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Reports

FIRST REPORT OF CO-HARBORING BLEOMYCIN RESISTANCE GENE (ble_{MBL}) AND CARBAPENEMASE RESISTANCE GENE (bla_{NDM-1}) KLEBSIELLA PNEUMONIAE IN IRAQ WITH COMPARISON STUDY AMONG THE SENSITIVITY TEST, THE BD PHOENIX CPO DETECT TEST, AND THE RAPIDEC® CARBA NP TEST FOR DETECTING CARBAPENEM RESISTANT GRAM NEGATIVE BACTERIA

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Two hundred and fifty samples were collected from burn and wound patients between January and July 2023 in Plastic Reconstructive and Burn Surgery Hospital at Sulaymaniyah city/Kurdistan region of Iraq, in addition to the Burn Unit and Wound Care Units of the Azadi Teaching Hospital in Kirkuk, Iraq. We performed a comparison study among (Antibiotic sensitivity test, BD Phoenix CPO detect test and RAPIDEC CARBA NP test), this was the first study of its kind to be performed in Iraq. The findings were supported by molecular detection of two of the most common major carbapenemases in the country, NDM-1 and OXA-48, as well as this study detected five of the recently identified minor/rare carbapenemases worldwide for the first time in Middle East (BKC-1, FRI-1, LMB-1 ,OXA-426, and OXA-198). We recorded five isolates containing chromosomal NDM-1 and OXA-48 genes. The findings indicated that the RAPIDEC CARBA NP test serves as a quick, accurate and sensitive means of detecting all five isolates containing the bla_{NDM-1} and bla_{OXA-48} genes. The antibiotic sensitivity test was insufficient in detecting the CPOs while phoenix NMIC-413 AST panel was revealed to be more reliable and accurate in identifying carbapenem resistant isolates in clinical settings than antibiotic sensitivity test and the BD Phoenix CPO detect test report was found to have low detection specificity, further confirmatory tests are required. The current study recorded first report of co-harboring bleomycin resistance gene (ble_{MBL}) and

carbapenemase resistance gene (bla_{NDM-1}) Klebsiella pneumoniae in Iraq with accession NO. PP411935. The development of new routine tests for identifying isolates resistant to carbapenem in Iraqi hospitals and other developing countries is very essential. Gram-negative isolates from Iraq that were resistant to carbapenem did not harbor the recently found minor carbapenemase.

Keywords: *OXA-48; OXA-426; BKC-1; FRI-1; OXA-198; Brilliance CRE Agar; report*

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Introduction

There has been an alarming rise in the prevalence of carbapenemase-producing gram-negative bacteria throughout the world over the last ten years [1]. Carbapenems (such as imipenem, meropenem, doripenem and ertapenem) are highly effective and safe antibiotics and used as last-line drugs for controlling multidrug-resistant gram-negative pathogens (including extended-spectrum beta-lactamase-producing *Enterobacteriaceae*) [2]. Carbapenem antibiotics are often used to treat serious infections because of their broad-spectrum activity, but the resistance towards carbapenem has increased significantly because of the overprescription of these antibacterial drugs, and this considered as a main cause of the carbapenemase gene expression in members of this family [3; 4]. Carbapenemase enzymes considered as major and most common mechanism of carbapenem resistance [3; 5].

There are two types of these wide-ranging enzymes, namely by “Big Five” carbapenemases and the “rare” carbapenemases , and this has been evidenced in 30 years of carbapenemase epidemiology, according to David et al. (2019) [6] the term “Big Five” refers to the five primary carbapenemases that identified globally, the Ambler classes A, B, and D beta-lactamases; class A carbapenemases, *Klebsiella pneumoniae* carbapenemase (KPC) enzymes; class B metallo-beta-lactamases (MBLs) such as New Delhi metallo-beta-lactamases (NDM), Verona integron-encoded metallo-beta-lactamase (VIM), and imipenemase (IMP) type enzymes; and class D carbapenem-hydrolyzing oxacillinase (OXA) such as OXA-48 enzymes.

The four classes of β-lactamases include a broad set of enzymes including the rare carbapenemases, their lower prevalence may be caused by underdetection since there are no specific diagnostic tests that target these rare enzymes , also, it could be due to genetic features leading to a lower spread [7].

Brazilian *Klebsiella c*arbapenemase, **BKC-1** is a newly-discovered carbapenemase , it was first identified in São Paulo, Brazil in three *K. pneumoniae* strains [8]. The synthesis of BKC-1 enzyme causes a reduction in susceptibility to carbapenems and resistance to aztreonam, penicillins and broad-spectrum cephalosporins. BKC-1 is similar to ESBLs; it has no effect on the effectiveness of cefoxitin. The reported phenotype with hydrolysis of penicillins, carbapenems and cephalosporins (but not cefoxitin) could be validated by purifying this enzyme [9].

French imipenemase FRI-1 gene is considered as another class A carbapenemase family present in Enterobacterales, which is resistant to imipenem [10]. The blaFRI−1 gene was detected in an *E.cloacae* obtained from a patient in Paris who had previously visited Switzerland, the production of FRI-1 reduced resistance to many antimicrobial agents such as penicillins, aztreonam, cephalosporins, and carbapenems. The results indicated that all of the tested β-lactams were hydrolyzed by the purified FRI-1 enzyme with the exception of ceftazidime. At present time, a total of nine variants were reported, FRI-1 has been discovered in France [10], FRI-2 is present in the United Kingdom [11], FRI-3 has been identified in Germany [12], FRI-4 in Japan [13], FRI-5 in Japan too (MH208723), FRI-6 in Canada [14], and FRI-7/-8/-9 in Japan (with accession numbers AP019534, AP019635, AP019633) [7].

Linz metallo-β-lactamase LMB-1 is the latest metallo-β-lactamase that present in Enterobacterales [15]. In 2013, this enzyme was initially identified in Salzburg/Austria from *E. cloacae* isolate, LMB-1 is reported to have high resistance to penicillins, broad-spectrum cephalosporins and carbapenems. Recently, this carbapenemase is detected in Buenos Aires, Argentina in the *C. freundii* isolate [16]. Various kinds of tests revealed that all β-lactams were hydrolyzed by the LMB-1, with the exception of cefepime and aztreonam [7].

