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Original Article

Susceptibility of carbapenem-resistant Enterobacterales isolates to new antibiotics from a tertiary care hospital, Egypt: A matter of hope

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Abstract

Introduction: Carbapenem-resistant *Enterobacterales* (CRE) are particularly worrisome pathogens because of their resistance to last-resort antibiotics, significant morbidity, and mortality. With limited treatment options, new therapeutic choices have become available for the management of CRE infections. Data regarding the efficacy of these novel agents are still limited particularly in a low-middle-income country like Egypt. This study aims to assess the prevalence of different carbapenemase genes among CRE isolates and the susceptibility of these isolates to novel antibiotics for improving antibiotic policy and infection control strategies in Egypt.

Methodology: In this cross-sectional study, 260 *Enterobacterales* were recovered from patients admitted to intensive care units between January and June 2021. Susceptibility testing was conducted using Kirby-Bauer method. Molecular detection of five carbapenemase genes, namely *bla*$_{KPC}$, *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{NDM}$, *bla*$_{OXA-48}$ was done using polymerase chain reaction (PCR).

Results: Of the 260 *Enterobacterales*, 34.6% were found to be carbapenems resistant. All of the CRE isolates were multi-drug resistant exhibiting resistance to most antibiotics. All isolates harbored one or more carbapenemases genes. The most prevalent was *bla*$_{NDM}$ (84.4%), followed by *bla*$_{OXA-48}$ (73.3%), *bla*$_{KPC}$ (13.3%), *bla*$_{IMP}$ (2.2%), while *bla*$_{VIM}$ gene wasn't detected. Among 62.2% of the CRE isolates, two or more carbapenemase genes co-existed. For the new antibiotics tested, 100% of CRE resisted ceftolozane/tazobactam, 86.7% resisted ceftazidime/avibactam, 51.1% were resistant to eravacyclin, and 42.2% were resistant to cefiderocol.

Conclusions: A high percentage of resistance to carbapenems among *Enterobacterales* isolates was revealed. *bla*$_{NDM}$ was found to be the most predominant carbapenemase gene. A high rate of CRE resistance to novel agents signifies a major threat.

Key words: *Enterobacterales*; carbapenem resistance; carbapenemases;novel antibiotics; multidrug resistance.

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Introduction

Being one of the last-line antibiotics for treating infections caused by multi-drug resistant (MDR) Gram-negative bacteria, the extensive use of carbapenems has led to the surge of carbapenem-resistant *Enterobacterales* (CRE) [1]. Not only the increasing prevalence of CRE across the world that is worrying but also the alarming mortality rate, which prompted the Centers for Disease Control and Prevention (CDC) to consider CRE as one of the three most critical antimicrobial-resistant threats with an associated mortality rate up to 50% [2].

Enterobacterales commonly cause a wide variety of community- and healthcare-associated infections including community-acquired pneumonia, ventilator-associated pneumonia, urinary tract infections as well as bloodstream infections. Thus, the emergence of resistance among these bacteria holds considerable clinical as well as socioeconomic impacts [3].

Although *Enterobacterales* resist carbapenems by different strategies, the production of carbapenemase remains the most important, mainly mediated by three primary groups of enzymes: KPC (*Klebsiella pneumoniae* carbapenemase) (Ambler class A), MBLs (Metallo-ß-Lactamases) (Ambler class B) and OXA-48-like (Ambler class D) [4]. Carbapenemases detection and precise characterization are important not only for infection control and public health but also for clinical practice since they influence therapeutic decisions; each novel agent has been designed with a unique range of activity against different carbapenemases produced by *Enterobacterales* [5].

In a limited resources setting of a low-middle income country like Egypt, the issue of carbapenem

resistance continues to worsen. We are challenged by the lack of a national antimicrobial surveillance system making it difficult to evaluate the problem of carbapenem resistance. The issue of resistance is also made worse by the absence of a comprehensive antimicrobial stewardship program as well as non-adherence to recommendations related to antibiotics' prescription [6].

