Common Antibiotic Combinations for Multidrug-Resistant Gram-Negative Bacilli Isolated from Intensive Care Unit Infected Patients, Egypt.

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ABSTRACT
Background: Infections with multidrug-resistant Gram-negative bacilli (MDR GNB) represent a principal threat particularly in intensive care unit (ICU) patients as the lack of effective antimicrobial therapy constitutes a serious challenge. Antimicrobial combinations could be a possible alternative. However, the most effective combination remains to be determined.
Objectives: Aims of this study were to detect the incidence of MDR GNB infections among ICU patients in Al-Ahrar Teaching Hospital, Zagazig, Egypt, to determine the most effective antibiotic combination against them, and to screen them for the presence of five major carbapenemase genes.
Subjects and methods: In a cross-sectional study, GNB were isolated and identified from different clinical specimens collected from ICU patients diagnosed with healthcare-acquired infections (HAIs) in Al-Ahrar hospital over the period from May 2017 to Mars 2021. Isolates were tested for antibiotic susceptibility by the disc diffusion method. MDR strains were tested against three antimicrobial combinations by Epsilometer (E-test) and screened for the presence of five carbapenemase genes by polymerase chain reaction (PCR).
Results: A total of 55 MDR isolates were obtained representing 58% of the isolated GNB. Ceftazidime/avibactam inhibited more than half of MDR isolates recording a 61.8% sensitivity ratio. The use of colistin/amikacin had a slightly higher synergistic effect on MDR isolates (27.4%) compared to amikacin/aztreonam combination (23.6%). One or more carbapenemase gene have been detected in 52.7% of MDR isolates. bla_{KPC} was detected in 29.1% of MDR isolates and was the most frequent among the five tested carbapenemase genes.
Conclusion: The incidence of MDRGNB infections is remarkably high in the ICU of Al-Ahrar Hospital with a high prevalence of carbapenemase genes. Among the tested antibiotic combinations, ceftazidime/avibactam demonstrated the best in-vitro performance against isolated MDR GNB.
Keywords: Multidrug-resistant gram-negative bacteria, ICU, antibiotic combination, carbapenemase genes.

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INTRODUCTION

The emergence of multidrug-resistant organisms (MDROs) has become a significant public health threat as there are fewer, or even sometimes no, effective antibiotics available for infections caused by these bacteria (1).

Although many new drugs have been introduced commercially, the development of resistance is

increasing especially among Gram-negative bacilli (GNB) that have developed multiple mechanisms to overcome the effectiveness of antibiotics **(2, 3).**

Among the important mechanisms causing antibiotics inactivation in GNB, is the production of carbapenemases which have a potential significance as carbapenems are often considered the last line of effective treatment available for infections with MDROs. Five major carbapenemases that belong to classes A, B, and D beta-lactamases are of major concern. They include; Klebsiella pneumonia carbapenemase (KPC), Imipenemase metallo-beta-lactamase (IMP), New Delhi metallo-beta-lactamase (NDM), Verona integron-encoded metallo-beta-lactamase (VIM) and Oxacillincarbapenemases (OXA) **(4).**

Unfortunately, more than 50% of healthcare-associated infections (HAIs) are caused by resistant bacterial strains. The tendency of increasing resistance is most critical in intensive care unit (ICU) patients who are 5 to 10 times more likely to acquire HAIs than those in general wards **(5, 6).**

The treatment of MDR GNB infections in critically ill patients represents many challenges since an effective treatment should be administered as soon as possible. Resistance to many antimicrobial classes almost invariably reduces the probability of adequate empirical coverage, with possible unfavorable consequences, additional costs, prolonged lengths of stay, higher morbidity, and mortality **(7, 8).**

Though antimicrobial combination therapy remains controversial and has not proven superior to monotherapy regarding cure rates in meta-analysis studies, it remains a possible alternative when effective monotherapy is lacking and can be used for severe infections caused by MDR GNB **(9, 10).**

This study aimed to detect the incidence of MDR GNB infections among ICU patients in Al-Ahrar Teaching Hospital, Zagazig, Egypt, to determine the most effective antibiotic combination against them, and to screen them for the presence of five major carbapenemase genes.

