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Phenotypic and molecular characterization of multidrug-resistant *Enterobacterales* isolated from clinical samples in Palestine: a focus on extended-spectrum β-lactamase- and carbapenemase-producing isolates

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Abstract

Background Infections resulting from multidrug-resistant *Enterobacterales* (MDR-E) pose a growing global threat, presenting challenges in treatment and contributing significantly to morbidity and mortality rates. The main objective of this study was to characterize phenotypically and genetically extended-spectrum β-lactamase- and carbapenemase- producing *Enterobacterales* (ESBLE and CPE respectively) isolated from clinical samples in the West Bank, Palestine.

Methods A cross sectional study was conducted in October 2023 on clinical bacterial isolates collected from five governmental hospitals in the West Bank, Palestine. The isolates obtained from the microbiology laboratories of the participating hospitals, underwent identification and antibiotic susceptibility testing (AST) using the VITEK® 2 Compact system. ESBL production was determined by the Vitek2 Compact system. A modified carbapenem inactivation method (mCIM) was employed to identify carbapenemase-producing Enterobacterales (CPE). Resistance genes were detected by real-time PCR.

Results Out of the total 1380 collected isolates, we randomly selected 600 isolates for analysis. Our analysis indicated that 287 (47.83%) were extended-spectrum beta-lactamase producers (ESBLE), and 102 (17%) as carbapenem-resistant *Enterobacterales* (CRE) isolates. A total of 424 isolates (70.67%) were identified as multidrug-resistant *Enterobacterales* (MDRE). The most prevalent ESBL species were *K. pneumoniae* ($n=124$; 43.2%), *E. coli* ($n=119$; 41.5%) and *E. cloacae* ($n=31$; 10.8%). Among the CRE isolates, 85 (83.33%) were carbapenemase-producing *Enterobacterales* (CPE). The most frequent CRE species were *K. pneumoniae* ($n=63$; 61.7%), *E. coli* ($n=25$; 24.5%) and *E. cloacae* ($n=13$; 12.8%). Additionally, 47 (7.83%) isolates exhibited resistance to colistin (CT), with 38 (37.62%) being CT-resistant CRE and 9 (3.14%) being CT-resistant ESBLE while sensitive to carbapenems. We noticed that 11

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isolates (6 *Klebsiella pneumoniae* and 5 *Enterobacter cloacae* complex) demonstrated sensitivity to carbapenems by phenotype but carried silent CPE genes (1 *bla*OXA48, and 6 *bla*NDM, 4 *bla*OXA48, *bla*NDM). ESBL-producing *Enterobacterales* strains exhibited varied resistance patterns across different antibiotic classes. *E. coli* isolates showed notable 48% resistance to trimethoprim/sulfamethoxazole. *K. pneumoniae* isolates displayed a significant resistance to trimethoprim/sulfamethoxazole, nitrofurantoin, and fosfomycin (54%, 90%, and 70% respectively). *E. cloacae* isolates showed complete resistance to nitrofurantoin and fosfomycin. *P. mirabilis* isolates exhibited high resistance against fluoroquinolones (83%), and complete resistance to trimethoprim/sulfamethoxazole, nitrofurantoin and fosfomycin.

Conclusion This study showed the high burden of the ESBLE and CRE among the samples collected from the participating hospitals. The most common species were *K. pneumoniae* and *E. coli*. There was a high prevalence of *bla*CTXm. Adopting both conventional and molecular techniques is essential for better surveillance of the emergence and spread of antimicrobial-resistant *Enterobacterales* infections in Palestine.

Keywords Antimicrobial Resistance, Extended-spectrum β-lactamase, Carbapenem-Resistant, Colistin-resistant, Silent (cryptic) antimicrobial resistance genes, Transmissible infections, Transmitted drug resistance

Introduction

ESBL-producing *Enterobacterales* (ESBLE) present a significant clinical challenge due to their capacity to generate extended-spectrum β-lactamases, enzymes capable of breaking down a broad spectrum of β-lactam antibiotics. These bacteria, containing species like *Escherichia coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis*, have acquired genes encoding ESBLs, resulting in resistance to commonly utilized antibiotics such as penicillins and cephalosporins [1]. Multidrug-resistant *Enterobacterales* (MDRE) strains commonly show resistance to widely used antibiotics, including penicillin's, cephalosporins, fluoroquinolones, aminoglycosides, and sometimes carbapenems, which are considered last-resort antibiotics [2]. The rise of colistin resistance in *Enterobacterales* constitutes a growing concern in healthcare settings worldwide. Colistin resistance mechanisms often involve chromosomal mutations and the acquisition of mobile genetic elements carrying resistance genes [3–5].

Infections caused by ESBLE and MDRE are linked to increased morbidity, mortality, and healthcare spending, causing prolonged hospitalizations and the use of more costly antibiotics [6].

ESBLE contains various genes conferring resistance to antimicrobial agents, with common ones including CTXm, TEM, and SHV. MDRE strains frequently carry genes encoding resistance mechanisms against other antibiotic classes, such as aminoglycosides, fluoroquinolones, and carbapenems. Examples include genes encoding aminoglycoside-modifying enzymes (e.g., AAC, ANT), fluoroquinolone resistance determinants (e.g., qnr genes), and carbapenemases (e.g., KPC, NDM, OXA) [7].

On a global scale, ESBL-positive *E. coli* (23.7%) and *K. pneumoniae* (35.1%) predominantly contained the CTXm-15 variant (*E. coli*: 53.9%; *K. pneumoniae*: 80.0%), with the highest incidence observed in the Africa/Middle East (AfME) region [8].

The prevalence of MDRE, particularly those carrying extended-spectrum β-lactamases, constitutes a significant challenge in the West Bank, Palestine. In the Gaza Strip, ESBL prevalence ranges from 35 to 54% [9]. The prevalent genes include CTXm, found in 45–60% of cases, followed by TEM and SHV genes, with prevalence rates ranging from 16.8 to 57.6% and 5.2–38.3%, respectively [9]. Conversely, research on ESBL prevalence in the other region of Palestine, the West Bank, is limited. One study reported a prevalence of 38% [10], while another found that ESBL-producing *E. coli* accounted for 62.8% of healthcare-acquired multidrug-resistant gram-negative bacilli [5]. Moreover, there have been no studies characterizing the phenotypic and genotypic traits of *Enterobacterales* in the West Bank. Despite efforts to combat antimicrobial resistance, the exact burden and genetic composition of ESBL-producing strains remain poorly understood. This research aims to address this knowledge gap by conducting a comprehensive phenotypic and genotypic assessment of ESBL-producing and multidrug-resistant *Enterobacterales* in the West Bank.

