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Original Research Article

Detection of *bla$_{NDM}$* -Metallo-β-Lactamase Genes in *Klebsiella pneumonia* Strains Isolated From Burn Patients in Baghdad Hospitals

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Abstract

From the period from March to August 2016, 210 swabs were collected from the burn patients hospitalized in different hospitals in Baghdad City: Al-Karama Teaching Hospital, Special Burn Hospital, Central Teaching Laboratories, Child protection Teaching Hospital, Imam Ali Hospital. Out of 210 clinical isolates, 42 (37.5 %) had been shown a single isolated of pathogenic bacteria *K. pneumoniae* and the others were belonged to other bacteria and mixed growth isolates. Identification of all isolates were carried out depending on macroscopic, microscopic characterizations, conventional biochemical tests and Api 20E system.Metallo-β lactamase (MBL) enzymes were screen by two phenotypic methods(Meropenem-EDTA double disks method and Modified Hodg test). Susceptibility testing were used with The following antibiotic disks:Imipenem,Meropenem,Ceftazidime,Cefotaxime,Pipracillin,Gentamicin,Amikacin andCiprofloxacin.The percentage of resistance isolates were as followed:Imipenem (21.42%),Meropenem (19.04%),Ceftazidime(69.04%), Cefotaxime (85.71%), Pipracillin(85.71%), Gentamicin (26.19%), Amikacin (19.04 %) andCiprofloxacin(59.52%).The percentage of the prevalence of *blaNDM*-1 and *blaNDM*-2 genes in *K. pneumoniae* isolates from burn patients in Baghdad hospitals were as followed:20(100 %) for*blaNDM*-1 genes and 6 (30 %) for *blaNDM*-2 genes.

Key Words: Burn Patients, *bla$_{NDM}$* -Metallo-β-Lactamase Genes, *Klebsiella pneumonia*.

الخلاصة

جمعت 210 مسحات قطنية للفترة من اذار ولغاية اب 2016 من مرضى الحروق الراقدين في مستشفيات مختلفة في مدينة بغداد:مستشفى الكرامة التعليمي, مستشفى الحروق التخصصي, المختبرات التعليمية المركزية, مستشفى حماية الاطفال التعليمي ومستشفى الامام علي , تم تشخيص 42 عزلة (37,5 %) للكلبسيلا الرئوية كعزلات مفردة من 210 عزلة,اما بقية العزلات فكانت لبكتيريا اخرى اعتمادا على الصفات المظهرية والمجهرية والفحوصات الكيموحياتية ,وتم تاكيد التشخيص باستعمال نظامApi 20 E تم استخدام طريقتينمن التوصيف المظهري للكشف عن انزيمات البيتالاكتميز المعدنيةCMDT, MHT.اظهرت عزلات الكلبسيلا الرئوية نسبة مقاومة للمضادات الحيوية الاتية وعلى التوالي:اميبينيم, ميروبينيم, سفتادزيم, سيفوتاكسيم, ببراسلين, جنتامايسين , اميكاسين و سبروفلوكساسين :(59.52%).(19.04 %),(26.19%),(85.71%),(85.71%),(69.04%),(19.04%),(21.42%)وكانت نسبة انتشار جينات انزيمات البيتالاكتميز المعدنية –1 بلغت 20 (100%) ونسبة انتشار جينات انزيمات البيتالاكتميز المعدنية–2 بلغت 6 (30 %).

الكلمات المفتاحية : مرضى الحروق , جينات انزيمات البيتالاكتميز المعدنية , الكليبسيلا الرئوية.