PATIENTS AND METHODS

This study was conducted in the Medical Microbiology and Immunology Department, Faculty of Medicine, Zagazig University, and ICU of Al-Ahrar Teaching Hospital, Zagazig, Egypt during the period from May 2017 to Mars 2021.Ninety-five patients including 40 males and 55 females with ages ranging from 1 day to 80 years were included in this cross-sectional study. They were recruited from the ICU of Al-Ahrar Teaching Hospital by simple random sampling.

Inclusion and Exclusion Criteria

Patients who developed different infections at least 48 h after admission to the ICU were included in the study. Patients who were admitted to the ICU for less than 48 h and patients who are already infected upon admission were excluded.

Ethical consideration

This study was approved by the Institutional Review Board of Zagazig University Hospital (Approval number 4039-8-10-2017) and was conducted in accordance with the updated 2013 Declaration of Helsinki.A written or verbal consent was obtained from each patient or his/her guardian before sample collection.

History taking: All patients were subjected to careful history taking through a worksheet that included personal data, cause of admission, name of the department, data about antibiotic therapy, length of hospital stay, associated comorbidities, surgical procedures, and data about devices application including their type and duration of application. Data were obtained from patients' medical records as well as from the medical staff involved in their care.

Isolation and Identification: Specimens were collected from patients diagnosed with different HAIs under complete aseptic conditions and were subjected to Gram staining and cultivation on appropriate media. GNB isolates were further identified using conventional biochemical reactions including triple sugar iron, indole, oxidase, urease , citrate utilization, H_2S production, and motility tests **(11, 12).**

Antibiotic susceptibility testing (Kirby-Bauer method) (13): Disc diffusion method was used for antibiotic susceptibility testing using Mueller-Hinton agar. Antibiotic susceptibility testing procedures were performed according to CLSI 2020 guidelines. The following antibiotic discs were applied; Ampicillin (10 µg), Amoxicillin-clavulanic acid (30 µg), Ampicillin-sulbactam (20 µg), Tobramycin (10 µg), Amikacin (30 µg), Piperacillin/tazobactam (100/10 µg), Piperacillin (100 µg), Aztreonam (10 µg), Cefepime (30 µg), Cefoxitin (30µg), Ceftriaxone (30µg), Ciprofloxacin(5 µg), Ceftazidime(30 µg), Levofloxacin (5 µg), Imipenem(10 µg), and Meropenem (10 µg). All were supplied from Oxoid. Results were interpreted according to CLSI 2020 guidelines. MDR isolates were identified by non-susceptibility (intermediate or resistance) to at least one agent in at least 3 different antibiotic categories or groups **(14).** They were further tested for the effect of antimicrobial combinations using E-test and for the presence of carbapenemases genes using polymerase chain reaction (PCR) reactions. Till that, strains were preserved at -20˚C in nutrient broth with 20% glycerol.

E-test for antibiotic combinations (BioMerieux, Marcy L Étoile, France): This was carried out according to **(15).** Three antimicrobial combinations were tested which are colistin/amikacin, amikacin/aztreonam, and ceftazidime/avibactam. With the first two combinations, the minimal inhibitory concentration (MIC) was determined for each antibiotic alone. Then, to test the effect of the antibiotic combination, the strip of the first antibiotic (in each combination) was placed onto the agar plate and incubated for 1 hour at 37˚C, then removed, cleaned properly with ethyl alcohol 70%, and kept for reading on the following day. The second antibiotic strip was placed on

top of the gradient of the first strip and incubated at 37˚C for 24h. The MIC was measured for the second strip as the value at which the inhibition zone intersected the scale on the E strip. Then the second strip was used to measure its MIC in the same way as the first strip. The effect of antibiotic combinations whether synergy, indifference, addition, or antagonism was determined using the following equation: FIC index=FIC of drug A+FIC of drug B, where FIC of drug A=MIC of drug A in combination/MIC of drug A alone; and FIC of drug B=MIC of drug B in combination/MIC of drug B alone **(16).** Synergism was defined as an FIC index of ≤0.5, addition as an FIC index of 0.51–0.99, indifference as an FIC index of 1–2, and antagonism as an FIC index of >2.

