


Antibiogram and Genetic Characterization of Carbapenem-Resistant Gram-Negative Pathogens Incriminated in Healthcare-Associated Infections

This article was published in the following Dove Press journal:
Infection and Drug Resistance

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Purpose: Carbapenems are considered the most efficient antibiotic used in the treatment of nosocomial infections. Carbapenem-resistant Gram-negative rods are becoming a serious hazard in hospitals threatening public health. The aim of the current study was to investigate the prevalence of carbapenem-resistant Gram-negative pathogens incriminated in healthcare-associated infections, along with antimicrobial resistance profiles, carbapenemase and metallo- β -lactamase production, and their molecular characterization.

Methods: A total of 186 clinical specimens were collected from 133 patients at various hospitals in Cairo, Egypt. The obtained specimens were subjected to bacteriological examination, antimicrobial susceptibility testing, detection of carbapenemase production using the modified Hodge test (MHT), the metallo- β -lactamase production using the EDTA combined disc test (CDT), and PCR-based detection of the

*bla*_{KPC} and

*bla*_{GES} resistance genes. The identification of the highly resistant retrieved isolates was then confirmed by 16S rRNA gene sequencing.

Results: The most common isolated Gram-negative species was *Klebsiella pneumoniae* (40.9%), followed by *Acinetobacter baumannii* (18.8%), *Pseudomonas aeruginosa* (17.3%), *Escherichia coli* (15.4%), *Enterobacter aerogenes* (5.3%), and *Proteus mirabilis* (2.4%). The prevalence of carbapenem-resistant isolates was 36.1% (n=75). However, 86.5% of the recovered clinical isolates were susceptible to colistin. The MHT revealed that 33.6% (n=70) of the tested strains were positive for carbapenemase production, while the CDT showed that 33.17% (n=69) of the examined strains were metallo- β -lactamase producers. The PCR revealed that 98.6% (74/75) of the tested strains possessed the *bla*_{KPC} gene; moreover, 97.3% (73/75) of the examined strains harbored the *bla*_{GES} gene.

Conclusion: This study displayed the emergence of carbapenem-resistant Gram-negative pathogens incriminated in healthcare-associated infections. The accurate identification of carbapenem-resistant bacterial pathogens is pivotal for the treatment of patients, in addition to propelling appropriate contamination control measures to restrain the fast spread of such pathogens. Colistin showed a potent in vitro antimicrobial activity against the carbapenem-resistant strains.

Keywords: gram-negative rods, antibiotic resistance, carbapenem-resistance, *bla*_{KPC}, *bla*_{GES}, 16S rRNA gene

Introduction

The ongoing global rise of resistance to carbapenems in Gram-negative rods is considered a public health threat. Infections caused by carbapenem-resistant

bacteria (CR) are extremely hard to treat and have been related to a high death rate.¹ Multidrug-resistant Gram-negative pathogens are turning into an inexorably troublesome issue in hospitals and semi-permanent care services.^{2–4} Resistance to broad-spectrum antimicrobials such as cephalosporins is a noteworthy issue among Gram-negative rods, such as *Enterobacteriales*, *Pseudomonas sp.* and *Acinetobacter sp.* On the other hand, carbapenems are considered as the last line of treatment for nosocomial infections and community-acquired diseases.⁵

Enterobacteriales are significant opportunistic pathogens; they are ordinary occupants of the intestinal microflora but can cause severe illness in humans; including meningitis, pneumonia, pyelonephritis, septicemia, peritonitis, and device-related contaminations. They are among the major etiologic causes that lead to healthcare-associated infections (HAIs).⁶ In addition, the existence of multidrug-resistant Gram-negative bacterial pathogens from animal origins was reported by previous studies.^{7–10}

β -Lactams for instance cephalosporins, carbapenems, penicillins, and monobactams represent 60% of all antimicrobial agents utilized.^{11,12} They are favored due to their safety and adequacy and since their activity may be extended or renovated by chemical manipulation, nonetheless, their significant usage led to resistance. Such antimicrobial agents are essential for controlling and curing the grave hospital-acquired infections such as those connected with medical procedures, transplantation and hospitalizations in escalated care-units, and the development of carbapenem-resistant microorganisms leaves the healthcare facilities with no powerful medications.^{11,13}

Carbapenem-resistance mechanisms comprise the production of β -lactamases as well as the genetic mutations that affect the expression of Penicillin-Binding Proteins (PBPs) and porins.^{14,15} The synergism of these mechanisms could cause a remarkable carbapenem-resistance in numerous pathogens, including; *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Acinetobacter baumannii*.^{12,16–18} Carbapenemases are placed into three Ambler classes; Class-A (penicillinases), Class-B (metallo- β -lactamases), and class-D (oxacillinases).^{19,20} The combination of carbapenems with the extended-spectrum beta-lactams was considered as powerful antimicrobial agents for the treatment of infections caused by multidrug-resistant Gram-negative rods.²¹

