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ORIGINAL RESEARCH

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

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Omneya M Helmy
Mona T Kashef

Department of Microbiology and Immunology, Faculty of Pharmacy, Cairo University, Cairo, Egypt

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 $bla_{OXA\text{-}23\text{-like}}$ 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, qnr*B*, and qnr*S) were detected in 57.6% and 83.33% of quinolone-resistant MDR *Escherichia coli* and *Klebsiella pneumoniae* isolates, respectively. The β-lactamase-resistance gene $bla_{SHV\text{-}31}$ was detected for the first time in one MDR *K. pneumoniae* isolate from an endotracheal tube specimen in Egypt, accompanied by $bla_{TEM\text{-}1}$, $bla_{CTX\text{-}M\text{-}15}$, $bla_{CTX\text{-}M\text{-}14}$, *aac*(6')-*Ib-cr, qnr*S, 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

Correspondence: Omneya M Helmy
Department of Microbiology and Immunology, Faculty of Pharmacy, Cairo University, Kasr el-Aini Street, Cairo 11562, Egypt
Tel +20 2 2535 3100
Email omnia.helmy@pharma.cu.edu.eg

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for infections caused by these bacteria.[2] MDR organisms, such as MDR carbapenemase-producing *Klebsiella pneumoniae*, and *Acinetobacter* spp., can be resistant to all currently available antimicrobial agents. Sometimes, they may remain susceptible only to older, potentially more toxic agents, such as polymyxins, leaving limited and suboptimal options for treatment.[3] The problem of increasing antimicrobial resistance is even more threatening when considering the very limited number of new antimicrobial agents in development.[4]

Several biochemical mechanisms can account for the antimicrobial resistance in GNB. These mechanisms include the enzymatic degradation of antibacterial agents, as in case of β-lactam resistance due to β-lactamases or modification of the antimicrobial agent by modifying enzymes, as in the case of aminoglycosides. It may also result from the alteration of antimicrobial targets in such organisms, as the in case of topoisomerase IV gene mutations that mediate resistance to fluoroquinolones. Moreover, changes in bacterial membrane permeability to antibiotics caused by mutations resulting in the loss of outer-membrane porin or overexpression of an efflux pump can lead to resistance to many effective antimicrobials. Efflux pumps, which expel multiple kinds of antibiotics, are now recognized as major contributors to MDR in bacteria: they can pump out most of the antibiotics in use.[5]

MDR has been reported to be highly prevalent among different clinical isolates in Egyptian patients;[6,7] however, few studies have examined the underlying resistance mechanisms.[7] Third-generation cephalosporins are among the most commonly used antibiotics in Egypt.[8] Therefore, resistance to third-generation cephalosporin will present a major problem in infection control, especially if accompanied with MDR. The aim of the present study was to detect the prevalence, molecular mechanisms of resistance, and clonal relatedness of MDRGNB among third-generation cephalosporin-resistant GN clinical isolates from Egypt.

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.[9] 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.

The antibiotic susceptibility of each isolate against its assigned categories of antimicrobials, as suggested by Magiorakos et al,[1] was determined using Kirby–Bauer disk diffusion method following Clinical and Laboratory Standards Institute guidelines.[10] *Stenotrophomonas maltophilia* was tested against the antimicrobial categories suggested by Milne and Gould.[11] The antibiotics included in the study were gentamicin 10 μg, tobramycin 10 μg, amikacin 30 μg, ciprofloxacin 5 μg, cefoxitin 30 μg, piperacillin 100 μg, piperacillin–tazobactam 100 and 10 μg, sulfamethoxazole–trimethoprim 1.25 and 23.75 μg, imipenem 10 μg, ofloxacin 5 μg, cefepime 30 μg, aztreonam 30 μg, ampicillin–sulbactam 10 μg each, cefotaxime 30 μg, and ceftazidime 30 μg (all Oxoid; Thermo Fisher Scientific, Waltham, MA, USA). Isolates were classified as MDR and non-MDR according to Magiorakos et al.[1] Intermediate susceptibility to any tested antibiotic was counted as resistant during the classification.