As reported by Garch et al. (2011) [17], the CHDL **OXA-198** is described in *P. aeruginosa*. Recently, it is detected in clinical isolate of *Citrobacter pasteurii* in France [18]. At present time, this kind of carbapenamase was only reported in France and Belgium, with MICs of 0.38 mg/L, 0.5 mg/L, and 1 mg/L for meropenem, ertapenem and imipenem, respectively, *C. pasteurii* clinical isolate reported to confer a slight decrease in susceptibility against carbapenems. Furthermore, this kind of carbapenemases preserves ceftazidime but conferring resistance against penicillin/inhibitor combinations.

The last CHDL family reported in Enterobacterale is **OXA-427** [19]. In Belgium, this kind of carbapenemase was isolated from a number of various kinds of Enterobacterales, including *K. pneumoniae, K. oxytoca, E. coli, Providencia rettgeri*, and *S. marcescens*. Moreover, this carbapenemase is widly resistant to penicillin (i.e., ceftazidime, temocillin, aztreonam, and ertapenem), but not cefotaxime. Bogaerts et al. (2017) [19] conducted a biochemical analysis and found that ceftazidime and imipenem can be hydrolysed by OXA-427 but Frère et al. (2020) [20] found that it was inhibited by avibactam.

Most beta-lactam antimicrobial drugs (including carbapenems) are hydrolyzed by MBLs. At present time, there are no clinically effective inhibitors for MBLs, based on their sequence, structure, and zinc ion site(s), MBLs are further divided into B1, B2, and B3 subclasses. They also have different substrate profiles or β-lactam specificities [21; 22; 23]. The subclass B2 enzymes contain a narrow substrate spectrum towards the carbapenem antibiotics, B1 and B3 MBLs have a large substrate spectrum; including the carbapenems and other β-lactam antibiotics [24], B1 enzyme subclass is considered as the most clinically prevalent MBL, which includes imipenemase (IMP), New Delhi metallo-β-lactamase (NDM) and Verona integron-encoded metallo-β-lactamase (VIM) [25].

Recently, a new gene identified from *Acinetobacter baumannii* and carbapenem-resistant *Enterobacteriaceae*, named bleomycin resistance gene(ble_{MBL}) which located downstream of the bla_{NDM-1} gene as part of the same operon, this gene coded for protein, called by BRP_{MBL} which recently demonstrated its function in decreasing the susceptibility against bleomycin and bleomycin-like molecules (zeocin)[26]. Dortet et al. (2017) [26] has shown that bleomycin-like molecules are inactivated by (i) bleomycin hydrolases; (ii) bleomycin N-acetylating enzymes; and (iii) bleomycin-binding proteins (BMLA and BMLT), which work by trapping the bleomycin-like molecules.

This study aimed to determine the prevalence of carbapenem resistance gram-negative bacteria in burn and wound patients, and determined the efficiency of some carbapenem resistant methods in detecting the carbapenemase-producing organisms (CPOs), so we performed a comparison study among (Antibiotic sensitivity test, BD Phoenix CPO detect test and RAPIDEC CARBA NP test), this was the first study of its kind to be performed in Iraq. The findings were supported by molecular detection of two of the most common major carbapenemases in the country (NDM-1 and OXA-48), as well as this study detected five of the recently identified minor/rare carbapenemases worldwide for the first time in Middle East (BKC-1, FRI-1, LMB-1, OXA-426,

and OXA-198) to provide information on the extent of these new minor genes from an epidemiological and microbiological perspective in Iraq.

Methodology

A cross-sectional study was carried out in the Plastic Reconstructive and Burn Surgery Hospital in Sulaymaniyah, in the Kurdistan region of Iraq, in addition to the Burn Unit and Wound Care Units of the Azadi Teaching Hospital in Kirkuk, Iraq. The study conducted on burn and wound patients between January and July 2023.

1. Sample Collection

Using a sterile cotton swab, 250 samples (110 burn swabs and 140 wound swabs) were collected, after which they were processed under aseptic conditions in the Microbiology Laboratory. Data on patients' gender, age, and type of burn and wound were recorded in current study.

2. Laboratory Analysis (Culturing)

The swabs were cultured on MacConkey agar, Cetrimide agar, and also on Drigalski Lactose agar upon arrival in the laboratory. This was the first study of its kind to use Drigalski Lactose agar in the context of Iraq. This medium is both selective and differential when isolating Enterobacteriaceae and specific non-fermenters from clinical samples. Subsequently, they were incubated for 18 to 24 hours at 37°C. The colour, form, and overall appearance of each colony on each plate were assessed after a 24-hour period, as part of the colonial morphology analysis.

3. The BD Phoenix™ M50 for chemical identification and Susceptibility testing:

Combination ID/AST panels are available with the BD PhoenixTM, which uses 51 wells for identifying substrates. The majority of clinically relevant Gram-positive and Gram-negative bacteria and yeast can be quickly identified using this device. We picked colonies in the culture then suspended them in ID broth, recapped the tube to vortex them then inserted the tube and read with the BD phoenixSpect™ nephelometer then transported 25 micron into AST broth tube and added BD Phoenix AST indicator to AST broth tube, recapped the tube and inverted to mix then poured the content of ID broth and AST tube inside the panel, finally capped the panel and placed it in BD Phoenix instrument.

4. Carbapenem Detection Approaches

1- RAPIDEC® CARBA NP Test: This test was conducted according to the instructors of Kit. It provided accurate results in 30 minutes to 2 hours.

2- BD Phoenix CPO detect test NMIC/ID-431 was employed to detect carbapenemase-producing organisms (CPOs) in gram-negative bacteria depending on the report results of this device.

3- Susceptibility testing (Kirby Bauer disc diffusion method) was performed using imipenem, meropenem, ertapenem discs (10 µg) in accordance with the CLSI 2018 guidelines.

5. Genotypic Identification of Carbapenemase-Encoding Genes

DNA and Plasmid Isolation of Bacteria

In accordance with the manufacturer's instructions, pure cultures of bacterial isolates were left overnight in a liquid nutrient broth medium (NB) to cultivate. The purpose of this was to isolate bacterial genomic DNA and plasmids using bacterial genomic DNA and plasmid purification kit (ADDBIO, Korea). Additionally, the PCR amplification method was employed to perform molecular screening on each isolate to identify genes encoding carbapenemase. In this work, carbapenemase-encoding genes from clinical isolates were identified using traditional PCR, and the PCR results were sequenced.