Polymerase Chain Reactions: The genes coding for five different carbapenemases were detected among MDR GNB using two multiplex PCR reactions for each isolate according to **(17).**

Statistical analysis:
All data were collected, tabulated, and statistically analyzed using SPSS 26.0 for windows (SPSS Inc., Chicago, IL, USA). Quantitative data were expressed as the mean ± SD and range. Qualitative data were expressed as absolute frequencies (number) and relative frequencies (percentage). Percent of categorical variables were compared using Chi-square test. Multivariate logistic regression was done to detect the risk factors for developing infection by MDR GNB.

RESULTS
A total of 95 unrepeated different GNB were obtained from an equal number of ICU patients (n=95) in this study. The mean age of the studied patients was 42.91±16.85 years (range2 days-67 years)and more than half of them (57.9%) were females (n=55).Most cases (72.6%) were hospitalized for more than 14 days and all of them received antibiotic therapy upon ICU admission and were exposed to invasive procedures. However, most studied patients (78.9%) were not ventilated. Concerning the presence of comorbidities, most of the cases (75.8%) were diabetic and 68.4% were hepatic. About one-fourth (25.3%) were hypertensive and 13.7% suffered from renal disease.
When multivariate logistic regression analysis was applied, the results demonstrated that prolonged hospitalization and ventilator use remained to be significant risk factors (P 0.008 and 0.028, respectively) that increased the susceptibility to infection by MDR GNB (Table 1).

Table (1): Multivariate logistic regression analysis of risk factors for developing MDR GNB infection in the studied patients

Factors	B	SE	Wald	Sig.	OR	95% CI	
						Lower	Upper
Presence of multiple risk factors	-2.661	1.675	2.523	0.112	.070	0.003	1.864
Prolonged hospitalization (> 14 days)	4.204	1.588	7.006	0.008*	66.9	2.977	150.6
Ventilator use	2.425	1.103	4.837	0.028*	11.2	1.302	98.067
Chronic disease	1.126	0.686	2.691	0.101	3.0	0.803	11.833

*; significant

Abbreviations: B; coefficient for the constant, SE; standard error, Sig; significance, OR; Odds ratio, CI; confidence interval

The different species of isolated GNB and their distribution among different HAIs are demonstrated in Table (2).

Table (2): Types and distribution of isolated GNB species (n=95) among different HAIs infections

Organism	Type of Infection									
	Sepsis		Meningitis		Wound infection		LRTI		UTI	
	No.	%	No.	%	No.	%	No.	%	No.	%
E. coli (n=33)	2	6.1	0	0	0	0	3	9.1	28	84.8
Klebsiella spp.(n=27)	9	33.4	0	0	0	0	11	40.7	7	25.9
Pseudomonas spp.(n=13)	0	0	1	7.6	5	38.5	5	38.5	2	15.4
Citrobacter spp.(n=12)	2	16.7	0	0	3	25.0	3	25.0	4	33.3
Proteus spp.(n=4)	1	25.0	0	0	1	25.0	0	0	2	50.0
Acinetobacter spp. (n=4)	1	25.0	0	0	0	0	3	75.0	0	0
Enterobacter spp. (n=2)	0	0	0	0	0	0	1	50.0	1	50.0

Abbreviations: LRTI; lower respiratory tract infection, UTI; urinary tract infection

Table (3) shows that 59% and 53% of the isolated Enterobacteriaceae and non-Enterobacteriaceae, respectively exhibited the MDR phenotype. All Enterobacter isolates, most of Proteus and Acinetobacter isolates (75% for each), and more than half of E. coli, Klebsiella, and Citrobacter were MDR (57.6%, 55.6%, and 58.3%, respectively). Among all isolated GNB, the least MDR ratio was recorded in Pseudomonas isolates (46.2%).