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.[12] The minimum inhibitory concentration (MIC) of CPZ was determined by the microdilution method as per Clinical and Laboratory Standards Institute guidelines in all tested MDR clinical isolates.[13] Efflux-pump inhibitor-based microplate assays using half the minimum inhibitory concentration of CPZ were performed in 24-well microplates (Thermo Fisher Scientific). Negative bacterial growth in a well containing an antibiotic disk besides CPZ and positive growth in a well containing the same antibiotic disk alone indicated efflux pump-mediated resistance to that antibiotic.[14]

Detection of antibiotic-resistance genes

Genomic DNA was extracted from MDR clinical isolates by the boiling method.[15] Polymerase chain reaction (PCR) identification of aminoglycoside-resistance genes (*arm*A and *aac*(6')-*Ib*), β-lactamase-resistance genes ((*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$ group 1 and group 9), metallo-β-lactamase-resistance genes (*bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{SPM-1}$, *bla*$_{NDM}$, *bla*$_{OXA-23-like}$)

and quinolone-resistance genes (*qep*A, *qnr*A, *qnr*B and *qnr*S) was performed as previously described.[16–23] 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.[18] 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.[24–27] Sequences of RAPD primers used in the study are provided

Table 1 Primers used for detection of resistance genes and RAPD typing, annealing temperatures (T_a), and expected product sizes

Primer	Sequence (5'-3')	Target gene	T_a	Product size	Reference
armA-F	ATT CTG CCT ATC CTA 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	CTCGAATGCCTGGCGTGTTT				
MultiTSO-T-F	CATTTCCGTGTCGCCCTTATTC	TEM variants, including TEM1 and TEM2	60°C	800 bp	20
MultiTSO-T-R	CGTTCATCCATAGTTGCCTGAC				
MultiTSO-S-F	AGCCGCTTGAGCAAATTAAAC	SHV variants, including SHV1	60°C	713 bp	20
MultiTSO-S-R	ATCCCGCAGATAAATCACCAC				
MultiCTXMGp1-F	TTAGGAARTGTGCCGCTGYA[a]	Variants of CTXM group 1	60°C	688 bp	20
MultiCTXMGp1-R	CGATATCGTTGGTGGTRCCAT[a]				
MultiCTXMGp9-F	TCAAGCCTGCCGATCTGGT	Variants of CTXM group 9	60°C	561 bp	20
MultiCTXMGp9-R	TGATTCTCGCCGCTGAAG				
MultiIMP-F	TTGACACTCCATTTACDG[a]	IMP variants	55°C	139 bp	20
MultiIMP-R	GATYGAGAATTAAGCCACYCT[a]				
MultiVIM-F	GATGGTGTTTGGTCGCATA	VIM variants	55°C	390 bp	20
MultiVIM-R	CGAATGCGCAGCACCAG				
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 TTG GCG ATC TGG TTT TC	NDM variants	52°C	621 bp	21
NDM-R	CGG AAT GGC TCA 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 TCC AT				
qepA-F	GCA GGT CCA GCA GCG GGT AG	*qep*A	60°C	199 bp	17
qepA-R	CTT CCT GCC CGA GTA TCG TG				
QnrA-F	AGAGGATTTCTCACGCCAGG	*qnr*A	54°C	580 bp	19
QnrA-R	TGCCAGGCACAGATCTTGAC				
QnrB-F	GGMATHGAAATTCGCCACTG[b]	*qnr*B	54°C	264 bp	19
QnrB-R	TTTGCYGYYCGCCAGTCGAA[b]				
QnrS-F	GCAAGTTCATTGAACAGGGT	*qnr*S	54°C	428 bp	19
QnrS-R	TCTAAACCGTCGAGTTCGGCG				
208	ACGGCCGACC	RAPD for *Pseudomonas aeruginosa*	36°C		
272	AGCGGGCCAA		36°C		24
ERIC1	ATGTAAGCTCCTGGGGATTCAC		35°C		
ERIC2	AAGTAAGTGACTGGGGTGAGCG	RAPD for *Klebsiella pneumoniae*	25°C		25
RAPD7	GTGGATGCGA		35°C		26
1247	AAGAGCCCGT		36°C		
1281	AACGCGCAAC	RAPD for *Escherichia coli and Acinetobacter baumannii*			27
1283	GCGATCCCCA				