Polymerase Chain Reaction (PCR)

When carrying out this test, seven pairs of primers (Macrogen, Seoul, Korea) as seen in Table 1 were employed. 2 µl of template DNA, 12.5 µl of Master Mix, and 1 µl of each prime were used in the PCR reaction mixture. The volume was filled to 25 µl using nuclease-free water. The following cycle parameters were employed: five minutes at 95°C, followed by 39 amplification cycles of 20 seconds at 95°C, 30 seconds at 58°C, and 30 seconds at 72°C. The final extension lasted five minutes at 72°C. Electrophoresis was used to resolve the amplification products on a 1.5% agarose gel for 50 minutes at 90 volts. The ethidium bromide-stained gels were seen under UV light, displaying as a light fluorescent band, and visually captured and recorded on a high-resolution digital camera (Hasan et al., 2021).

Table 1.

The primers used in this study

Gene	Primer Sequence (5' - 3')	Product size (b)	Reference
bla_{NDM-1}	Forward AGCAAATGGAAACTGGCGAC Reverse AAAACGCCTCTGTCACATCG	766	This study
bla_{OXA-48}	Forward TCATCAAGTTCAACCCAACCG Reverse GGTAGCAAAGGAATGGCAAGAA	629	This study
bla_{BKC-1}	Forward CGGCAATGCGACCAATCTC Reverse ATCACATTTTCGCGCCGG	925	This study

bla_{FRI-1}	Forward TTACTGCTTCGTCATGTTTGTC Reverse GCTCTGTCTTCCATTGAACTCA	791	This study
bla_{LMB-1}	Forward AAGTTCGATGGCTATGCTGG Reverse CTCGAGCCGCTGTGTTATC	837	This study
$bla_{OXA-427}$	Forward GTCCCGCATTCTGTTATCCA Reverse AAACACCTCTTCCCTGGCC	760	This study
$bla_{OXA-198}$	Forward CTTTCTGCTGTCGGTGCC Reverse CGATGATCCCCTTTGCTTGT	743	This study

6. Phylogenetic Analysis

The nucleotide sequences of the genes encoding carbapenemase were ascertained by DNA sequencing by Macrogen Company (South Korea). MEGA X was used to create a phylogenetic tree after the sequencing data were analysed using the NCBI-based BLAST website.

7. Genbank Accession Numbers

In this work, the bla_{OXA-48} gene and bla_{NDM-1} gene sequences were deposited in the Genbank database under accession numbers PP411933, PP411934, PP411935, PP411936 and PP411937.

8. Statistical Analysis

The 23rd edition of Statistical Package for Social Science (SPSS) was used in this work to analyse the data. Moreover, the gathered data was subjected to a chi-square test, where a P-value of < 0.05 was deemed to be statistically significant.

Results

1. Prevalence of Gram Negative Bacteria among Burn and Wound Patients

250 clinical samples were collected from the Burn Unit and wound units of Azadi Teaching Hospital in Kirkuk, Iraq and the Plastic Reconstructive and Burn Surgery Hospital in Sulaymaniyah, Kurdistan region, Iraq, 100 (40%) Gram negative bacteria were isolated from the total medical cases as mentioned below in Figure 1, distributed 42(42%) isolates from burn patients and 58(58%) from wound patients.

Table 2 showed the rate of gram negative bacteria isolates according to patients' gender and their age group in burn patients, the results demonstrated high rate of gram negative bacteria among the female than male (59.52% and 40.47% respectively), and the high prevalence of these isolates were reported among 1 to 10 years old group (30.95%) and the low prevalence among 51-60 years old group (2.38%). There was no a statistically significant difference in

rate of gram negative bacteria according to patients' gender and age group in burn patients ($p > 0.05$).

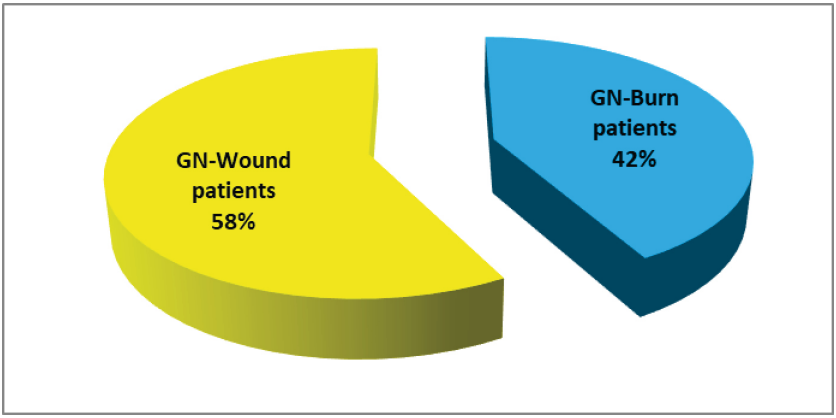

Fig. 1. Prevalence of Gram Negative Bacteria among total of positive isolates

Table 2.

The rate of gram negative bacteria according to patients' gender and age group in burn patients

Gender of Burn Patients	**NO. of Burn Samples (total. No=110)**	**NO. of positive Samples(%) (total. No=42)**
Male	44 (40%)	17(40.47%)
Female	66 (60%)	25(59.52%)
Age of Burn Patients	**NO. of Burn Samples (total. No=110)**	**NO. of positive Samples(%) (total. No=42)**
1-10 Years	37	13(30.95%)
11-20 Years	15	7(16.66%)
21-30 Years	23	7(16.66%)
31-40 Years	10	3(7.14%)
41-50 Years	12	6(14.28%)
51-60 Years	5	1(2.38%)
More than 61	8	5(11.9%)

Table 3 showed that the Scalds were the main cause of burn injuries in 62(56.36%) patients, while the highest rate of gram negative bacterial isolates were from patients with flame as the source of burn injuries 25 (59.52%) followed by scalds and electricity (38.09 and 2.38% respectively). The majority of gram negative isolates were from third burn degree (57.14%) followed by

second burn degree (40.47%) and first burn degree (2.38%). There was no a statistically significant difference in rate of gram negative bacteria according to source of burn ($p > 0.05$), but statistically significant difference was observed in rate of gram negative bacteria according to degree of burns ($p < 0.05$).

Table 3.

The rate of gram negative bacteria Mechanism of burn injury and type of burn degree

Source of Burn	**NO. of Burn Samples (Total. No=110)**	**NO. of positive Samples(%) (Total. No=42)**
Flame	45 (40.9%)	25(59.52%)
Scalds	62 (56.36%)	16(38.09%)
Electricity	3 (2.72%)	1(2.38%)
Degree of burns	**NO. of Burn Samples (Total. No=110)**	**NO. of positive Samples(%) (Total. No=42)**
First Degree	8 (7.72%)	1(2.38%)
Second Degree	71 (64.54%)	17(40.47%)
Third Degree	31 (28.18%)	24(57.14%)

Table 4 showed the rate of gram negative bacterial isolates according to patients' age group and their gender in wound patients. This study demonstrated high rate of isolated gram negative bacteria were among the male than female (58.62% and 41.37% respectively), and the high prevalence of these isolates were reported among more than 61 years old group (37.93%) and the low prevalence were among 1-10 years old group (1.72%). There was no a statistically significant difference in rate of gram negative bacteria according to patients' gender and age group in wound patients ($p > 0.05$).