Various kinds of carbapenem antibiotics including ertapenem, imipenem, meropenem, doripenem, and faropenem are most frequently used for treating severe nosocomial

infections yielded by β -lactamase producing *Enterobacteriaceae*.²² Resistance to carbapenems are usually intervened through β -lactamases and in addition due to carbapenemases enzymes (KPC, IMP, VIM, NDM, and OXA-48), encoded by the *bla* genes typically situated on transmissible-components “plasmid-mediated”, encouraging the transmission of resistance among various gram-negative pathogens.²³ Accurate investigation of carbapenem-resistance is essential for directing the line of treatment and additionally establishing a reliable infection control.^{24,25}

Molecular techniques that are used for precise detection of carbapenemases genes remain the gold standard for specificity and sensitivity. Consequently, PCR-based assays are usually used to avoid the problems of phenotypic identifications.²⁴ Amongst class-A β -lactamases, the most clinically important enzymes are KPC enzymes which encoded by *K. pneumoniae* carbapenemase-gene (KPC).^{26–29} The Guiana extended-spectrum β -lactamase (encoded by GES gene) involved up to 20 variants.³⁰ The *bla*_{GES} genes are generally carried on integrons found in different members of family *Enterobacteriaceae*. All the GES type variations can break-down the cephalosporins, however, certain variants have amino acid substitutions which amplify their broad effect against carbapenems.^{29,31,32}

The present study aims to determine the prevalence, antimicrobial resistance profiles, carbapenemases and metallo- β -lactamase production, and molecular characterization of carbapenem-resistant Gram-negative pathogens incriminated in healthcare-associated infections.

Materials and Methods

Specimens Collection

All methods were carried out in accordance with relevant guidelines and regulations. Sampling and all the experimental protocols were carried out by well-trained scientists and were approved by the Ethics committee, Specialist Hospital, Ain Shams University, Cairo, Egypt. The collected specimens were a part of the routine hospital laboratory procedures. A total of 186 clinical specimens: sputum (n=44), urine (37), blood (n=33), pus (n=27), wound (n=17), endotracheal tube (n=8), stool (n=7), drains (n=5), 3 central line (n=3), cerebrospinal fluid (n=3), bronchial alveolar lavage (n=1), and 1 pleural fluid (n=1) were collected from 133 different patients admitted in governmental and private hospitals

in Cairo, Egypt. Those patients were suffering from various diseases: 27 performed liver transplantation surgery, 21 were suffering from Diabetes mellitus (D.M), 18 chronic obstructive pulmonary disease (C.O.P.D), 18 urinary tract infection (UTI), 14 bronchogenic carcinoma (B.C), 12 chest infection, 8 Liver Cell Failure (L.C.F), 8 respiratory failure, and 7 were suffering from pneumonia. The samples were collected during the period from June to October 2017. All samples were collected in a clean and sterilized container under complete aseptic conditions and transported immediately to the Microbiology laboratory of Suez Canal University. Each specimen was labeled with the patient's age, gender, site of infection, and the source of specimen, and then transported to the laboratory.³³

Isolation and Phenotypic Identification of Gram-Negative Rods

The obtained samples were inoculated on MacConkey's agar, blood agar, cetrinide agar, nutrient agar, trypticase soy agar, and eosin methylene blue agar plates (Oxoid, UK) and incubated at 37°C for 24 h. A loopful of each pure colony was inoculated in trypticase soy broth (Oxoid, UK) and incubated on a shaker at 37°C for 24 h., 0.8 mL of the obtained culture was mixed with 0.2 mL of sterile glycerol in sterile vials and immediately placed in -20° C for later use. The suspected colonies were identified according to Tille,³⁴ Bergey, Holt³⁵ upon Gram's stain, colonial characteristics, motility, and biochemical reactions (oxidase, catalase, lactose, sorbitol, mannitol, and sucrose fermentation, indole, methyl red, Voges-Proskauer, citrate utilization, H₂S production, gelatin hydrolysis, urease production, and nitrate reduction tests).

Antimicrobial Susceptibility Testing (AST) and Minimal Inhibitory Concentration (MIC)

The susceptibility of the retrieved isolates to various commercial antimicrobial agents: imipenem (10µg), meropenem (10µg), amoxicillin-clavulanic acid (20/10µg), piperacillin-tazobactam (100/10µg), amikacin (30µg), tetracycline (30µg), ciprofloxacin (5µg), levofloxacin (5µg), trimethoprim-sulfamethoxazole (1.25/23.75µg), cefepime (30µg), ceftazidime (30µg), ceftriaxone (30µg), aztreonam (30µg), gentamycin (10µg), and colistin (10µg) (Oxoid, UK) were investigated using the disc

diffusion method (Modified Kirby-Bauer method). Additionally, the Minimal Inhibitory Concentration (MIC) for imipenem was performed using the broth dilution technique. Both tests were applied as described by CLSI.³⁶

Phenotypic Detection of Carbapenemases Production

In order to determine if strains produce carbapenemases, the retrieved Carbapenem-resistant isolates were subjected to Modified Hodge Test (MHT) as described by Clinical and Laboratory Standards Institute.³⁶ *E. coli* ATCC 25922 was used as an indicator strain. The turbidity of an overnight broth culture of the indicator strain was adjusted to 0.5 McFarland standards then diluted to 1:10 broth. Using a sterile cotton swab the indicator strain was inoculated on a Mueller Hinton agar plate (Oxoid, UK), as with the routine disc diffusion technique. Then the ertapenem disc (10 µg) was positioned in the center of the agar plates. Three to five colonies of the tested organism from overnight blood agar culture were picked by sterile swab and inoculated in straight-line, from the edge of the disc, up to a distance of at least 25 mm, then plates were incubated at 37 °C overnight then were examined. The Positive result is indicated by a clover-leaf-like indentation zone of inhibition from the indicator strain and the streak of inoculum.³⁷

