Different phenotypic and molecular mechanisms associated with multidrug resistance in Gram-negative clinical isolates from Egypt

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Objectives: We set out to investigate the prevalence, different mechanisms, and clonal relatedness of multidrug resistance (MDR) among third-generation cephalosporin-resistant Gram-negative clinical isolates from Egypt.

Materials and methods: A total of 118 third-generation cephalosporin-resistant Gram-negative clinical isolates were included in this study. Their antimicrobial susceptibility pattern was determined using Kirby–Bauer disk diffusion method. Efflux pump-mediated resistance was tested by the efflux-pump inhibitor-based microplate assay using chlorpromazine. Detection of different aminoglycoside-, β-lactam-, and quinolone-resistance genes was done using polymerase chain reaction. The genetic diversity of MDR isolates was investigated using random amplification of polymorphic DNA.

Results: Most of the tested isolates exhibited MDR phenotypes (84.75%). The occurrence of efflux pump-mediated resistance in the different MDR species tested was 40%–66%. Acinetobacter baumannii isolates showed resistance to most of the tested antibiotics, including imipenem. The blaOXA-23-like gene was detected in 69% of the MDR A. baumannii isolates. The MDR phenotype was detected in 65% of Pseudomonas aeruginosa isolates, of which only 23% exhibited efflux pump-mediated resistance. On the contrary, efflux-mediated resistance to piperacillin and gentamicin was recorded in 47.5% of piperacillin-resistant and 25% of gentamicin-resistant MDR Enterobacteriaceae. Moreover, the plasmid-mediated quinolone-resistance genes (aac(6')-Ib-cr, qnrB, and qnrS) were detected in 57.6% and 83.33% of quinolone-resistant MDR Escherichia coli and Klebsiella pneumoniae isolates, respectively. The β-lactamase-resistance gene blaSHV-31 was detected for the first time in one MDR K. pneumoniae isolate from an endotracheal tube specimen in Egypt, accompanied by blaTEM-1, blaCTX-M-15, blaCTX-M-14, aac(6')-Ib-cr, qnrS, and multidrug efflux-mediated resistance.

Conclusion: MDR phenotypes are predominant among third-generation cephalosporin-resistant Gram-negative bacteria in Egypt and mediated by different mechanisms, with an increased role of efflux pumps in Enterobacteriaceae.

Keywords: multidrug resistance, efflux pump, Egypt, Gram-negative bacilli, RAPD typing

Introduction

Effective treatment of infections is compromised worldwide by the emergence of multidrug resistance (MDR). According to the European Centre for Disease Prevention and Control, MDR is defined as unsusceptibility to at least one agent in three or more of the specified antimicrobial categories used in treatment.1

MDR Gram-negative bacteria (MDRGNB) have become a major public health threat, as there are fewer or even sometimes no effective antimicrobial agents available
Materials and methods
Bacterial strains and antibiotic susceptibility testing
A total of 118 GN clinical isolates collected during 2009–2010, previously identified with API 20E and API 20NE systems (BioMérieux, France) with an identity of not less than 80%, were included in this study. They were selected from our culture collection based on their resistance to at least one of the third-generation cephalosporins. All isolates were from children with suspected infections in Abu El-Rish Children’s Hospital, Cairo, Egypt. The isolates had been taken from different specimens: blood (n=3), catheter tips (n=3), cerebrospinal fluid (n=8), ear discharge (n=1), endotracheal tubing (n=20), midline subumbilical gaps (n=1), peritoneal discharge (n=4), pus (n=4), sputum (n=18), stool (n=9), urine (n=43), and wounds (n=5). All experiments in this study were conducted in accordance with and approval of the ethical committee at the Faculty of Pharmacy, Cairo University.

Identification of efflux pump-mediated resistance using efflux-pump inhibitor-based microplate assays
Chlorpromazine (CPZ; Hongda Pharmaceutical, Donggang, China) acts as an efflux-pump inhibitor in GN bacteria. The minimum inhibitory concentration (MIC) of CPZ was determined by the microdilution method as per Clinical and Laboratory Standards Institute guidelines. All tested MDR clinical isolates were tested against the antimicrobial categories suggested by the boiling method. Polymerase chain reaction (PCR) identification of aminoglycoside-resistance genes (armA and aac(6’)-Ib), β-lactamase-resistance genes (blaTEM, blaSHV, blaCTX-M group 1 and group 9), metallo-β-lactamase-resistance genes (blaIMP, blaVIM, blaARM, blaOXA-23-like) from different specimens: blood (n=3), catheter tips (n=3), cerebrospinal fluid (n=8), ear discharge (n=1), endotracheal tubing (n=20), midline subumbilical gaps (n=1), peritoneal discharge (n=4), pus (n=4), sputum (n=18), stool (n=9), urine (n=43), and wounds (n=5). All experiments in this study were conducted in accordance with and approval of the ethical committee at the Faculty of Pharmacy, Cairo University.

Detection of antibiotic-resistance genes
Genomic DNA was extracted from MDR clinical isolates by the boiling method. Polymerase chain reaction (PCR) identification of aminoglycoside-resistance genes (armA and aac(6’)-Ib), β-lactamase-resistance genes (blaTEM, blaSHV, blaCTX-M group 1 and group 9), metallo-β-lactamase-resistance genes (blaIMP, blaVIM, blaARM, blaOXA-23-like)
and quinolone-resistance genes (*qep*A, *qnr*A, *qnr*B and *qnr*S) was performed as previously described. Sequences of the resistance-genes primers used in the study and their annealing temperatures are provided in Table 1. When necessary, PCR products were purified with a GeneJet PCR purification kit (Thermo Fisher Scientific). PCR products of *aac*(6')*-Ib* positives were analyzed further by digestion with BstF5I (Thermo Fisher Scientific) to detect the cr variant. The purified PCR products were sequenced by an ABI 3730 XL DNA sequencer (Thermo Fisher Scientific). Detection of similarity for nucleotide sequences was performed using the BLAST program (http://www.ncbi.nlm.nih.gov/blast) with default settings.

**Detection of genetic diversity of MDR isolates using random amplification of polymorphic DNA**

Clonal relatedness between isolates from the same species was assessed by random amplification of polymorphic DNA (RAPD) using at least two primers for each tested species. Sequences of RAPD primers used in the study are provided in Table 1.