Introduction

*Klebsiella pneumoniae*was an opportunistic gram-negative pathogenic bacterium associated with a range of nosocomial infections (e.g. septicemia, pneumonia, bacteremia, meningitis, urinary tract, burn and wound infections) [1]. Furthermore it was the most medically important species of the genus *Klebsiella*. In recent years, *Klebsiella* have become important pathogens in nosocomial infections [2]. It was also a potential community – acquired pathogen [3]. Antibiotic therapies are widely used for treating infectious diseases. Nowadays, antibiotic-resistant bacteria are a great concern of worldwide public health [4]. The problem of antimicrobial resistance is highlighted by a recent increase of carbapenem-resistant *K. pneumoniae,* which has largely been driven by the emergence and spread of mobile genetic elements carrying carbapenemase resistance genes including the metallo-beta-lactamase [5, 6].Meropenem and imipenem are carbapenems that remain active against organisms carrying most Ambler classes of β-lactamases which include many Gram-negative bacilli, including *Klebsiella* spp. One of the major mechanisms of carbapenem resistance in this pathogen is the production of carbapenem hydrolyzing β-lactamases. These specific groups of β-lactamases are categorized into class B metallo β-lactamases (MBLs) includingImipenemase (*IMP*)and Verona integrin encoded metallob-lactamase (*VIM*), New Delhi metallo-beta-lactamase (*NDMs*) and class D (Oxacillinases) including*OXA*-23-like, OXA-24/40-like and *OXA*-58 [7, 8]. The new MBL, New Delhi metallo-β-lactamase (*NDM*-1), initially reported in *K. pneumoniae* and *E. coli* recovered from a Swedish patient who was previously hospitalized in India in 2008 [9]. The rapid emergence spread of *NDM*positive bacteria has a complex epidemiology involving a variety of harboring species (principally *Klebsiella pneumoniae*and *E. coli*), inter-strain, inter-species, and inter-genus transmission,which has been related to a diverse moveable plasmid that can be transferred from one bacteria to another, from man to man and even from country to country in more than 40 countries worldwide [10,11]. The bacteria with *NDM-1* gene are known as superbugs and public health must pay more attention to them [12]. Many phenotypic, genotypic, phylogenic and molecular methods used to detect the production of enzymes by bacteria that responsible about drug resistant which causes increased morbidity and mortality among patients with infections caused by these bacteria and increased healthcare costs due to the extended hospital stay [13]. In recent years, many Iraqi patients were travelled to India and to other countries for medical care purpose which may helped in acquiring *NDM* gene. In Iraq there were no information about the occurrence of *NDMK. pneumoniae* producing clinical isolates. So the proposed aim of this study was to detect MBL genes ^{bla}*NDM*-1,2among resistant isolates of *K. pneumoniae* obtained from burn patients in Baghdad Hospitalsbypolymerase chain reaction(PCR).

Material and Methods

Isolation and Identification

During the period from March to August 2016, 42 *K. pnuemoniae* strains were isolated from 210 swabs of burn patients hospitalizedin different hospitals in Baghdad City: Al-Karama Teaching Hospital, Special Burn Hospital, Central Teaching Laboratories, Child protection Teaching Hospital, Imam Ali Hospital. Specimens were collected by sterile swabs after the removal of dressing and cleaning the wound surface by 70% alcohol. The isolation and identification of *K. pnuemoniae*from wound specimens were streaked on blood agar, MacConkey agar and Eosin methylene blue(EMB) agar (Biomark Lab. Pune. India) and incubated at 37℃ for 24hrs. The isolates were identified as *K. pnuemoniae*by manual biochemical tests that were used in accordance with the manufacturer's instructions; based on Gram staining,

catalase test, oxidase test, triple sugar iron (TSI) fermentation, Indole test,Voges-Proskauer (VP) test, Methyl red (MR) test, Simmons Citrate test,Urease test, motility test, and string test [14].For final confirmation, biochemical tests embedded in the API-20E biochemical kit system (Bio-Merieux, France).

Antimicrobial Susceptibility Testing

The susceptibility pattern of isolates to different antibiotics were examined using disk diffusion method (Kirby-Bauer) on Muller-Hinton agar plates(Biomark Lab.,Pune. India) according to guidelines of CLSI [15]. The antimicrobial disks were included: Imipenem (10µg), Meropenem (10µg), Ceftazidime(30µg), Cefotaxime (30µg), Pipracillin (100µg), Gentamicin (10µg), Amikacin (30µg) and Ciprofloxacin (5µg) (MAST Co. UK). *Pseudomonas aeruginosa* ATCC27853 were used as a control strain[16].