Exhibiting resistance to various classes of antibiotics, still no consensus exists for the optimal treatment for CRE infections. Options for managing CRE had been limited to repurposing of already existing antibiotics such as colistin, fosfomycin, tigecycline, and carbapenem in selected cases [5].

Not long ago, several novel agents with activity against carbapenem-resistant pathogens have been approved, others are in their late-stages of clinical development. These novel agents are expected to widen the already present armamentarium for CRE, which will in turn improve the outcome of patients affected by these resistant pathogens. Novel β-lactam/β-lactamase inhibitors such as ceftolozane/tazobactam (C/T), ceftazidime/avibactam (CZA), meropenem/vaborbactam have brought additional choices for clinicians treating these MDR organisms. With a broader spectrum of activity, the clinicians' repertoire furtherly welcomed newer agents including cefiderocol (FDC), eravacycline (ERV), plazomicin and others [7].

Despite the promising results exhibited by these novel agents, data on their clinical efficacy are still limited and slowly emerging, also several concerns about their use still exist, including some gaps in their spectrum of activity as well as the development of resistance [5]. In Egypt and to the best of our knowledge, only a few scattered studies have been conducted evaluating these new antibiotics. These gaps in knowledge are curbing the clinical integration of these agents into the patient's care.

Methodology

Study design

This observational cross-sectional study was carried out in Medical Microbiology and Immunology Department, Faculty of Medicine, Zagazig University.

Collection of data

A worksheet was filled for every patient including demographic data (age, sex, etc.), clinical data (diagnosis, comorbid conditions, etc.), previous laboratory investigations as well as the presence of any risk factors e.g. length of hospital stay, duration of intensive care unit admission, previous antibiotics intake, etc.).

Specimens' collection

Two-hundred sixty (260) *Enterobacterales* isolates were obtained from 558 patients admitted at different intensive care units of Zagazig University Hospitals. Different clinical samples (urine, endotracheal aspirate, sputum, and wound swabs) were collected under complete aseptic conditions in a sterile container to be examined.

Microbiological identification

For isolation of *Enterobacterales*, all bacterial isolates were cultured on blood agar and MacConkey agar (Oxoid) plates, incubated overnight at 35 °C–37 °C [8]. For identification, Gram's staining, and API 20E (BioMérieux Inc., France) were used [9].

Detection and antibiotic susceptibility of carbapenem-resistant Enterobacterales

As stated by the CDC, *Enterobacterales* that test resistant to at least one of the carbapenem antibiotics are called CRE [10]. For the 260 *Enterobacterales*, phenotypic screening for carbapenem resistance was performed by Kirby-Bauer disc diffusion method on Mueller- Hinton agar (Oxoid, UK) incubated at 35ºC ± 2˚C for 16-18 hours as per the Clinical and Laboratory Standards Institute (CLSI). Four carbapenem disks were used (imipenem - 10 µg, meropenem - 10 µg, ertapenem - 10 µg, and doripenem - 10 µg). Results were interpreted according to the CLSI 2021 guidelines [11]. Detected CRE isolates were subjected to antibiotic susceptibility testing by a standardized Kirby–Bauer disk diffusion method [8]. The used antibiotics- chosen based on the latest recommendations of CLSI (CLSI 2021) - included: Amoxicillin-clavulanate 20/10µg (AMC), aztreonam 30µg (ATM), trimethoprim-sulfamethoxazole 1.25/23.75µg (SXT), cefepime 30µg (FEP), cefoxitin 30µg (FOX), ceftazidime 30µg (CAZ), tetracycline 30µg (TE), levofloxacin 5µg (LEV) gentamycin 10µg (CN), and nitrofurantoin 300 µg (F). Nitrofurantoin was used only for CRE urinary pathogens. All these discs are manufactured by (Oxoid, UK) [11].