| Primer | Sequence (5'→3') | Target gene | T<sub>a</sub> | Product size | Reference |
|--------|------------------|-------------|--------------|-------------|-----------|
| armA-F | ATT CTG CCT ATC ATA ATT GG | 16S RNA methylase armA | 55°C | 315 bp | 16 |
| armA-R | ACC TAT ACT TTA TCG TCG TC | | | | |
| *aac*(6')*-Ib-F | TTGCGATGCTCTATGAGTGGCTA | *aac*(6')*-Ib | 54°C | 482 bp | 18 |
| *aac*(6')*-Ib-R | CTCGAATGCCTGGCGTTTT | | | | |
| MultiTSO-T-F | CATTTCGGTGTCGCCCCTAAC | TEM variants, including TEM1 and TEM2 | 60°C | 800 bp | 20 |
| MultiTSO-T-R | GTCTGATCTCATATGGTGGCGTCGAC | | | | |
| MultiTSO-S-F | AGCCGCTTGAAGCAAATTAAC | SHV variants, including SHV1 | 60°C | 713 bp | 20 |
| MultiTSO-S-R | ATCCCGCAGATAATCACCAC | | | | |
| MultiCTXMGp1-F | TTAGGAATGTGCTGCCCTGYA | Variants of CTX group 1 | 60°C | 688 bp | 20 |
| MultiCTXMGp1-R | CGATCTGTTTTGGTGGTRCCAT | | | | |
| MultiCTXMGp9-F | TCAAGCCTGCCGATCTGGT | Variants of CTX group 9 | 60°C | 561 bp | 20 |
| MultiCTXMGp9-R | TGATTCTCCGGCCTGAAG | | | | |
| MultiIMP-F | TTAGACTCTCATTATTACDG | IMP variants | 55°C | 139 bp | 20 |
| MultiIMP-R | GATYGAGAATTAAATGCGCACTY | | | | |
| MultiVIM-F | GATGTTTGGTTGGTGCATATA | VIM variants | 55°C | 390 bp | 20 |
| MultiVIM-R | CGATGGCCAGACACACAG | | | | |
| Spm-F | AAA ATC TGG GTA CGC AAA CG | SPM1 | 52°C | 271 bp | 23 |
| Spm-R | ACA TTA TCC GCT GGA ACA GG | | | | |
| NDM-F | GGT TGG GCG ATC TGG TTT TC | NDM variants | 52°C | 621 bp | 21 |
| NDM-R | CGG AAT GCC TCA CGA TC | | | | |
| OXA-23-like-F | GAT CGG ATT GGA GAA CCA GA | OXA23-like | 53°C | 501 bp | 22 |
| OXA-23-like-R | ATT TCT GAC CGC ATT TCT AT | | | | |
| qepA-F | GCA GGT CCA GCA GGG GGT AG | qepA | 60°C | 199 bp | 17 |
| qepA-R | CTT CCT GGC GTA TCG TGG | | | | |
| QnrA-F | AGAGGATTTCACGCGCCAGG | *qnr*A | 54°C | 580 bp | 19 |
| QnrA-R | TGCCAGGACAGATTTGAC | | | | |
| QnrB-F | GMTHAGAAATTGCCGTATG | *qnr*B | 54°C | 264 bp | 19 |
| QnrB-R | TTTCGGYCGCGGTGGCAAG | | | | |
| QnrS-F | GCCAGTCTACGAGGCGGT | qnrS | 54°C | 428 bp | 19 |
| QnrS-R | CTCAACGTGGTCGGTCCG | | | | |
| 208 | AGGCGGCACC | RAPD for *Pseudomonas aeruginosa* | 36°C | | 36 |
| 272 | AGGCGCGCAA | | | | |
| ERIC1 | ATGTAAGCTCTGGGGGATTAC | RAPD for *Klebsiella pneumoniae* | 35°C | | 25 |
| ERIC2 | AAGTAAAGCTCTGGGGGATTAC | | | | |
| RAPD7 | GTGGATGCAGT | | | | |
| 1247 | AAGAGGCGCAGT | RAPD for *Escherichia coli* and *Acinetobacter baumannii* | 36°C | | 27 |
| 1281 | ACGCCGCAA | | | | |
| 1283 | GCAGGCACCA | | | | |

Notes: *Y* = T or C; *R* = A or G; *D* = A or G or T; *M* = A or C; *H* = A or C or T; *Y* = C or T.

Abbreviation: RAPD, random amplification of polymorphic DNA.
in Table 1. Amplicons were separated by 1.5% agarose-gel electrophoresis using a GeneRuler 100 bp ladder (Thermo Fisher Scientific) as a molecular size standard in each gel. Gels were stained with ethidium bromide and photographed under ultraviolet transillumination. Gel images were analyzed by GelAnalyzer 2010. The absence or presence of a band of a certain size was recorded as 0 or 1. For each strain, the RAPD type was defined as the combined band patterns obtained with the tested primers. The relationship between the RAPD types of isolates of the same species were calculated by unweighted pair-group (UPG) averages and represented as a dendrogram using UPGMA algorithms. In any tested isolate, banding patterns differing by two or more bands represented different strains, while banding patterns that differed by fewer than two bands were the same strain.25

Results
Bacterial strains and antibiotic-susceptibility testing
A total of 118 GN clinical isolates characterized as being resistant to at least one of the third-generation cephalosporins were included in the study, and 100 isolates (84.75%) were classified as MDR: Acinetobacter baumannii (13 of 15, 86.6%), Escherichia coli (37 of 38, 97.37%), K. pneumoniae (21 of 22, 95.45%), Pseudomonas aeruginosa (17 of 26, 65.38%), S. maltophilia (three of four, 75%), and other Enterobacteriaceae (nine of 13, 69.23%). MDR and non-MDR distribution among third-generation cephalosporin-resistant GN clinical isolates from different infection sites is shown in Figure 1. The antibiotic-susceptibility profile of each tested isolate is shown in Table S1.

A. baumannii isolates were resistant to most of the tested antibiotics. Imipenem was the most effective antibiotic against tested Enterobacteriaceae and P. aeruginosa. All S. maltophilia isolates were susceptible to ofloxacin, ciprofloxacin, cefepime, piperacillin, piperacillin–tazobactam and sulfamethoxazole–trimethoprim. The number of resistant isolates in every tested bacterial species for each of the tested antibiotics is shown in Table 2 and Figure 2.

Identification of efflux pump-mediated resistance using efflux-pump inhibitor-based microplate assays
Efflux pump-mediated resistance was recorded in 46.1% (six of 13), 41.1% (seven of 17), 40.54% (15 of 37), 66.67% (14 of 21), 66.67% (two of three), and 66.67% (six of nine) of MDR A. baumannii, P. aeruginosa, E. coli, K. pneumoniae, S. maltophilia, and other Enterobacteraeae, respectively. Efflux pump-mediated resistance for more than one antibiotic was recorded in five of 13 and nine of 21 of MDR A. baumannii and K. pneumoniae, respectively. However, this multidrug efflux pump-mediated resistance was of lower incidence in other tested species. The number of isolates in each tested species displaying different patterns of efflux-mediated resistance is shown in Table 3. Efflux pump-mediated resistance to different antibiotics in each MDRGNB isolate is shown in Table S2.

Antibiotic-resistance genes
The sequenced products were deposited in the GenBank under accession numbers KY640457–KY640597. The incidence of each tested gene in the different species of MDRGNB clinical isolates tested is recorded in Table 4, and their distribution in the different MDRGNB isolates is shown in Table S3. All detected blaTEM were TEM1 variants, while, blaSHV were SHV1, SHV11, SHV12, and SHV31 variants. Group 1 blaCTXM ESBL-resistance genes belonged to type CTXM15, while blaCTXM group 9 belonged to type
MDR in Gram-negative clinical isolates

The metallo-β-lactamase resistance genes \( \text{bla}_{\text{IMP}} \), \( \text{bla}_{\text{SPM-1}} \), and \( \text{bla}_{\text{NDM}} \) and quinolone-resistance genes: \( \text{qepA} \) and \( \text{qnrA} \) were not detectable in our tested MDRGNB clinical isolates.

### Determination of genetic diversity of MDR isolates using RAPD

The number of clonal patterns detected in MDRGNB isolates was 34 of 37, ten of 13, 18 of 21, and 17 of 26 patterns in \( \text{E. coli} \), \( \text{A. baumannii} \), \( \text{K. pneumoniae} \), and \( \text{P. aeruginosa} \) isolates, respectively. No predominant clonal type was detectable with \( \text{E. coli} \) or \( \text{P. aeruginosa} \) isolates. However, five of 13 of \( \text{A. baumannii} \) isolates belonged to two clonal types, and three of 21 of \( \text{K. pneumoniae} \) isolates belonged to one clonal type. Clonally identical isolates shared the same antibiotic-resistance pattern (8, 27, and 146; 150, and 179 in \( \text{A. baumanii} \) and 161, 163, and 223 in \( \text{K. pneumoniae} \)), although they had different infection sites. Phenograms constructed using UPGMA algorithms for MDR isolates are shown in Figure S1.