Table 4.

The rate of gram negative bacteria according to patients' gender and age group in wound patients

Gender of Wound Patients	**NO. of Wound Samples (Total. No=140)**	**NO. of positive Samples (%) (Total. No=58)**
Male	99 (70.71%)	34(58.62%)
Female	41 (29.28%)	24(41.37%)
Age of Wound Patients	**NO. of Wound Samples (Total. No=140)**	**NO. of positive Samples (%) (Total. No=58)**
1-10 Years	9	1(1.72%)
11-20 Years	17	5(8.62%)

21-30 Years	19	7(12.06%)
31-40 Years	14	4(6.89%)
41-50 Years	13	3(5.17%)
51-60 Years	29	16(27.58%)
More than 61	39	22(37.93%)

Table 5 showed that the highest rate of isolated gram negative bacteria were from patients with Diabetic wound followed by Bed sore (29.31%and 24.13% respectively), while the lowest rate was from the dog bite wound (1.72%). There was no a statistically significant difference in rate of gram negative bacteria according to wound specimens' types ($p > 0.05$).

Table 5.

The rate of gram negative bacteria according to wound specimens' types

Source of Wound specimens'	NO. of Wound Samples (Total. No=140)	NO. of positive Samples (%) (Total. No=58)
Accidents wound	21	4(6.89%)
Surgery wound	36	10(17.24%)
Diabetic wound	37	17(29.31%)
Bed sore	16	14(24.13%)
Plastic surgery	28	12(20.68%)
Dog bite wound	2	1(1.72%)

In this study and for the first time in Iraq, the BD Phoenix was used for accurate and timely identification of whole 100 gram negative bacterial isolates; we obtained 21 different species of gram negative bacteria as mentioned in Table 6. The highest rate of identified gram negative bacteria from burn and wound patients were *Pseudomonas aeruginosa* 31(31%) followed by *Escherichia coli* 16(16%), *Enterobacter cloacae* 13(13%) and *Klebsiella pneumonia* 11(11%) distributed in burn patients as 15(35.71%), 6(14.28%), 3(7.14%) and 5(11.9%) respectively, while distributed in burn patients as 16(27.58%), 10(17.24%), 10(17.24%) and 6(10.34%) respectively.

The other species of gram negative bacteria that isolated from burn patients were *Acinetobacter baumannii* 4(9.52%), *Klebsiella oxytoca* 2(4.76%), *Citrobacter koseri* 1(2.38%), *Acinetobacter calcoaceticus–baumannii complex* 1(2.38%), *Serratia marcescens* 1(2.38%), *Pantoea agglomerans* 1(2.38%), *Pasteurella pneumotropica* 1(2.38%), *Pseudomonas fluorescens* 1(2.38%) and *Klebsiella aerogenes* 1(2.38%). While the gram negative bacterial species that isolated from wound patients were *Proteus mirabilis* 4(6.89%), *Morganella mor-*

ganii 4(6.89%), *Citrobacter freundii* 2(3.44%), *Citrobacter koseri* 1(1.72%), *Citrobacter farmeri* 1(1.72%), *Stenotrophomonas maltophilia* 1(1.72%), *Kluyvera ascorbata* 1(1.72%), *Escherichia vulneris* 1(1.72%) and *Pseudomonas putida* 1(1.72%). There was no a statistically significant difference in rate of identified gram negative bacteria isolates by using BD Phoenix™ M50 ($p > 0.05$).

Table 6.

Identified gram negative bacteria isolates by using BD Phoenix™ M50

Isolated bacteria by BD phoenix	NO. of Isolated bacteria from Burn Patients (%)	NO. of Isolated bacteria from Wound Patients (%)	Total (%)
Pseudomonas aeruginosa	15(35.71%)	16(27.58%)	31(31%)
Escherichia coli	6(14.28%)	10(17.24%)	16(16%)
Enterobacter cloacae	3(7.14%)	10(17.24%)	13(13%)
Klebsiella pneumonia	5(11.9%)	6(10.34%)	11(11%)
Acinetobacter baumannii	4(9.52%)	0	4(4%)
Proteus mirabilis	0	4(6.89%)	4(4%)
Morganella morganii	0	4(6.89%)	4(4%)
Klebsiella oxytoca	2(4.76%)	0	2(2%)
Citrobacter freundii	0	2(3.44%)	2(2%)
Citrobacter koseri	1(2.38%)	1(1.72%)	2(2%)
Citrobacter farmeri	0	1(1.72%)	1(1%)
Acinetobacter calcoaceticus–baumannii complex	1(2.38%)	0	1(1%)
Serratia marcescens	1(2.38%)	0	1(1%)
Pantoea agglomerans	1(2.38%)	0	1(1%)
Stenotrophomonas maltophilia	0	1(1.72%)	1(1%)
Kluyvera ascorbata	0	1(1.72%)	1(1%)
Pasteurella pneumotropica	1(2.38%)	0	1(1%)
Escherichia vulneris	0	1(1.72%)	1(1%)
Pseudomonas fluorescens	1(2.38%)	0	1(1%)
Pseudomonas putida	0	1(1.72%)	1(1%)
Klebsiella aerogenes	1(2.38%)	0	1(1%)
Total (%)	42	58	100

2. Prevalence of Carbapenem Resistance Gram Negative Bacteria among Burn and Wound Patients

The prevalence of carbapenem resistance gram negative bacteria were 23(23%), 21(21%) and 61(61%) by using various methods (antibiotic sensi-

tivity test, BD Phoenix CPO detect test NMIC/ID-431 and RAPIDEC CARBA NP Test) respectively, in this study, the total rate of carbapenem resistance was 66(66%) distributed as following: 24(77.41%), 10(62.5%), 6(15.38%), 5(45.45%) and 2(50%) for *Pseudomonas aeruginosa*, *Escherichia coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae* and *Proteus mirabilis* respectively, for the first time in Iraq, the results of RAPIDEC CARBA NP test recorded carbapenemase producing *Citrobacter freundii,* carbapenemase producing *Citrobacter farmeri,* carbapenemase producing *Escherichia vulneris* and carbapenemase producing *Pseudomonas putida* isolated from wound patients, carbapenemase producing *Acinetobacter calcoaceticus–baumannii complex* and carbapenemase producing *Pseudomonas fluorescens* isolated from Burn patients as mentioned in Table 7.