Four novel antibiotics were used: C/T 30/10µg, CZA 30/10µg, FDC 30µg, and ERV 20µg (Liofilchem, Italy). Clinical and Laboratory Standards Institute and Food and Drug Administration (FDA) breakpoint criteria were used for CZA, C/T, ERV and FDC [11,12]. *Pseudomonas aeruginosa* ATCC 27853 and

Escherichia coli ATCC 25922 were used as quality controls [11].

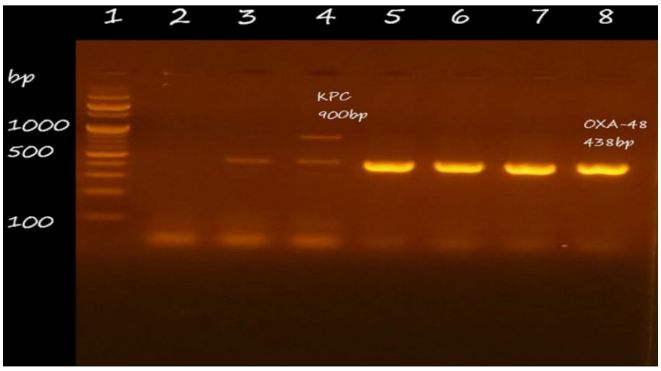
For colistin susceptibility, colistin broth disk elution method was used [11]. For each isolate to be tested, 4 tubes of cation-adjusted Mueller Hinton broth were labeled as 1, 2, and 4 µg/mL and growth control. Aseptically the following were added: 1 colistin disk to the tube labeled "1µg/mL", 2 colistin disks to the tube labeled "2µg/mL", 4 colistin disks to the tube labeled "4µg/mL". Tubes were vortexed and colistin was allowed to elute from disks for 30 minutes at room temperature. 50µL of the standardized inoculum was added to each of the 4 tubes. Tubes were incubated at 35 ºC for 20 hours. *Pseudomonas aeruginosa* ATCC 27853 was used as a quality control. By visual inspection, minimum inhibitory concentration (MIC) is read as the lowest concentration that inhibits the growth of the test isolate. Enterobacterial isolates with colistin MICs of ≤ 2 µg/mL were categorized as intermediate, and those with MICs of ≥ 4 µg/mL were categorized as resistant [11].

Detection of carbapenemase production

All the CRE isolates, detected by screening by carbapenem disks, were further confirmed by detection of carbapenemases genes by multiplex PCR as follows:

DNA extraction was performed using G-spinTM Genomic DNA Extraction Kit (iNtRON Biotechnology, Inc., Korea) as per the manufacturer's instructions. Two multiplex PCR reactions were executed for detection of the five predominant carbapenemases (*bla*$_{KPC}$, *bla*$_{NDM}$, *bla*$_{OXA-48}$, *bla*$_{VIM}$, and *bla*$_{IMP}$) [13,14]. The 1st reaction for *bla*$_{KPC}$, *bla*$_{IMP}$, *bla*$_{OXA-48}$ detection and the 2nd for *bla*$_{NDM}$, *bla*$_{VIM}$. Each of the PCR reaction mixtures was prepared in a total volume of 30µL including 5µL of template DNA, 15 µL of PCR master mix, and 2 µL of each primer then the volume was completed with nuclease-free water up to 30µL. The following thermal cycling conditions were used for amplification: an initial denaturation step at 95 °C for 5 minutes and 15 cycles of DNA denaturation at 95 °C for 30 seconds, annealing at 52, or 58 °C for 30 seconds, and extension at 72°C for 1 minute, for 30 cycles.

Figure 1. Agarose gel electrophoresis of *bla*$_{KPC}$ and *bla*$_{OXA-48}$ genes amplicons.



Lane (1): DNA ladder 100 bp; lane (2): negative control; lanes (3-8): positive for *bla*$_{OXA-48}$ gene (438bp); lane (4): positive for *bla*$_{KPC}$ gene (900bp).