Phenotypic Detection of Metallo-β-Lactamase Production

The isolated species were tested for metallo-β-lactamase (MBL) production using imipenem/EDTA combined disc test (CDT) as previously described by Yong et al³⁸ imipenem/EDTA disc was prepared by adding 750 µg of a sterile 0.5 M EDTA solution to an imipenem disc-10 µg, then discs were dried immediately in a 37°C incubator. A 0.5 McFarland-suspension of the tested isolate was inoculated on the surface of the Muller Hinton agar plate then a disc of imipenem and another disc of imipenem/EDTA were placed at suitable distances on the inoculated plate. Then plates were incubated for 24 h at 37°C. The positive result is indicated by the increase of the inhibition zone diameter (≥17 mm) of imipenem/EDTA disc compared to the imipenem disc alone. The negative result is indicated by no difference in the diameter of the zones around the two discs.

Genotypic Detection of Class-A Carbapenemases Genes

The extraction of genomic DNA was conducted using Bacterial DNA Preparation Kit (Jena Bioscience, GmbH, Germany) according to the manufacturer's instructions. The used oligonucleotide primers (Metabion International AG, Germany) were previously reported in the detection of Class-A carbapenemases.³⁹ Primer-sequences, cycling conditions, and the specific amplicon size are listed in Table 1.

PCR reaction mixtures of 25 µL (12.5 µL of Master Mix (Promega, WI, USA), 0.5 µL of each primer forward and reverse, 2.5 µL of the DNA, and up to 25 µL deionized water) were amplified in the MJ Mini™ Gradient Thermocycler apparatus (Biometra Göttingen, Germany). The PCR protocol was carried out as described by.²⁸ A reaction with no DNA-template was used as a negative control, while reference strains, kindly supplied by the Microbiology Department, Faculty of Science, Suez Canal University, Egypt, were used as positive controls. PCR products were screened using 1.5% (w/v) agarose gel electrophoresis (Applichem GmbH, Darmstadt, Germany) for 45 m at 100 V in 1× TAE buffer (0.04 M Tris, 0.0001 M EDTA, pH 8.0), visualized using 15µL of DNA gel stain and photographed under UV transilluminator. A 100 bp ladder (Thermo Scientific, Darmstadt, Germany) was used.³⁹

Genotypic Identification of the Retrieved Carbapenem-Resistant Isolates by 16S rRNA-Sequencing

The Molecular identification was performed by 16S rRNA sequencing for Carbapenem-resistant isolates which recorded the highest MIC value for imipenem and were positive for *bla*_{KPC} and *bla*_{GES} genes (n=13) including: *K. pneumoniae* (n=7), *A. baumannii* (n=2), *E. coli* (n=2), *E. aerogenes* (n=1), and one carbapenem susceptible *P. mirabilis* (n=1).

The universal 16S rRNA specific primers 27F-YM (5'-AGAGTTTGATYMTGGCTCAG-3') and 1492R (5'-TACCTTGTTACGACTT-3') were used in the PCR as previously described.⁴⁰ 1500 bp amplicons were produced and DNA sequenced between position 27 and 1492 of bacterial 16S rRNA genes numbered according to *E. coli* rRNA. PCR products were purified and sequenced by MacroGen Inc., (Seoul, Korea).

The sequences obtained were compared with those deposited in the public databases using the Basic Local Alignment Search Tool (BLAST) provided by the National Center for Biotechnology Information (NCBI) at (<http://www.ncbi.nlm.nih.gov/BLAST>). Sequences were submitted to NCBI and GenBank nucleotide sequence database and deposited under accession numbers KY856923 to KY856935.

Phylogenetic Analysis

The newly generated sequences of the current study as well as other related sequences downloaded from the GenBank were downloaded and concatenated into a balanced dataset for the phylogenetic analysis. The nucleotide sequences of the 16SrRNA genes of the retrieved isolates were aligned using ClustalW from MEGA software, version 5.05.⁴¹

The Juke and Cantor model of sequence similarity was conducted to estimate sequence relatedness.⁴² The dendrogram was constructed using Neighbor-Joining (NJ) method. Maximum Parsimony (MP) was applied to infer the phylogenetic relationships with bootstrapping of 100 replicates to get the branch support and testing the tree stability.