Materials and methods

Study design and setting

A cross-sectional study was conducted from October 1st to October 31st, 2023, on clinical bacterial isolates collected from five governmental Palestinian hospitals situated throughout the West Bank. These hospitals include Martyr Khalil Suleiman Hospital (MKSH) in Jenin, Rafidia Surgical Hospital (RSH) in Nablus, both located in the northern region of the West Bank. Palestinian Medical Complex (PMC) is positioned in the city of Ramallah, centrally within the West Bank. Additionally, Al-Hussain Hospital is situated in Beit Jala, and Alia Governmental Hospital (AGH) is located in Hebron, situated in the southern part of the West Bank.

Sources of bacterial isolates

The research was conducted on 600 isolates chosen at random from a pool of 1380 isolates collected from patients during October 2023. Bacterial isolates were collected from both female and male patients (367(61%) and 233/600 (39%), respectively). The patients from whom the samples were collected range in age from one to 92 years old. Bacterial isolates were cultivated from different patient samples: 364 urine samples, 120 wound samples, 68 blood samples, 45 sputum samples and 3 CSF samples.

Microbiological methods

Bacterial identification and antimicrobial susceptibility testing

All collected isolates (600 isolates from1380 patients) were analyzed at the microbiology labs of the participating hospitals. The VITEK® 2 Compact system from bioMérieux, France, was used to identify all isolates. Bacterial identification utilized VITEK 2 GN cards, while susceptibility testing for both beta-lactam and non-beta-lactam antibiotics was carried out using VITEK 2 GN AST 204 and 417 cards. These antibiotic panels are suitable for testing *Enterobacterales* isolates in line with the 2023 guidelines of the Clinical Laboratory Standards Institute (CLSI) [11].

All bacterial isolates were sent to the microbiology laboratory of An-Najah National University for confirmation of ESBLE and CPE phenotyping, genotyping, and testing for colistin resistance. The Double Disc Synergy Test (DDST)was employed to retest all ESBL-producing bacteria identified by the VITEK 2 Compact system. Antibiotics were used for DDST namely: Amoxicillin-Clavulanic acid (20/10 µg), Ceftriaxone (30 µg), and Cefotaxime (30 µg). At center Amoxicillin-Clavulanic acid disc was placed and these discs were placed at a distance of 1.5 cm. Development of the zone of inhibition towards the Clavulanate disc at 37 °C after 24 h. incubation was indicative of a potential ESBL-positive organism, this involved placing antibiotic discs containing ceftriaxone 30 µg and cefotaxime 30 µg, with and without amoxicillin clavulanic acid 20/10µg, on a Mueller Hinton agar plate [12].

We classified bacterial isolate as MDRE when they showed resistance to at least one antibiotic in three distinct classes of antibiotics. Results were interpreted following the CLSI 2023 guideline [11], and *E. coli* American type culture collection (ATCC) 25,922 was utilized as a quality control for antibiotic disc potency.

Phenotypic carbapenemases detection

The modified carbapenem inactivation method (mCIM) was conducted on all CRE and ESBLE isolates to test for carbapenemase production. We conducted mCIM on ESBLE isolates to assess for the absence or presence of slow carbapenemase enzyme activity among these isolates.

This assay operates on the principle that when a 10-µg meropenem disk is exposed to a cation adjusting Mueller Hinton broth (CAMHB) of a carbapenemase-producing microorganism for 4 h, the carbapenemase degrades the carbapenem in the disk. Conversely, if the test microorganism does not produce carbapenemase, meropenem maintains its antimicrobial activity after being incubated in the bacterial suspension. Subsequently, the disk is removed from the suspension and transferred onto a Mueller-Hinton agar (MHA) plate that has been inoculated with a suspension of a carbapenem-susceptible indicator organism (*E. coli* ATCC 25922). After overnight incubation, the zone of inhibition is measured to determine whether the meropenem has been hydrolyzed (indicated by the growth of the indicator organism close to the disk) or remains active (evidenced by a large zone of inhibition around the disk). CRE isolates were classified into carbapenemase producer and non-producer *Enterobacterales* (CPE and none CPE).

Colistin minimum inhibitory concentration (MIC)

Colistin susceptibility test was conducted using the agar dilution method (ADM) where all isolates were streaked on four plates of Mueller Hinton agar containing 0, 1, 2, 4 µg/ml of colistin sulfate (Thero Fisher Scientific). Results were interpreted as sensitive or resistant according to CLSI 2023 guidelines [11].

Colistin resistance by agar dilution method (MIC>4 µg/ml) was confirmed using two other methods: broth microdilution method (BMD) and disk elution method.

BMD was conducted according to CLSI 2017 M52-Ed 1 guidelines [13], briefly:

A) Preparation of Colistin Solutions: Colistin sulfate powder and CAMHB were used to prepare 5120 µg/ml stock solutions. From this stock solution, a 512 µg/ml concentration was prepared by mixing 1 ml of the stock solution with 9 ml of CAMHB. Using the 512 µg/ml concentration, subsequent concentrations of 256, 128, and 64 µg/ml were prepared in separate test tubes. From the 64 µg/ml test tube, further dilutions of 32, 16, and 8 µg/ml were made. Using the 8 µg/ml tube, additional concentrations of 4, 2, and 1 µg/ml were prepared. From the 1 µg/ml tube, further dilutions of 0.5, 0.25, and finally 0.125 µg/ml were made. We then dispensed 100 µl of each of the following concentrations into separate wells: 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, and 0.125 µg/ml.
B) Preparation of Bacterial Isolates: Isolates were prepared to a 0.5 McFarland standard and then

Table 1 Primers used for the detection of the ESBL and CRE genes with their amplicon size