Multiple annealing temperatures were used with the gradient thermocycler in an attempt to amplify related, but nonidentical, sequences. Gel electrophoresis and visualization of PCR products under UV light was performed. Primers used are listed in Table 1.

Statistical analysis

All data were collected, tabulated, and statistically analyzed using SPSS 26.0 for windows (SPSS Inc., Chicago, IL, USA). Qualitative data were expressed as absolute frequencies (number) and relative frequencies (percentage).

Results

Two hundred sixty *Enterobacterales* were isolated from 558 different clinical specimens (46.6%). From the 260 *Enterobacterales*, 180 isolates were identified as *Klebsiella pneumoniae*, 72 as Escherichia coli, and 8 as Proteus mirabilis. By disc diffusion method,

Table 1. PCR primers.

Target	Sequence	Amplicon size (bp)	Annealing temp. (°C)	Reference
KPC	F: TGTCACTGTATCGCCGTC R: CTCAGTGCTCTACAGAAAACC	900	58	[13]
IMP	F: CTACCGCAGCAGAGTCTTTG R: AACCAGTTTTGCCTTACCAT	587	55	[14]
VIM	F: AGTGGTGAGTATCCGACAG R: ATGAAAGTGCGTGGAGAC	261	52	[13]
NDM	F: GGTTTGGCGATCTGGTTTTC R: CGGAATGGCTCATCACGAT	621	50	[13]
OXA-48	F: GCGTGGTTAAGGATGAACAC R: CATCAAGTTCAACCCAACCG	438	52	[14]

IMP: imipenemase; KPC: *Klebsiella pneumoniae* carbapenemases; NDM: New Delhi metallo—lactamase; VIM: Veronese imipenemase. OXA-48: oxacillinase-48.

carbapenem resistance was detected among 34.6% (90/260) of the isolated *Enterobacterales*.

Distribution of carbapenemase genes among CRE isolates

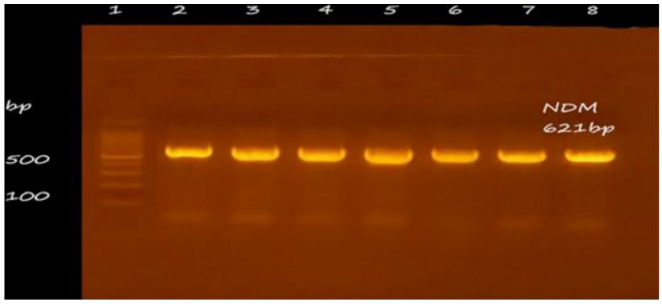
All CRE isolates (100%) harbored one or more of the carbapenemases genes, (56/90) 62.2% carried 2 or more genes (Figure 1, Figure 2). The most prevalent gene detected was bla_{NDM} (76/90) 84.4%, followed by bla_{OXA-48} (66/90) 73.3 %, bla_{KPC} (12/90) 13.3%, bla_{IMP} (2/90) 2.2% while bla_{VIM} gene couldn't be detected (Figure 3). Co-existence of carbapenemase genes was observed in which, (10/90) 11.1% of isolates carried 3 genes (bla_{NDM}, $bla_{OXA-48,}$ and bla_{KPC}), while (46/90) 51.1% of the isolates carried 2 genes mostly of bla_{NDM} and bla_{OXA-48} types.

Antibiotic susceptibility testing

Guided by CLSI breakpoints, all CRE isolates were resistant to aztreonam, ceftazidime, cefoxitin, cefepime, amoxicillin-clavulanate, nitrofurantoin, trimethoprim-sulfamethoxazole, and tetracycline. Eighty-six isolates (95.6 %) were resistant to levofloxacin and (80/90) 88.9% were resistant to gentamicin. For colistin, 77.8% of the isolates were of intermediate susceptibility (MIC ≤ 2 µg/mL) while 22.2% were resistant (MIC ≥ 4 µg/mL).

