Original Article

Carbapenem and colistin-resistant bacteria in North Lebanon: Coexistence of mcr-1 and NDM-4 genes in Escherichia coli

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Abstract

Introduction: The increasing incidence of infections caused by multidrug-resistant bacteria is considered a global health problem. This study aimed to investigate this resistance in Gram-negative bacteria isolated from patients hospitalized in North-Lebanon.

Methodology: All isolates were identified using the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Antibiotic susceptibility testing was achieved using disk diffusion, E-test and Broth microdilution methods. Phenotypic detection of carbapenemase was carried out using the CarbaNP test. RT-PCR, standard-PCR and sequencing were performed to detect resistance genes and oprD gene. Conjugal transfer was carried out between our isolates and Escherichia coli J53 to detect the genetic localization of resistance genes. MLST was conducted to determine the genotype of each isolate.

Results: Twenty-three carbapenem-resistant Enterobacterales of which eight colistin-resistant Escherichia coli, and Twenty carbapenem-resistant Pseudomonas aeruginosa were isolated. All isolates showed an imipenem MIC greater than 32 µg/mL with MICs for colistin greater than 2 mg/L for E. coli isolates. All the Enterobacterales isolates had at least one carbapenemase-encoding gene, with E. coli isolates cohaboring blaNDM-4 and mcr-1 genes. Moreover, 16/20 Pseudomonas aeruginosa harbored the blaVIM-2 gene and 18/20 had mutations in the oprD gene. MLST revealed that the isolates belonged to several clones.

Conclusions: We report here the first description in the world of clinical E. coli isolates cohaboring blaNDM-4 and mcr-1 genes, and K. pneumoniae isolates producing NDM-6 and OXA-48 carbapenemases. Also, we describe the emergence of NDM-1-producing E. cloacae in Lebanon. Screening for these isolates is necessary to limit the spread of resistant microorganisms in hospitals.

Key words: Extremely drug resistant bacteria; NDM-4, mcr-1; Lebanon.

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Introduction

The dramatic increase in the prevalence of infections caused by multi-drug resistant (MDR) bacteria has become a major global health concern. Carbapenems are the β-lactam group of drugs used to treat infections caused by resistant Gram-negative bacteria such as extended-spectrum (ESBL) and AmpC β-lactamases producing strains [1,2]. However, in recent years, this evidence has changed with the emergence of carbapenem-resistant bacteria in both non-fermenters (Acinetobacter baumannii and Pseudomonas aeruginosa) [3,4] and fermenters (Enterobacterales) Gram-negative bacteria (GNB) [1,4]. This has led to the renewal of colistin as the last treatment option especially for intensive care units (ICU) patients [5]. Despite that, the intensive use of colistin to treat patients infected with MDR bacteria has been followed by an increase in the number of colistin-resistant bacteria, not to forget that the clinical use of this antibiotic has shown to have significant problems such as nephrotoxicity and neurotoxicity [6].

Carbapenemase related resistance is now described in the majority of enterobacterial species but mainly found in K. pneumoniae and E. coli. This resistance is mediated by carbapenem-hydrolyzing β-lactamases, including class A β-lactamases (KPC, IMI, and GES),
class B metallo-β-lactamases (NDM, IMP, and VIM), and class D β-lactamases (OXA-48) [7-9]. Moreover, among the most reported multi-drug resistant bacteria, \textit{P. aeruginosa} occupies an important place. Various types of metallo-β-lactamases (MBLs) have been described in \textit{P. aeruginosa} isolates (IMP, VIM, SPM, GIM, SIM, AIM, FIM, and NDM), with VIM-2 being the most detected one [3,10]. However, mutations in the outer membrane porin (OprD) gene represent the major molecular mechanism conferring resistance to carbapenems in \textit{P. aeruginosa} [3]. As for colistin, the main mechanism of resistance in \textit{Enterobacteriales} occurs by the alteration of the negatively charged lipopolysaccharide (LPS), by the addition of phosphoethanolamine (PEtN) or 4-amino-4-deoxy-L-arabinose (L-Ara4N), to the lipid A moiety of the LPS. This can be insured by specific mutations of the two-component systems (TCSs) (pmrA/pmrB, phoP/phoQ), or its negative regulator mgrB. The modified LPS with this positive charge reduces its binding to polymyxins and generates this resistance [6]. Besides, it has been shown that mutations in lpxACD genes leading to the loss of LPS production are also responsible for colistin resistance mainly in \textit{A. baumannii} [11]. Finally, colistin resistance in \textit{Enterobacteriales} may be also due to the presence of a plasmid-mediated mcr gene that encodes for the phosphoethanolamine transferase enzyme, which modifies the lipid A moiety of LPS with the addition of phosphoethanolamine [12].