Gene	Primer reverse	Primer forward	Amplicon size Pb	Reference
bla_{TEM}	R: (5′-CTGACAGTTACCAATGCTTA-3′).	F: (5′-ATGAGTATTCAACATTTCCG-3′)	431	www.ecdc.europa.eu/en and [14]
bla_{SHV}	R: (5′- TTAGCGTTGCCAGTGCTC – 3′).	F: (5′- GGGTTATTCTTATTTGTCGC – 3′)	214	www.ecdc.europa.eu/en and [15]
bla_{CTXm}	R: (5′- TGGGTRAARTARGTSACCAGA – 3′).	F (5′- ATGTGCAGYACCAGTAARGT – 3′)	501	www.ecdc.europa.eu/en and [15]
bla_{OXA48}	R: (5′-GAGCACTTCTTTTGTGATGGC-3′).	F: (5′-TTGGTGGCATCGATTATCGG-3′)	744	www.ecdc.europa.eu/en and [16]
bla_{NDM}	R: (5′-AGATTGCCGAGCGACTTG-3′).	F: (5′-TGGCAGCACACTTCCTATC-3′)	488	www.ecdc.europa.eu/en and [17]
bla_{KPC}	R: (5′-CCTCGCTGTRCTTGTCATCC-3′).	F: (5′-CTGTCTTGTCTCTCATGGCC-3′)	796	www.ecdc.europa.eu/en and [17]
bla_{VIM}	R: (5′-TCAATCTCCGCGAGAAG-3′).	F: (5′-AGTGGTGAGTATCCGACAG-3′)	212	www.ecdc.europa.eu/en and [16]

diluted to a concentration of 1:1000 (100,000 cfu/ml). Subsequently, 100 µl of the diluted bacteria were dispensed into all wells except for the well dedicated to negative growth control.

The plates were incubated at 35 °C±2 for 18–24 h, and the results were interpreted as either sensitive or resistant.

In the disk elution method (DE), all isolates demonstrating resistance to colistin by ADM (MIC>4 µg/ml) underwent retesting with DE. Specifically, four 10 ml tubes were filled with 5 ml of CAMHB, and 50 µl of 0.5 McFarland of the isolates were introduced into each of the four tubes. Subsequently, commercially available 1 µg colistin disks from Thermo Fisher Scientific were placed into the tubes. The first tube contained no colistin disc (0 µg/ml CT), the second tube contained one colistin disc (1 µg/ml CT), the third tube contained two colistin discs (2 µg/ml CT), and the fourth tube contained four colistin discs (4 µg/ml CT).

Following this, all tubes were incubated at 35 °C±2 for 18–24 h, and the results were interpreted as either sensitive or resistant following the CLSI 2023 guidelines [11].

Antibiogram of all ESBLE isolates with non-beta lactam antibiotics and CRE combination antibiotics

Susceptibility testing of all ESBLE isolates was examined by VITEK 2 compact system from bioMerieux, using AST N204 (ESBL test, ampicillin, amoxicillin-clavulanic acid, piperacillin-tazobactam, cefotaxime, ceftazidime, cefepime, meropenem, ertapenem, imipenem, ciprofloxacin, gentamycin, amikacin, nitrofurantoin, fosfomycin, and trimethoprim-sulfamethoxazole), AST N417(amoxicillin-clavulanic acid, piperacillin-tazobactam, cefazolin, cefuroxime, ceftriaxone, ceftazidime, cefepime, meropenem, ertapenem, imipenem, ciprofloxacin, gentamycin, amikacin, nitrofurantoin, fosfomycin, and trimethoprim-sulfamethoxazole), and AST NX20 cards (ampicillin-sulbactam, Aztreonam, cefotaxime, cefoxitin, ceftazidime-avibactam, colistin, doripenem, levofloxacin, meropenem-vaborbactam, tetracycline, doxycycline, minocycline, tigecycline, and tobramycin). Disk Diffusion Method (DDM) was used to determine antibiotic susceptibility against the following antibiotic categories, Aminoglycosides (30-µg amikacin, 10-µg gentamicin, 10-µg tobramycin), fluoroquinolones (5-µg ciprofloxacin, 5-µg levofloxacin, 5-µg moxifloxacin), other categories (1.25/23.75 µg trimethoprim/sulfamethoxazole, 200 µg fosfomycin and 300 µg nitrofurantoin) and CRE combinations antibiotics (30/20-µg ceftazidime-Avibactam, 20/10-µg meropenem-vaborbactam, 30-µg cefiderocol).

Table 2 The details of the programs used for detection of carbapenemases and ESBL -encoding genes as recommended by EUCAST

Section A: Detection of carbapenemases-encoding genes			
Cycle step	**Temperature (°C)**	**Time**	**Number of cycles**
Initial denaturation	94	5 min	1
Denaturation	94	30 s	30
Annealing	60	30 s	30
Extension	72	1 min	30
Hold	72	10 min	1
Section B: Detection of ESBL-encoding genes			
Cycle step	**Temperature (°C)**	**Time**	**Number of cycles**
Initial denaturation	95	3 min	1
Denaturation	95	30 s	24
Annealing	65	30 s	24
Extension	72	1 min	1
Hold	4	10 min	

Molecular characterization of ESBLE and CPE

DNA extraction was performed using a column-based DNA isolation kit (DNA mini kit, Qiagen, Germany). The DNA from all ESBLE isolates was tested for the presence of ESBL genes (blaCTXm, blaSHV, and blaTEM) and the presence of CRE genes (blaKPC, blaOXA48, blaNDM, blaVIM). Additionally, the DNA from CRE isolates was tested for the presence of CRE genes (blaKPC, blaOXA48, blaNDM, blaVIM). The primers used for the detection of the ESBL and CRE genes are listed in Table 1. The presence of ESBL genes and carbapenemase genes was examined using a Quanta Stadium 5 real-time PCR system with primer sets and a specialized program designed for the detection of carbapenemases, as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST), as shown in Table 2. (Accessible at www.ecdc.europa.eu/en.)