In current study four gram negative bacterial species were not carbapenemase producing organisms *(Klebsiella oxytoca, Citrobacter koseri, Pasteurella pneumotropica* and *Klebsiella aerogenes),* as mentioned in table 7. There was a statistically significant difference among the three methods that used to detect carbapenem resistant gram negative bacteria ($p < 0.05$).

Table 7.

The prevalence of carbapenem resistant gram negative bacteria by using antibiotic sensitivity test, BD Phoenix- CPO detect test and RAPIDEC CARBA NP Test

Carbapenem Resistant GN bacteria	Total	Detection method of Carbapenem Resistant bacteria			
		Sensitivity Test(Meropenem 10ug)	BD Phoenix-CPO marker	RAPIDEC CARBA NP Test	Total (%)*
Pseudomonas aeruginosa	31(31%)	11(35.48%)	9(29.03%)	22(70.96%)	24(77.41%)**
Escherichia coli	16(16%)	1(6.25%)	1(6.25%)	10(62.5%)	10(62.5%)
Enterobacter cloacae	13(13%)	0	0	6(15.38%)	6(15.38%)
Klebsiella pneumoniae	11(11%)	2(18.18%)	2(18.18%)	5(45.45%)	5(45.45%)
Acinetobacter baumannii	4(4%)	4(100%)	4(100%)	3(75%)	4(100%)*
Proteus mirabilis	4(4%)	0	1(25%)	2(50%)	2(50%)
Morganella morganii	4(4%)	0	1(25%)	4(100%)	4(100%)
Klebsiella oxytoca	2(2%)	0	0	0	0

Citrobacter freundii	2(2%)	1(50%)	1(50%)	2(100%)	2(100%)
Citrobacter koseri	2(2%)	0	0	0	0
Citrobacter farmeri	1(1%)	0	0	1(100%)	1(100%)
Acinetobacter calcoaceticus–baumannii complex	1(1%)	1(100%)	1(100%)	1(100%)	1(100%)
Serratia marcescens	1(1%)	0	0	1(100%)	1(100%)
Pantoea agglomerans	1(1%)	1(100%)	0	1(100%)	1(100%)
Stenotrophomonas maltophilia	1(1%)	1(100%)	0	0	1(100%)
Kluyvera ascorbata	1(1%)	1(100%)	0	0	1(100%)
Pasteurella pneumotropica	1(1%)	0	0	0	0
Escherichia vulneris	1(1%)	0	1(100%)	1(100%)	1(100%)
Pseudomonas fluorescens	1(1%)	0	0	1(100%)	1(100%)
Pseudomonas putida	1(1%)	0	0	1(100%)	1(100%)
Klebsiella aerogenes	1(1%)	0	0	0	0
Total (%)	100(100%)	23(23%) ***	21(21%)	61(61%)	66(66%)

* One isolate of *Acinetobacter baumannii* with** two isolates of *Pseudomonas aeruginosa* were resistant by sensitivity test but they gave negative results by RAPIDEC CARBA NP Test.

***Total results of sensitivity Test by using Imipenem and Ertapenem were 29(29%) and 38(38%) respectively.

The prevalence of carbapenem resistant gram negative bacteria according to the source of specimens among 66 CRGN isolates was distributed as following: 24(36.36%) from burn patients and 42(63.63%) from wound patients as mentioned in Figure 2.

The current study, for the first time reported the prevalence of carbapenem resistance GN isolates according to the cause of burn, degree of burns and the types of wounds, distributed as following: 12(50%), 12(50%) and 0% detected from Flame as cause of Burn in patients, scalds and electricity respectively, while 1(4.16%), 9(37.5%) and 14(58.33%) were determined from first degree,

second degree and third degree burn patients respectively. The highest rate of carbapenem resistance isolates were among third degree burn isolates

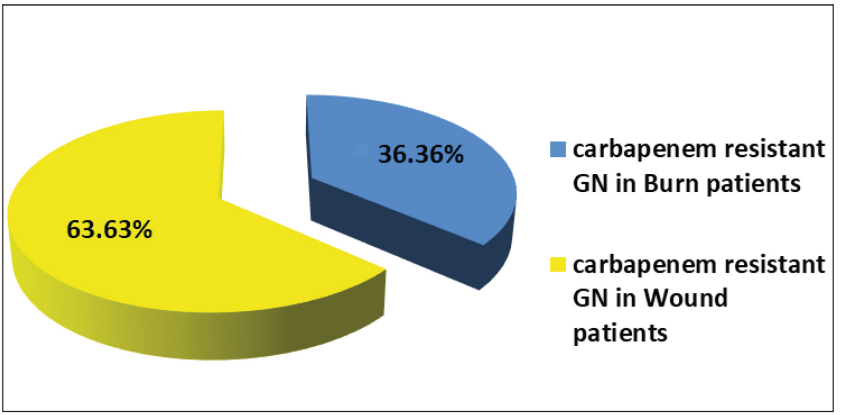

Fig. 2. Prevalence of carbapenem resistant gram negative according to the source of specimens

While 3(7.14%), 4(9.52%), 15(35.71%), 13(30.95%), 7(16.66%) and 0% of carbapenem resistance GN isolates detected from various kinds of wounds, distributed as accidents wound, surgery wound, diabetic wound, bed sore, plastic surgery and dog bite wound respectively. The highest rate of carbapenem resistance isolates were among diabetic wound isolates followed by bed sore isolates as mentioned in (Table 4.7). There was a statistically significant difference in the prevalence rates of CRGN bacteria according to cause of burns, degree of burns and source of wounds ($p< 0.05$).

Table 8.