Notes: [a]Y = T or C; R = A or G; D = A or G or T; [b]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. baumanii 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 Enterobacteriaceae, 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 bla_{TEM} were TEM1 variants, while, bla_{SHV} were SHV1, SHV11, SHV12, and SHV31 variants. Group 1 bla_{CTX-M} ESBL-resistance genes belonged to type CTXM15, while bla_{CTX-M} group 9 belonged to type

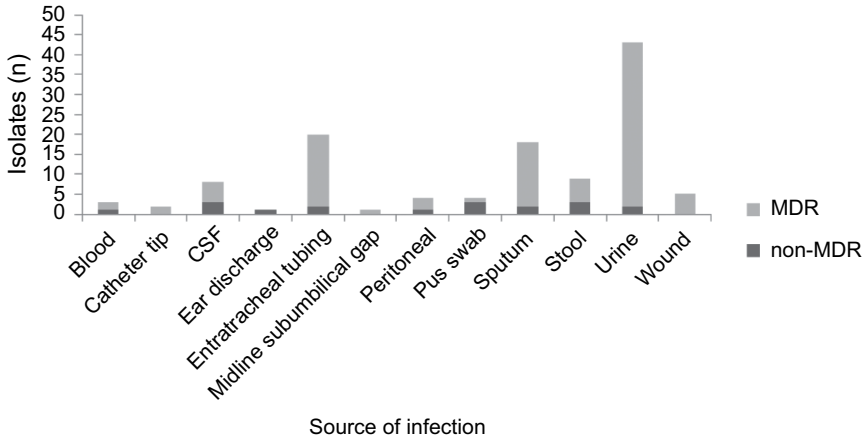

Figure 1 Distribution of MDR and non-MDR phenotypes among third-generation cephalosporin resistant Gram-negative clinical isolates from different infection sites.
Abbreviations: MDR, multidrug-resistant; CSF, cerebrospinal fluid.

Table 2 Resistance to different antibiotics in the tested species of Gram-negative clinical isolates

Bacterial species	CN	AK	TOB	OFX	CIP	FOX	FEP	PRL	PT	SXT	IMP	AO	AS	CTX	CAZ
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
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
Enterobacter cloacae	1/3	1/3	1/3	0/3	1/3	2/3	1/3	1/3	1/3	1/3	1/3	1/3	2/3	1/3	1/3
Escherichia coli	19/38	3/38	17/38	33/38	32/38	31/38	25/38	32/38	3/38	37/38	1/38	28/38	24/38	31/38	30/38
Klebsiella pneumoniae	17/22	9/22	18/22	12/22	12/22	12/22	20/22	22/22	11/22	17/22	2/22	20/22	18/22	20/22	21/22
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
Pseudomonas aeruginosa	11/26	10/26	10/26	13/26	11/26	20/26	11/26	12/26	7/26	15/26	4/26	17/26	19/26	19/26	21/26
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
Stenotrophomonas maltophilia	1/4	1/4	1/4	0/4	0/4	4/4	0/4	0/4	0/4	0/4	3/4	3/4	1/4	3/4	3/4
Serratia marcescens	1/2	2/2	1/2	0/2	0/2	1/2	0/2	2/2	0/2	1/2	0/2	0/2	1/2	2/2	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.