### Discussion

Few reports are available on the prevalence and mechanisms of MDR in GNB in developing countries including Egypt.\(^6\)\(^7\) Therefore, our study was carried out to determine the prevalence, molecular resistance mechanisms, and clonal relatedness of MDRGNB among third-generation cephalosporin-resistant isolates from Egypt. Our findings showed that 84.75% of the third-generation cephalosporin-resistant isolates were classified as MDR, with the highest percentage of MDR recorded in \( \text{E. coli} \), followed by \( \text{K. pneumoniae} \) and \( \text{A. baumannii} \). Various international surveys have reported an increase in the number of MDRGNB in the last few years.\(^2\)\(^8\)

One of the alarming results was the resistance of \( \text{A. baumannii} \) isolates to most of the antibiotics tested, including imipenem. Carbapenems are considered one of the last-resort antimicrobials for GNB,\(^29\) and resistance to carbapenems leaves few effective therapeutic options, such as polymyxins or tigecycline.\(^3\) This high level of imipenem resistance (ten of 13) may result from the high number of \( \text{bla}_{\text{OXA-23}} \)-like genes detected among MDR \( \text{A. baumannii} \) (nine of 13), as previously reported.\(^3\) This is in accordance with the results of Al-Agamy et al from Egypt, where \( \text{bla}_{\text{OXA-23}} \) and \( \text{bla}_{\text{OXA-24}} \)-like genes were found to be the most prevalent type of β-lactamase-encoding genes in \( \text{A. baumannii} \).\(^3\) Efflux-mediated resistance accounted for this MDR phenotype in \( \text{A. baumannii} \) (six of 13), half of which (three of six) contained multidrug-efflux pumps that mediated resistance to

| Bacterial species | Resistance to different antibiotics in the tested species of Gram-negative clinical isolates |
|------------------|--------------------------------------------------------------------------------------------------|
| \( \text{Acinetobacter baumannii} \) | 12/15 12/15 10/15 13/15 12/15 14/15 13/15 13/15 13/15 11/15 10/15 15/15 11/15 14/15 15/15 |
| \( \text{Citrobacter freundii} \) | 2/4 0/4 2/4 0/4 0/4 1/4 1/4 2/4 1/4 1/4 0/4 2/4 2/4 1/4 2/4 |
| \( \text{Enterobacter cloacae} \) | 1/3 1/3 1/3 0/3 1/3 2/3 1/3 1/3 1/3 1/3 1/3 2/3 1/3 1/3 |
| \( \text{Escherichia coli} \) | 19/38 3/38 17/38 33/38 32/38 31/38 31/38 32/38 3/38 37/38 11/38 28/38 24/38 31/38 30/38 |
| \( \text{Klebsiella pneumoniae} \) | 17/22 9/22 8/22 12/22 12/22 12/22 12/22 12/22 11/22 17/22 11/22 17/22 17/22 17/22 17/22 |
| \( \text{Morganella morganii} \) | 1/2 0/2 0/2 0/2 0/2 1/2 0/2 0/2 0/2 1/2 0/2 0/2 1/2 0/2 0/2 |
| \( \text{Pseudomonas aeruginosa} \) | 11/24 10/24 10/24 13/24 11/24 20/24 11/24 12/24 7/24 15/24 4/24 17/24 19/24 19/24 21/24 |
| \( \text{Proteus mirabilis} \) | 0/2 0/2 0/2 1/2 0/2 0/2 0/2 1/2 1/2 1/2 0/2 2/2 1/2 1/2 1/2 |
| \( \text{Stenotrophomonas maltophilia} \) | 1/4 1/4 1/4 0/4 0/4 4/4 0/4 0/4 0/4 3/4 3/4 1/4 3/4 3/4 |
| \( \text{Serratia marcescens} \) | 1/2 1/2 1/2 1/2 1/2 1/2 1/2 1/2 1/2 1/2 1/2 1/2 1/2 1/2 1/2 |

**Abbreviations:** CN, gentamicin; AK, amikacin; TOB, tobramycin; OFX, ofloxacin; CIP, ciprofloxacin; FOX, cefoxitin; FEP, cefepime; PRL, piperacillin; PT, piperacillin–tazobactam; SXT, sulfamethoxazole–trimethoprim; IMP, imipenem; AO, aztreonam; AS, ampicillin–sulbactam; CTX, cefotaxime; CAZ, ceftazidime.
gentamicin, ciprofloxacin, sulfamethoxazole–trimethoprim, and piperacillin. A previous study in Egypt reported a higher percentage of efflux pumps (77.8%) in *A. baumannii* isolates. In accordance with previous studies,  aminoglycoside resistance was common among our isolates. This may have been due to the presence of *aac-(6’)-Ib* gene-and efflux pump-mediated gentamicin resistance in nine of 12 and five of 12 of aminoglycoside-resistant MDR *A. baumannii* isolates, respectively.

In agreement with the reported susceptibility pattern of *P. aeruginosa*, most of our isolates were sensitive to imipenem (84%) and piperacillin–tazobactam (73%). On the contrary, 65% of *P. aeruginosa* isolates were MDR, of which only 23.5% showed multidrug efflux-mediated resistance. This is in contrast to the known major contribution of efflux pumps in MDR *P. aeruginosa*. In addition, efflux-mediated resistance to piperacillin (β-lactam) was recorded in 47.5% of piperacillin-resistant MDR Enterobacteriaceae. This highlights the major role played by efflux pumps in resistance to β-lactams in MDR Enterobacteriaceae. A lower predominance of efflux pump-mediated resistance (39%) was reported among MDR *K. pneumoniae* isolates in Turkey. The *bla*<sub>TEM-1</sub> gene was common in our MDR Enterobacteriaceae isolates and was the only detected β-lactamase-resistance gene in 6% of them. This is in agreement with previous studies showing the high persistence of the *bla*<sub>TEM-1</sub> gene among Enterobacteriaceae worldwide. The β-lactamase-resistance gene *bla*<sub>SHV</sub> was detected in 28.3% of MDR Enterobacteriaceae and identified by sequencing as variants SHV1, SHV11, SHV12 and SHV31 in 79%, 10.5%, 5%, and 5% of *bla*<sub>SHV</sub>-positive isolates, respectively. This was in contrast to another study from Egypt that detected only SHV1 and SHV11 in 57% and 29% of *bla*<sub>SHV</sub>-containing isolates, respectively. To the best of our knowledge, this is the first report on the occurrence of SHV31 in MDR *K. pneumoniae* isolates from Egypt, Africa, and the Middle East. Isolates were recovered from an endotracheal tube specimen, and were also positive for...
### Table 3

Number of isolates in each tested species displaying different patterns of efflux-mediated resistance