The prevalence of carbapenem resistant gram negative bacteria according to Cause of Burns, degree of Burns and Source of Wounds

Source of specimens (Burn and Wound)	Gram negative isolates (%)	Carbapenem resistant GN isolates (%)
Cause of Burns Flame	Total 42 25(59.52%)	Total 24 12(50%)
Scalds	16(38.09%)	12(50%)
Electricity	1(2.38%)	0
Degree of Burns First degree burn patients	Total 42 1(2.38%)	24 1(4.16%)
Second degree burn patients	17(40.47%)	9(37.5%)

Third degree burn patients	24(57.14%)	14(58.33%)
Source of Wounds Accidents wound	Total 58 4(6.89%)	Total 42 3(7.14%)
Surgery wound	10(17.24%)	4(9.52%)
Diabetic wound	17(29.31%)	15(35.71%)
Bed sore	14(24.13%)	13(30.95%)
Plastic surgery	12(20.68%)	7(16.66%)
Dog bite wound	1(1.72%)	0

3. Prevalence of Carbapenemase genes

All 100 gram negative bacterial isolates were detected genotypically based on the primers described previously for two common major carbapenemase genes in Iraq (NDM-1 and OXA-48), and five latest minor carbapenemases in the world BKC-1, FRI-1, MBL-1, OXA-426 and OXA-198 by PCR technique (Figure 3), 4/66 (6.06%) of the isolates were harboring $bla_{\text{NDM-1}}$ gene among the overall carbapenem resistant gram negative (two isolates of *Pseudomonas aeruginosa* with two isolates of *Klebsiella pneumoniae*), and one isolate 1/66 (1.51%) of *Klebsiella pneumoniae* was carried $bla_{\text{OXA-48}}$ gene. While the latest minor carbapenemase genes were absent in all of studied isolates.

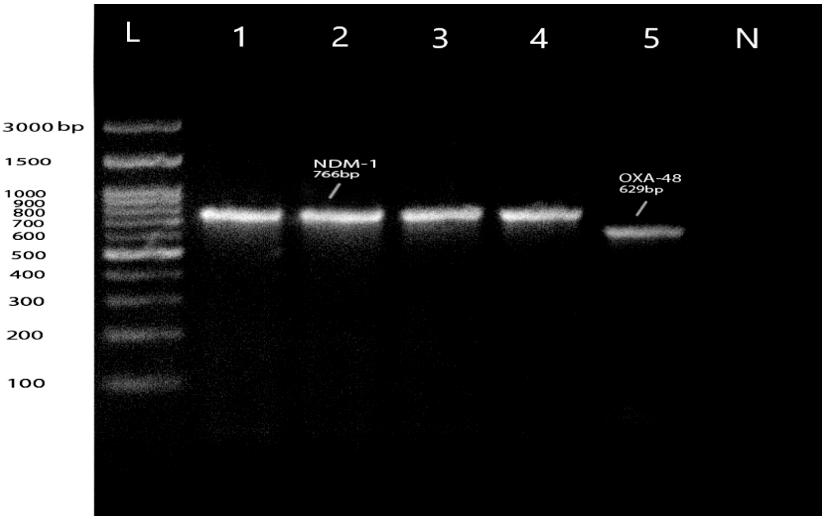

Fig. 3. PCR product of NDM-1 and OXA-48 genes isolated from carbepenem-resistant gram negative bacteria by gel electrophoresis. Lane L: 100bp DNA ladder; Lane N: negative control; Lanes 1-5: clinical isolates

These findings compared with the three tests' results that used in this study to detect the carbapenem resistant gram negative isolates, routine sensitivity test was able to detect two CPO isolates, BD Phoenix CPO detect test NMIC-431 was able to detect three carbapenemase producer, while all the 5 isolates that were carried bla_{NDM-1} gene and bla_{OXA-48} gene were detected by RAPIDEC CARBA NP Test.

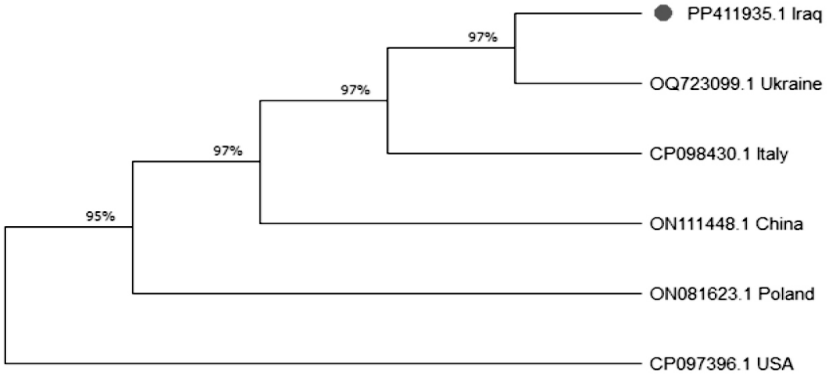

Fig. 4. The phylogenetic tree of ble_{MBL} and bla_{NDM-1} producing *Klebsiella pneumoniae*

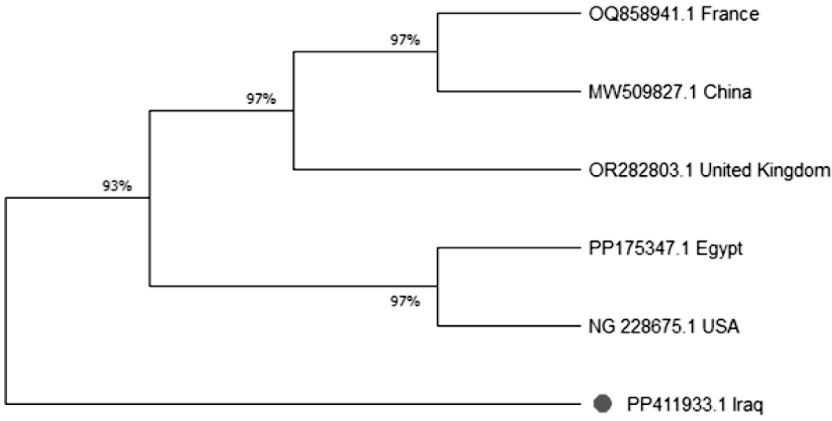

Fig. 5. The phylogenetic tree of bla_{OXA-48} producing *Klebsiella pneumoniae*

Discussion

1. Prevalence of Gram Negative Bacteria among Burn and Wound Patients

Morbidity and mortality are mainly caused by infectious diseases [27]. The skin and mucous membranes damaging can compromise the body's defense

and enable microbes to enter the body and cause infections [28]. Patients with burn injuries have more complications in infection, and wound infections can decrease the healing process of wound patients [29].