CTXM14. The metallo-β-lactamase resistance genes bla_{IMP}, bla_{SPM-1}, and bla_{NDM} and quinolone-resistance genes: *qep*A and *qnr*A 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 *E. coli*, *A. baumannii, K. pneumoniae*, and *P. aeruginosa* isolates, respectively. No predominant clonal type was detectable with *E. coli* or *P. aeruginosa* isolates. However, five of 13 of *A. baumannii* isolates belonged to two clonal types, and three of 21 of *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 *A. baumanii* and 161, 163, and 223 in *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 *E. coli*, followed by *K. pneumoniae* and *A. baumannii*. Various international surveys have reported an increase in the number of MDRGNB in the last few years.[28]

One of the alarming results was the resistance of *A. baumanii* 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.[5] This high level of imipenem resistance (ten of 13) may result from the high number of bla_{OXA-23}-like genes detected among MDR *A. baumanii* (nine of 13), as previously reported.[5] This is in accordance with the results of Al-Agamy et al from Egypt, where bla_{OXA-23} and bla_{OXA-24}-like genes were found to be the most prevalent type of β-lactamase-encoding genes in *A. baumannii*.[30] Efflux-mediated resistance accounted for this MDR phenotype in *A. baumannii* (six of 13), half of which (three of six) contained multidrug-efflux pumps that mediated resistance to

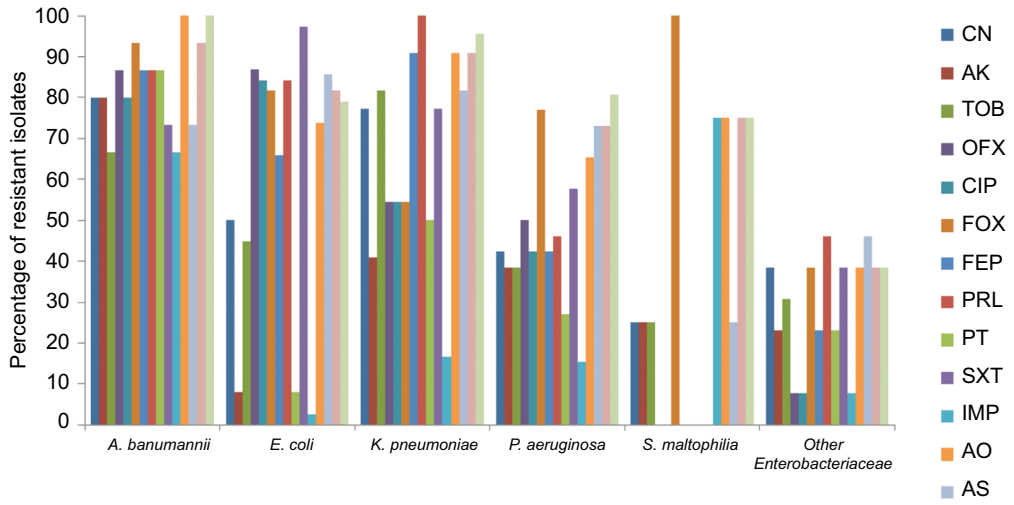

Figure 2 Percentage of isolates resistant to each antimicrobial tested within the different bacterial species.
Abbreviations: CN, gentamicin; AK, amikacin; TOB, tobramycin; OFX, ofloxacin; CIP, ciprofloxacin; FOX, cefoxitin; FEP, cefepime; PRL, piperacillin; PT, piperacillin–tazobactam; SXT, sulfamethoxazole–trimethoprim; IMP, imipenem; AO, aztreonam; AS, ampicillin–sulbactam.

gentamicin, ciprofloxacin, sulfamethoxazole–trimethoprim, and piperacillin. A previous study in Egypt reported a higher percentage of efflux pumps (77.8%) in *A. baumannii* isolates.[31] In accordance with previous studies,[5] 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. baumanii* isolates, respectively.

In agreement with the reported susceptibility pattern of *P. aeruginosa*,[5] 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*.[5] The metallo-β-lactamase-resistance gene bla_{VIM} was detected in one *P. aeruginosa* isolate. This represented 5.88% of MDR *P. aeruginosa* clinical isolates and 33.33% of *P. aeruginosa* isolates resistant to imipenem. Other studies in Egypt reported higher prevalence of bla_{VIM} in *P. aeruginosa* clinical isolates.[32,33]

