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NEW DELHI METALLO-B-LACTAMASE 1 (NDM-1) PRODUCING *ESCHERICHIA COLI* IN BASRAH HOSPITALS. IRAQ

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ABSTRACT
Resistance to carbapenems is developing around the world *Escherichia coli(E.coli*) 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-resistant *E.coli* isolates from six hospitals 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. Phylogenetic analysis showed very less variation in bla NDM-1 gene with respect to bla NDM-1 possessing by different bacteria Conclusions: Our findings highlight the incidence of bla NDM-1 in *E. coli* isolates with a reduced susceptibility to Imipenem or meropenem.

KEYWORDS: NDM-1, carbapenems, MβLs, *Escherichia coli*, antibiotics resistance.

INTRODUCTION
Carbapenem resistance caused by acquiring the Metallo-beta-lactamases (MBL) is considered to be more serious than other resistance mechanisms because MBLs can almost hydrolyses all beta-lactam antibiotics except monobactams (Rahmati *et al*., 2013). Recently identified New Delhi MBL-1(NDM-1) is a new type of carbapenemasebelongs to the class B of Ambler *β*-lactamases produced by certain strains of bacteria, and is able to inactivate all β-lactams except aztreonam(Walsh *et al*., 2005). It was first reported in *K. pneumoniae* and *Escherichia coli* derived from a Swedish patient of Indian origin who was admitted to hospital in New Delhi, India in 2009 (Yonge *et al*., 2009) 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 clinical samples in Some Basrah Hospitals \Iraq during the period from April to August 2016. 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**.**

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).

Phenotype detection of Metallo-β lactamase (MβLs). Detection of MBLs production among the *Escherichia coli* isolates was done using combined-disc synergy test (CDST). As an inhibition zone with the imipenem-EDTA disc was >7 mm than the zone of inhibition of imipenem alone. Shown in Figure 1.

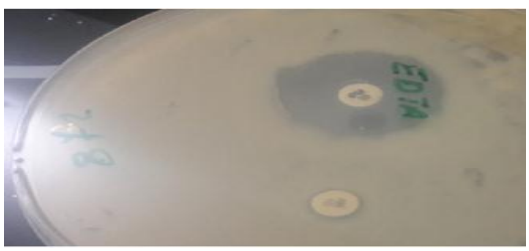

Figure.1Combined disk synergy test (CDST) positive for MBL production in *Escherichia coli* isolates,

upper disk, is imipenem+EDTA, and the lower disk is imipenem only.

The detection of carbapenemase production among the *Escherichia coli* isolates was done using Chrom ID Carba agar. A positive result. Which appear as red colonies on Chrom ID Carba agar shown in Figure 2.

Figure. 2 Chrom ID Carba agar positive for carbapenemase producing in *Escherichia coli* isolates,

The other method detection of carbapenemase production among the *Escherichia coli* isolates was done by using RAPIDEC CARBA NP test a positive result which appear as orange or yellow colure in Test well **e** Figure 3

Figure 3 RAPIDEC CARBA NP test positive for carbapenemase producing in *Escherichia coli* isolates

Preparation of primers suspension

The DNA primers were re suspended by dissolving the lyophilized primers after spinning down with TE buffer depending on manufacturer instruction as stock suspension. Working primer tube was prepared by diluted with TE buffer.

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 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).

The DNA sequences of blaNDM-1

The DNA sequences of blaNDM-1 amplicons obtained in the present study were compared with the four isolates retrieved from theNCBI GenBank database. Nucleotide BLAST (BLASTN) was used for the sequence searches with default parameters.