In Lebanon, the first resistance to carbapenem described in Gram-negative bacteria dates back to 2008, when Matar et al. reported the presence of carbapenem-resistant \textit{Klebsiella pneumoniae} isolates producing OXA-48 [13]. Also, Zarrilli et al. described the presence of carbapenem-hydrolyzing oxacillinases OXA-58 in carbapenem-resistant \textit{Acinetobacter baumannii} collected from a Lebanese hospital [14]. On the other hand, Carrè et al. reported the spread of OXA-48-encoding plasmid in several countries, including Lebanon [8]. This study was followed by several other studies describing the dissemination of this type of carbapenemase in Lebanon [15–17]. The other carbapenemases of the Ambler class D (OXA-23, OXA-24, and OXA-58) type were mainly detected in \textit{A. baumannii} [17–19]. Moreover, several studies have described the emergence of carbapenem-resistant \textit{Enterobacteriales} producing other types of carbapenemase, such as the NDM-1 [15] and NDM-5 [20]. On the other hand, carbapenem-resistant \textit{P. aeruginosa} has been described in few studies in Lebanon. Al-Bayssari et al. reported in 2014 for the first time in Lebanon, the emergence of VIM-2 producing \textit{P. aeruginosa} isolates [3]. This study has been followed recently by another study conducted by Nawfal Dagher et al. describing the emergence of plasmid-mediated VIM-2 carbapenemase in \textit{P. aeruginosa} [21].

Few studies have described colistin resistance in clinical settings in Lebanon. Okdah et al. were the first to report colistin-resistant \textit{Klebsiella pneumoniae} strains having mutations in mgrB and phoQ genes [12]. This was followed by two other studies describing colistin resistance in Gram-negative bacteria in Saint George hospital in Beirut due to mutations in mgrB, pmrA, pmrB, phoP and phoQ genes [20,22]. Recently, Salloum et al. reported the emergence of plasmid-mediated colistin resistance mcr-8 gene from a clinical \textit{Klebsiella pneumoniae} isolate [23]. However, no previous study has described the presence of colistin resistance in \textit{Acinetobacter baumannii} and \textit{Pseudomonas aeruginosa} in Lebanon.

This study aims to investigate the molecular support of carbapenem and colistin resistance in gram-negative bacteria collected from clinical samples, between June 2018 and April 2019, from patients hospitalized in Saydet Zgharta University Medical Center, in North Lebanon.

**Methodology**

**Study Design**

Between June 2018 and April 2019, carbapenem and colistin-resistant bacteria were collected from different pathological specimens, from patients hospitalized at Saydet Zgharta hospital, located in North Lebanon. Some isolates were collected from a patient infected upon admission and without prior exposure to antibiotics, and others were collected from patients remaining at the hospital for more than one day and treated with antibiotics or had a nosocomial transmission. Patients who were positive for carbapenem-resistant bacteria were treated with colistin. All isolates were kept at -80°C before being transported to the laboratory in Marseille, France.