Table 1 : Antibiotic disks used in this study

Antibiotic disks	Symbol	Disks potency (µg/ disk)	Company (origin)
Imipenem	IMP	10 µg	MAST Co. UK.
Meropenem	MEM	10 µg	
Ceftazidime	CAZ	30µg	
Cefotaxime	CTX	30µg	
Pipracillin	Pip	100µg	
Gentamicin	GM	10 µg	
Amikacin	AK	30µg	
Ciprofloxacin	CIP	5µg	

Screening for metallo β-lactamases (MBL)

Meropenem-EDTA double disks methodwas performed using disks containing 1900 µg of EDTA plus 10 µg of Meropenem disks were placed on inoculated Muller Hinton agar plates. After 24hr. incubation, an increase of ≥ 17 mm in zone diameter in the presence of 1900 µg of EDTA compared to Meropenem disk alone were considered as MBL producing *K. pnuemoniae*strains (positive results) [17].

Modified Hodge test(MHT)

Tested isolates were exposed to MHT test as recommended by [18]; Inoculating an overnight culture suspension of *E.coli* ATCC 25922 was streaked across the entire plate of Mueller-Hinton agar (MHA)plate.After drying 10 µg of Meropenem disk was placed at the center of the plate and up to 4 different isolates of tested organisms were streaked linearly from the periphery of the plate into the direction of Meropenem disk at the center then the test plate was incubated at 37ºC for 18 hours. The presence of a clover leaf-like shaped zone of inhibition around each tested strain is interpreted as Carbapenemases producing strain.

PCR amplification:

DNA was extracted from the isolates by using genomic extraction mini kit according to the manufacture instructions (Promega company, USA). To amplify the genes encoding carbapenemases, a PCR was run using the primers of NDM-1 and NDM-2 gene (Table-2) as described by [19]. Amplification was performed in a 20µl volume as recommended by Promega Master mix instruction. PCR amplifications were carried out on a thermal cycler (ESCO/USA). The cycling conditions for amplification were as follows: for *bla*NDM-1 and 2 genes, initial denaturation at 95°C for 5 min.,1 cycle,Denaturation at 95° C, 30 sec., Annealing at 55° C,30 sec.,Extension at 72°C for 30 sec.,30-35cycles and Final Extension at 72 °C. for 5 min., 1 cycle.

Table 2 : The sequences of primers used in PCR to detect *bla* NDM-1&2 [19].

Primer	Sequence	Amplicon size (bp)
*bla*NDM-1 Forward	'5-ATG GAA TTG CCC AAT ATT ATG C-3'	500bp
Reverse	'5-CGA AAG TCA GGC TGT GTT G-3'	
*bla*NDM-2 Forward	'5-CAC CTC ATG TTT GAA TTC GCC-3'	1000bp
Reverse	'5-CTC TGT CAC ATC GAA ATC GC-3'	

Agarose Gel Electrophoresis

Amplified products were detected by agarose gel electrophoresis in 1% Tris-borate-EDTA (TBE) agarose (Promega USA), and staining with ethidium bromide, electric current was allowed at 70 volts for 2 hrs. DNA bands were observed using UV-Transilluminator and photographed with Gel documentation system. 100 bp DNA Ladder (Promega) was used to assess PCR product size [20].

Results and Discussion

Bacterial strains, antibiotic susceptibility and MBL phenotypic test.

In this study a total of 210 sample swabs of clinical isolates of burn wound infections were cultured , examined and identified. Out of 210 clinical isolates, 42 (37.5 %) had been shown a single isolated of pathogenic bacteria *K. pneumonia* and the others were belonged to other bacteria: 36 (32.14%) *Pseudomonas* spp., 20 (17.86%) *E. coli*, 10(8.93%) *S.aureus* and 4 (3.57 %) *Proteus* spp.,while mixed growth isolates frequency as the following:*K. pneumoniae* and *Pseudomonas* spp.64 (65.31),*Pseudomonas* spp. and *E. coli* 18 (18.37%), *K. pneumoniae* and *E. coli* 7(7.14%), *Pseudomonas* spp. and *Proteus* spp. 4 (4.08 %), *K. pneumoniae* and *S.aureus* 3 (3.06 %) and *Proteus* spp. and *E. coli* 2 (2.04 %).

In a local study done by Mohammed(2007),who isolated *K. pneumoniae* from burn wound infection (36.7%) 20[21]; whileAssal, (2010) isolated *K. pneumoniae* from wound (31.25%).These results were agreement with this study. Kehined*et.al*(2004) also found that *Klebsiella spp.* (34.4%) was the most common isolate from infected burn wounds [22].*K. pneumoniae* associated with hospital-acquired infection accounting for 34–36% of cases of *K. pneumoniae* bacteremia [23].