| Pattern                        | Acinetobacter baumannii | Citrobacter freundii | Escherichia coli | Enterobacter cloacae | Klebsiella pneumoniae | Morganella morganii | Pseudomonas aeruginosa | Proteus mirabilis | Stenotrophomonas maltophilia | Serratia marcescens |
|--------------------------------|-------------------------|----------------------|-----------------|----------------------|----------------------|---------------------|------------------------|-------------------|---------------------------|---------------------|
| No efflux-mediated resistance  | 7/13                    | 2/3                  | 22/37           | 0/1                  | 7/21                 | 0/1                 | 10/17                  | 1/2               | 1/3                       | 0/2                 |
| CN, CIP, SXT, PRL              | 3/13                    | 0/3                  | 1/37            | 0/1                  | 3/21                 | 0/1                 | 0/17                   | 0/2              | 0/3                       | 0/2                 |
| CN, CIP, SXT                   | 1/13                    | 0/3                  | 0/37            | 0/1                  | 1/21                 | 0/1                 | 0/17                   | 0/2              | 0/3                       | 0/2                 |
| CN, CIP, PRL                   | 1/13                    | 0/3                  | 0/37            | 0/1                  | 1/21                 | 0/1                 | 0/17                   | 0/2              | 0/3                       | 0/2                 |
| CN, SXT, PRL                   | 0/13                    | 0/3                  | 0/37            | 0/1                  | 1/21                 | 0/1                 | 0/17                   | 0/2              | 0/3                       | 0/2                 |
| CIP, SXT, PRL                  | 0/13                    | 0/3                  | 1/37            | 0/1                  | 0/21                 | 0/1                 | 0/17                   | 0/2              | 0/3                       | 0/2                 |
| CN, CIP                        | 0/13                    | 0/3                  | 1/37            | 0/1                  | 1/21                 | 0/1                 | 0/17                   | 0/2              | 0/3                       | 0/2                 |
| CN, PRL                        | 0/13                    | 0/3                  | 0/37            | 0/1                  | 0/21                 | 0/1                 | 0/17                   | 0/2              | 0/3                       | 0/2                 |
| SXT, PRL                       | 0/13                    | 0/3                  | 1/37            | 0/1                  | 1/21                 | 0/1                 | 0/17                   | 0/2              | 0/3                       | 0/2                 |
| CN                             | 0/13                    | 0/3                  | 0/37            | 1/1                  | 1/21                 | 0/1                 | 0/17                   | 0/2              | 1/3                       | 0/2                 |
| CIP                            | 1/13                    | 0/3                  | 0/37            | 0/1                  | 0/21                 | 0/1                 | 0/17                   | 0/2              | 0/3                       | 0/2                 |
| SXT                            | 0/13                    | 1/3                  | 5/37            | 0/1                  | 1/21                 | 1/1                 | 1/17                   | 1/2              | 0/3                       | 0/2                 |
| PRL                            | 0/13                    | 0/3                  | 3/37            | 0/1                  | 2/21                 | 0/1                 | 2/17                   | 0/2              | 1/3                       | 1/2                 |

**Abbreviations:** CN, gentamicin; CIP, ciprofloxacin; PRL, piperacillin; SXT, sulfamethoxazole-trimethoprim.

### Table 4

Resistance genes in the different species of multidrug-resistant Gram-negative clinical isolates

| Bacterial species          | amA | acrA-Lb | acrA-Lb-cr | blaTEM-1 | blaSHV | blaCTX-M-15 | blaCTX-M-14 | blaIMP | blaVIM | blaOXA-23 | qepA | qnrA | qnrB | qnrS |
|----------------------------|-----|---------|------------|----------|--------|-------------|-------------|--------|--------|-----------|------|------|------|------|
| Acinetobacter baumannii    | 0/13| 9/13    | 0/13        | 7/13     | 3/13   | 4/13        | 1/13        | 0/13   | 0/13   | 9/13      | 0/13 | 0/13 | 0/13 | 1/13 |
| Citrobacter freundii       | 0/3 | 0/3     | 0/3         | 1/3      | 1/3    | 1/3         | 1/3         | 1/3    | 0/3    | 0/3       | 0/3  | 0/3  | 0/3  | 0/3  |
| Enterobacter cloacae       | 0/1 | 1/1     | 0/1         | 0/1      | 0/1    | 0/1         | 0/1         | 0/1    | 0/1    | 0/1       | 0/1  | 0/1  | 0/1  | 0/1  |
| Escherichia coli           | 1/37| 2/37    | 18/37       | 22/37    | 0/37   | 23/37       | 8/37        | 0/37   | 0/37   | 0/37      | 0/37 | 0/37 | 0/37 | 0/37 |
| Klebsiella pneumoniae      | 0/21| 8/21    | 8/21        | 12/21    | 17/21  | 15/21       | 7/21        | 0/21   | 0/21   | 0/21      | 2/21 | 0/21 | 0/21 | 1/21 |
| Morganella morganii        | 0/1 | 0/1     | 0/1         | 0/1      | 0/1    | 0/1         | 0/1         | 0/1    | 0/1    | 0/1       | 0/1  | 0/1  | 0/1  | 0/1  |
| Pseudomonas aeruginosa     | 0/17| 4/17    | 1/17        | 3/17     | 2/17   | 5/17        | 1/17        | 0/17   | 0/17   | 0/17      | 0/17 | 0/17 | 0/17 | 0/17 |
| Proteus mirabilis          | 0/2 | 0/2     | 1/2         | 1/2      | 1/2    | 0/2         | 0/2         | 0/2    | 0/2    | 0/2       | 0/2  | 0/2  | 0/2  | 0/2  |
| Stenotrophomonas maltophilia| 0/3 | 0/3     | 0/3         | 0/3      | 0/3    | 0/3         | 0/3         | 0/3    | 0/3    | 0/3       | 0/3  | 0/3  | 0/3  | 0/3  |
| Serratia marcescens        | 0/2 | 0/2     | 0/2         | 0/2      | 0/2    | 0/2         | 0/2         | 0/2    | 0/2    | 0/2       | 0/2  | 0/2  | 0/2  | 0/2  |

**Notes:** 1. Two of three of the detected SHVs were the variant SHV1 and one of three the variant SHV11; 2. one detected SHV = SHV1; 3. 13 of 17 of the detected SHVs were SHV1, two of 17 SHV11, one of 17 SHV12, and one of 17 SHV31; 4. one of two of the detected SHVs was SHV1 and one of two SHV11; 5. one detected SHV = SHV1.
blaTEM-1, blaCTX-M-15, blaCTX-M-14, aac(6’)-Ib-cr, qnrS, and multidrug efflux-mediated resistance. The SHV31 variant has limited dissemination worldwide. It has been detected only in K. pneumoniae in the Netherlands (2001), Brazil (2005–2007), Iran (2006–2007), and Taiwan.37

ESBL-resistance genes blaCTX-M-15 and blaCTX-M-14 were detected in 60%, and 24% of our MDR Enterobacteriaceae. This is in agreement with the worldwide prevalence of CTXM15 and CTXM14.38 Our findings are comparable with another study conducted in Egypt on β-lactamase prevalence in Enterobacteriaceae.39 In a similar study conducted in India, 66% of third-generation cephalosporin-resistant E. coli and K. pneumoniae isolates had blaCTX-M-15.40 Moreover, blaOXA-23-like, mainly detectable in A. baumannii,30 was detected in two of 21 K. pneumoniae isolates. The detection of blaOXA-23-like in K. pneumoniae has previously been reported in the literature.41

Fluoroquinolone resistance in Enterobacteriaceae results mainly from mutations in DNA gyrase and topoisomerase genes.5 It was surprising to detect the plasmid-mediated quinolone-resistance genes (aac(6’)-Ib-cr, qnrB, and qnrS) in 57.6% (19 of 33) and 83.33% (ten of 12) of quinolone-resistant MDR E. coli and K. pneumoniae, respectively. These determinants have been detected worldwide with high prevalence among K. pneumoniae.42 The aac(6’)-Ib-cr gene, which confers resistance to ciprofloxacin and norfloxacin besides aminoglycosides, was prevalent in MDR E. coli isolates (48.6%), although lower incidence has previously been detected in Egypt (23.3%).43

The aminoglycoside-modifying enzyme (aac (6’)-Ib) was detected in 84.4% of aminoglycoside-resistant Enterobacteriaceae. The role of modifying enzymes in aminoglycoside resistance has been documented.4 However, efflux-mediated gentamicin resistance was detected in 26.6% of aminoglycoside-resistant MDR Enterobacteriaceae. This again reflects the growing role of efflux pumps in mediating MDR among members of Enterobacteriaceae in Egypt.