**Identification of carbapenem and colistin-resistant bacteria**

The collected bacteria were cultivated on the Trypticase Sodium Agar medium (TSA) at 37°C for 24h. The identification of bacteria at the species level was achieved using the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (MicroflexTM; Bruker Daltonic, Bremen, Germany) with flex control and biotyper 3.0 software (Bruker Daltonic).
Antibiotic susceptibility testing and CarbaNP test

To determine the antibiotic susceptibility of the strains, the standard disk diffusion method was performed on Mueller-Hinton agar, as recommended by the European Committee of Antimicrobial Susceptibility Testing (EUCAST) guidelines. Sixteen different antibiotics were tested for the *Enterobacterales* species: amoxicillin (20 µg), amoxicillin-clavulanic acid (20-10 µg), piperacillin-tazobactam (30-6 µg), cefalotin (30 µg), ceftazidime (10 µg), cefepime (30 µg), ertapenem (10 µg), imipenem (10 µg), amikacin (30 µg), gentamicin (10 µg), ciprofloxacin (5 µg), Fosfomycin (200 µg), nitrofurantoin (100 µg), tobramycin (10 µg), trimethoprim-sulfamethoxazole (1.25-23.75 µg) and colistin (10 µg). Whereas for *P. aeruginosa* isolates, sixteen different antibiotics were tested: Ticarcillin (75 µg), Ticarcillin-clavulanic acid (75-10 µg), piperacillin-tazobactam (30-6 µg), ceftazidime (10 µg), cefepime (30 µg), imipenem (10 µg), ertapenem (10 µg), amikacin (30 µg), gentamicin (10 µg), tobramycin (10 µg), ciprofloxacin (5 µg), Fosfomycin (200 µg), nitrofurantoin (100 µg), trimethoprim-sulfamethoxazole (1.25-23.75 µg) and rifampicin (5 µg). Minimal inhibitory concentration (MIC) of imipenem was determined using the E-test, and the MIC of colistin was performed using the broth microdilution method, as recommended by the EUCAST (2017, version 2). Strains were considered resistant to colistin when the MIC was greater than 2mg/L. Phenotypic detection of carbapenemase production was performed by the CarbaNP test, as previously described [24].

DNA extraction

The DNA of the bacteria was extracted using the automatic robot EZ1 (Qiagen BioRobot EZ1-, Tokyo, Japan), with the extraction kit EZ1 DNA, Qiagen, Hilden, Germany, according to the manufacturer's guidelines. They were eluted in 200 µL of elution buffer and kept stored at -20°C.

Screening of isolates by real-time PCR, standard PCR, and sequencing of the resistance genes and oprD gene

The real-time PCR assay was performed to assess the presence of carbapenemase encoding genes, using specific primers, such as *bla*OXA-23, *bla*OXA-24, *bla*OXA-48, *bla*OXA-58, *bla*KPC, *bla*NDM, and *bla*VIM genes. Colistin-resistant bacteria were also screened for the presence of mcr-1, 2, 3, 4, and 5 genes. All probes and primers used have been previously described [20,25,26]. Sequencing of PCR products was performed by using the BigDye terminator chemistry on an automated ABI 3130 sequencer (PE Applied Biosystems, Foster City, CA). Analysis of the sequenced genes was carried out using the ARG-ANNOT database [27]. Comparison with other genes was performed using the BlastN and BlastP analysis of the National Center for Biotechnology Information (NCBI) database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Molecular characterization of the oprD gene was also performed for *P. aeruginosa* isolates [28]. The sequenced oprD gene was analyzed and compared against the oprD gene of the reference strain *P. aeruginosa* PA01, using multalin alignment software (http://multalin.toulouse.inra.fr/multalin/).