Table 3 Sources of bacterial isolates

Source	Number (*n*, %)	*Enterobacterales* isolates (*N*, %)						
		E. coli	*K. pneumoniae*	*E. cloacae*	*P. mirabilis*	*S. marcescens*	*C. freundii*	*Salmonella group*
Blood	68 (11.3)	5 (7.5)	31 (45.5)	12 (17.5)	2 (3)	17 (25)	0	1 (1.5)
CSF	3 (0.5)	1 (33)	2 (67)	0	0	0	0	0
Urine	364 (60.7)	211 (58)	97 (27)	36 (10)	18 (4.5)	0	2 (0.5)	0
Sputum	45 (7.5)	1 (2.3)	37 (82)	5 (11.1)	1 (2.3)	0	1 (2.3)	0
Wound	120 (20)	5 (4.0)	82 (68.0)	30 (25.0)	1 (1.0)	0	2 (2.0)	0
Total	600 (100)	223 (37.2)	249 (41.5)	83 (13.8)	22 (3.7)	17 (2.8)	5 (0.8)	1 (0.2)

CSF: cerebrospinal fluid

Table 4 Phenotypic and genotypic characteristics of ESBLE isolates

Species	ESBL (*n*, %)	ESBL phenotype							ESBL genotype (*N*, %)		
		Resistance rates to beta lactam antibiotics [*n* (%)]							bla_{TEM}	bla_{SHV}	bla_{CTXm}
		CTX	CRO	CAZ	FEP	ATM	TPZ	AMC			
E. coli	119 (41.5)	119 (100)	119 (100)	50 (42)	43 (36.1)	46 (38.7)	4 (3.4)	74 (62.2)	38 (32)	34 (29)	92 (77)
K. pneumoniae	124 (43.2)	124 (100)	124 (100)	66 (53.2)	54 (43.6)	63 (50.8)	459 (36.3)	64 (51.6)	56 (45)	39 (31)	103 (83)
E. cloacae	31 (10.8)	31 (100)	31 (100)	19 (61.3)	18 (58)	18 (58)	5 (16.1)	31 (100)	7 (23)	9 (29)	25 (81)
P. mirabilis	6 (2.1)	6 (100)	6 (100)	0	0	0	0	6 (100)	2 (33)	3 (50)	3 (50)
S. marcescens	4 (1.4)	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)	0	4 (100)	2 (50)	1 (25)	1 (25)
C. freundii	2 (1.0)	2 (100)	2 (100)	0	0	0	0	2 (100)	0	1 (50)	1 (50)
Salmonella group	1 (0.4)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	0	1 (100)	0	0	1 (100)
Total (*n*, %)	287 (47.8)	287 (100)	287 (100)	140 (48.8)	120 (41.8)	132 (46)	54 (18.8)	182 (63.4)	105 (36.6)	87 (30.3)	226 (78.7)

R: resistant, ESBL: extended spectrum beta lactamase, CTX: cefotaxime, CRO: ceftriaxone, CAZ: ceftazidime, ATM: aztreonam, FEB: cefepime, TPZ: piperacillin-tazobactam, AMC: amoxicillin-clavulanic acid

Ethical considerations and consent to participate

Ethical approval (Ref. Mas. Oct. 2023/7) was obtained from the Institutional Review Board (IRB) of An-Najah National University, which approved all aspects of the study design, including the collection of bacterial isolates from microbiology laboratories, and the patient demographic data. The need for informed consent was waived by the IRB of An-Najah National University. Approval to collect demographic data and bacterial isolates from the governmental hospitals was also obtained from the Palestine Ministry of Health (MOH). The data collected were used exclusively for research, kept confidential, and not used for any other purpose.

Statistical analysis

Data were coded, categorized, and entered using Microsoft Office Excel (2010). Descriptive statistics were conducted with frequencies and percentages for categorical variables.

Results

Distribution of *Enterobacterales* isolates across different sources

Five government Palestinian hospitals spread around the West Bank were the source of bacterial isolates. 367/600 (61%) were from female and 233/600 (39%) were from male patients. The patients' ages went from one day to 92 years with a median age of 41.5 years.

As shown in Table 3, there is a diversity in the distribution of *Enterobacterales* isolates across different sources. Urine samples accounted for the highest proportion of *Enterobacterales* isolates, with 364 (60.7%) isolates identified. This was followed by isolates from wounds (120, 20%) and blood (68, 11.3%). Among the *Enterobacterales* isolates, *K. pneumoniae* and *E. coli* were the most prevalent species across all sources. However, their distribution varied depending on the sample source. For instance, *K. pneumoniae* was predominant in sputum (82%) and wound (68%) samples, while *E. coli* was more prevalent in urine samples (58%).

Phenotypic and genotypic characterization of ESBL among isolated *Enterobacterales*

Table 4 shows the distribution of ESBL phenotypes and genotypes among different species of gram-negative bacteria. Among the total isolates tested, 287 (47.8%) exhibited the ESBL phenotype. The most prevalent species showing the ESBL phenotype were *K. pneumoniae* (43.2%) followed by *E. coli* (41.5%).

The majority of ESBL-producing isolates demonstrated resistance to various antibiotics, including cefotaxime (100%), ceftriaxone (100%), amoxicillin-clavulanic acid (63.4%), ceftazidime (48.8%), cefepime (41.8%), and piperacillin-tazobactam (18.8%). All ESBL isolates tested negative with mCIM.

The prevalence of ESBL genotypes varied among species; ESBL genotypes identified were bla_{TEM}, bla_{SHV}, and

bla_{CTXm}. Notably, bla_{CTXm} was the predominant genotype across all species.

Each species showed specific patterns of ESBL genotype distribution. For instance, *E. coli* predominantly carried bla_{TEM} and bla_{CTXm}, while *K. pneumoniae* exhibited a higher prevalence of bla_{TEM} and bla_{SHV}. *E. cloacae* had a notable presence of bla_{CTXm}, and *Salmonella* group showed a unique pattern with only bla_{CTXm} detected.

Phenotypic and genotypic characteristics of carbapenem-resistant *Enterobacterales* (CRE) isolates

The total number of CRE isolates phenotyping based on carbapenem resistance (at least resistant or intermediate to one agent of the four carbapenems used in the study) was 102 (17%), while out of them 85 (83.3%) isolates, based on the mCIM were identified as CPE. As shown in Table 5, among the total CRE isolates, *K. pneumoniae* had the highest proportion with 63 (61.7%) isolates, followed by *E. coli* with 25 (24.5%) isolates and *E. cloacae* with 13 (12.5%) isolates. *P. mirabilis* had the lowest proportion with only 1 (1%) isolate. Among the CRE genotypes, bla_{NDM} was the most prevalent, with 30 (35%) isolates, followed by OXA48 with 24 (28%) isolates and KPC with 23 (27%) isolates. Some isolates also harbored multiple carbapenemase genes, such as KPC and NDM, KPC and OXA48, and NDM and OXA48, as shown in Table 5.