Statistical Analysis

The Chi-square was conducted to analyze the results using the statistical analysis software (SAS software version 9.4,

Table 1 Oligonucleotide Primers and Their Recycling Conditions Used for Detection of Class-A Carbapenemases Genes

Target Gene	Primer	Primer Sequence (5' - 3')	PCR Conditions				No of Cycles	Amplicon Size (bp)
			Denaturation	Annealing	Extension	Final Extension		
KPC	<i>bla</i> _{KPC} -F	GTATCGCCGTCTAGTTCTGC	94 °C for 30 sec	55 °C for 30 sec	72°C for 1 min	72°C for 10 min	25	638
	<i>bla</i> _{KPC} -R	GGTCGTGTTCCCTTTAGCC						
GES	<i>bla</i> _{GES} -F	GCTTCATTCACCGCACTATT	52 °C for 30 sec				30	323
	<i>bla</i> _{GES} -R	CGATGCTAGAAACCGCTC						

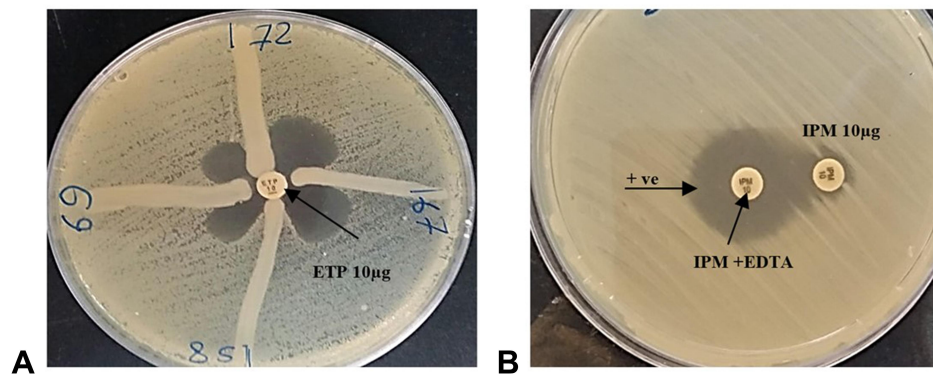


Figure 1 (A) Modified Hodge Test; Positive result appeared as a cloverleaf-like indentation of the zone of inhibition of the *E. coli* ATCC 25922 (indicator organism) along with the streak of inoculum. (B) Combined Disk Test; An inhibition zones with ≥ 17 mm in the inhibition zone diameter of EDTA-containing imipenem disc compared to imipenem disc alone was a positive MBL producer. The negative result appeared as no difference in the zones diameter around the two discs.

SAS Institute, Cary, NC, USA). The significance level was ($P < 0.05$).

Results

Prevalence Gram-Negative Rods

The bacteriological examination revealed that 208 isolates clinical isolates were retrieved from different examined specimens. The most predominant isolated pathogens were *K. pneumoniae* (40.9%), followed by *A. baumannii* (18.8%), *P. aeruginosa* (17.3%), *E. coli* (15.4%), *E. aerogenes* (5.3%), and *P. mirabilis* (2.4%) (Table 2). Statistically, there is a significant difference in the

prevalence of different bacterial pathogens recovered from various types of samples ($p < 0.05$).

Antimicrobial Resistance Profiles of the Retrieved Strains

In the present study, 86.5% of the recovered clinical isolates ($n=180$) exhibited remarkable sensitivity to colistin, while 13.5% of the obtained strains ($n= 28$) were resistant to colistin. On the other hand, the retrieved *K. pneumoniae* strains exhibited remarkable resistance to ceftazidime and levofloxacin (89.4%), ceftriaxone and trimethoprim-sulfamethoxazole (87.1%). Furthermore, the isolated

Table 2 The Prevalence of Different Bacterial Pathogens Isolated from Various Types of Samples

Samples	<i>K. pneumoniae</i> n (%)	<i>E. coli</i> n (%)	<i>A. baumannii</i> n (%)	<i>P. aeruginosa</i> n (%)	<i>E. aerogenes</i> n (%)	<i>P. mirabilis</i> n (%)	Total Isolates n (%)
Sputum	21 (40.4)	1 (1.9)	13 (25)	11 (21.2)	3 (5.8)	3 (5.8)	52 (25)
Urine	18 (45)	11 (27.5)	4 (10)	4 (10)	2 (5)	1 (2.5)	40 (19.2)
Blood	17 (50)	3 (8.8)	7 (20.6)	4 (11.8)	3 (8.8)	-	34 (16.3)
Pus	9 (28.1)	9 (28.1)	4 (12.5)	8 (25)	2 (6.3)	-	32 (15.4)
Wound	6 (28.6)	3 (14.3)	5 (23.8)	5 (23.8)	1 (4.8)	1 (4.8)	21 (10)
ETT	6 (66.7)	1 (11.1)	1 (11.1)	1 (11.1)	-	-	9 (4.3)
Stool	2 (28.6)	3 (42.9)	1 (14.3)	1 (14.3)	-	-	7 (3.4)
Drain	1 (20)	1 (20)	2 (40)	1 (20)	-	-	5 (2.4)
CSF	1 (33.3)	-	2 (66.7)	-	-	-	3 (1.4)
CL	3 (100)	-	-	-	-	-	3 (1.4)
BAL	-	-	-	1 (100)	-	-	1 (0.5)
Pleural	1 (100)	-	-	-	-	-	1 (0.5)
Chi-square	95.6278	60.00	53.1189	50.1818	20.1322	23.3455	223.3895
P value	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0436$	$P < 0.0158$	$P < 0.0001$
Total	85 (40.9)	32 (15.4)	39 (18.8)	36 (17.3)	11 (5.3)	5 (2.4)	208 (100)
Chi-square				138.5077			
P value				$P < 0.0001$			

A. baumannii strains were resistant to trimethoprim-sulfamethoxazole (89.7%), levofloxacin (87.2%), aztreonam (84.6%), tetracycline, amoxicillin-clavulanic acid, ciprofloxacin, and cefepime (82.1%).