The copresence of different classes of resistance genes was common among our isolates (Table S3). This is alarming, as it presents an antibiotic selection advantage for these isolates to predominate as MDR. It is also worth noting that 17 of the MDRGNB isolates carried none of the tested β-lactamase genes nor exhibited efflux pump-mediated resistance. It is likely that these isolates carry one or more β-lactamase genes not tested in this study or contain efflux pumps that could not be detected by the efflux-pump inhibitor used.

The MDR species tested were genotypically variable. This suggested that multiple subtypes of the species were involved in MDR and opposed the probability that MDR may have resulted from clonal spread. The only limitation of this study was the small number of isolates tested in some species, which made it difficult to draw solid conclusions about these organisms.

**Conclusion**

MDR is predominant among third-generation cephalosporin-resistant GNB in Egypt. In most cases, resistance is caused by different mechanisms. This study highlighted the increasing role of efflux pumps and the increase in plasmid-mediated quinolone resistance among MDR Enterobacteriaceae. Therefore, new treatment strategies need to be implemented. The use of an efflux-pump inhibitor combined with old antibiotics can provide a possible treatment for infections caused by efflux-mediated resistant bacteria, maintaining the effectiveness of old antibiotics. Moreover, antibiotic misuse needs to be stopped to avoid the selection of MDR species.

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**Disclosure**

The authors report no conflicts of interest in this work.

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### Supplementary material

**Table S1** Identification, source, susceptibility pattern, and multidrug-resistant phenotype of each tested isolate

| Isolates (n) | Source  | CN | AK | TOB | OFX | CIP | FOX | FEP | PRL | PT | SXT | IMP | AO | AS | CTX | CAZ | MDR |
|--------------|---------|----|----|-----|-----|-----|-----|-----|-----|----|-----|-----|----|----|-----|-----|-----|
| Acinetobacter baumanii | 8 | Sputum | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Acinetobacter baumanii | 27 | ETT | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Acinetobacter baumanii | 82 | ETT | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Acinetobacter baumanii | 136 | Sputum | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Acinetobacter baumanii | 141 | Peritoneal | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Acinetobacter baumanii | 145 | Sputum | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Acinetobacter baumanii | 146 | ETT | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Acinetobacter baumanii | 149 | Peritoneal | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Acinetobacter baumanii | 150 | Sputum | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Acinetobacter baumanii | 152 | Peritoneal | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Acinetobacter baumanii | 162 | ETT | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Acinetobacter baumanii | 179 | Urine | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Acinetobacter baumanii | 203 | ETT | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Acinetobacter baumanii | 217 | Wound | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Acinetobacter baumanii | 226 | CSF | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Acinetobacter baumanii | 236 | Wound | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Citrobacter freundii | 72 | Stool | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Citrobacter freundii | 202 | Wound | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Citrobacter freundii | 217 | Stool | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Citrobacter freundii | 252 | Sputum | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Enterobacter cloacae | 87 | Stool | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Enterobacter cloacae | 117 | Sputum | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Enterobacter cloacae | 147 | ETT | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Escherichia coli | 9 | Sputum | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Escherichia coli | 25 | Urine | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Escherichia coli | 70 | Urine | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Escherichia coli | 71 | Urine | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Escherichia coli | 74 | Urine | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Escherichia coli | 78 | Urine | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Escherichia coli | 81 | Urine | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Escherichia coli | 94 | Urine | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Escherichia coli | 113 | Urine | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Escherichia coli | 121 | Urine | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Escherichia coli | 122 | Urine | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Escherichia coli | 123 | Urine | + | + | + | + | + | + | + | + | + | + | + | + | + | + |

(Continued)
| Isolates (n) | Source       | CN | AK | TOB | OFX | CIP | FOX | FEP | PRL | PT | SXT | IMP | AO | AS | CTX | CAZ | MDR |
|-------------|--------------|----|----|-----|-----|-----|-----|-----|-----|----|-----|-----|----|----|-----|-----|-----|-----|
| 124         | Urine        | +  |    |     |     |     |     |     |     |    |     |     |    |    |     |     |     |     |
| 135         | Urine        | +  |    |     |     |     |     |     |     |    |     |     |    |    |     |     |     |     |
| 137         | Urine        | +  |    |     |     |     |     |     |     |    |     |     |    |    |     |     |     |     |
| 140         | Urine        | +  |    |     |     |     |     |     |     |    |     |     |    |    |     |     |     |     |
| 174         | ETT          | +  |    |     |     |     |     |     |     |    |     |     |    |    |     |     |     |     |
| 177         | Urine        | +  |    |     |     |     |     |     |     |    |     |     |    |    |     |     |     |     |
| 181         | Urine        | +  |    |     |     |     |     |     |     |    |     |     |    |    |     |     |     |     |
| 183         | Urine        | +  |    |     |     |     |     |     |     |    |     |     |    |    |     |     |     |     |
| 184         | Urine        | +  |    |     |     |     |     |     |     |    |     |     |    |    |     |     |     |     |
| 186         | Pus swab     | -  |    |     |     |     |     |     |     |    |     |     |    |    |     |     |     |     |
| 188         | Sputum       | +  |    |     |     |     |     |     |     |    |     |     |    |    |     |     |     |     |
| 192         | Urine        | +  |    |     |     |     |     |     |     |    |     |     |    |    |     |     |     |     |
| 195         | Stool        | +  |    |     |     |     |     |     |     |    |     |     |    |    |     |     |     |     |
| 199         | Wound        | +  |    |     |     |     |     |     |     |    |     |     |    |    |     |     |     |     |
| 204         | Urine        | +  |    |     |     |     |     |     |     |    |     |     |    |    |     |     |     |     |
| 206         | Urine        | +  |    |     |     |     |     |     |     |    |     |     |    |    |     |     |     |     |
| 214         | Urine        | +  |    |     |     |     |     |     |     |    |     |     |    |    |     |     |     |     |
| 219         | Urine        | +  |    |     |     |     |     |     |     |    |     |     |    |    |     |     |     |     |
| 227         | Urine        | +  |    |     |     |     |     |     |     |    |     |     |    |    |     |     |     |     |
| 229         | Urine        | +  |    |     |     |     |     |     |     |    |     |     |    |    |     |     |     |     |
| 231         | Urine        | +  |    |     |     |     |     |     |     |    |     |     |    |    |     |     |     |     |
| 232         | Urine        | +  |    |     |     |     |     |     |     |    |     |     |    |    |     |     |     |     |
| 246         | Urine        | +  |    |     |     |     |     |     |     |    |     |     |    |    |     |     |     |     |
| 247         | Sputum       | +  |    |     |     |     |     |     |     |    |     |     |    |    |     |     |     |     |
| 249         | Urine        | +  |    |     |     |     |     |     |     |    |     |     |    |    |     |     |     |     |
| 255         | Stool        | +  |    |     |     |     |     |     |     |    |     |     |    |    |     |     |     |     |

**Klebsiella pneumoniae**

| Isolates (n) | Source       | CN | AK | TOB | OFX | CIP | FOX | FEP | PRL | PT | SXT | IMP | AO | AS | CTX | CAZ | MDR |
|-------------|--------------|----|----|-----|-----|-----|-----|-----|-----|----|-----|-----|----|----|-----|-----|-----|-----|
| 3           | Sputum       | +  |    |     |     |     |     |     |     |    |     |     |    |    |     |     |     |     |
| 7           | Urine        | +  |    |     |     |     |     |     |     |    |     |     |    |    |     |     |     |     |
| 12          | CSF          | +  |    |     |     |     |     |     |     |    |     |     |    |    |     |     |     |     |
| 39          | Catheter tip | +  |    |     |     |     |     |     |     |    |     |     |    |    |     |     |     |     |
| 68          | Wound        | +  |    |     |     |     |     |     |     |    |     |     |    |    |     |     |     |     |
| 75          | Midline subumbilical gapped | + | | | | | | | | | | | | | | | |
| 83          | Urine        | +  |    |     |     |     |     |     |     |    |     |     |    |    |     |     |     |     |
| 100         | Urine        | +  |    |     |     |     |     |     |     |    |     |     |    |    |     |     |     |     |
| 114         | Sputum       | +  |    |     |     |     |     |     |     |    |     |     |    |    |     |     |     |     |
| 134         | Sputum       | +  |    |     |     |     |     |     |     |    |     |     |    |    |     |     |     |     |

