, CRKP, harbor NDM-1 gene

Introduction
The first KPC producer (KPC-2 in *K. pneumoniae*) was identified in 1996 in the eastern United States (Yigit et al., 2001).Within a few years, KPC producers had spread globally and have been described across the contiguous United States (still mostly in eastern coast states) and, in particular, in Puerto Rico, Colombia, Greece, Israel, and the People's Republic of China ((Nordmann et al., 2009),(Navon-Venezia et al., 2009)
New Delhi MBL-1(NDM-1) is a new type of carbapenemase belongs to the class B of Ambler *β*-lactamases produced bysome strains of bacteria, and is able to inactivate all β-lactams (Walsh et al., 2005).
Although NDM-1producers have been described worldwide, they are mainly recovered from patients who had relationship with the Indian subcontinent (Nordmann et al., 2011) and in some cases with the Balkan states (Livermore *et al*., 2011) and the Middle East (Poirel *et al*., 2011).

METHOD AND MATERIAL
Bacterial isolates: Bacterial isolates were recovered from different swage water sample in Basrah Iraq during the period from April to August 2019. Bacteria were cultured on MacConkey agar in aerobic condition at 37 °C for 24-48 h, and then identified by conventional biochemical tests and by using of VITEK 2 Automated system using (GN) cards

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Antibiotic susceptibility testing

VITEK 2 system using (AST- GN30) was used, and the MIC values for these isolates were obtained .The susceptibility of the isolates was determined against 15 antibiotics included: Priperacillin(PRL), Piperacillin/Tazobactam(TPZ-TZP), Ceftazidime(CAZ)Cefepime, (FEP) Aztreonam(ATM), imipenem(IMP), Meropenem(MEM, Amikacin(AK) Gentamicin(CN), Netilmicin(NTE), Tobramycin(TOP), Ciprofloxacin(CIP), Levofloxa(LVE), Tetracycline(TE) Trimethoprim/Sulfamethoxazoleremaining(SXT**).**

Carbapenemase-producing Detection by Disc Difussion

The recognition of carbapenemase-producing bacteria was achieved using the Kirby-Bauer disk-diffusion method using 10 µg ertapenem discs, 10 µg meperenem and 10 µg imipenem, on Muller-Hinton agar. Any strain having a decreased sensitivity to mropenem (inhibition zone <22 mm) was considered suspicious of being carbapenemase producing and was verified by other phenotypic tests as recommended by CLSI (Dortet et al., 2015).

Carbapenemase-producing Detection by Modified Hodge Test (MHT):

A 0.5 MacFarland turbidity suspension of the E. coli strain E. coli ATCC 25922 was set and injected onto Muller Hinton Agar medium. The medium was dried and a disk of meropenem (10µg) was sited in the center of the test zone. Using a straightline swab, the test strain was distributed from the middle edge to the center and this was repeated with each of the test strains plus the control strains (positive and negative) in different directions. The samples were incubated overnight at 35°C +/- 2°C. The results were read after incubation; if bacterial growth in the form of flooding occured at the intersection of the E. coli 25922 inhibition zone with the line of the test strain (Dortet et al., 2015)

Carbapenemase-producing Detection by CHROMID®.CARBA agar

The CHROMID®CARBA agar (Ref 414012) bi-plate comprises one half specific for blaOXA-like producing organisms while the other half is specific for blaVIM, blaNDM, blaKPC and blaIMP producing organisms.(Dortet et al., 2015)

Carbapenemase-producing Detection by RAPIDEC®.CARBA NP

The RAPIDEC®.CARBA NP is a ready-to-use strip and employs the principles of the Carba NP test, a novel phenotypic carbapenem hydrolysis test . It was achieved as advised by the manufacturer. A loopful (10 loop) of a bacterial colony was handpicked from overnight- incubated Mueller-Hinton agar plates and combined into API suspension medium(provided with kit); the bacterial suspension was then putted to wells with check strip and incubated at 37°C. Optical studying of the test strip was finished wards 30 min and later 2 h, if essential. a +ve matched to a shade alter from red to yellow-orange, whereas a red color indicated a –ve results (Dortet et al., 2015).