Moreover, the recovered *P. aeruginosa* strains exhibited resistance to cefepime (72.2%), and trimethoprim-sulfamethoxazole and ceftriaxone (66.9%). Besides, the isolated *E. coli* strains exhibited resistance to ceftriaxone and ceftazidime (90.6%), levofloxacin and aztreonam (87.5%). The retrieved *E. aerogenes* strains exhibited resistance to ciprofloxacin (72.7%), aztreonam, cefepime, piperacillin-tazobactam, trimethoprim-sulfamethoxazole, ceftriaxone, gentamycin, ceftazidime, and levofloxacin (63.5% for each). Furthermore, the isolated *P. mirabilis* were resistant to ceftazidime, ciprofloxacin, cefepime, and trimethoprim-sulfamethoxazole (80% for each). Statistically, there is a significant difference in the resistance of different recovered bacterial pathogens to the tested antimicrobial agents ($p < 0.05$). The isolated *E. aerogenes* strains exhibited non-significant differences for resistance to various tested antimicrobial agents ($p > 0.05$) (Table 3).

Concerning the carbapenem-resistance, 36% of the recovered strains (75/208) were carbapenem-resistant (resistant to both imipenem and meropenem), including: *K. pneumoniae* (n=36),

A. baumannii (n=19), *P. aeruginosa* (n=10), *E. coli* (n=7), and *E. aerogenes* (n=3) (Table 3). MIC values (using imipenem) for the 75 CR-strains ranged from 2 to $>128 \mu\text{g mL}^{-1}$ (Table 4).

Phenotypic Detection of Carbapenem-Resistant Gram-Negative Bacterial Pathogens

Prevalence of Class-A Carbapenemases-Producing Bacterial Pathogens Using the Modified Hodge Test (MHT)

The MHT revealed that 70/208 tested strains (33.6%) were class A-Carbapenemases producing bacterial pathogens (were resistant to imipenem and meropenem) including: *K. pneumoniae* (n=36), *A. baumannii* (n=19), *P. aeruginosa* (n=7), *E. coli* (n=7), and *E. aerogenes* (n=1) (Table 5 and Figure 1A).

Prevalence of Metallo- β -Lactamase-Producing Bacterial Pathogens Using Combined Disk Test (CDT)

The CDT proved that 69/208 tested strains (33.17%) were metallo- β -lactamase-producing bacterial pathogens (were resistant to imipenem and meropenem) including: *K. pneumoniae* (n=36), *A. baumannii* (n=19), *P. aeruginosa* (n=6), *E. coli* (n=7), and *E. aerogenes*

Table 3 The Antimicrobial Resistance Profiles of the Retrieved Gram-Negative Rods

Antimicrobial Agents	<i>K. pneumoniae</i> (n=85)	<i>E. coli</i> (n=30)	<i>A. baumannii</i> (n=39)	<i>E. aerogenes</i> (n=11)	<i>P. mirabilis</i> (n=5)	<i>P. aeruginosa</i> (n=36)
	R	R	R	R	R	R
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Imipenem (IMP)	36 (42.4)	7 (21.9)	19 (48.7)	3 (27.3)	0 (0)	10 (27.8)
Meropenem (MEM)	36 (42.4)	7 (21.9)	19 (48.7)	3 (27.3)	0 (0)	10 (27.8)
Colistin (CT)	16 (18.8)	1 (3.1)	8 (20.5)	1 (9.1)	0 (0)	2 (5.6)
Amoxicillin-clavulanic (AUG)	66 (77.6)	20 (62.5)	32 (82.1)	7 (63.5)	2 (40)	2 (5.6)
Piperacillin-tazobactam (TZP)	56 (65.9)	19 (59.4)	31 (79.5)	7 (63.5)	1 (20)	25 (69.4)
Cefepime (FEP)	75 (88.2)	27 (84.4)	32 (82.1)	7 (63.5)	4 (80)	26 (72.2)
Ceftazidime (CAZ)	76 (89.4)	29 (90.6)	30 (76.9)	7 (63.5)	4 (80)	22 (61.1)
Ceftriaxone (CRO)	74 (87.1)	29 (90.6)	30 (76.9)	7 (63.5)	3 (60)	24 (66.7)
Aztreonam (ATM)	75 (88.2)	28 (87.5)	33 (84.6)	6 (54.5)	3 (60)	18 (50)
Amikacin (AK)	54 (63.5)	21 (65.6)	27 (69.2)	3 (27.3)	0 (0)	16 (44.4)
Gentamycin (CN)	62 (72.9)	23 (71.9)	29 (74.4)	7 (63.5)	2 (40)	23 (63.9)
Tetracycline (TE)	71 (83.5)	25 (78.1)	32 (82.1)	5 (45.5)	3 (60)	21 (58.3)
Ciprofloxacin (CIP)	75 (88.2)	26 (81.3)	32 (82.1)	8 (72.7)	4 (80)	18 (50.0)
Levofloxacin (LEV)	76 (89.4)	28 (87.5)	34 (87.2)	7 (63.5)	2 (40)	21 (58.3)
Trimethoprim-sulfamethoxazole (SXT)	74 (87.1)	27 (84.4)	35 (89.7)	7 (63.5)	4 (80)	24 (66.7)
Chi-square	292.6699	182.4720	96.6036	23.0515	30.8477	98.2789
P value	P<0.0001	P<0.0001	P<0.0001	0.0594	0.0058	P<0.0001