(Continued)
Table S1 (Continued)

| Isolates (n) | Source     | CN | AK | TOB | OFX | CIP | FOX | FEP | PRL | PT | SXT | IMP | AO | AS | CTX | CAZ | MDR |
|-------------|------------|----|----|-----|-----|-----|-----|-----|-----|----|-----|-----|----|----|-----|-----|-----|
| 153         | ETT        |    |    |     |     |     |     |     |     |    |     |     |    |    |     |     | +   |
| 157         | ETT        |    |    |     |     |     |     |     |     |    |     |     |    |    |     |     | +   |
| 161         | Catheter tip |    |    |     |     |     |     |     |     |    |     |     |    |    |     |     | +   |
| 163         | ETT        |    |    |     |     |     |     |     |     |    |     |     |    |    |     |     | +   |
| 165         | ETT        |    |    |     |     |     |     |     |     |    |     |     |    |    |     |     | +   |
| 210         | Sputum     |    |    |     |     |     |     |     |     |    |     |     |    |    |     |     | +   |
| 216         | Sputum     |    |    |     |     |     |     |     |     |    |     |     |    |    |     |     | +   |
| 220         | CSF        |    |    |     |     |     |     |     |     |    |     |     |    |    |     |     | +   |
| 223         | CSF        |    |    |     |     |     |     |     |     |    |     |     |    |    |     |     | +   |
| 243         | Urine      |    |    |     |     |     |     |     |     |    |     |     |    |    |     |     | +   |
| 251         | ETT        |    |    |     |     |     |     |     |     |    |     |     |    |    |     |     | +   |
| 254         | ETT        |    |    |     |     |     |     |     |     |    |     |     |    |    |     |     | +   |
| *Morganella morganii* | 176 | Stool |    |    |     |     |     |     |     |    |     |     |    |    |     |     | -   |
|             | 224 | Blood |    |    |     |     |     |     |     |    |     |     |    |    |     |     | +   |
| *Proteus mirabilis* | 96 | Urine |    |    |     |     |     |     |     |    |     |     |    |    |     |     | +   |
|             | 182 | Stool |    |    |     |     |     |     |     |    |     |     |    |    |     |     | +   |
| *Pseudomonas aeruginosa* | 11 | CSF |    |    |     |     |     |     |     |    |     |     |    |    |     |     | +   |
|             | 14 | ETT |    |    |     |     |     |     |     |    |     |     |    |    |     |     | +   |
|             | 15 | Urine |    |    |     |     |     |     |     |    |     |     |    |    |     |     | +   |
|             | 28 | ETT |    |    |     |     |     |     |     |    |     |     |    |    |     |     | +   |
|             | 29 | ETT |    |    |     |     |     |     |     |    |     |     |    |    |     |     | +   |
|             | 38 | Pus swab |    |    |     |     |     |     |     |    |     |     |    |    |     |     | +   |
|             | 56 | Urine |    |    |     |     |     |     |     |    |     |     |    |    |     |     | +   |
|             | 58 | Urine |    |    |     |     |     |     |     |    |     |     |    |    |     |     | +   |
|             | 59 | ETT |    |    |     |     |     |     |     |    |     |     |    |    |     |     | +   |
|             | 88 | Sputum |    |    |     |     |     |     |     |    |     |     |    |    |     |     | +   |
|             | 102 | Sputum |    |    |     |     |     |     |     |    |     |     |    |    |     |     | +   |
|             | 104 | Pus swab |    |    |     |     |     |     |     |    |     |     |    |    |     |     | +   |
|             | 106 | Pus swab |    |    |     |     |     |     |     |    |     |     |    |    |     |     | +   |
|             | 107 | Peritoneal |    |    |     |     |     |     |     |    |     |     |    |    |     |     | +   |
|             | 127 | Urine |    |    |     |     |     |     |     |    |     |     |    |    |     |     | +   |
|             | 138 | Wound |    |    |     |     |     |     |     |    |     |     |    |    |     |     | +   |
|             | 155 | CSF |    |    |     |     |     |     |     |    |     |     |    |    |     |     | +   |
|             | 158 | Urine |    |    |     |     |     |     |     |    |     |     |    |    |     |     | +   |
|             | 167 | ETT |    |    |     |     |     |     |     |    |     |     |    |    |     |     | +   |
|             | 170 | ETT |    |    |     |     |     |     |     |    |     |     |    |    |     |     | +   |
|             | 180 | Urine |    |    |     |     |     |     |     |    |     |     |    |    |     |     | +   |
|             | 198 | Urine |    |    |     |     |     |     |     |    |     |     |    |    |     |     | +   |
| Isolates (n) | Source          | CN | AK | TOB | OFX | CIP | FOX | FEP | PRL | PT | SXT | IMP | AO | AS | CTX | CAZ | MDR |
|-------------|-----------------|----|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|----|----|-----|-----|-----|-----|
| 209         | Stool           |    |    |     |     |     |     |     |     |     |     |     |    |    |     |     |     |     |
| 234         | Ear discharge   |    |    |     |     |     |     |     |     |     |     |     |    |    |     |     |     |     |
| 244         | Blood           |    |    |     |     |     |     |     |     |     |     |     |    |    |     |     |     |     |
| 256         | Blood           |    |    |     |     |     |     |     |     |     |     |     |    |    |     |     |     |     |
| *Serratia marcescens* | 79 | ETT |     |     |     |     |     |     |     |     |     |     |    |    |     |     |     |     |
|             | Sputum          |    |    |     |     |     |     |     |     |     |     |     |    |    |     |     |     |     |
|             | *Stenotrophomonas maltophilia* | 211 | CSF |     |     |     |     |     |     |     |     |     |    |    |     |     |     |     |
|             | CSF             |    |    |     |     |     |     |     |     |     |     |     |    |    |     |     |     |     |
|             | Stool           |    |    |     |     |     |     |     |     |     |     |     |    |    |     |     |     |     |
|             | Sputum          |    |    |     |     |     |     |     |     |     |     |     |    |    |     |     |     |     |

Notes: Black cells, resistance to tested antibiotic; white cells, sensitive to tested antibiotic; gray cells, intermediate result to tested antibiotic.