In current study, the most common gram-negative bacteria isolated from burn and wound patients were *Pseudomonas aeruginosa* due to its high antibiotic resistance and ability to thrive in different conditions, it raises mortality rates and hospitalizations [30; 31]. The results demonstrated higher rate of gram negative bacteria was among the female burn patients than male because the majority of studied burn specimens were collected from female patients, this agreed with the findings of other studies conducted in Iraq [32; 33], which revealed that burn injuries were more common in women than in men because women are more participated in cooking activities, which could explain why they have a comparatively higher risk of burn injuries [32]. The ages of studied burn patients were ranged from 1 to > 61 years; the age group of 1 to 10 years had the highest number of burn injuries, this is because of the risky behaviors of children when exploring their surrounding environment (i.e., playing with fire), and delayed defense reflex mechanisms [34]. This group was followed by the 21–30 age groups because they are the most age group that participated in outdoor activities.

This study showed higher rate of gram negative bacteria was among patients burned with flame, while the scalds were the main cause of burn injuries, this result disagreed with those of Hasan and Abass (2019) [32] because they recorded the flame as the main cause of burn injuries, this is because of their studied burn patients were adults with 21–30 age group who were suffered from flame burns, while in current study; the largest number of our studied burn patients were from children under 4 years and this agreed with the findings of Pruitt et al. (2012) [35], published in France, which showed children ages 4 and below who had been hospitalized for burn-related injuries were suffered from scald burns (65%) or contact burns (20%).

We in The current study recorded third burn degree patients with the majority of gram negative isolates followed by second burn degree and first burn degree. While, an Iraqi study performed in 2019 [32] found the majority of gram negative isolates was isolated from second-degree burns patients, followed by third-degree burns and lastly first-degree burns patients, the differences in results can be related to the timing of specimen collection from the burn patients under study, the bacterial growth is significantly more common in newer burns (burns less than 20 days old), than older burns [36].

While in wound patients, the highest rate of gram-negative bacteria was isolated from male patients because the majority of studied wound specimens

were collected from male patients with fewer samples taken from female wound patients. Also this study revealed the highest prevalence of these isolates in individuals over 61 years of age because of the declining immune responses in older individuals [37; 38].

According to type of wound, the highest rate of gram-negative bacteria was isolated from patients with diabetic wounds, because the diabetic patients tend to develop foot infections because of the neuropathy, diminished neutrophil function and vascular insufficiency. Between 30 and 50% of patients with diabetes develop foot ulcerations [39]. The second highest rate of gram-negative bacteria was identified in patients with bed sores because the bacterial infections were found to be the most common complication associated with pressure ulcers, and infections here can lead to soft tissue and bone infections, such as cellulitis, abscesses, bursitis, and osteomyelitis of the bone underneath the bed of the wound. Furthermore, pressure ulcers are a well-known cause of bacteremia [40]. While, dog bites were found to have the lowest rate of gram-negative bacteria, because there were only two studied patients with dog bite wound; one of them was dog bite wound patient with burn injury at the same site of dog biting. The findings of this study showed that *Pasteurella pneumotropica* was isolated from a dog bite patient with burn injury; this agreed with study of Lahlou et al. (2023) [41] revealed that the soft tissue infection after dog/cat bites or scratches is the most cause of pasteurellosis in humans.

2. Prevalence of Carbapenem Resistance Gram Negative Bacteria among Burn and Wound Patients

There are many reports of carbapenemases in Enterobacteriaceae in countries around the world [42], but not much research has been conducted in Iraq to study the gram-negative bacteria resistant to carbapenem. In order to study the efficacy of different techniques to detect the carbapenem resistant bacteria and provide important medical recommendations to improve the health situation in Iraq, we performed a comparison study among three medical methods: routine sensitivity tests, BD Phoenix CPO detect tests and RAPIDEC CARBA NP test for detecting carbapenem-resistant gram-negative bacteria, this was the first study of its kind to be performed in Iraq.

Based on the results of the RAPIDEC CARBA NP test (the most reliable, internationally-administered test), the carbapenemase production rate was 61 (61%), this rate is considered high and concerning rate, revealed that carbapenemase production plays a significant role in determining carbapenem resistance, these findings agreed with those revealed by other researchers in Spain [43], Iraq [44] and Italy [45]. Comparing the results of the most accurate test (RAPIDEC

CARBA NP) to those of the antibiotic sensitivity test using carbapenem discs revealed inefficiencies, it recorded 23(23%), 29(29%) and 38(38%) to meropenem, imipenem and ertapenem respectively, this agreed with the fact that the susceptibility pattern by disc diffusion was considered as first-line method for early detection of carbapenemases, our findings revealed Ertapenem as the most sensitive indicator for the activity of carbapenemases compared with Imipenem and Meropenem, this agreed with many studies [46; 47]. In contrast to disc diffusion method, BD phoenix reported 40 (61%), 35 (54%) and 28 (42%) resistance to ertapenem, imipenem and meropenem respectively; phoenix NMIC-413 AST panel was revealed to be more reliable and accurate in identifying carbapenem producing organisms (CPOs) in clinical settings, this agreed with study conducted by Zhang et al in 2021 [48], in addition to that, this device reported 21(21%) isolates as carbapenem producing organisms (CPOs), thus, this medical device was considered to be low specificity device for detecting carbapenemase-producing organisms (CPOs); when a carbapenemase-producer (CP) test yields positive results, the device quickly reports the data as "carbapenemase producer". However, it needs to be confirmed by confirmatory tests because of its relatively low detection specificity. Furthermore the new panel of BD Phoenix NMIC-500 appeared significant potential for CPO and AST [49]; as well as the Phoenix NMIC-502 panel for detecting Ambler class carbapenemases in CPO isolates [50].

From the total rate of carbapenem resistance 66 (66%), two isolates of *P. aeruginosa* and one isolate of *Acinetibacter baumanii*, *Stenotrophomonas maltophilia*, and *Kluyvera ascorbata* were negative for the RAPIDEC CARBA NP test, they showed resistance towards carbapenem by the antibiotic sensitivity test, which indicates other mechanisms of carbapenem resistance these isolates own, such as loss of porins that work to reduce the uptaking of carbapenems and/or efflux pump mechanism, which pumps the carbapenem drugs outside the cells [44; 45; 51].

As mentioned in Table 7, the results of RAPIDEC CARBA NP test recorded new bacterial species with carbapenemase enzyme in Iraq such as carbapenemase-producing *Citrobacter freundii,* carbapenemase-producing *Citrobacter farmeri,* carbapenemase-producing *Escherichia vulneris*, and carbapenemase-producing *Pseudomonas putida* isolated from wound patients*,* carbapenemase-producing *Acinetobacter calcoaceticus–baumannii* complex, and carbapenemase-producing *Pseudomonas fluorescens* isolated from Burn patients These results demonstrate the extensive transmission of carbapenemase genes through transmissible genetic factors (i.e., insertion sequences and plasmids) between different genera and even between gram-negative bacteria species.