Conjugal transfer and plasmid extraction

Conjugation experiments were performed using sodium azide-resistant *E. coli* J53 as a recipient strain. Transconjugants were selected on Mueller-Hinton agar supplemented with sodium azide (100 mg/L) and ertapenem (0.5 µg/L) or colistin (4 µg/ml). PCR for the carbapenemase and/or colistin-resistance encoding genes was performed on all imipenem-resistant and/or colistin-resistant *E. coli* transconjugants. Plasmid DNA extraction from transconjugants was performed using a NucleoBond Xtra Plus kit (Macherey-Nagel, Dueren, Germany) according to the manufacturer’s instructions and was separated by agarose gel electrophoresis. Gel slices containing individual plasmid bands were excised with a razor blade for the isolates that harbored more than one plasmid. These plasmids were extracted from gel slices using the GeneClean Turbo glass milk spin kit (Qbiogene, Inc., Carlsbad, CA) following the manufacturer’s instructions. PCR was performed on all extracted plasmids to verify the conjugation experiments.

Multilocus sequence typing (MLST)

The analysis of the genotype was performed using the housekeeping genes for *E. coli, K. pneumoniae, E. cloacae*, and *P. aeruginosa* to determine the genetic relationship between isolates, as described on the Web site of Institute Pasteur’s MLST (www.pasteur.fr/mlst) and PubMLST (https://pubmlst.org/).

Results

Twenty-three carbenem-resistant *Enterobacterales* and twenty carbapenem-resistant *P. aeruginosa* were collected from patients admitted to Saydet Zgharta hospital in Lebanon. No *A. baumannii* strains were detected. Among the 23 carbapenem-resistant *Enterobacterales*, strains were identified as
follows: 8 *E. coli*, 5 *E. cloacae*, and 10 *K. pneumoniae*. All the strains were susceptible to colistin except *E. coli* strains. The antibiotic resistance profile of all Extremely drug-resistant (XDR) isolates is detailed in Table 1 and Table 2. E-tests for all isolates revealed a high level of resistance to imipenem with MIC greater than 32 µg/ml. All *E. coli* isolates were resistant to colistin with MIC equal to 32 µg/ml. Phenotypic detection of carbapenemase production by the carbaNP test showed a positive result for all isolates, except for four *P. aeruginosa* (PA-1, PA-4, PA10, PA-18).

Table 1. Phenotypic and genotypic features of the XDR *Enterobacterales* isolates.