All our *S. maltophilia* isolates were sensitive to sulfamethoxazole–trimethoprim, the cornerstone in the treatment of this pathogen,[5] and to the tested fluoroquinolones (ciprofloxacin and ofloxacin). Most isolates (three of four) were sensitive to β-lactam/β-lactamase inhibitor combinations. Fluoroquinolones and β-lactam/β-lactamase inhibitor combinations have been reported to be among the most effective agents against *S. maltophilia*.[5] Although *S. maltophilia* are known to be aminoglycoside-resistant,[5] only one isolate (of three) was resistant to the three tested aminoglycosides, and showed efflux-mediated resistance to aminoglycosides. Efflux pumps are one of the known resistance mechanisms in *S. maltophilia*.[5] Predominant resistance to aztreonam, cephalosporins, and imipenem in *S. maltophilia*, has been reported in the literature.[5]

About 76% of the MDR Enterobacteriaceae contained at least one of the tested β-lactam-resistance genes, where β-lactamases are commonly reported among Enterobacteriaceae.[5] 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.[34]

The bla_{TEM-1} 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_{TEM-1} gene among Enterobacteriaceae worldwide.[35] The β-lactamase-resistance gene bla_{SHV} 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_{SHV}-positive isolates, respectively. This was in contrast to another study from Egypt that detected only SHV1 and SHV11 in 57% and 29% of bla_{SHV}-containing isolates, respectively.[36] 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	1/17	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	1/17	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	1/21	0/1	0/17	0/2	0/3	0/2
CIP, SXT	0/13	0/3	1/37	0/1	0/21	0/1	0/17	0/2	0/3	0/2
CIP, PRL	0/13	0/3	2/37	0/1	0/21	0/1	1/17	0/2	0/3	0/2
SXT, PRL	0/13	0/3	1/37	0/1	1/21	0/1	1/17	0/2	0/3	1/2
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	1/21	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	*armA*	*aac-Ib*	*aac-Ib-cr*	*bla*$_{TEM-1}$	*bla*$_{SHV}$	*bla*$_{CTX-M-15}$	*bla*$_{CTX-M-14}$	*bla*$_{IMP}$	*bla*$_{VIM}$	*bla*$_{SPM-1}$	*bla*$_{OXA-23}$	*qepA*	*qnrA*	*qnrB*	*qnrS*
Acinetobacter baumannii	0/13	9/13	0/13	7/13	3/13[a]	4/13	1/13	0/13	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[b]	1/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	1/3	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	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	1/37
Klebsiella pneumoniae	0/21	8/21	8/21	12/21	17/21[c]	15/21	7/21	0/21	0/21	0/21	2/21	0/21	0/21	1/21	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	0/1
Pseudomonas aeruginosa	0/17	4/17	1/17	3/17	2/17[d]	5/17	1/17	0/17	1/17	0/17	0/17	0/17	0/17	0/17	0/17
Proteus mirabilis	0/2	0/2	1/2	1/2	1/2[e]	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	0/3
Serratia marcescens	0/2	0/2	0/2	0/2	0/2	0/2	1/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2

Notes: [a]Two of three of the detected SHVs were the variant SHV1 and one of three the variant SHV11; [b]one detected SHV – SHV1; [c]13 of 17 of the detected SHVs were SHV1, two of 17 SHV11, one of 17 SHV12, and one of 17 SHV31; [d]one of two of the detected SHVs was SHV1 and one of two SHV11; [e]one detected SHV – SHV1.

bla$_{TEM-1}$, *bla*$_{CTX-M-15}$, *bla*$_{CTX-M-14}$, *aac(6')-Ib-cr, qnr*S, 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 *bla*$_{CTX-M-15}$ and *bla*$_{CTX-M-14}$ 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 *bla*$_{CTX-M-15}$.[40] Moreover, *bla*$_{OXA-23}$-like, mainly detectable in *A. baumannii*,[30] was detected in two of 21 *K. pneumoniae* isolates. The detection of *bla*$_{OXA-23}$-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, qnr*B, and *qnr*S*)* 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.[5] 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	8	Sputum																+
baumanii	27	ETT																+
	82	ETT																+
	136	Sputum																+
	141	Peritoneal																+
	145	Sputum																+
	146	ETT																+
	149	Peritoneal																+
	150	Sputum																+
	152	Peritoneal																–
	162	ETT																+
	179	Urine																+
	203	ETT																+
	226	CSF																–
	236	Wound																+
Citrobacter freundii	72	Stool																+
	202	Wound																+
	217	Stool																–
	252	Sputum																+
Enterobacter cloacae	87	Stool																+
	117	Sputum																–
	147	ETT																–
Escherichia coli	9	Sputum																+
	25	Urine																+
	70	Urine																+
	71	Urine																+
	74	Urine																+
	78	Urine																+
	81	Urine																+
	94	Urine																+
	113	Urine																+
	121	Urine																+
	122	Urine																+
	123	Urine																+