Antibiotic Susceptibility Testing

Antimicrobial resistance to the carbapenems(e.g. imipenem and meropenem) mediated by metallo-βlactamase(MBL) enzymes hasremarkable clinical implications since the carbapenems are usually the last options of treatment for bacterial infections caused by multidrug resistant organisms (e.g. producers of extended spectrum B-lactamases) [24].Eight antibiotic disks were used in this study included two types of Carbapenems antibiotics; Imipenem (IPM), Meropenem (MEM) and two types of third generation Cephalosporins included; Ceftazidime (CAZ), Cefotaxime (CTX). Table(1) summarizes the results of antibiotic susceptibility test and reflects forty-two isolates were resistance to the following antibiotics; Imipenem (21.42%), Meropenem (19.04%), Ceftazidime (69.04%),Cefotaxime(85.71%),Pipracillin(85.71%)),gentamicin(26.19%),Amikacin(1 9.04%)andCiprofloxacin(59.52).

Furthermore, some isolates exhibited intermediate susceptibility to Imipenem(9.52%), Meropenem(4.76%), Ceftazidime(11.90%), Pipracillin (4.76%), Amikacin(4.76%) and Ciprofloxacin(2.38%).While some isolates showed susceptibility to the antibiotics as the following: Gentamycin and Amikacin(78.57%), Meropenem(76.19%), Imipenem(69.04%), Ciprofloxacin(38.09%), Ceftazidime(19.04%)and both Cefotaxime, Pipracillin (9.52%).

A high degree of resistance to the tested antibiotics was noted among the bacteria isolates especially to the third-generation cephalosporins; Cefotaxime(85.71%), Ceftazidime (69.04%),this results of the study agreement with Ejikeugwu*et al.,* 24[25]who reportedthat *K. pneumoniae* show a resistance rates for CTX were (61.5 %), CAZ(38.5 %)and Fluoroquinolones (Ciprofloxacin; CIP show a resistance rate(53.8 %). While the carbapenems used, IPM and MEM, the resistance rates of the *K. pneumonia* was (12.8 %),(7.7 %) respectively.

Table 3: Antimicrobial susceptibility pattern of the bacterial isolates

Antibiotic	Susceptible	Intermediate	Resistant
Imipenem (IPM)	(69.04%)	(9.52%)	(21.42%)
Meropenem (MEM)	(76.19%)	(4.76%)	(19.04%)
Ceftazidime (CAZ)	(19.04 %)	(11.90%)	(69.04%)
Cefotaxime (CTX)	(9.52%)	-	(85.71%)
Pipracillin (Pip)	(9.52%)	(4.76%)	(85.71%)
Gentamicin(GM)	(78.57%)	-	(26.19%)
Amikacin(AK)	(78.57%)	(4.76%)	(19.04 %)
Ciprofloxacin(CIP)	(38.09%)	(2.38%)	(59.52%)

Also studies contacted in Iraq reported that the susceptibility of *K. pneumoniae* isolates collected from clinical and environmental samples to imipenem was (100%) (Al-Asady, 2009; Al-Hilli,2010).These results relatively in agreement to the present study . Reasons of resistance may be due to inappropriate duration of antibiotic therapy and sub-therapeutic concentrations of the drug [26] or due to other resistance mechanisms including conformational changes in PBPs, permeability changes in the outer membrane and active efflux of the antibiotic [27].

In local studies; at Baghdad city, Rhumaid and Al-Mathkhury [28] referred that the isolates were resistance to antibiotics; Imipenem (5.6%), Meropenem (9.4%), Ceftazidime (58.5%), and Cefotaxime (43.3%).While Al-Qafaji [29] referred that (100%) and (94.5%) of *K. pneumoniae* isolates were resistance to Cefotaxime and Ceftazidime respectively. The present study exhibited sensitivity to Amikacin and Gentamicin (78.57%), Meropenem (76.19%), Imipenem (69.04%) andCiprofloxacin(38.09%).The results of this study belong to Cefotaxime and Imipenem in compared to local study [30] was not agree. Results from table (1) revealed that higher resistant rate was found for Piperacillin (85.71%);this result in agreement with a previous studies; Al-Asady (2009) and Al–Hilli (2010) who found that *Enterobacteriaceae* isolates were resistant to piperacillin (100%)and (81%) respectively. High resistance to this class of antibiotics may be due to widespread use of antibiotics in hospitals [31,32].