| Strain name | Source | Antibiotic Susceptibility Profile | T/NT | IMP MIC (µg/ml) | CS MIC (µg/ml) | Carba NP test | blaOXA-48 | blaNDM-1 | blaNDM-4 | blakPC | Genes | Genetic Localization | ST |
|-------------|--------|----------------------------------|------|----------------|---------------|--------------|------------|-----------|-----------|--------|-------|---------------------|-----|
| *E. coli* EC-1 | Urine  | TOB, AK, GEN                     | NT   | > 32           | > 32          | +            | -          | -         | -         | +       | +       | P 405                |     |
| *E. coli* EC-2 | Urine  | TOB, AK, GEN                     | NT   | > 32           | > 32          | +            | -          | -         | +         | +       | +       | P 405                |     |
| *E. coli* EC-3 | Urine  | TOB, AK, GEN                     | NT   | > 32           | > 32          | +            | -          | -         | +         | +       | +       | P 405                |     |
| *E. coli* EC-4 | Urine  | TOB, AK, GEN                     | NT   | > 32           | > 32          | +            | -          | -         | +         | +       | +       | P 405                |     |
| *E. coli* EC-5 | Urine  | TOB, AK, GEN                     | NT   | > 32           | > 32          | +            | -          | -         | +         | +       | +       | P 405                |     |
| *E. coli* EC-6 | Urine  | TOB, AK, GEN                     | NT   | > 32           | > 32          | +            | -          | -         | +         | +       | +       | P 405                |     |
| *E. coli* EC-7 | Urine  | TOB, AK, GEN                     | NT   | > 32           | > 32          | +            | -          | -         | +         | +       | +       | P 405                |     |
| *E. coli* EC-8 | Urine  | TOB, AK, GEN                     | NT   | > 32           | > 32          | +            | -          | -         | +         | +       | +       | P 405                |     |
| *E. cloacae* Eclo-1 | Urine  | CS                             | NT   | > 32           | < 2           | +            | -          | +         | -         | -       | +       | P 120                |     |
| *E. cloacae* Eclo-2 | Catheter  | CS                         | NT   | > 32           | < 2           | +            | -          | +         | -         | -       | +       | P 120                |     |
| *E. cloacae* Eclo-4 | Urine  | TOB, AK, GEN, CS                | T    | > 32           | < 2           | +            | -          | +         | -         | -       | +       | P 109                |     |
| *E. cloacae* Eclo-5 | Urine  | CS                             | NT   | > 32           | < 2           | +            | -          | +         | -         | -       | +       | P 135                |     |
| *E. cloacae* Eclo-6 | Urine  | CS                             | NT   | > 32           | < 2           | +            | -          | +         | -         | -       | +       | P 66                 |     |
| *K. pneumoniae* KP-1 | BAL  | TOB, AK, GEN, CS               | T    | > 32           | < 2           | +            | -          | +         | -         | -       | +       | P 11                  |     |
| *K. pneumoniae* KP-2 | Wound | AK, GEN, CS                    | NT   | > 32           | < 2           | +            | -          | +         | -         | -       | +       | P 14                  |     |
| *K. pneumoniae* KP-4 | Urine  | TOB, AK, GEN, CS               | T    | > 32           | < 2           | +            | -          | +         | -         | -       | +       | P 17                  |     |
| *K. pneumoniae* KP-5 | Sputum | TOB, AK, GEN, CS              | NT   | > 32           | < 2           | +            | -          | +         | -         | -       | +       | P 101                 |     |
| *K. pneumoniae* KP-6 | Sputum | AK, CS                       | T    | > 32           | < 2           | +            | -          | +         | -         | -       | +       | P 3367                |     |
| *K. pneumoniae* KP-8 | Wound | TOB, AK, GEN, CS               | NT   | > 32           | < 2           | +            | -          | +         | -         | -       | +       | P 13                  |     |
| *K. pneumoniae* KP-9 | Wound | TOB, AK, GEN, CS               | NT   | > 32           | < 2           | +            | -          | +         | -         | -       | +       | P 13                  |     |
| *K. pneumoniae* KP-10 | Catheter | TOB, AK, GEN, CS            | NT   | > 32           | < 2           | +            | -          | +         | -         | -       | +       | P 17                  |     |
| *K. pneumoniae* KP-3 | Urine  | CS                             | NT   | > 32           | < 2           | +            | -          | +         | -         | -       | +       | P 147                 |     |
| *K. pneumoniae* KP-7 | Urine  | CS                             | NT   | > 32           | < 2           | +            | -          | +         | -         | -       | +       | P 147                 |     |

XDR: Extremely drug resistant; BAL: Bronchoalveolar Lavage; IPM: Imipenem; MIC: Minimum Inhibitory Concentration; T: Patient previously treated with antibiotics; NT: Patient not previously treated with antibiotics; P: Plasmidic; ST: Sequence type; TOB: Tobramycin; AK: Amikacin; GEN: Gentamicin; CS: Colistin.
substitution mutations, leading to a premature stop codon TGA in oprD, resulting in the formation of a truncated polypeptide made of 345 amino acid residues. Conjugal transfer between VIM-2-producing *P. aeruginosa* (PA-2 and PA-19) and *Escherichia coli* (J53) failed, to yield *E. coli* transconjugants resistant to imipenem suggesting a chromosomal localization of VIM-2. On the other hand, the same experiment was performed between *E. coli* EC-1, *E. cloacae* Eclo-1, *K. pneumoniae* KP-3, and *Escherichia coli* (J53), to determine if NDM-4, mcr-1, NDM-1 and NDM-6 genes are chromosomal or plasmidic. This experiment succeeded to yield *E. coli* transconjugants resistant to imipenem (MIC > 32 μg/ml) and/or colistin (MIC > 2 mg/L) and harboring plasmids of ~46.5 kb (NDM-4) and ~ 62 kb (mcr-1); ~ 340 Kb (NDM-1); ~ 62 kb (OXA-48) and ~ 110 Kb (NDM-1) respectively. PCR performed on extracted plasmids from *E. coli* (J53) transconjugants were positive for the above carbapenemase genes and mcr-1 gene. Conjugation experiments on the *Enterobacterales* isolates and positive PCR results assume that these isolates had plasmid-mediated carbapenemase and mcr-1 genes.