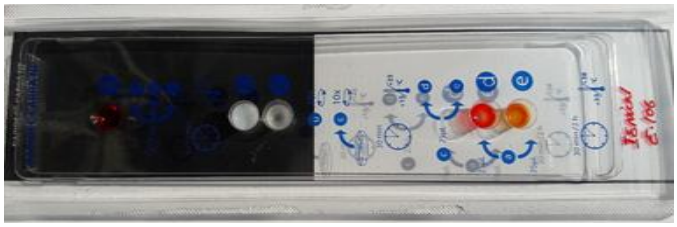

Figure (1): RAPIDEC®.CARBA NP panel

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Detection of New Delhi Metallo-β-Lactamase (NDM-1) gene by using PCR

Detection of NDM-1 gene was conducted by using primers for amplification. A fragment 621 bp of NDM -1 was amplified using a forward primer (NDM-1 F: 5GGTTTGGCGATCTGGTTTTC 3′) and a reverse primer (NDM-1 R: 5′ CGGAATGGCTCATCACGATC 3′) (Primers set supplied by IDT (Integrated DNA Technologies company, Canada.).

The PCR amplification was performed in a total volume of 25µl containing 1.5µl DNA, 5 µl Tag PCR Premix (Intron, Korea), 1µl of each primer (10 pmol) then distilled water was added into tube to a total volume of 25µl.The thermal cycling conditions were done as follows: Denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 45s, 48°C for 35 sec and 72 °C for 35 sec with final incubation at 72 °C for 7 min using a thermal Cycler (Gene Amp, PCR system 9700; Applied Bio system).

The PCR products were separated by 1.5% agarose gel electrophoresis and visualized by exposure to ultraviolet light (302nm) after red stain staining (Intron Korea).

Results and Discussion

five (5) Carbapenem resistant *Klebsiella pneumoniae* were recovered from 154 swage sample Basrah ,Iraq

The MIC of 15 antibiotics listed in Table 1 was done using VITEK 2-Compact using Interpreted according to the CLSI breakpoints

The Carbapenem-resistant *Klebsiella pneumoniae* (CKP) in this study differed in the level of resistance to different antibiotics including the carbapenems (Table 1).

Table: 1 Antibiotic susceptibility of carbapenem-resistant *K.pneumoniae* isolates

Bacteria No	**PRL**	**TPZ-TZP**	**CAZ**	**FEP**	**ATM**	**IMP**	**MEM**	**AK**	**CN**	**NET**	**TOB**	**CIP**	**LEV**	**TE**	**SXT**
K. pneumoniae1	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
K. pneumoniae2	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**S**	**R**	**R**	**R**	**R**	**R**	**R**	**S**
K. pneumoniae3	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**S**	**S**	**R**	**R**
K.	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**S**	**S**	**R**	**R**	**R**	**R**	**R**	**R**

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pneumoniae4															
K. pneumoniae5	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**S**	**R**

All 5(100%) showed resist for both imipenem and Meropenem and for, Piperacillin/Tazobactam, Ceftazidime Cefepime, Aztreonam, Netilmicin, Tobramycin while (80%) isolates showed resist for Gentamicin, Ciprofloxacin, Levofloxa, Trimethoprim/Sulfamethoxazoleremaining and Tetracycline

(60%) isolates showed resist for Amikacin So in the present study, most isolates show a high level of resistance to all antibiotics including β-lactamase inhibitor, aminoglycosides, and quinolones. This is because β -lactamase producers have enzymes that hydrolysis the active site of the antibiotics, thus making the organism resistance to virtually all β -lactam antibiotics. This enzyme has spread worldwide with intra and interspecies transfer being facilitated by plasmid encoded enzyme. β - lactams are the most widely used antibiotics all over the world and resistance to this antibiotics has resulted in a major clinical crisis(Spanu et al., 2002)

Resistance to Meropenem and Imipenem to was present in all isolates 5(100%) There is an increase in the resistance against the powerful carbapenems antibiotics because long-term hospitalization. And long term antibiotic use. Some other studies(Abderrahim et al., 2017),(Al-Agamy et al., 2018) reported *Klebsiella pneumonia* with high percentage of resistance against doripenem and imipenem and these are also in accordance with our present study with both antibiotics having resistance percentages of 89% and 86%, respectively.