Screening for metallo β-lactamases (MBL)

Detection of metallo β-lactamases (MBL) were performed by Meropenem-EDTA double disks method and modified Hodge test. Some carbapenem resistance *K. pneumoniae* isolates were MBL producers. 20 from 30 of isolates(66.6%) showed overnight growth an increase of ≥ 17 mm in zone diameter in the presence of 1900 µg of EDTA compared to Meropenem disk alone (Figure-1). Also all these isolates showed the presence of a clover leaf-like shaped zone of inhibition around each tested strain, which was interpreted as a phenotypic evidence of MBL production (Figure-2).

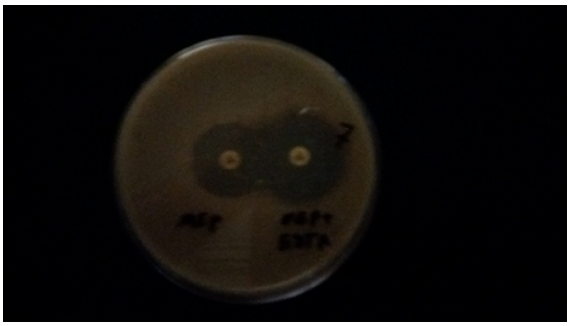

Figure 1: Meropenem-EDTA double disks method.

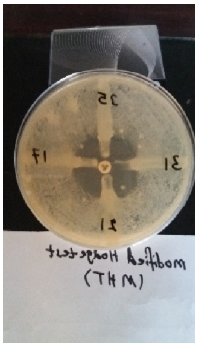

Figure 2: Modified Hodge test (MHT)

Genotypic detection of *blaNDM*-1,2 genes:

PCR was carried out on the DNA of 20 carbapenem resistance *K. pneumoniae* isolates for *blaNDM*-1,2, using specific primer for *blaNDM*-1,2 forward and *blaNDM*-1,2 reserve (Table-2). Amplification was performed in a 25µl volume as recommended by Promega Master mix instruction. DNA molecular size marker (500-bp ladder for *NDM*-1gene and 1000-bp for *NDM*-2gene). PCR revealed Lanes (K1 to 20) of *K. pneumoniae* isolates showed positive results with *blaNDM*-1gene (100 %) (table 5) (Fig.3a,b). Lanes (K 2, 10,16,17,18,and 20) show negative results with *blaNDM*-2 genes 6 (30 %). (Fig.4a,b.).

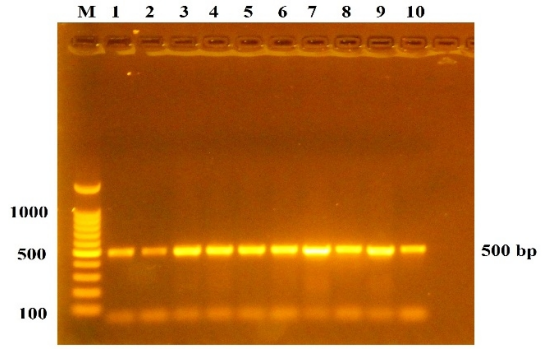

Figure 3a.: Agarose gel electrophoresis in 1% for *bla NDM*-1gene product show positive results(1-10).Ethidium bromide stain (0.5%), Amplicon size (500bp), DNA Ladder (100bp), the electric current at 100 volt for 1hr.

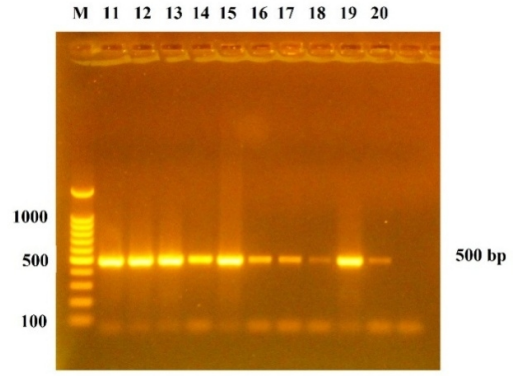

Figure 3 b.: Agarose gel electrophoresis in 1% for *blaNDM*-1gene product show positive results(11-20). Ethidium bromide stain (0.5%),Ampliconsize (500bp),DNA Ladder (100bp),the electric current at 100 volt for 1hr.