Table 4 The MIC Value of Carbapenem-Resistant Species Against Different Concentrations of Imipenem ($\mu\text{g mL}^{-1}$)

Species	Imipenem Concentration $\mu\text{g mL}^{-1}$						
	2	4	8	16	32	64	≥ 128
	Number of Isolates						
<i>K. pneumoniae</i> (n=36)	36	36	30	20	15	9	3
<i>A. baumannii</i> (n=19)	19	19	17	16	5	2	0
<i>E. coli</i> (n=10)	10	10	7	4	2	0	0
<i>P. aeruginosa</i> (n=7)	7	7	6	5	4	3	0
<i>E. aerogenes</i> (n=3)	3	3	3	2	1	0	0
Total number	75	75	63	47	27	14	3

Table 5 The Phenotypic and Genotypic Detection of Carbapenem-Resistant Strains

CR Species	Phenotypic Detection		Genotypic Detection	
	MHT n (%)	CDT n (%)	<i>bla</i> _{KPC} n (%)	<i>bla</i> _{GES} n (%)
<i>K. pneumoniae</i> (n=36)	36 (48)	36 (48)	36 (48)	36 (48)
<i>A. baumannii</i> (n=19)	19 (25.4)	19 (25.4)	19 (25.4)	19 (25.4)
<i>E. coli</i> (n=7)	7 (9.3)	7 (9.3)	7 (9.3)	7 (9.3)
<i>E. aerogenes</i> (n=3)	1 (1.3)	1 (1.3)	2 (2.6)	1 (1.3)
<i>P. aeruginosa</i> (n=10)	7 (9.3)	6 (8)	10 (13.3)	10 (13.3)
Chi-square	71.845	74.714	60.17	63.039
P value	P<0.0001	P<0.0001	P<0.0001	P<0.0001

Note: The percentage was calculated according to the number of the phenotypic carbapenem-resistant strains (which were resistant to imipenem and meropenem) (n=75).

(n=1) (Table 5 and Figure 1 B). Statistically, there is a remarkably significant difference in the prevalence of class A-Carbapenemases and metallo- β -lactamase producing bacterial pathogens ($p < 0.0001$).

Genotypic Detection of Class-A Carbapenemases-Producing Carbapenem-Resistant Gram-Negative Bacterial Pathogens

The genotypic investigation of the presence of Class-A carbapenemases *bla*_{KPC} and *bla*_{GES} genes were performed by PCR for the carbapenem-resistant strains (n=75). The PCR revealed that 98.6% (74/75) of the tested strains

possessed the *bla*_{KPC} gene including: *K. pneumoniae* (36/75), *A. baumannii* (n=19), *P. aeruginosa* (n=10), *E. coli* (n=7), and *E. aerogenes* (n=2). In addition, 97.3% (73/75) of the examined strains harbored the *bla*_{GES} gene including: *K. pneumoniae* (36/75), *A. baumannii* (n=19), *P. aeruginosa* (n=10), *E. coli* (n=7), and *E. aerogenes* (n=1) (Table 5 and Figure 2). Statistically, there is a highly significant difference in the prevalence of *bla*_{KPC} and *bla*_{GES} genes among tested strains ($p < 0.0001$).

16S rRNA Gene Sequencing and Phylogenetic Analysis

Thirteen isolates were identified with 16S rRNA sequencing. Twelve of these isolates were CR: *K. pneumoniae* (n=7), *A. baumannii* (n=2), *E. coli* (n=2), *E. aerogenes* (n=1), and one carbapenem susceptible *P. mirabilis* (n=1). Sequences were submitted to NCBI and Gene Bank nucleotide sequence database and were deposited under accession numbers (KY856923 to KY856935).

The phylogenetic relationships and placement among the isolated CR species were retrieved based on the obtained 16S rRNA sequences. The obtained phylogenetic tree has a well-supported phylogeny, providing a maximum and decisive resolution of the relationships among the examined species.

The 16S rRNA gene sequences of the bacterial isolates diverged into five phylogenetic clades: *Acinetobacter* clade, composed of five accessions, two of which were new to GenBank (KY856930 and KY856931); *Escherichia coli* clade, composed of four accessions, two new to GenBank (KY856932, and KY856933); *Klebsiella* clade, of nine accessions, seven new to GenBank (KY856923, KY856924, KY856925, KY856926, KY856927, KY856928, and KY856929); *Enterobacter* clade of four accessions, one new to GenBank (KY856934); and *Proteus* clade, of five accessions, only one new to GenBank (KY856935) (Figure 3).