Regarding susceptibility to new antibiotics, (90/90) 100% of CRE isolates were resistant to C/T, (78/90) 86.7% were resistant to CZA, (46/90) 51.1% were resistant to ERV, and (38/90) 42.2% were resistant to FDC (Figure 4). The sensitivity of each of the detected carbapenemase classes to each novel antibiotic tested is illustrated in (Table 2).

Figure 2. Agarose gel electrophoresis of bla_{NDM} gene amplicons.



Lane (1): DNA ladder 100 bp; lanes (2-8): positive for bla_{NDM} gene (621bp).

Discussion

Carbapenem-resistant *Enterobacterales* can be considered a serious health burden threatening the whole world. With higher estimates than reported from other Arab, African, or Asian countries, carbapenem resistance among *Enterobacterales* is ubiquitous in Egypt and on the rise [15]. With limited therapeutic options available for treatment of CRE in Egypt, ceftazidime avibactam (CZA) was recently introduced

Figure 3. Prevalence of carbapenemase genes.

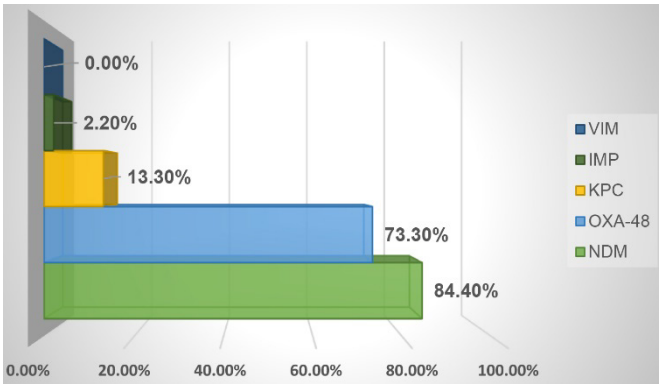
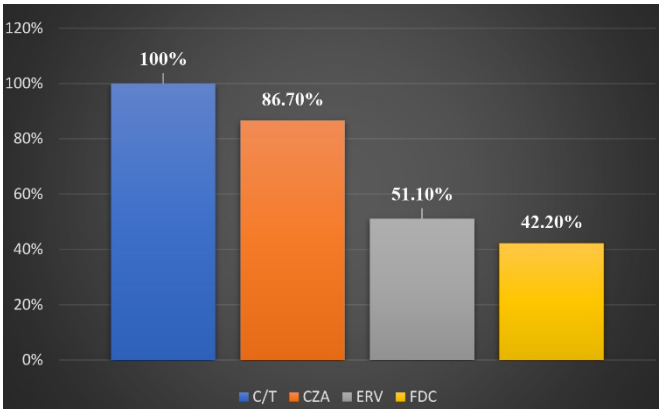


Figure 4. Resistance pattern of CRE to new antibiotics.



(C/T) Ceftolozane/tazobactam; (CZA) Ceftazidime/avibactam; (ERV) Eravacycline; (FDC) Cefiderocol.

Table 2. Susceptibility of each carbapenemase class to novel antibiotics.

	Cefiderocol	Eravacycline	Ceftolozane-tazobactam (C/T)	Ceftazidime/ avibactam (CZA)
NDM harboring enterobacterales (n = 76)	55.3% (42/76)	47.4% (36/76)	0 (0/76)	2.6% (2/76)
OXA-48 harboring enterobacterales (n = 66)	60.6% (40/66)	51.5% (34/66)	0 (0/66)	18.2% (12/66)
KPC harboring enterobacterales (n = 12)	33.3% (4/12)	16.7% (2/12)	0 (0/12)	0 (0/12)
IMP harboring enterobacterales (n = 2)	100% (2/2)	100% (2/2)	0 (0/2)	0 (0/2)

IMP: imipenemase; KPC: *Klebsiella pneumoniae* carbapenemases; NDM: New Delhi metallo—lactamase, VIM: Veronese imipenemase; OXA-48: oxacillinase-48.

into the Egyptian market. It has been reserved for patients showing resistance to the last line of traditional CRE treatment (e.g. colistin and tigecycline). In our institution, and in an unexpected speediness, resistance to CZA was reported. Concerned about this drug's future effectiveness and the need for alternatives, this study was conducted.