Many report show carbapenem-resistance in environment solates of *Klebsiella pneumonia* is increasing(Poirel et al., 2011)(Hentschke et al., 2015)(Walthall et al., 2018)

The low susceptibility of the Klebsiella pneumoniae isolates against some antibiotics in this study may be due to extensive using of these antibiotics in clinical practice in Iraq. Excessive use of broad-spectrum antibiotics in hospitals has led to the emergence of highly resistant strains of *Klebsiella pneumoniae* and strong selective pressure posed on bacteria by the use of carbapenems has facilitated the emergence and spread of carbapenemases in β-lactamase classes A, B and D in clinical settings ((Nordmann et al., 2011)(Cantón et al., 2012)

To reduce the selection pressure for resistance, it is an important issue to determine the antibiotic susceptibility pattern of bacteria, so that hospital patients can be treated with more narrow-spectrum and target-specific

The increasing reports on NDM-1 producing *Enterobacteriaceae* have addressed a potential threat to global health. In Iraq NDM-1gene was report in *Klebsiella pneumoniae*, *Pseudomonas aeruginosa, Acinetobacter baumannii* (Alshara et al., 2014) (Anoar et al., 2014) (Al-Harmoosh & Jarallah, 2015)

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The presence of NDM-1 gene was detected by conventional PCR technique. PCR analysis for NDM-1 gene was accomplished for the five *Klebsiella pneumoniae* isolates only one isolates was harbor NDM-1 gene

. This increase in gene frequency because gene for this enzymes are often carried on the plasmid, facilitating rapid spread between microorganisms (Chagas et al., 2011)and NDM-enzymes are very frequent in the human population and can even be found in the environment(Nordmann et al., 2011) The distribution of plasmid-mediated NDM-1 gene within study isolates are shown in Figures 2, The NDM-1 gene was not detected in the remaining isolate that identified as MBLs producer by the phenotypic method. As the PCR is the gold standard technique. So, the phenotypic result may be a false positive result, or the isolates had MBLs variants or other carbapenemase genes not detected by the primers used in this study.

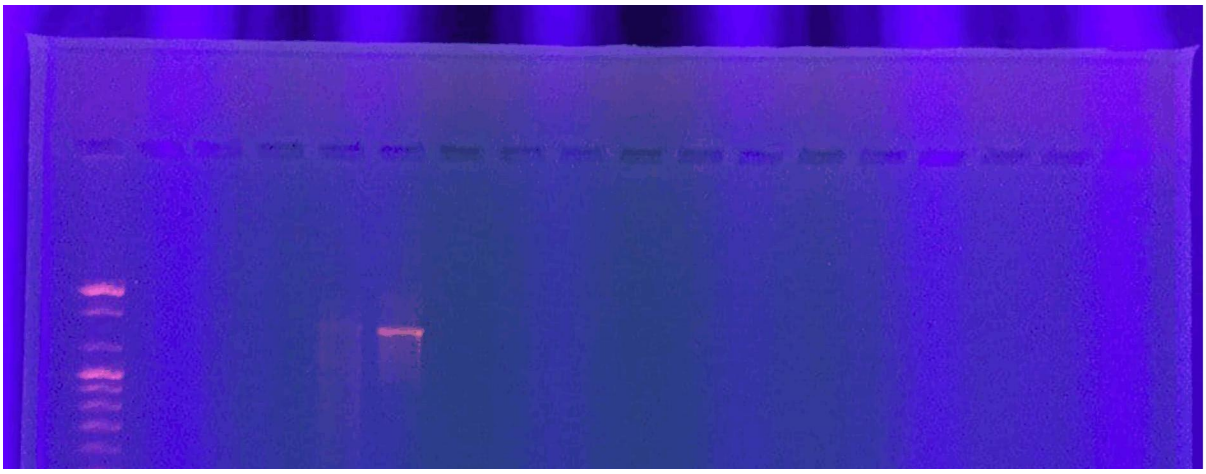

Figure. 2 Ethidium bromide stained agarose gel showing PCR Amplification products with NDM -1gene (621bp).

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