As mentioned in Table 8, the prevalence of carbapenem-resistant GN isolates has been determined for the first time in this study according to the cause of burns, degree of burns, and types of wounds (accident, surgery, diabetic, bed sore, plastic surgery, and dog bite wounds), The differences in the rate of carbapenem-resistant isolates among many local and international studies belong to many important points such as the geographic region, hospital cleanliness, infection type from which samples were obtained, the technique that used to detect carbapenem-resistant bacterial isolates, and the hygiene of the population under study [52; 53; 54].

3. Prevalence of Carbapenemase genes

In this study, Special primers were designed and employed in this work to detect the two major carbapenemse genes (NDM-1 and OXA-48) with the latest discover minor/rare carbapenemase genes (BKC gene, FRI gene, LMB-1, OXA-426 and OXA-198), making the present study the first of its kind to be performed in the Middle East. The findings revealed that the studied carbapenem resistant gram negative isolates were lacked the recently identified minor carbapenemase, this could indicate that the genes have been recently developed and less widely distributed among gram-negative bacteria, making them less transmitted by plasmids and transposons. Also, their lower prevalence is due to their genetic features which limited their spread [7]. This study chose two major carbapenemase genes ($bla_{\text{OXA-48}}$ and $bla_{\text{NDM-1}}$) for molecularly detection because of the epidemiological fact that both of them are the most spread genes in Iraq, as reported in a systematic review on carbapenem resistance in Iraq [55], five bacterial isolates were found to be carrying these two genes in there chromosomes because of the fact that the two gene sequences employed in this study were not among the most common variant genes, the $bla_{\text{OXA-48}}$ gene was detected from the *Klebsiella pneumoniae* in a third-degree burn patient from Sulaymaniyah City in Kurdistan region of Iraq (accession NO. PP411933). The two NDM-1-producing *Klebsiella pneumoniae* isolates were obtained from skin graft wounds in Sulaymaniyah City (accession NO. PP411934). While the other isolate was obtained from a hand-wound patient in Iraq's Kirkuk City, this isolate carried the chromosomal $bla_{\text{NDM-1}}ble_{\text{MBL}}$, revealed the coexistence of $bla_{\text{NDM-1}}$ gene subclass B1 and the bleomycin binding protein gene (*ble*) as a first Iraqi report recorded in NCBI with accession NO. PP411935 (These two isolates; PP411934&PP411935 were carried the $bla_{\text{NDM-1}}$ gene subclass B, which defined as gene subclass with a wide substrate spectrum towards carbapenems among other β-lactam antibiotics). The two isolates of NDM-1-producing *Pseudomonas aeroginosa* were taken from bed-sore patients, one of whom was from

Kirkuk city in Iraq (accession NO. PP411936). This isolate also contained the NDM-1 gene subclass B, while the other isolate was from a patient in Sulaymaniyah City (accession NO. PP411937).

In this study, the results of three methods: Antibiotic sensitivity test, BD Phoenix CPO detects test NMIC/ID-431, and RAPIDEC CARBA NP test were investigated with the results of the PCR method. The antibiotic sensitivity test identified two CPOs out of 5 isolates that where harboring carbapenemase gene according to the molecularly test (PCR), BD Phoenix CPO detect test NMIC 431 identified three CPOs, thus, this medical device was considered to be low specificity device for detecting carbapenemase-producing organisms (CPOs), so it is required to use additional confirmatory methods to detect CPO accurately, our findings agreed with many international studies including [50; 56; 57]. All five CPOs were identified using the RAPIDEC CARBA NP test making it a great option for use in clinical laboratories, this finding agreed with those revealed in a number of international studies [58; 59; 60].

High rates of morbidity and mortality are associated with carbapenem resistant bacterial infections due to limited treatment options. Developing new routine method for detecting carbapenem-resistant organisms can help medical professionals to make important decisions about patient treatment and infection prevention. Therefore, this study recommended the Iraqi Ministry of Health to enter new, basic method to health system of laboratories in order to detect the carbepenem-resistant bacteria.

The RAPIDEC CARBA NP test is a great choice for clinical laboratories because it is an accurate method that can be completed in less than 2.5 hours, but it cost 15$ for a single test, it is expensive. Therefore, we suggest the use of Brilliance CRE Agar in all Iraqi hospitals; this medium facilitates the growth of isolates that are resistant to carbapenems with low detection limits and it is considered as a useful screening tool for non-fermentative gram-negative strains of bacteria that are resistant to carbapenems as well as enterobacteria. The medium is less expensive than the RAPIDEC CARBA NP test and permitted the growth of nearly all carbapenem-resistant non-fermenting isolates. Also, non-fermenters were easily distinguished from Enterobacteriaceae based on colony colour and form [61; 62].

The $bla_{\text{OXA-48}}$ gene is endemic in neighboring Turkey [63], whereas $bla_{\text{NDM-1}}$ is endemic in India [64], and many cases have been reported from Saudi Arabia, Turkey, and Iran [65; 66; 67]. This has an impact on Iraqi people who are visiting these endemic nations for tourism, medical care, and pilgrimage as well as the acquisition of these resistance genes from hospital settings [68]. Also, every

year, over 10 million tourists visit Iraq each year to participate in the fortieth day, which is known as the "Arba'een of Imam Hussein (Peace Be Upon him)." This helps bring carbapenem resistance genes by sick visitors from different countries to Iraq. Therefore, it is necessary to consider wearing a mask and using alcohol to sterilize the hands, in addition to avoiding using other personal tools. There is an urgent need to find guidelines and appropriate infection control procedures to prevent such infections among patients.[69]

Conclusions

The findings of this study demonstrated a considerable rise in the percentage of gram-negative isolates that produce carbapenemase among burn and wound patients in Iraq, Therefore it is recommended that the Iraqi Ministry of Health include new routine method in health laboratory system for detecting the carbapenem resistant bacteria such as the Brilliance CRE Agar or RAPEDIC CARBA NP tests. Compared to RAPEDIC CARBA NP, Brilliance CRE Agar is less expensive and the Iraqi hospitals recommended to use ertapenem discs because ertapenem is most sensitive indicator for detecting the activity of carbapenemases compared with imipenem and meropenem. It is imperative that medical staff wash their hands before and after using the restroom and interacting with patients to minimize the transmission of infectious pathogens to patients. Contamination control measures should also be implemented to prevent the spread of these microorganisms.

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