Frequency of multidrug-resistant Enterobacterales

The total number of MDRE was 424 isolates (70.67%) of them 396 isolates (66%) were eligible for colistin testing.

Among the isolates eligible for colistin testing 396 (66%), *K. pneumoniae isolates* exhibiting the highest percentage of MDRE (50.5%), followed by *E. coli* (37%) and then *E. cloacae* (12.5%) as depicted in Table 6.

Colistin resistance among multidrug-resistant Enterobacterales

As discussed in the previous sections and shown in Table 7, of our 600 selected isolates, 396 (66%) were MDRE, with 47 of these (11.9%) being colistin-resistant. Additionally, 287 isolates (48%) were ESBL, of which 9 (3.1%) were colistin-resistant. Furthermore, 102 isolates (17%) were CRE, with 38 of them (37.6%) were colistin-resistant.

Colistin resistance among carbapenem-resistant *Enterobacterales* phenotypes

Both the broth microdilution and disc elution methods were utilized to evaluate colistin resistance phenotypically. Thirty-eight out of 101 (38%) isolates demonstrated resistance to colistin using both techniques, while 84 (83%) were identified as carbapenemase-producing *Enterobacterales* by the mCIM. Among the total 101 CRE

Table 5 Phenotypic and genotypic characteristics of carbapenem-resistant *Enterobacterales* (CRE) isolates

Species	CRE (*n*, %)	CRE phenotype (*N*, %)					CRE genotype (*N*, %)						
		Antimicrobial Resistance (*n*, %)				mCIM							
		MEM	IPM	ETP	DOR		KPC	NDM	OXA48	VIM	KPC, NDM	OXA48, KPC	NDM, OXA48
E. coli	25 (24.5)	23 (27)	24 (24)	24 (24)	23 (23)	15 (15)	3 (3)	7 (7)	4 (4)	0	1 (1)	1 (1)	1 (1)
K. pneumoniae	63 (61.7)	52 (51)	54 (53)	55 (54)	53 (52)	56 (55)	17 (17)	16 (16)	16 (16)	0	1 (1)	4 (4)	5 (5)
E. cloacae	13 (12.5)	10 (10)	10 (10)	13 (13)	11 (11)	13 (13)	3 (3)	7 (7)	3 (3)	0	0	0	0
P. mirabilis	1 (1)	1 (1)	1 (1)	1 (1)	1 (1)	1 (1)	0	0	1 (1)	0	0	0	0
Total (n, %)	102 (17)	86 (84)	89 (87)	93 (91)	88 (86)	85 (83)	23 (27)	30 (35)	24 (28)	0	2 (2)	5 (4.9)	6 (7)

mCIM: modified carbapenem inactivation method. CRE: carbapenem resistant *Enterobacterales*, MEM: meropenem, IPM: imipenem, ETP: ertapenem, Dor: Doripenem

Table 6 Frequency of MDR *enterobacterales* among isolates eligible for colistin testing

Species	*N*	%
E. coli	146	37
K. pneumoniae	200	50.5
E. cloacae	50	12.5
Total	396	100

Table 7 Colistin resistance among *Enterobacterales*

Enterobacterales	ESBL (*N*, %)	CRE (*N*, %)	MDRE (*N*, %)
Total isolates (N)	287 (48)	102 (17)	396 (66)
Species	**Colistin resistance rates**		
E. coli	0	8 (7.8)	8 (2.0)
K. pneumoniae	9 (3.1)	25 (24.5)	34 (8.6)
E. cloacae	0	5 (4.9)	5 (1.3)
Total	9 (3.1)	38 (37.6)	47 (11.9)

ESBL: extended spectrum beta lactamase, MDRE: multidrug resistant *Enterobacterales*. CRE: carbapenem resistant *Enterobacterales*

isolates, *K. pneumoniae* had the highest proportion with 63 (62%) isolates, followed by *E. coli* with 25 (25%) isolates, and *E. cloacae* with 13 (13%) isolates, as depicted in Table 8.

Detection of silent carbapenemases genes among ESBLE

Sixty-five isolates were chosen at random from the pool of 287 ESBLE isolates for CRE genotyping. Despite the absence of any CRE phenotypes among them, eleven ESBLE isolates were discovered to have CRE genes. Within this group, 6 (54%) contained the NDM gene, 4 (36%) harbored the OXA48 gene in conjunction with NDM, and 1 (9%) carried only the OXA48 gene. These genetic markers were identified in 6 isolates of ESBL-producing *K. pneumoniae* and 5 isolates of ESBL-producing *E. cloacae*. As shown in Table 9.

Colistin resistance among ESBL-positive Carbapenems sensitive and bacterial isolates

Nine isolates (3.14%) were found to be ESBL-producing *Enterobacterales* that were resistant to colistin but sensitive to carbapenems. All of these isolates were members of the *K. pneumoniae* species. However, one *K. pneumoniae* ESBL producer that was resistant to colistin according to broth microdilution (BMD) testing was found to be sensitive to colistin according to disk diffusion (DE) testing as shown in Table 10.

Table 10 Colistin resistance among ESBL-positive Carbapenems sensitive *enterobacterales*

Species	ESBL (*N*, %)	Colistin resistance		Susceptibility to carbapenems (*N*, %)			
		BMD (*N*, %)	DE (*N*, %)	MEM	IPM	ETP	DOR
E. coli	119 (43)	0	0	119 (43)	119 (43)	119 (43)	119 (43)
K. pneumoniae	124 (46)	9 (3.3)	8 (3)	124 (46)	124 (46)	124 (46)	124 (46)
E. cloacae	31 (11)	0	0	31 (11)	31 (11)	31 (11)	31 (11)
Total (n, %)	274 (100)	9 (3.3)	8 (3)	274 (100)	274 (100)	274 (100)	274 (100)

CT: colistin, BMD: broth micro dilution method, ESBL: extended spectrum beta lactamase, DE: disc elution method, mCIM: modified carbapenemase inactivation method, MEM: meropenem, IPM: imipenem, ETP: ertapenem, Dor: Doripenem

Antibiogram of ESBL-producing *Enterobacterales*

Table 11 shows the varying resistance rates of ESBL-producing *Enterobacterales* to non-beta-lactam antibiotics, as well as to combinations of beta-lactam antibiotics with beta-lactamase inhibitors.