Direct evidence for CRE treatment is still lacking and therapeutic options are limited. Furtherly, data regarding novel agents are still limited and slowly emerging. Therefore, continuous surveillance and epidemiological investigation of carbapenemases are of great importance to control infections.

In this study, a total of 260 *Enterobacterales* were isolated at a rate of 46.6 %. Nearly similar results were reported [16]. Higher results (55.8%) were also reported [17]. *Klebsiella pneumoniae* was the most commonly isolated organism (69.2%), predominantly from patients with urinary tract infections.

Of the isolated *Enterobacterales*, 34.6% were carbapenem-resistant. This rate was in line with that of Reheel *et al.* 2020 from Egypt [16] (34.1%). Carbapenem-resistant *Enterobacterales* at lower rates; 20.4% and 8.27 %, respectively were reported by Muhammad *et al.* 2021 from Egypt and Logman *et al.* 2021 from Morocco [18,19]. On the other hand, Tawfick *et al.* 2020 in an Egyptian study recorded a much higher rate (89.6%) [20].

Prevalence rates of CRE may vary among different studies owing to a variety of factors including, the sampled population, carbapenem resistance mechanism studied, the use and misuse of carbapenems as well as the applied infection control practices [21]. Undoubtedly resistance to carbapenems is obviously increasing in Egypt, probably because of the unrestricted usage of these agents even without a clinical prescription.

Being the gold standard, molecular detection of carbapenemases was performed. By PCR, all CRE isolates harbored one or more of the carbapenemases genes, similar results were reported [22].

The most prevalent gene detected in the current study was bla_{NDM} (84.4%) followed by the $bla_{\text{OXA-48}}$ with an incidence of (73.3%). This finding was in total conformity with Tawfick *et al.*, Aamer *et al*., Eldomany *et al.*, and Shawky *et al.* from Egypt [20,22-24]. On the other hand, Reheel *et al.* and El-Badawy *et al.* from Egypt [16,25] reported $bla_{\text{OXA-48}}$ as the most commonly present gene followed by bla_{NDM} as well as Al-Abdely *et al.* [26] from Saudi Arabia. *Bla* $_{\text{KPC}}$ as the most common gene was reported by Emira *et al.* and Amer *et al.* from Egypt [17,27].

The predominance of bla_{NDM} could be related to the fact that they are encoded on a variety of highly mobile conjugative plasmids, allowing for horizontal inter and intra-species transfer between bacteria rather than clonal spread [28]. The dominance of NDM-producing isolates is considered a critical situation since plasmids with the bla_{NDM} often carry resistance genes to most antibiotics conferring pan-drug resistance [29].

Low incidence rate of bla_{IMP} and absence of bla_{VIM} genes in this study were also reported by others [20,23,24] confirming that both genes are not prevalent in our geographical region [24]. In addition, bla_{VIM} genes are uncommon among *Enterobacterales* [30].

A high rate of carbapenemases genes coexistence was also detected in this study in which 62.2% of the isolates carried 2 or more genes. To the best of our knowledge, this is the highest rate reported by an Egyptian study. Emira *et al.* and El-Domany *et al*. from Egypt [17,23] also reported high rates of isolates harboring multiple carbapenemases genes at 48% and 57.9 %, respectively.

Despite the low prevalence of bla_{KPC} in the current study (13.3%), the uncommon triple gene combination of (bla_{NDM}, $bla_{\text{OXA-48}}$, and bla_{KPC}) at a rate of 11.1 %, was among our concerning findings being not reported before among isolates in Egypt. At a much higher rate (80.9 %) this combination was also recently reported in Saudi Arabia [31].