Discussion

The existence of carbapenem-resistant bacterial pathogens is mainly accompanied by failure of medication, leading to significant morbidity and mortality. The on-going overall rise of carbapenem-resistant Gram-negative rods causes expanding public health threats.⁴ The laboratory detection

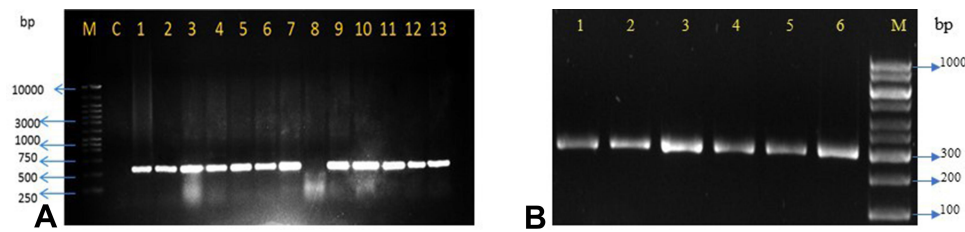


Figure 2 Gel electrophoresis analysis; **(A)** *bla_{KPC}* gene (638bp); M 100 bp DNA ladder; C: Negative control; lane 1: Positive control; Lanes: 2, 3, 4, 5, 6, 7, 9, 10, 11, 12, 13: positive isolates for *bla_{KPC}* gene with specific amplicon size 638 bp; Lane 8: negative isolate. **(B)** *bla_{GES}* gene (323 bp); M 100 bp DNA ladder; lane 1: Positive control; Lanes: 2–6 positive isolates for *bla_{GES}* gene with specific amplicon size 323 bp.

of carbapenemases-producers among clinical isolates is crucial to limit its rapid spread. In the present study, the bacteriological examination revealed that 208 isolates were retrieved from different examined specimens. The most predominant recovered pathogen was *K. pneumoniae* (40.9%), followed by *A. baumannii* (18.8%), *P. aeruginosa* (17.3%), *E. coli* (15.4%), *E. aerogenes* (5.3%), and *P. mirabilis* (2.4%) (Table 2). Statistically, there is a significant difference in the prevalence of different bacterial pathogens recovered from various types of samples ($p < 0.05$). These findings are in agreement with those obtained by Talaat et al⁴³ who illustrated that the most predominant Gram-rods in hospital

settings were *K. pneumoniae* (28.7%) and *Acinetobacter* sp. (13.7%).

Concerning the carbapenem-resistance, in the present study, 36% (75/208) of recovered strains were carbapenem-resistant (resistant to both imipenem and meropenem) including: *K. pneumoniae* (n=36), *A. baumannii* (n=19), *P. aeruginosa* (n=10), *E. coli* (n=7), and *E. aerogenes* (n=3). These findings agree with those obtained by Fattouh et al⁴⁴ and Ramana et al⁴⁵ who reported that among the tested members of *Enterobacteriaceae*, *K. pneumoniae* is the most predominant carbapenem-resistant pathogen (44.1%). Centers for Disease Control Prevention, CDC⁴⁶ reported that the prevalence of

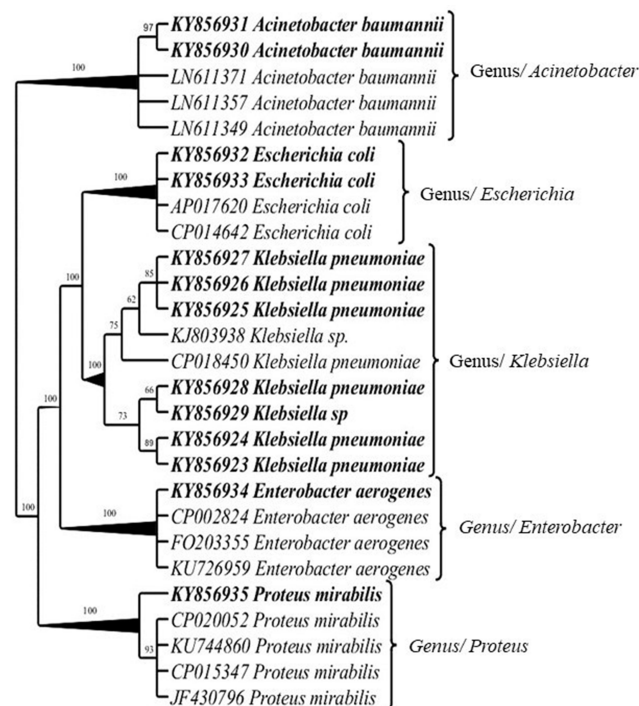


Figure 3 Dendrogram of the carbapenem-resistant bacterial pathogen isolated from clinical samples, constructed based on the 16S rRNA gene sequences. Bootstrap support value above branches, newly generated sequences of the isolated carbapenem-resistant Gram-negative bacterial pathogens in bold.

carbapenem-resistant pathogens elevated from 1.2% (2001) to 4.2% (2011) in intensive-care hospitals in the United States.