Table 8 Colistin resistance among CRE phenotypes

Species	CRE (*N*, %) By phenotyping	mCIM phenotype (*n*, %)		Colistin Resistance (*n*, %)	
		CPE	NCPE	Broth microdilution	Disc elution
E. coli	25 (25)	15 (15)	10 (10)	8 (8)	8 (8)
K. pneumoniae	63 (62)	56 (55)	7 (7)	25 (25)	25 (25)
E. cloacae	13 (13)	13 (13)	0	5 (5)	5 (5)
Total	101 (99)	84 (83)	17 (17)	38 (38)	38 (38)

BMD: broth microdilution method, ESBL: extended spectrum beta lactamase, DE: disc elution method, mCIM: modified carbapenemase inactivation method. CPE: carbapenemases producer *Enterobacterales*, NCPE: none carbapenemases producers *Enterobacterales*, CRE: carbapenem resistant *Enterobacterales*

Table 9 Detection of silent genes among ESBLE

Species	Number	ESBL Phenotype (*N*, %)	ESBL genotype (*N*, %)				CRE genes (*N*, %)				
			TEM	SHV	CTX_m	SHV, CTX_m	KPC	NDM	OXA48	VIM	OXA48, NDM
K. pneumoniae	28	6 (2.1)	0	1 (9)	3 (27)	2 (18)	0	4 (36)	0	0	2 (18)
E. cloacae	17	5 (1.7)	0	2 (18)	3 (27)	0	0	2 (18)	1 (9)	0	2 (18)
E. coli	20	0	5	12	3	0	0	0	0	0	0
Total (n, %)	65 (100)	11 (3.8)	0	3 (27)	6 (54)	2 (18)	0	6 (54)	1 (9)	0	4 (36)

Table 11 Antibiogram of ESBL producing *Enterobacterales*

Resistance rates of ESBL producing Enterobacterales				
	E. coli	***K. pneumoniae***	***E. cloacae***	***P. mirabilis***
Number of isolates	119	124	31	6
Aminoglycosides				
Amikacin	3 (2.5)	6 (5)	1 (3)	1 (17)
Gentamicin	21 (18)	11 (19)	4 (13)	3 (50)
Tobramycin	12 (10)	8 (6.5)	2 (6.5)	1 (17)
Fluoroquinolones				
Ciprofloxacin	38 (32)	27 (22)	9 (29)	5 (83)
Levofloxacin	32 (27)	27 (22)	9 (29)	5 (83)
Moxifloxacin	38 (32)	27 (22)	9 (29)	5 (83)
Trimethoprim/sulfamethoxazole	57 (48)	67 (54)	11 (35)	6 (100)
Nitrofurantoin	17 (14)	112 (90)	31 (100)	6 (100)
Fosfomycin	12 (10)	87 (70)	31 (100)	6 (100)
Beta Lactams				
Ceftazidime-avibactam	14 (12)	0	0	0
Meropenem-vaborbactam	0	0	0	0
Cefiderocol	0	0	0	0
Polymyxins				
Colistin	0	9 (7)	0	-

ESBL: extended spectrum beta lactamase

Antibiogram of ESBL-producing E. Coli isolates

ESBL-producing *E. coli* isolates exhibited notably high resistance rates for fluoroquinolones with 32% resistance to ciprofloxacin, levofloxacin, and moxifloxacin. Additionally, a high resistance rate of 48% was observed against trimethoprim/sulfamethoxazole.

Antibiogram of ESBL-producing K. pneumoniae isolates

Resistance rates of ESBL-producing *K. pneumoniae* isolates were high against trimethoprim/sulfamethoxazole 54%. Extremely high resistance to nitrofurantoin was noted, affecting 90% of isolates. Resistance to fosfomycin was prevalent, with 70% of isolates showing resistance.

Antibiogram of ESBL-producing E. Cloacae isolates

ESBL-producing *E. cloacae* isolates exhibited resistance rate of 35% against trimethoprim/sulfamethoxazole. Notably, all isolates demonstrated total resistance to both nitrofurantoin and fosfomycin.

Antibiogram of ESBL-producing P. mirabilis isolates

ESBL-producing *P. mirabilis* isolates showed resistance to gentamicin with 50% resistance rat. High resistance rates to fluoroquinolones were observed for ciprofloxacin, levofloxacin, and moxifloxacin, each at 83%. All isolates exhibited total resistance to trimethoprim/sulfamethoxazole, nitrofurantoin and fosfomycin.

Discussions

Infections caused by ESBLE, CRE, and colistin-resistant *Enterobacterales* pathogens, are becoming a top noticeable issue worldwide, this is due to the dramatically increased isolation of these pathogens from clinical samples, and their threat to human wellbeing [18]. Our study found that the overall percentage of multidrug-resistant *Enterobacterales* (MDRE) among the tested clinical isolates was 70.67% (424 out of 600). Our results agree with a 2021 report from Nablus, Palestine by Aiesh et al. which concluded that infections caused by ESBL-producing and carbapenem-resistant *E. coli* and *K. pneumoniae* have resulted in increased morbidity and mortality [5].

The biological differences, social inequities and restrictive cultural norms, make women more vulnerable to infectious diseases than men, this is highlighted in a study conducted by Cataldo, C. 2023, who found that women were at higher risk for many infectious diseases, they had more chance to infections than men [19]. In addition to that, most of the samples used in this study were urine samples, since females are more vulnerable to UTIs than males, (because the normal female urinary tract has a comparatively short urethra) therefore, carring an inherent predisposition to proximal seeding of bacteria. This anatomy increases the frequency of infections. Due to the fact that, females use more antibiotics than males especially third-generation cephalosporin like cefixime, ceftriaxone, and cefotaxime. As a consequence they become more vulnerable to selective pressure and harboring MDRE [20]. Approximately two-thirds of the bacterial isolates (61%) came from female patients, and the patients' age was distributed from 1 day to 92 years old, similar to a study from the same region regarding infections with ESBLE at an institutional level that showed female predominance with 51.6%, and the median age

of patients was 53 years [5]. Consistently, other studies from India showed that ESBL *E. coli* was predominate in female patients [12, 21].