Co-presence of genes could be attributed to the existence of several gene cassettes encoding other carbapenemase previously described among bla_{NDM} bearing isolates [32]. Combined carbapenemases in a single isolate render it extremely resistant by expanding its overall hydrolytic spectrum thereby limiting the treatment options [33].

Results of antibiotic susceptibility represent further evidence of the multi-resistance profile of CRE. As per the interim standard definitions of acquired resistance [34], all CRE isolates in the current study were MDR exhibiting resistance to most antimicrobial agents, a finding that was also reported in other studies [23,31,35].

The emergence of carbapenemases in *Enterobacterales* is cause for concern, not only because it provides carbapenems with a more efficient and stable mechanism of resistance, but also because non-beta lactam resistance genes are frequently co-acquired on the same mobile genetic elements carrying these resistance determinants, further limiting the therapeutic options [36]. Additionally, antibiotic resistance is also being exacerbated by over-the-counter prescriptions and antimicrobial misuse, particularly in Egypt [37].

As regards susceptibility to new antibiotics, all isolates were resistant to C/T and nearly all resistant to CZA. This result was also consistent with that of Aamer *et al*. and Lutgring *et al.* [22,38]. On the contrary, Aamer *et al.* and Sader *et al.* [39,40] reported high levels of sensitivity to CZA. The lack of activity of these antibiotics against metallo-ß-lactamases could explain the high rate of resistance recorded in this study [37,41]. Enzymatic inactivation, chemical modification of the target or expressions of an alternative target, and changes in cell permeability or expression of efflux pumps are suggested causes for resistance exerted by non-metallo-ß-lactamase isolates [42].

Despite their unavailability in the Egyptian market, an unexpectedly high rate of resistance to ERV (51.1%) and FDC (42.2%) was also detected. In agreement with this study, resistance to ERV was also reported by Zheng *et al.* [43]. Inconsistently with this finding [44,45] stated retained activity of ERV against *Enterobacterales*

Overconsumption of tigecycline with a subsequent high rate of resistance could play a role in the emergence of ERV-resistant isolates in our hospitals. Moreover, ERV-inactivating enzymes have now been discovered in bla_{NDM} harboring isolates [46].

Although infrequent, resistance to FDC was also reported by Mushtaq *et al.* and Ito *et al.* [47,48]. Regarding cefiderocol, resistant mutants as well as cross-resistance with CZA have been described [49]. Yamano [50] also proposed that cefiderocol resistance could be linked to the co-existence of NDM and serine-lactamases at the same time.

The previous findings point to an alarming situation that might be encountered in the future, particularly with the continuous worsening of the issue of carbapenem resistance in Egypt.

Conclusions

In this study, a high percentage of resistance to carbapenems as well as most tested antimicrobials was detected among *Enterobacterales* isolates from Zagazig University Hospitals. bla_{NDM} was found to be the most prevalent carbapenemase gene which necessitates a reconsideration of the value of using the newly introduced CZA as an option for CRE, being not effective against metallo-beta lactamases. A high rate of co-harboring 2 or more carbapenemase genes was also detected. A high rate of resistance to eravacycline and cefiderocol requires prospective thinking of alternatives. We are obliged to wisely use the current therapeutic options that seem to be our only resort for a while.

Findings from this study probably reflect injudicious use of antibiotics as well as serious breaches in the infection control measures in our institute. The only hope for facing the present and the upcoming MDR including carbapenem resistance is the consistent application of infection control standards to disrupt the cycle of transmission of MDR bacteria. In addition to the implementation of comprehensive antimicrobial stewardship programs including molecular characterization of the prevalent type of resistance to address proper antimicrobial use.

Limitations

In view of limited available resources, we couldn't proceed to further advanced diagnostics, indispensable for a more comprehensive and accurate insight. Thus, this study can be considered as no more than a preliminary study that could form a platform for further in-depth studies.

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