Furthermore, in the current study, the retrieved isolates exhibited remarkable multidrug resistance to most tested antimicrobial classes including cephalosporins, monobactams, tetracycline, quinolones, aminoglycosides, and beta-lactam-beta-lactamase inhibitor combinations, while showed high sensitivity to lipopeptides (colistin) as described in (Table 3). Colistin is considered the most effective antimicrobial against carbapenemases-producing bacterial pathogens. The standard therapeutic strategy of the carbapenem-resistant strains has yet to be identified. Nevertheless, the current strategy depends upon the results of the in vitro antibiogram: tigecycline, polymyxins, and combinations of carbapenems with other antibiotics were involved.^{47,48}

In the current study, 13.5% (28/208) of the retrieved clinical isolates were resistant to colistin. The emergence of such strains is considered a public health threat. Earlier study reported that prevalence of colistin resistance among the KPC-producers was 22.4% among the recovered species.⁴⁹ The emergence of multidrug-resistant Gram-negative rods is also reported by previous studies.^{44,50} The occurrence of such strains is always accompanied with bad prognosis as well as medication-failure.⁵¹

Regarding the prevalence of class A-Carbapenemases and metallo- β -lactamase producing bacterial pathogens, the MHT revealed that 33.6% (n=70) of the tested strains were positive for carbapenemases production, while the CDT proved that 33.17% (n=69) of the examined strains were metallo- β -lactamase production. This prevalence is similar to that reported by Fattouh et al.⁴⁴ Although, the MHT is considered a simple, effective, reliable, and cheap diagnostic tool that could be used in monitoring for carbapenemases-production in KPC, it exhibited a lower specificity and sensitivity in other species such as *Enterobacter*.⁵²

The combination of MHT, CDT, and the antibiotic sensitivity test is an effective tests for the phenotypic screening of carbapenem-resistant strains.^{37,53} The class-A- β -lactamases (especially GES and KPC enzymes) are the most prevalent type of the β -lactamase-group; which may attribute to the frequent occurrence of their specific encoding genes in various pathogenic bacterial species.⁵⁴ These enzymes break down the β -lactam antibiotics and are stable to clavulanate, sulbactam and tazobactam.²⁷

PCR is considered the most significant specific technique used for the determination of carbapenem-resistance isolates

in clinical specimens. In the current study, the genotypic investigation of the presence of Class-A carbapenemases *bla*_{KPC} and *bla*_{GES} genes was performed by PCR for the carbapenem-resistant strains (n=75). The PCR revealed that 98.6% (74/75) of the tested strains possessed the *bla*_{KPC} gene; moreover, 97.3% (73/75) of the examined strains harbored the *bla*_{GES} gene. The results of the *bla*_{KPC} gene are similar to those obtained by Raghunathan et al,⁵⁵ who reported that prevalence of the *bla*_{KPC} gene was 82% in examined strains. Jeong et al⁵⁶ reported the first *K. pneumoniae* expressing GES-5 in Korea. Nordmann et al²⁴ and Yigit et al²⁶ reported the emergence of carbapenem-resistant *K. pneumoniae* that produce the new carbapenemase, KPC, as the first report globally. Since then, KPC-producing isolates have become more widespread globally.

In the current study, the identification of 13 isolates; *K. pneumoniae* (n=7), *A. baumannii* (n=2), *E. coli* (n=2), *E. aerogenes* (n=1), and *P. mirabilis* (n=1) was confirmed by 16S rRNA sequencing. The obtained phylogenetic tree has a very well supporting phylogeny, providing a maximum and decisive resolution of the relationships among the examined species. It was divergent into five Genera; *Acinetobacter*, *Escherichia*, *Klebsiella*, *Enterobacter*, and *Proteus* as illustrated in Figure 3.

The conventional phenotypic identification of bacterial pathogens is not 100% exact. The 16S rRNA gene sequencing has elicited a specific, reliable molecular technique; which is useful in the accurate identification of scarcely isolated strains as well as phenotypically eccentric ones. Furthermore, the 16S rRNA gene is the most conserved gene in various bacterial pathogens; beneficial in phylogenetic analyses. The 16S rRNA sequencing gives a complete conceive of whether the tested strains belonged to distantly or closely related bacterial lineages.³⁷

In conclusion, the present study emphasized that the most predominant carbapenem-resistant bacterial pathogens incriminated in healthcare-associated infections were *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *E. coli*. The retrieved clinical isolates exhibited remarkable multidrug resistance to cephalosporins, monobactams, tetracycline, quinolones, aminoglycosides, and beta-lactam-beta-lactamase inhibitor combinations that is considered a public health threat. The carbapenem-resistance is commonly associated with class-A carbapenemases and metallo- β -lactamases production as well as the presence of both *bla*_{KPC} and *bla*_{GES} resistance-genes. The combination of phenotypic and genotypic detection of carbapenem-resistant

strains is a reliable effective epidemiological tool. The continuous application of antimicrobial susceptibility testing is essential to detect multidrug-resistant strains. The accurate identification of carbapenem-resistant bacterial pathogens is pivotal for the treatment of patients, in addition to propelling appropriate contamination control measures to restrain the fast spread of these pathogens.

Acknowledgments

The authors gratefully acknowledge Dr. Ahmed EL-Banhawy, Suez Canal University, Faculty of Science, Botany Department, Ismailia, Egypt for his help in the phylogenetic analysis. We also appreciate the help of Dr. Oliver Harry Ellingham, Fungiscape, UK, for the help in improving the manuscript language.

Author Contributions

All authors contributed to data analysis, drafting or revising the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors declare no conflict of interest.

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