MLST analysis reveals that all *E. coli* isolates belonging to the ST405 sequence type, two *E. cloacae* isolates have the sequence type ST120 and the remaining three isolates have different sequence types: ST109, ST135 and ST66. Analysis of *K. pneumoniae* revealed that all the isolates harboring the bla<sub>oxa-48</sub> gene have several sequence types (ST11, ST14, ST17, ST101, ST3367, and ST13), whereas those harboring the bla<sub>oxa-48</sub> and bla<sub>ndm-6</sub> (KP-3, and KP-7) have the sequence type ST147. Results for *P. aeruginosa* showed that fourteen *P. aeruginosa* (PA-2, PA-3, PA-5, PA-9, PA-20, PA-22, PA-6, PA-7, PA-11, PA-12, PA-13, PA-15, PA-16, PA-17) have the sequence type ST233. The remaining *P. aeruginosa* isolates have several sequence types: ST1329 (PA-1), ST253 (PA-4), ST622 (PA-10), ST1701 (PA-18) and ST357 (PA-19 and PA-21) (Table 2).

**Discussion**

The Multi-Drug Resistant (MDR) Gram-negative bacteria has multiplied worldwide, leading to serious clinical problems. This study reports the presence of carbapenem and colistin-resistant *Enterobacterales* and *P. aeruginosa* collected from patients at Saydet Zgharta Hospital. These isolates were collected from patients upon admission or from patients who remained in the hospital for more than one day. In this study, all isolates were resistant to the majority of antibiotics tested. PCR, sequencing, and MLST results showed that all *E. coli*

### Table 2. Phenotypic and genotypic features of the carbapenem-resistant *P. aeruginosa* isolates.

| Strain name | Source | Antibiotic Susceptibility Profile | T/NT | IMP MIC (μg/ml) | CS MIC (μg/ml) | Carba NP test | VIM-2 | Genetic localization | OprD Mutational Groups | ST |
|-------------|--------|----------------------------------|------|----------------|---------------|---------------|--------|---------------------|------------------------|----|
| *P. aeruginosa* PA-1 | Urine | TOB, AK, GEN, CS | T | >32 | <2 | - | - | C | G1 | 1329 |
| *P. aeruginosa* PA-4 | Tracheal | TOB, AK, GEN, CS | NT | >32 | <2 | - | - | C | G6 | 253 |
| *P. aeruginosa* PA-10 | Wound | CS | T | >32 | <2 | - | - | C | G2 | 622 |
| *P. aeruginosa* PA-18 | BAL | AK, GEN, CS | T | >32 | <2 | - | - | C | G3 | 1701 |
| *P. aeruginosa* PA-19 | Sputum | CS | NT | >32 | <2 | - | + | C | G4 | 357 |
| *P. aeruginosa* PA-21 | Sputum | CS | NT | >32 | <2 | + | + | C | G4 | 357 |
| *P. aeruginosa* PA-2 | Urine | CS | NT | >32 | <2 | - | - | C | G5 | 233 |
| *P. aeruginosa* PA-3 | Sputum | CS | NT | >32 | <2 | - | - | C | G5 | 233 |
| *P. aeruginosa* PA-5 | BAL | CS | NT | >32 | <2 | - | - | C | G5 | 233 |
| *P. aeruginosa* PA-9 | Wound | CS | NT | >32 | <2 | - | - | C | G5 | 233 |
| *P. aeruginosa* PA-22 | Sputum | CS | NT | >32 | <2 | - | - | C | G5 | 233 |
| *P. aeruginosa* PA-6 | Urine | CS | NT | >32 | <2 | - | - | C | G5 | 233 |
| *P. aeruginosa* PA-7 | BAL | CS | NT | >32 | <2 | - | - | C | G5 | 233 |
| *P. aeruginosa* PA-11 | BAL | CS | NT | >32 | <2 | - | - | C | G5 | 233 |
| *P. aeruginosa* PA-12 | BAL | CS | NT | >32 | <2 | - | - | C | G5 | 233 |
| *P. aeruginosa* PA-13 | BAL | CS | NT | >32 | <2 | - | - | C | G5 | 233 |
| *P. aeruginosa* PA-15 | Wound | CS | NT | >32 | <2 | - | - | C | G5 | 233 |
| *P. aeruginosa* PA-16 | Sputum | CS | NT | >32 | <2 | - | - | C | G5 | 233 |
| *P. aeruginosa* PA-17 | Wound | CS | NT | >32 | <2 | - | - | C | G5 | 233 |
| *P. aeruginosa* PA-20 | Wound | CS | NT | >32 | <2 | - | - | C | G5 | 233 |