Many reports demonstrated variations in the distribution of ESBLE isolates across different sources worldwide, a study conducted in India showed that ESBLE were most commonly isolated from urine (40%) followed by pus samples (22%) [21]. Another study conducted in south Asia regions, Bangladesh, and India, 2023, found that ESBLE were commonly isolated from urine samples (80%) in Bangladesh, and from skin and soft tissues in India (70%) [6]. Another study conducted in Palestine, Gaza Strip showed that ESBLE were commonly isolated from pus samples (55.2%) followed by urine samples (53.3%) [9]. Our study found that wound swabs were a major source of ESBLE (68/120) (56.6%), followed by urine samples (194/364) (53%).

A study conducted in the Gulf Cooperation Council countries showed that, among Gram-negative bacterial infections, *E. coli* (33%) was the most isolated organism followed by *K. pneumoniae* (19.2%) [22],, this is consistent with our study which found *E. coli* and *K. pneumoniae* were major causative agents of infections among *Enterobacterales*..

This study highlights the challenges between the detection of ESBLE and CRE by phenotypic and genotypic methods, it also highlights the prevalence rate of MDRE, ESBLE, CRE, and colistin resistance, in Palestine, and provide an antibiograms for *Enterobacterales* species with beta-lactam and none beta-lactam antibiotics.

Identifying ESBLE isolated from clinical samples is imperative for disease monitoring and the provision of efficacious treatments, although phenotypic method such as antimicrobial susceptibility profiling is still the most commonly used method to detect MDRE, ESBLE, CRE, and colistin-resistant bacteria. Some ESBLE harboured slow activity CRE genes called cryptic or silent genes, that thought to be a problematic issue, that lead to under-reporting or misdiagnosis of CRE as carbapenems sensitive bacteria. This highlights the importantce of the integration between phenotypic and genotypic to characterize MDRE [23]. Palestine like other developing countries, mainly depends on phenotyping characterizations for the detection of MDRE, ESBL and CRE, this is considered a challenge that increases the chance of missing the detection of *Enterobacterales* with cryptic mobile genomic elements, and could lead to failed treatment with prophylactics or drugs of choices among hospitalized patients. Our study found that among the randomly selected 65 ESBLE isolates from clinical samples, 11(17%) isolates harbored cryptic CRE genes, this finding will not only highlight the prevalence of both ESBLE and CRE in Palestine, but also will impair the infection control activities, therapeutic plan for infected patients, and the appropriate detection of CRE. Phenotypic detections alone could lead to failure in the treatment of patients with serious infections, that might increase the morbidity and mortality rates.

Similar to our study, a study conducted in Nigeria 2023, showed that (33/49) (67.35%) of isolates harbored carbapenemase genes, which were primarily bla_{NDM} (60.0%), of which three (3/33) (9%) were susceptible to carbapenems by phenotyping, this could be explained by the fact that carbapenemase were not expressed in vitro but could expressed in vivo [23]. In the same context a study conducted in Egypt 2022, showed that, among the phenotypically carbapenem-sensitive isolates, 42.5% were carrying carbapenem resistance genes, bla_{NDM} (80.5%) was the most prevalent, this finding also, demonstrate the importance of integrations between phenotype and molecular techniques to identify MDRE to CRE and ESBLE, to avoid failure treatment, and impaired infection control [24].

The high prevalence of ESBLE in Palestine and worldwide, lead us to think more if these isolates harbored cryptic multi-drug-resistant genes (MDRG). Our study found that (287/600) 47.8% of *Enterobacterales* isolates were ESBLE, a study conducted in Gaza Strip 2023, found that the prevalence rate of ESBLE was increasing in the children in pediatric Gaza Strip hospitals with a prevalence rate of 51.6% [9].

Many studies in the early 2000s, suggested that bla_{CTXm}-producing isolates were becoming widespread in Europe, Latin America and the Asia-Pacific region [1]. Our study found that (226/287) 78.7% of ESBLE were positive for bla_{CTXm}, this evidence stresses the importance of the collaboration of epidemiological surveillance, and antimicrobial stewardship to reduce the burden of ESBL gene transmissions from bla_{TEM}, bla_{SHV}, to bla_{CTXm} [9]. A study conducted by Ghenea, A. E.2022, showed that bla_{CTXm} has shown a rapid spread in recent years among *Enterobacterales* and has become the most prevalent ESBL in many parts of the world [25]. Further investigations need to be addressed in the future to demonstrate the relationships between different ESBLE genes and the detection of slow activity of not only CRE genes, but also MDRG among *Enterobacterales*.

Confirmation of phenotypic detection of CRE by molecular methods could prevent treatment failure and provide an actual prevalence rate of CRE worldwide. Some carbapenemase genes like bla_{NDM}, and bla_{OXA48} have a slow activity and cannot be detected by phenotype alone, which lead to under-reporting of CRE to ESBLE, AmpC, or even sensitive strains [26]. Our study found that (102/600) 17% were CRE.