Abbreviations: MDR, multidrug resistance; CN, gentamicin; AK, amikacin; TOB, tobramycin; OFX, ofloxacin; CIP, ciprofloxacin; FOX, cefoxitin; FEP, cefepime; PRL, piperacillin; PT, piperacillin–tazobactam; SXT, sulfamethoxazole–trimethoprim; IMP, imipenem; AO, aztreonam; AS, ampicillin–sulbactam; CTX, cefotaxime; CAZ, ceftazidime; ETT, endotracheal tube; CSF, cerebrospinal fluid.
### Table S2 Efflux-mediated resistance profile in each tested multidrug-resistant Gram-negative isolate

| Isolate number | Gentamicin | Ciprofloxacin | Trimethoprim–sulfamethoxazole | Piperacillin |
|----------------|------------|---------------|------------------------------|-------------|
| **Acinetobacter baumannii** | 8           |               |                              |             |
|                | 27         |               |                              |             |
|                | 82         |               |                              |             |
|                | 136        |               |                              |             |
|                | 141        |               |                              |             |
|                | 145        |               |                              |             |
|                | 146        |               |                              |             |
|                | 149        |               |                              |             |
|                | 150        |               |                              |             |
|                | 162        |               |                              |             |
|                | 179        |               |                              |             |
|                | 203        |               |                              |             |
|                | 236        |               |                              |             |
| **Citrobacter freundii** | 72          |               |                              |             |
|                | 202        |               |                              |             |
|                | 252        |               |                              |             |
| **Enterobacter cloacae** | 87          |               |                              |             |
| **Escherichia coli** | 9           |               |                              |             |
|                | 25         |               |                              |             |
|                | 70         |               |                              |             |
|                | 71         |               |                              |             |
|                | 74         |               |                              |             |
|                | 78         |               |                              |             |
|                | 81         |               |                              |             |
|                | 94         |               |                              |             |
|                | 113        |               |                              |             |
|                | 121        |               |                              |             |
|                | 122        |               |                              |             |
|                | 123        |               |                              |             |
|                | 124        |               |                              |             |
|                | 135        |               |                              |             |
|                | 137        |               |                              |             |
|                | 140        |               |                              |             |
|                | 174        |               |                              |             |
|                | 177        |               |                              |             |
|                | 181        |               |                              |             |
|                | 183        |               |                              |             |
|                | 184        |               |                              |             |
|                | 188        |               |                              |             |
|                | 192        |               |                              |             |
|                | 195        |               |                              |             |
|                | 199        |               |                              |             |
|                | 204        |               |                              |             |
|                | 206        |               |                              |             |
|                | 214        |               |                              |             |
|                | 219        |               |                              |             |
|                | 227        |               |                              |             |
|                | 229        |               |                              |             |
|                | 231        |               |                              |             |
|                | 232        |               |                              |             |

(Continued)
| Isolate number | Gentamicin | Ciprofloxacin | Trimethoprim–sulfamethoxazole | Piperacillin |
|----------------|------------|---------------|-------------------------------|-------------|
| 246            |            |               |                               |             |
| 247            |            |               |                               |             |
| 249            |            |               |                               |             |
| 255            |            |               |                               |             |
| 3              |            |               |                               |             |
| 7              |            |               |                               |             |
| 12             |            |               |                               |             |
| 39             |            |               |                               |             |
| 68             |            |               |                               |             |
| 75             |            |               |                               |             |
| 83             |            |               |                               |             |
| 100            |            |               |                               |             |
| 114            |            |               |                               |             |
| 134            |            |               |                               |             |
| 153            |            |               |                               |             |
| 157            |            |               |                               |             |
| 161            |            |               |                               |             |
| 163            |            |               |                               |             |
| 165            |            |               |                               |             |
| 210            |            |               |                               |             |
| 220            |            |               |                               |             |
| 223            |            |               |                               |             |
| 243            |            |               |                               |             |
| 251            |            |               |                               |             |
| 254            |            |               |                               |             |
| 224            |            |               |                               |             |
| 96             |            |               |                               |             |
| 182            |            |               |                               |             |
| 11             |            |               |                               |             |
| 14             |            |               |                               |             |
| 15             |            |               |                               |             |
| 28             |            |               |                               |             |
| 29             |            |               |                               |             |
| 38             |            |               |                               |             |
| 58             |            |               |                               |             |
| 88             |            |               |                               |             |
| 102            |            |               |                               |             |
| 107            |            |               |                               |             |
| 127            |            |               |                               |             |
| 138            |            |               |                               |             |
| 158            |            |               |                               |             |
| 167            |            |               |                               |             |
| 170            |            |               |                               |             |
| 198            |            |               |                               |             |
| 256            |            |               |                               |             |
| 79             |            |               |                               |             |
| 143            |            |               |                               |             |
| 211            |            |               |                               |             |
| 240            |            |               |                               |             |
| 245            |            |               |                               |             |

**Notes:** Black cells, presence of efflux-mediated resistance; white cells, absence of efflux-mediated resistance.
Table S3 Distribution of different resistance genes in each tested multidrug-resistant Gram-negative clinical isolate

| Isolate number | armA | aac(6’)-Ib | aac(6’)-Ib-cr | bla*TEM-1 | bla*SHV | bla*CTX-15 | bla*CTX-14 | bla*IMP | bla*VIM | bla*SPM-1 | bla*OXA-23 | qepA | qnrA | qnrB | qnrS |
|----------------|------|-------------|--------------|-----------|---------|-----------|-----------|---------|---------|-----------|-----------|-------|------|-------|------|
| Acinetobacter baumannii |     |             |              |           |         |            |            |         |         |           |           |       |      |       |      |
| 8              | 27   |             |              |           |         |            |            |         |         |           |           |       |      |       |      |
| 82             |      |             |              |           |         |            |            |         |         |           |           |       |      |       |      |
| 136            |      |             |              |           |         |            |            |         |         |           |           |       |      |       |      |
| 141            |      |             |              |           |         |            |            |         |         |           |           |       |      |       |      |
| 145            |      |             |              |           |         |            |            |         |         |           |           |       |      |       |      |
| 146            |      |             |              |           |         |            |            |         |         |           |           |       |      |       |      |
| 149            |      |             |              |           |         |            |            |         |         |           |           |       |      |       |      |
| 150            |      |             |              |           |         |            |            |         |         |           |           |       |      |       |      |
| 162            |      |             |              |           |         |            |            |         |         |           |           |       |      |       |      |
| 179            |      |             |              |           |         |            |            |         |         |           |           |       |      |       |      |
| 203            |      |             |              |           |         |            |            |         |         |           |           |       |      |       |      |
| 236            |      |             |              |           |         |            |            |         |         |           |           |       |      |       |      |
| Citrobacter freundii |     |             |              |           |         |            |            |         |         |           |           |       |      |       |      |
| 72             |      |             |              |           |         |            |            |         |         |           |           |       |      |       |      |
| 202            |      |             |              |           |         |            |            |         |         |           |           |       |      |       |      |
| 252            |      |             |              |           |         |            |            |         |         |           |           |       |      |       |      |
| Enterobacter cloacae |    |             |              |           |         |            |            |         |         |           |           |       |      |       |      |
| 87             |      |             |              |           |         |            |            |         |         |           |           |       |      |       |      |
| Escherichia coli |     |             |              |           |         |            |            |         |         |           |           |       |      |       |      |
| 9              |      |             |              |           |         |            |            |         |         |           |           |       |      |       |      |
| 25             |      |             |              |           |         |            |            |         |         |           |           |       |      |       |      |
| 70             |      |             |              |           |         |            |            |         |         |           |           |       |      |       |      |
| 71             |      |             |              |           |         |            |            |         |         |           |           |       |      |       |      |
| 74             |      |             |              |           |         |            |            |         |         |           |           |       |      |       |      |
| 78             |      |             |              |           |         |            |            |         |         |           |           |       |      |       |      |
| 81             |      |             |              |           |         |            |            |         |         |           |           |       |      |       |      |
| 94             |      |             |              |           |         |            |            |         |         |           |           |       |      |       |      |
| 113            |      |             |              |           |         |            |            |         |         |           |           |       |      |       |      |
| 121            |      |             |              |           |         |            |            |         |         |           |           |       |      |       |      |
| 122            |      |             |              |           |         |            |            |         |         |           |           |       |      |       |      |
| 123            |      |             |              |           |         |            |            |         |         |           |           |       |      |       |      |
| 124            |      |             |              |           |         |            |            |         |         |           |           |       |      |       |      |
| 135            |      |             |              |           |         |            |            |         |         |           |           |       |      |       |      |
| 137            |      |             |              |           |         |            |            |         |         |           |           |       |      |       |      |
| 140            |      |             |              |           |         |            |            |         |         |           |           |       |      |       |      |
| 174            |      |             |              |           |         |            |            |         |         |           |           |       |      |       |      |
| 177            |      |             |              |           |         |            |            |         |         |           |           |       |      |       |      |
| 181            |      |             |              |           |         |            |            |         |         |           |           |       |      |       |      |
| 183            |      |             |              |           |         |            |            |         |         |           |           |       |      |       |      |