(*Continued*)

Table S1 (*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	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	11	CSF																+
aeruginosa	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																+

(Continued)

Table S1 (*Continued*)

	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																+
	143	Sputum																+
Stenotrophomonas maltophilia	211	CSF																+
	225	CSF																–
	240	Stool																+
	245	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 baumanii	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*)

Table S2 (*Continued*)

	Isolate number	Gentamicin	Ciprofloxacin	Trimethoprim–sulfamethoxazole	Piperacillin
Escherichia coli	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				
	251	■	■	■	■
	254	■	■	■	■
Morganella morganii	224			■	
Proteus mirabilis	96				
	182			■	
Pseudomonas aeruginosa	11				
	14			■	■
	15				
	28				
	29				
	38				
	58			■	
	88				■
	102				■
	107				
	127		■	■	■
	138				
	158		■		■
	167				
	170				
	198	■	■	■	
	256				
Serratia marcescens	79				■
	143			■	■
Stenotrophomonas maltophilia	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}[a]	$bla_{CTXM-15}$	$bla_{CTXM-14}$	bla_{IMP}	bla_{VIM}	bla_{SPM-1}	bla_{OXA-23}	*qepA*	*qnrA*	*qnrB*	*qnrS*
Acinetobacter baumannii	8		■													
	27		■									■				
	82															
	136				■	I						■				
	141				■		■					■				
	145		■		■							■				
	146		■		■		■					■				
	149		■													
	150		■		■							■				
	162		■		■	I	■	■								■
	179		■									■				
	203		■									■				
	236				■	II	■					■				
Citrobacter freundii	72				■										■	
	202															
	252					I	■									
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*)

Table S3 (*Continued*)

	Isolate number	*armA*	*aac(6')-Ib*	*aac(6')-Ib-cr*	bla_{TEM-1}	bla_{SHV}[a]	$bla_{CTXM-15}$	$bla_{CTXM-14}$	bla_{IMP}	bla_{VIM}	bla_{SPM-1}	bla_{OXA-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					1										
	39					1										
	68					1										
	75					1										
	83					1										
	100					1										
	114					12										
	134					1										
	153					1										
	157					1										
	161					1										
	163					31										
	165					1										
	210					1										
	220					11										
	223					11										
	243															

(*Continued*)

Table S3 (*Continued*)

	Isolate number	*armA*	*aac(6')-Ib*	*aac(6')-Ib-cr*	bla_{TEM-1}	bla_{SHV}[a]	$bla_{CTXM-15}$	$bla_{CTXM-14}$	bla_{IMP}	bla_{VIM}	bla_{SPM-1}	bla_{OXA-23}	*qepA*	*qnrA*	*qnrB*	*qnrS*
Klebsiella pneumoniae	251															
	254		■			1	■									
Morganella morganii	224															
Proteus mirabilis	182			■												
	96				■		■									
Pseudomonas aeruginosa	11		■													
	14		■			11	■									
	15															
	28															
	29															
	38															
	58															
	88															
	102															
	107						■									
	127															
	138															
	158		■		■	1	■									
	167		■		■		■	■								
	170															
	198									■						
	256			■	■		■									
Serratia marcescens	79															
	143							■								
Stenotrophomonas maltophilia	211															
	240															
	245															

Notes: [a]Gene variants detected. Black cells, presence of resistance genes; white cells, absence of resistance genes.