According to a 2022 special report by the Centers for Disease Control and Prevention, during the COVID-19 pandemic the rate of CRE infections in hospitals

increased by 35% in 2020 compared with 2019 [27]. In a study conducted in Thailand 72% *K. pneumoniae* and 22% *E. coli* were CRE by phenotyping [28]. A study conducted in southern Saudi Arabia found that, out of the 86 tested *K. pneumoniae* CRE isolates, 64 (74.4%) were CPE isolates [26]. In southern Saudi Arabia *K. pneumoniae* strain was reported with triple carbapenemase genes, the emergence of such an isolate could threaten patients and healthcare workers and requires great attention to rapid interventions to avoid further dissemination [26]. A similar study conducted in Thailand found that bla_{NDM} was more prevalent in several regions. CRE strains from urine, sputum and blood were collected in Thailand from 2016 to 2018 which were composed of 72% *K. pneumoniae* and 22% *E. coli*, 80% of the CRE strains produced carbapenemases, 17% (629/4296) produced more than one carbapenemases, and the most common type of carbapenemase was bla_{NDM}, accounting for 65% (2392/4296) [28]. Our study found that the prevalence of CRE in Palestine increased in the timeline determined during October 2023, (85/102) 83% were CPE, (30/85) 30% were bla_{NDM} further investigations based on our study finding need to be addressed, based on the most updated molecular techniques, way to understand the high prevalence of like these genes among MDRE. Also, the presence of cryptic genes among ESBLE will not only increase the actual prevalence of CRE, but also encourage our hypothesis to think about the presence of other cryptic MDRGs among CRE, to avoid treatment failures and infection control obstacles in the future.

Colistin is considered the last choice for carbapenem-resistant MDRE and it is often used for treatment of blood stream infections, wound infections, and respiratory infections caused by CRE. In recent years, there has been a marked increase in the incidence of colistin-resistant bacterial infections [4]. A similar study found that the overall resistance to colistin was high (41%) among tested clinical isolates, 61.0% of meropenem-resistant *Enterobacteriaceae* were resistant to colistin [4]. Another study showed that among 100 CRE isolates, 15% were resistant to colistin [29]. Another study conducted on 196 MDRE isolates, (19.9%) showed reduced susceptibility to colistin, which is an alarming sign of increasing colistin resistance rates among CRE isolates [30]. Our study found that colistin resistance is not only among CRE, but also, we found colistin resistance among ESBLE, this will highlight our hypothesis to test all MDRE, or even the whole *Enterobacterales* for colistin resistance, by the most appropriate and approved methods, that will reveal the actual colistin-resistant status not only in Palestine but also, worldwide. The phenotyping ADM explained in detail by CLSI 2023 guidelines is considered an excellent option for routine use, as it combines ease of performance with affordable cost [31]. Our study found 9 (3.14%) were colistin-resistant ESBLE producers and carbapenems sensitive *Enterobacterales* by phenotyping, all of them were *K. pneumoniae* ESBL producers carbapenems sensitive by phenotyping, one *K. pneumoniae* was an ESBL producer colistin-resistant by BMD and was colistin-sensitive by DE. DDM is not a recommended method by CLSI and EUCAST guidelines, for detecting colistin susceptibility, we should always depend on susceptibility testing by BMD or DE before releasing the final report even in resource-limited settings. This can preserve the therapeutic value of MDRE infections until we have newer treatment options available in the country [32]. Challenges in the methods of sensitivity testing of colistin along with an increase in the prevalence of colistin-resistant *Enterobacterales* needs to be addressed , our results showed that colistin performed by DE worked well compared to the reference BMD except for one isolate that was colistin-sensitive by DE (MIC=2 μg/mL), and colistin-resistant by BMD (MIC=4 μg/mL), since colistin sensitivity by BMD is considered as a gold standard and more reliable than DE we considered the isolate to be resistant to colistin [3, 33].

The increasing of ESBLE-producing bacteria have become a global concern because of their multi-resistance to most antibiotic classes, which makes the treatment difficult [2]. To achieve appropriate treatment choices, identification of the *Enterobacterales* species that generate ESBL as well as their antibiotic sensitivity pattern is essential worldwide [34]. A study by Ortiz de la Rosa, 2019 found that the ESBL bacterial isolates, may be a source of resistance to ceftazidime-avibactam [35], another study by Petty, L. A. 2018 found that, vaborbactam combined with meropenem had improved activity compared to meropenem alone across all isolates [36], another study by Yao, J. 2021 found that cefiderocol has demonstrated excellent activity against gram-negative bacilli including ESBLE, and CRE [19]. A study conducted in Iran showed the same results as our study, they found that aminoglycosides were confirmed as the most effective treatment option for ESBLE bacteria [34], while a study in Portugal showed that, a higher prevalence of resistance to fluoroquinolones was observed in ESBLE, 16.7% of ESBLE were resistant to fluoroquinolones [37], a study conducted at An-Najah National university hospital showed that ESBLE producing *K. pneumoniae* was highly resistant to fosfomycin, trimethoprim/sulfamethoxazole and nitrofurantoin [5]. A study conducted at the institutional level in Switzerland in 2022, found that ESBLE is an important reservoir for mobile colistin-resistant (mcr) genes [35]. Our study highlights that, for epidemiological purposes, the prevalence of colistin resistance should be checked among all *Enterobacterales* isolates not only among CRE isolates.

Conclusion

In conclusion, our study found a high burden of MDRE in West Bank, Palestine, this will limit and complicate treatment options for infections caused by *Enterobacterales*, which in turn calls for immediate actions to control and monitor the use of antimicrobials in general, it would be beneficial to incorporate both conventional laboratory techniques and modern molecular techniques for comprehensive characterization of the emergence silent carbapenemase genes among MDRE infections in Palestine, and over all the world. Resistant to colistin is not only among CRE bacteria, but some can also be detected even among ESBLE bacteria, combination of β-lactam beta-lactamases inhibitors with beta-lactam antibiotics is still useful for the treatment of various types of MDRE isolated from clinical samples.

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Author contributions
MI conceptualized and designed the study and did the literature search, lab work, and manuscript writing. MQ & AA conceptualized and designed the study and did the literature search, supervise the lab work, revise, and write the manuscript. All authors discussed the results and contributed to the final manuscript.

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Data availability
The data used to support the findings of this study are included within the article.

Declarations

Conflict of interest
The authors declare that there is no conflict of interest regarding the publication of this paper.

Ethical considerations and consent to participate
Ethical approval (Ref. Mas. Oct. 2023/7) was obtained from the Institutional Review Board (IRB) of An-Najah National University, which approved all aspects of the study design, including the collection of bacterial isolates from microbiology laboratories, and the patient demographic data. The need for informed consent was waived by the IRB of An-Najah National University. Approval to collect demographic data and bacterial isolates from the governmental hospitals was also obtained from the Palestine Ministry of Health (MOH). The data collected were used exclusively for research, kept confidential, and not used for any other purpose.

Consent for publication
Not applicable.

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