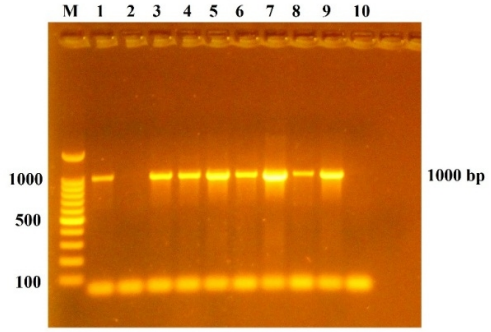

Figure 4 a.: Agarose gel electrophoresisin 1% for *blaNDM*-2gene product show negative results(2,10).Ethidium bromide stain (0.5%), Amplicon size(1000bp), DNA Ladder (100bp) ,the electric current at 100 volt for 1hr.

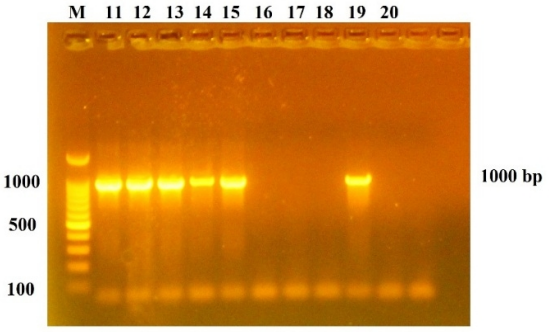

Figure 4 b.: Agarose gel electrophoresis in 1% for *blaNDM*-2gene product show negative results(16,17,18,20).Ethidium bromide stain (0.5%), Amplicon size (1000bp), DNA Ladder (100bp), the electric current at 100 volt for 1hr.

Table 5 : Occurrence of *blaNDM*-1 and 2 genes between *K. pneumonia* isolates

Frequency	isolates No.	*NDM*-1 gene	*NDM*-2 gene
1	1	+	+
2	2	+	-
3	3	+	+
4	4	+	+
5	5	+	+
6	7	+	+
7	8	+	+
8	9	+	+
9	11	+	+
10	12	+	-
11	13	+	+
12	14	+	+
13	15	+	+
14	16	+	+
15	17	+	+
16	21	+	-
17	31	+	-
18	35	+	-
19	39	+	+
20	40	+	-

This is the first report of *blaNDM*-1 and *blaNDM*-2 genes in Baghdad hospitals among *K. pneumoniae* isolates. There were many types of *blaNDM* gene which was located mostly onto conjugative plasmids belonging to several incompatibility groups [32].So an important consideration should be taken when designing genetic tools to the target carbapenem resistance genes. The occurrence of isolates contain *blaNDM* in Baghdad hospitals may be resulted from transfer of plasmid among resistant isolates from medical care purpose which may helped in acquiring *NDM* gene when many Iraqi patients were travelled to India and to other countries for medical care purpose **.**Comparing our results that showed high prevalence of *NDM*-1 and 2 genes, with a study was carried out in the period between April 2009 and February 2011 in Mubarak Al Kabeer Hospital in Kuwait agreed with our results; three isolates were *NDM*-1 positive in *K. pneumoniae* [33]. Multiple reports showed infected cases with *NDM*-1 positive organisms; 44 isolates with *NDM*-1 were identified in south India(Chennai), 26 in north India (Haryana), 37 in the UK, and 73 in other sites in India and Pakistan [34].While Molecular investigations revealed the first identification of the *NDM*-1 gene in Australia [35] from a man who had been previously hospitalized in Bangladesh and then transferred to Australia.

In local study which was carried out in Hillah hospital by AL-Harmoosh and Jarallah [36] revealed the first identification of the *NDM*-1,2 genes in Iraq harbored *Acinetobacter baumannii* isolates among patients with different infections.

Conclusion

The study had shown the spreading of *blaNDMK. pneumoniae* isolates among patients with burn infections. The rapid spread of *blaNDM* genes of *K. pneumoniae* isolates among patients with burn infections in this study poses an increased threat to hospitalized patients in Iraq and more importantly, avoiding misuse , overuse of antibiotics may converse the undesired effects of multidrug resistant and *NDM*producing bacteria. Furthermore we would expect more *NDM* variants to be discovered in the next years in Iraq.

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