RESULTS AND DISCUSSION

Eighteen (18) Carbapenem resistant *Escherichia coli* were recovered from different clinical samples urine (8)44.44%, burn (6) 33.33%, blood (2) 11.11% and wound (2) 11.11%. From different hospitals in Basrah ,Iraq The MIC of 15 antibiotics listed in Table 3 was done using VITEK 2-Compact using Interpreted according to the CLSI breakpoints

The Carbapenem-resistant *Escherichia coli* in this study differed in the level of resistance to different antibiotics including the carbapenems (Table 1). All isolates18(100%) showed resist for both imipenem and Meropenem and for Priperacillin, Piperacillin/Tazobactam, Ceftazidime Cefepime, Aztreonam, Netilmicin, Tobramycin while 15(83.3%) isolates showed resist for Gentamicin, Ciprofloxacin, Levofloxa, Trimethoprim/Sulfamethoxazoleremaining and 11 (61%) isolates showed resist for Tetracycline while 10(55%) 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 isolates16 (100%) There is an increase in the resistance against the powerful carbapenems antibiotics because long-term hospitalization. And long term antibiotic use. This is in agreement with the work done by Ullah *et al,* (2009) who reported susceptibility pattern to imipenem in *Escherichia coli* to be 98% while meropenem was 97%.Many report show carbapenem-resistance in clinical isolates of *E. coli* is increasing (Poirel *et al*., 2004; Hong *et al*., 2005; Lartigue *et al*., 2007; Oteo *et al*., 2008; Falagas andKarageorgopoulos, 2009),

The low susceptibility of the *E.coli* isolates against many 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 *E.coli*. 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, Akóva, *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.

Table: 1 Antibiotic susceptibility of carbapenem-resistant *E.coli* isolates

Bacteria No	PRL	TPZ-TZP	CAZ	FEP	ATM	IMP	MEM	AK	CN	NET	TOB	CIP	LEV	TE	SXT
E.coli 1	≥128 (R)	≥128 (R)	≥64 (R)	≥32 (R)	≥64 (R)	≥16 (R)	≥16 (R)	≤2 (S)	≥16 (R)	≥32 (R)	≥16 (R)	≥4 (R)	≥8 (R)	≥16 (R)	≥320 (R)
E.coli 2	≥128 (R)	≥128 (R)	≥64 (R)	≥32 (R)	32 (R)	≥16 (R)	≥16 (R)	≥64 (R)	≥16 (R)	≥32 (R)	≥16 (R)	≥4 (R)	≥8 (R)	2 (S)	≥320 (R)
E.coli 3	≥128 (R)	≥128 (R)	≥128 (R)	16 (R)	≥64 (R)	≥16 (R)	≥16 (R)	16 (I)	>=1 (S)	≥32 (R)	≥16 (R)	1 (S)	1 (S)	≥16 (R)	<=20 (S)
E.coli 4	≥128 (R)	≥128 (R)	≥128 (R)	≥32 (R)	≥64 (R)	≥16 (R)	8 (R)	≥16 (R)	≥16 (R)	≥32 (R)	≥16 (R)	≥4 (R)	≥8 (R)	≥16 (R)	≥320 (R)
E.coli5	≥128 (R)	≥128 (R)	≥128 (R)	16 (R)	≥64 (R)	≥16 (R)	≥16 (R)	16 (I)	>=1 (S)	≥32 (R)	≥16 (R)	1 (S)	1 (S)	≥16 (R)	<=20 (S)
E.coli 6	≥128 (R)	≥128 (R)	≥64 (R)	≥32 (R)	32 (R)	≥16 (R)	≥16 (R)	≥64 (R)	≥16 (R)	≥32 (R)	≥16 (R)	≥4 (R)	≥8 (R)	2 (S)	≥320 (R)
E.coli 7	≥128 (R)	≥128 (R)	≥64 (R)	≥32 (R)	≥64 (R)	≥16 (R)	≥16 (R)	≤2 (S)	≥16 (R)	≥32 (R)	≥16 (R)	≥4 (R)	≥8 (R)	≥16 (R)	≥320 (R)
E.coli 8	≥128 (R)	≥128 (R)	≥128 (R)	≥32 (R)	≥64 (R)	≥16 (R)	8 (R)	≥16 (R)	≥16 (R)	≥32 (R)	≥16 (R)	≥4 (R)	≥8 (R)	≥16 (R)	≥320 (R)
E.coli9	≥128 (R)	≥128 (R)	≥128 (R)	16 (R)	≥64 (R)	≥16 (R)	≥16 (R)	16 (I)	>=1 (S)	≥32 (R)	≥16 (R)	1 (S)	1 (S)	≥16 (R)	<=20 (S)
E.coli10	≥128 (R)	≥128 (R)	≥64 (R)	≥32 (R)	≥64 (R)	≥16 (R)	≥16 (R)	≤2 (S)	≥16 (R)	≥32 (R)	≥16 (R)	≥4 (R)	≥8 (R)	≥16 (R)	≥320 (R)
E.coli 11	≥128 (R)	≥128 (R)	≥128 (R)	≥32 (R)	≥64 (R)	≥16 (R)	8 (R)	≥16 (R)	≥16 (R)	≥32 (R)	≥16 (R)	≥4 (R)	≥8 (R)	≥16 (R)	≥320 (R)
E.coli 12	≥128 (R)	≥128 (R)	≥64 (R)	≥32 (R)	32 (R)	≥16 (R)	≥16 (R)	≥64 (R)	≥16 (R)	≥32 (R)	≥16 (R)	≥4 (R)	≥8 (R)	2 (S)	≥320 (R)
E.coli 13	≥128 (R)	≥128 (R)	≥64 (R)	≥32 (R)	32 (R)	≥16 (R)	≥16 (R)	≥64 (R)	≥16 (R)	≥32 (R)	≥16 (R)	≥4 (R)	≥8 (R)	2 (S)	≥320 (R)
E.coli 14	≥128 (R)	≥128 (R)	≥64 (R)	≥32 (R)	32 (R)	≥16 (R)	≥16 (R)	≥64 (R)	≥16 (R)	≥32 (R)	≥16 (R)	≥4 (R)	≥8 (R)	2 (S)	≥320 (R)
E.coli 15	≥128 (R)	≥128 (R)	≥64 (R)	≥32 (R)	32 (R)	≥16 (R)	≥16 (R)	≥64 (R)	≥16 (R)	≥32 (R)	≥16 (R)	≥4 (R)	≥8 (R)	2 (S)	≥320 (R)
E.coli 16	≥128 (R)	≥128 (R)	≥64 (R)	≥32 (R)	≥64 (R)	≥16 (R)	≥16 (R)	≤2 (S)	≥16 (R)	≥32 (R)	≥16 (R)	≥4 (R)	≥8 (R)	≥16 (R)	≥320 (R)
E.coli 17	≥128 (R)	≥128 (R)	≥64 (R)	≥32 (R)	≥64 (R)	≥16 (R)	≥16 (R)	≤2 (S)	≥16 (R)	≥32 (R)	≥16 (R)	≥4 (R)	≥8 (R)	≥16 (R)	≥320 (R)
E.coli 18	≥128 (R)	≥128 (R)	≥64 (R)	≥32 (R)	32 (R)	≥16 (R)	≥16 (R)	≥64 (R)	≥16 (R)	≥32 (R)	≥16 (R)	≥4 (R)	≥8 (R)	2 (S)	≥320 (R)