XDR: Extremely drug resistant; BAL: Bronchoalveolar Lavage; IPM: Imipenem; MIC: Minimum Inhibitory Concentration; T: Patient previously treated with antibiotics; NT: Patient not previously treated with antibiotics; P: Plasmidic; ST: Sequence type; TOB: Tobramycin; AK: Amikacin; GEN: Gentamicin; CS: Colistin. G1: G to A substitution in nucleotide position 830 leading to the premature stop codon TAG in oprD, resulting in a truncated polypeptide of 276 amino acid residues; G2: G to T substitution in nucleotide position 688 leading to the premature stop codon TAA in oprD, resulting in a truncated polypeptide of 229 amino acid residues; B: Several Substitution mutations leading to the premature stop codon TGA in oprD, resulting in a truncated polypeptide of 345 amino acid residues; G4: Full length oprD gene. No mutations; G5: Insertion sequence (ISPa1328) in nucleotide position 419 of the oprD gene; G6: Insertion sequence (ISRP10) in nucleotide position 503 of the oprD gene.

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isolates harbored the blaNDM-4 and mer-1 genes, and have the sequence type ST405, which is firstly reported in Lebanon and the world. However, the blaNDM-4 E. coli ST405 isolates have already been reported in various studies from hospitalized patients in China and Pakistan [29,30]. Also, the presence of plasmid-mediated mer-1 E. coli ST405 has been recently described in farmlands in Algeria [31]. To the best of our knowledge, the coexistence of mer-1 and NDM-4 genes has never been reported in E. coli, but it was described in a study done in Vietnam where Le et al. reported the presence of clinical K. pneumoniae isolates harboring these two genes [32]. From our results, it has been noticed that the first blaNDM-4 and mer-1 carrying E. coli ST405 (EC-1) has been detected in a patient upon his first day of admission. Following this initial detection, the same isolate was collected from a larger number of different patients who were hospitalized for several days and were not carriers of this bacteria at the time of admission. This means that this clone is of high concern because it was circulating in the hospital.

Moreover, the two E. cloacae isolates that belong to the ST120 and harbor the blaNDM-1 gene have been collected from two patients who were transferred to Saydet Zgharta hospital from another Lebanese peripheral hospital. To the best of our knowledge, no previous studies have reported the blaNDM-1 gene in E. cloacae in Lebanon. Yet, this gene has been detected in other bacterial species, such as E. coli, K. pneumoniae, and A. baumannii [18,33,34], suggesting the dissemination of the plasmid carrying this gene between Enterobacteriales species. This ST120 carbapenem-resistant E. cloacae producing NDM-1 has been identified as an endemic clone in Henan province, China [35]. This underscores the urgent need for action to prevent the spread of this clone having the plasmid-mediated NDM-1 gene in Saydet Zgharta hospital. Moreover, OXA-48-producing E. cloacae isolates have been previously described in Lebanon by Hammoudi et al. [16]. However, the clones ST109, ST135, and ST66 have been reported in patients from different countries in the world, including the Czech Republic, Ireland, and Croatia respectively [36–38].