(Continued)
| Isolate number | armA  | aac(6')-Ib | aac(6')-Ib-cr | blaTEM-1 | blashv | blaCTXM-15 | blaCTXM-14 | blaiso | blavim | blaSPM-1 | blaoxa-23 | qepA | qnrA | qnrB | qnrS |
|----------------|-------|------------|--------------|----------|--------|------------|------------|--------|--------|----------|------------|------|------|------|------|
| 184            |       |            |              |          |        |            |            |        |        |          |            |      |      |      |      |
| 188            |       |            |              |          |        |            |            |        |        |          |            |      |      |      |      |
| 192            |       |            |              |          |        |            |            |        |        |          |            |      |      |      |      |
| 195            |       |            |              |          |        |            |            |        |        |          |            |      |      |      |      |
| 199            |       |            |              |          |        |            |            |        |        |          |            |      |      |      |      |
| 204            |       |            |              |          |        |            |            |        |        |          |            |      |      |      |      |
| 206            |       |            |              |          |        |            |            |        |        |          |            |      |      |      |      |
| 214            |       |            |              |          |        |            |            |        |        |          |            |      |      |      |      |
| 219            |       |            |              |          |        |            |            |        |        |          |            |      |      |      |      |
| 227            |       |            |              |          |        |            |            |        |        |          |            |      |      |      |      |
| 229            |       |            |              |          |        |            |            |        |        |          |            |      |      |      |      |
| 231            |       |            |              |          |        |            |            |        |        |          |            |      |      |      |      |
| 232            |       |            |              |          |        |            |            |        |        |          |            |      |      |      |      |
| 246            |       |            |              |          |        |            |            |        |        |          |            |      |      |      |      |
| 247            |       |            |              |          |        |            |            |        |        |          |            |      |      |      |      |
| 249            |       |            |              |          |        |            |            |        |        |          |            |      |      |      |      |
| 255            |       |            |              |          |        |            |            |        |        |          |            |      |      |      |      |
| Klebsiella pneumoniae |       |            |              |          |        |            |            |        |        |          |            |      |      |      |      |
| 3              |       |            |              |          |        |            |            |        |        |          |            |      |      |      |      |
| 7              |       |            |              |          |        |            |            |        |        |          |            |      |      |      |      |
| 12             |       |            |              |          |        |            |            |        |        |          |            |      |      |      |      |
| 39             |       |            |              |          |        |            |            |        |        |          |            |      |      |      |      |
| 68             |       |            |              |          |        |            |            |        |        |          |            |      |      |      |      |
| 75             |       |            |              |          |        |            |            |        |        |          |            |      |      |      |      |
| 83             |       |            |              |          |        |            |            |        |        |          |            |      |      |      |      |
| 100            |       |            |              |          |        |            |            |        |        |          |            |      |      |      |      |
| 114            |       |            |              |          |        |            |            |        |        |          |            |      |      |      |      |
| 134            |       |            |              |          |        |            |            |        |        |          |            |      |      |      |      |
| 153            |       |            |              |          |        |            |            |        |        |          |            |      |      |      |      |
| 157            |       |            |              |          |        |            |            |        |        |          |            |      |      |      |      |
| 161            |       |            |              |          |        |            |            |        |        |          |            |      |      |      |      |
| 163            |       |            |              |          |        |            |            |        |        |          |            |      |      |      |      |
| 165            |       |            |              |          |        |            |            |        |        |          |            |      |      |      |      |
| 210            |       |            |              |          |        |            |            |        |        |          |            |      |      |      |      |
| 220            |       |            |              |          |        |            |            |        |        |          |            |      |      |      |      |
| 223            |       |            |              |          |        |            |            |        |        |          |            |      |      |      |      |
| 243            |       |            |              |          |        |            |            |        |        |          |            |      |      |      |      |

Table S3 (Continued)
| Isolate number | Isolate number | armA | aac(6')-Ib | aac(6')-Ib-cr | blaTEM-1 | blaDCR-1 | blaCTXM-15 | blaCTXM-14 | blaIMP | blaVIM | blaAPH-1 | qepA | qnrA | qnrB | qnrS |
|----------------|----------------|------|------------|----------------|-----------|-----------|------------|------------|--------|--------|----------|------|------|------|------|
| 251            | Klebsiella pneumoniae |      |            |                |           |           |             |             |        |        |          |      |      |      |      |
| 254            |                |      |            |                |           |           |             |             |        |        |          |      |      |      |      |
| 224            | Morganella morganii |      |            |                |           |           |             |             |        |        |          |      |      |      |      |
| 182            | Proteus mirabilis |      |            |                |           |           |             |             |        |        |          |      |      |      |      |
| 96             |                |      |            |                |           |           |             |             |        |        |          |      |      |      |      |
| 11             | Pseudomonas aeruginosa |      |            |                |           |           |             |             |        |        |          |      |      |      |      |
| 14             |                |      |            |                |           |           |             |             |        |        |          |      |      |      |      |
| 15             |                |      |            |                |           |           |             |             |        |        |          |      |      |      |      |
| 28             |                |      |            |                |           |           |             |             |        |        |          |      |      |      |      |
| 29             |                |      |            |                |           |           |             |             |        |        |          |      |      |      |      |
| 38             |                |      |            |                |           |           |             |             |        |        |          |      |      |      |      |
| 58             |                |      |            |                |           |           |             |             |        |        |          |      |      |      |      |
| 88             |                |      |            |                |           |           |             |             |        |        |          |      |      |      |      |
| 102            |                |      |            |                |           |           |             |             |        |        |          |      |      |      |      |
| 107            |                |      |            |                |           |           |             |             |        |        |          |      |      |      |      |
| 127            |                |      |            |                |           |           |             |             |        |        |          |      |      |      |      |
| 138            |                |      |            |                |           |           |             |             |        |        |          |      |      |      |      |
| 158            |                |      |            |                |           |           |             |             |        |        |          |      |      |      |      |
| 167            |                |      |            |                |           |           |             |             |        |        |          |      |      |      |      |
| 170            |                |      |            |                |           |           |             |             |        |        |          |      |      |      |      |
| 198            |                |      |            |                |           |           |             |             |        |        |          |      |      |      |      |
| 256            |                |      |            |                |           |           |             |             |        |        |          |      |      |      |      |
| 79             | Serratia marcescens |      |            |                |           |           |             |             |        |        |          |      |      |      |      |
| 143            |                |      |            |                |           |           |             |             |        |        |          |      |      |      |      |
| 211            | Stenotrophomonas maltophilia |      |            |                |           |           |             |             |        |        |          |      |      |      |      |
| 240            |                |      |            |                |           |           |             |             |        |        |          |      |      |      |      |
| 245            |                |      |            |                |           |           |             |             |        |        |          |      |      |      |      |

**Notes:** Gene variants detected. Black cells, presence of resistance genes; white cells, absence of resistance genes.
Figure S1  Phenogram of different multidrug resistant isolates constructed using UPGMA algorithms based on RAPD analysis.

Notes: (A) Phenogram of *Escherichia coli* using three different primers; (B) phenogram of *Klebsiella pneumoniae* using three different primers; (C) phenogram of *Acinetobacter baumannii* using three different primers; (D) phenogram of *Pseudomonas aeruginosa* using two different primers.

**Abbreviations:** RAPD, random amplification of polymorphic DNA; UPGMA, unweighted pair group method with arithmetic mean.