In the present study detection of MBLs production among the *Escherichia coli* isolates was done using combined-disc synergy test (CDST). Only 8 (44.44%) out of 18 *Escherichia coli* isolates showed a positive result. And to detection of carbapenemase production among the *Escherichia coli* isolates was done using Chrom ID Carba agar 17(94.44%) out of isolates showed a positive result while by using RAPIDEC CARBA NP

test that all isolated show positive for this test .Table 3, these results agreement with Simner *et al* 2015 who found that ChromID Carba was the most sensitive and specific chromogenic medium evaluated for the detection of carbapenemase gram negative bacteria.

Table 2 Prevalence of Phenotype method among Clinical isolates of *Escherichia coli*.

Test	CDST	Chrom ID Carba agar	RAPIDEC CARBA NP
Positive	8 (44.44%)	17(94.44%)	(100)100%
Negative	10(55.55%)	1(0.05%)	0

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* (Poireletal2011; AL-Harmoosh and Jarallah2014; Anoar eta 2014; Alshara *et al* 2013; Till now there are no published works in Iraq reporting NDM-1 gene in *E.coli* The present study gives an initial insight on the incidence of bla NDM-1 gene in the clinical isolates of *E. coli*. The presence of NDM-1 gene was detected by conventional PCR technique. PCR analysis for NDM-1 gene was accomplished for the 17 *E.coli* isolates only one isolates was lake to plasmid and the NDM-1 gene are found in 14 (77.77%) isolates table 4. 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 4, 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.