Also, results showed that 80% of carbapenem-resistant K. pneumoniae isolates harbor the blaOXA-48 gene and have different Sequence Types such as ST11, ST14, ST17, ST101, ST3367 and ST13, whereas 20% of K. pneumoniae isolates (KP-3 and KP-7) harbor the blaOXA-48 gene and the blaNDM-6 gene and belong to ST147. OXA-48 producing K. pneumoniae had been previously reported in different countries including Lebanon [20,34], whereas K. pneumoniae isolates harboring the blaOXA-48 and the blaNDM-6 gene simultaneously, have never been reported worldwide. OXA-48 producing K. pneumoniae (ST11, ST14, ST17, ST101, and ST3367) have been reported in different countries worldwide, such as Taiwan and Greece [39,40], Spain [41], Algeria and Tunisia [42,43] and Italy [44]. Furthermore, three OXA-48 producing K. pneumoniae have ST13. One isolate (KP-8) was collected from an injured patient on admission to the hospital and the other two isolates (KP-9 and KP-10) were collected from two different patients after several days of admission. This Sequence Type is of great concern as it has been identified as an internationally disseminated clone and has been reported in humans and animals in different countries such as Ireland [45], and Algeria [46]. Results also showed that the two OXA-48 and NDM-6 coproducing K. pneumoniae isolates having the sequence type ST147 were collected from two patients during their hospitalization without carrying this bacterium at the time of admission. This sequence type ST147 has been associated with different nosocomial outbreaks in different countries such as China [47] and Tunisia [42]. A strategy must be implemented to prevent the spread of these resistant microorganisms among patients.

Results also revealed the presence of carbapenem-resistant P. aeruginosa essentially due to mutations in the oprD gene, but also to the presence of the chromosomal blaVIM-2 gene. These findings are in concordance with previous studies carried out in Lebanon that reported the emergence of VIM-2 producing P. aeruginosa and having mutations in the oprD gene in both animals and humans [3,21,48]. In parallel, several worldwide studies have demonstrated that carbapenem-resistance in P. aeruginosa was mainly due to the mutations in the oprD gene, but also to the production of metallo-β-lactamase VIM-2 [49–51]. Genotyping results showed that P. aeruginosa belonged to different Sequence Types (ST1329, ST253, ST622, ST1701, ST357, and ST233), where the ST233 accounts for 70% of the total isolates, suggesting a link between P. aeruginosa ST233 isolated in the same hospital. Sequence Types ST1329, ST622, ST1701 and ST357 have been found in Lebanon [3,21] and in different countries, such as Netherlands [52] and Russia [49], whereas the ST253 has never been reported in Lebanon, but has been identified in China [53]. On the other hand, the ST233 that represents the major clone in the hospital has already been described in Lebanon and different countries worldwide [3,21,54–57]. This ST233 VIM-2-producing P. aeruginosa, which accounts for the majority of isolates is of great concern.
as it belongs to the international clone and is now an endemic clone in this hospital.

Conclusions
In conclusion, this study described the emergence of carbapenem and colistin-resistance in Gram-negative bacteria in North Lebanon. Our study reported for the first time in the world the coexistence of plasmid-mediated mer-1 and NDM-4 in E. coli isolates and OXA-48 and NDM-6 in K. pneumoniae isolates. Moreover, it described the emergence of NDM-1 producing E. cloacae in Lebanon. Also, MLST showed that there were major circulating clones such as ST405 for E. coli and ST233 for P. aeruginosa that become endemic in the hospital. A serious strategy must be implemented to inhibit the spread of these resistant microorganisms among patients hospitalized at Saydet Zgharta Hospital.

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