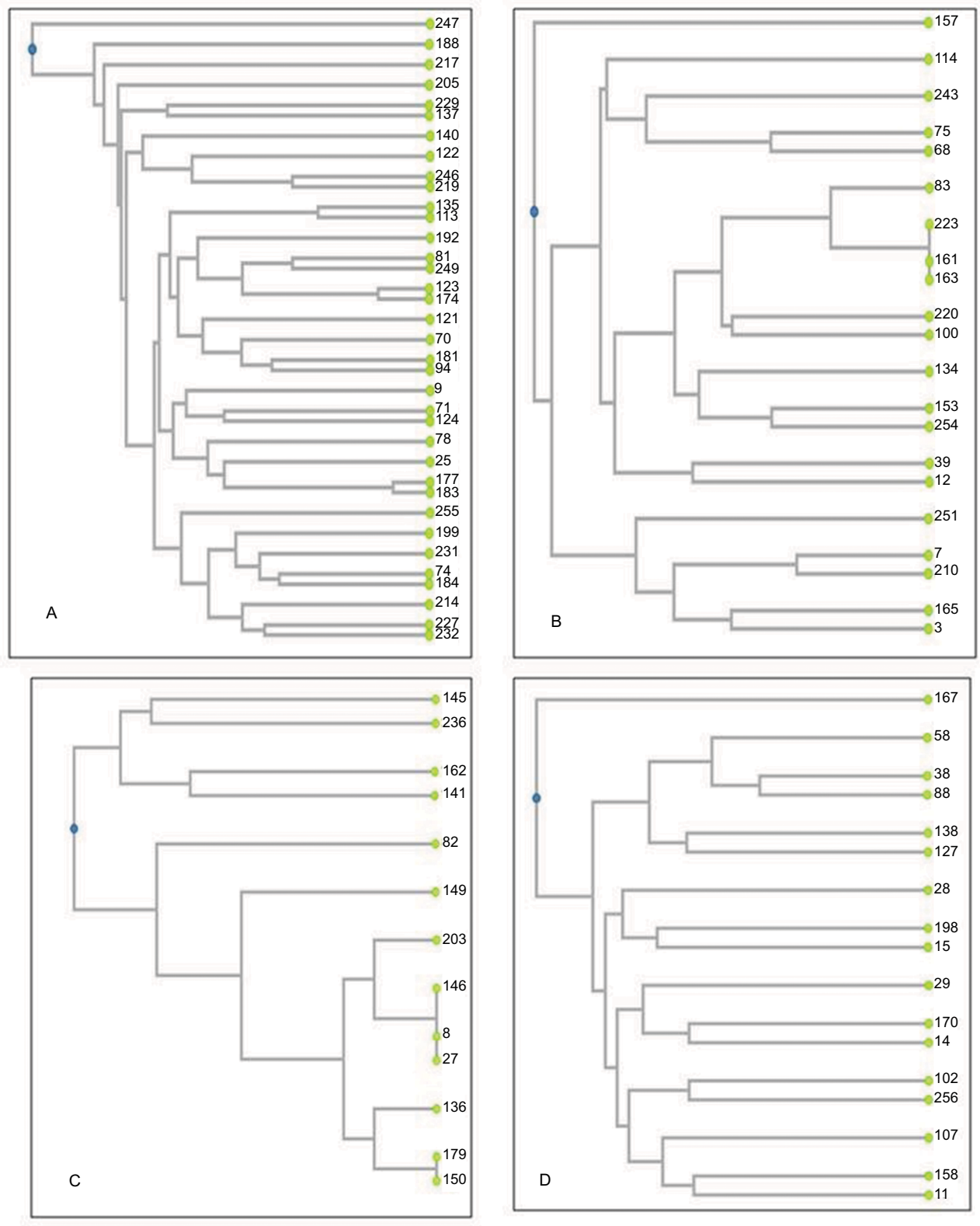

Figure S1 Phenogram of different multidrug resistant isolates constructed using UPGMA algorithms based on RAPD analysis.
Notes: (**A**) Phenogram of *Escherichia coli* using three different primers; (**B**) phenogram of *Klebsiella pneumoniae* using three different primers; (**C**) phenogram of *Acinetobacter 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.