Table 3 .Present of NDM-1 gene in *E.coli* isolates.

Total	NDM-1 gene present	NDM-1 gene absent	Plasmid absent
18(100%)	14(77.77%)	3(16.66)	1(5.55%)

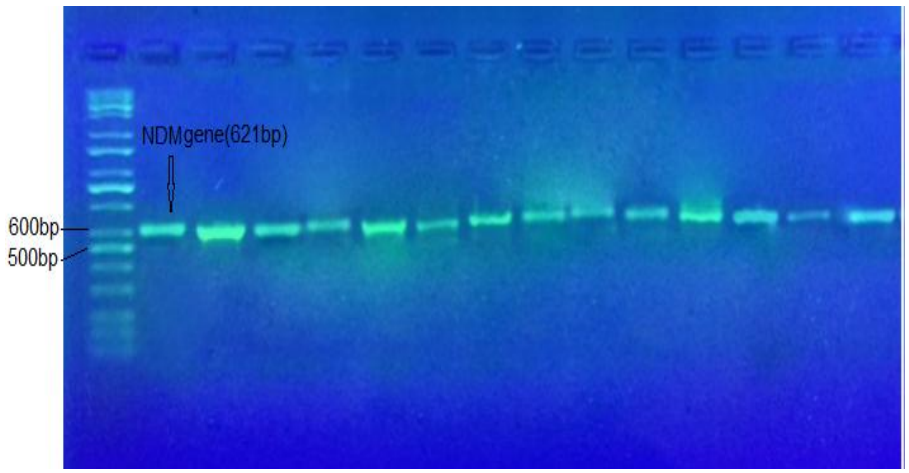

Figure. 4 Ethidium bromide stained agarose gel showing PCR Amplification products with NDM -1gene (621bp).

bla NDM-1 specific amplicons were sequenced only three isolates (*E.coli 1*, *E.coli 11 E.coli 15*) which have different pattern of antibiotic resistant. The DNA sequence identity was confirmed using BLASTN analysis. These three DNA sequences together with four similar sequences, retrieved from NCBI Gen Bank database under accession numbers (KR872634.1, KR872624.1, NC023908.1, JN255860) .were used to construct the phylogenetic tree in order to understand the nearest neighbor of the study sequences. The genetic divergence and homogeneity of the sequences are apparent in the phylogenetic tree Figure 5 these sequences were closely placed in the phylogenetic tree and their genetic similarity with four sequences reported from India.

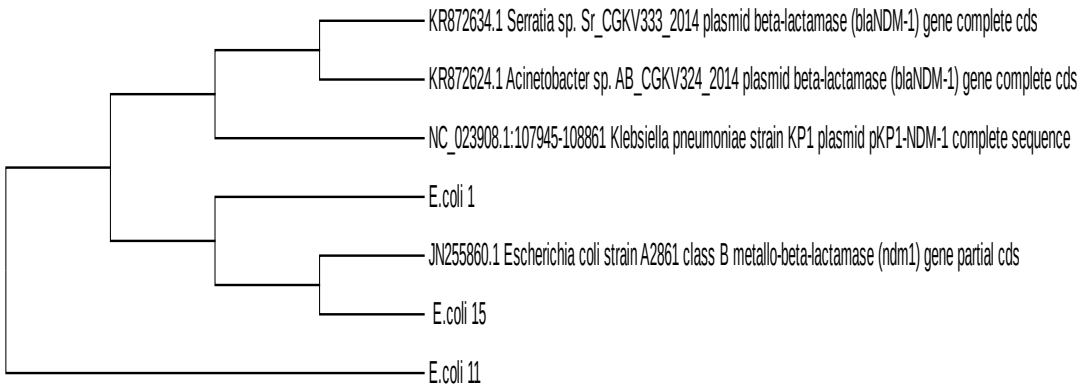

Figure: 5. Phylogenetic analysis based on bla NDM-1 gene sequences obtained from the three E. *coli* isolates in this study and four sequences retrieved from GenBank database (NCBI).

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