Table (3): Frequency distribution of MDR among isolated GNB

	MDR GNB			
	YES N=55		NO N=40	
	No	(%)	No	(%)
Enterobacteriaceae (n=78)	46	59	32	41
E. coli (n=33)	19	57.6	14	42.4
Klebsiella spp. (n=27)	15	55.6	12	44.4
Citrobacter spp. (n=12)	7	58.3	5	41.7
Enterobacter spp. (n=2)	2	100.0	0	0.0
Proteus spp. (n=4)	3	75	1	25
Non-Enterobacteriaceae (n=17)	9	53	8	47
Pseudomonas spp. (n=13)	6	46.2	7	53.8
Acinetobacter spp. (n=4)	3	75.0	1	25.0

The results of in-vitro testing of antimicrobial combinations demonstrated that the use of colistin/amikacin had a slightly higher synergistic effect on MDR isolates (27.4%) compared to amikacin/aztreonam (23.6%). Both combinations resulted in an indifferent effect on most MDR isolates (63.4% and 70.9%, respectively). An antagonistic effect was recorded with a few strains (9.2% and 5.5%, respectively). The combination ceftazidime/avibactam was shown to inhibit more than half of MDR isolates recording a 61.8% sensitivity ratio (Table 4).

Table (4): Effect of the tested antibiotic combinations on MDR GNB

Antibiotic combination	Effect on MDR isolates (n=55)					
	Antagonism		Indifference		Synergy	
	No.	%	No.	%	No.	%
Colistin + Amikacin	5	9.2	35	63.4	15	27.4
Amikacin + Aztreonam	3	5.5	39	70.9	13	23.6
	Resistant		Sensitive			
Ceftazidime / Avibactam	No.	%	No.	%		
	21	38.2	34	61.8		

Upon comparing the synergistic / susceptibility effect of the three antibiotic combinations on various MDR GNB species, ceftazidime/avibactam had recorded higher susceptibility ratios with all tested MDR GNB isolates compared to the other two combinations. However, its effect was comparable to the synergistic effect exhibited by colistin/amikacin (66.7%) on Proteus isolates as well as to the effect of amikacin/aztreonam on Pseudomonas isolates (33.3%) (Table 5).

Table (5): Comparison of the synergistic/susceptibility effect of the three antibiotic combinations on various MDR isolates

	Synergistic effect				Susceptibility effect	
	Colistin/Amikacin		Amikacin / Aztreonam		Ceftazidime / Avibactam	
	No.	(%)	No.	(%)	No.	(%)
Acinetobacter spp. (n=3)	0	0	0	0	3	100
E. coli (n=19)	5	26.3	4	21.1	13	68.4
Klebsiella spp. (n=15)	5	33.3	5	33.3	8	53.3
Citrobacter spp. (n=7)	0	0	2	28.6	5	71.5
Enterobacter spp. (n=2)	0	0	0	0	1	50.0
Proteus spp. (n=3)	2	66.7	0	0	2	66.7
Pseudomonas spp. (n=6)	3	50	2	33.3	2	33.3

The frequency of the studied genes coding for carbapenemases in MDR isolates is demonstrated in Figure (1). A total of 29 isolates (52.7%) were found to harbor one or more genes. Nine isolates (16.4%) were found to harbor more than one carbapenemase gene. bla_{KPC} was the most frequently detected gene being present in 29.1% of examined isolates. This was followed by bla_{OXA}, bla_{VIM}, bla_{IMP}, and bla_{NDM} which were detected in 18.2%, 14.5%, 7.3%, and 3.6% of the isolates, respectively.

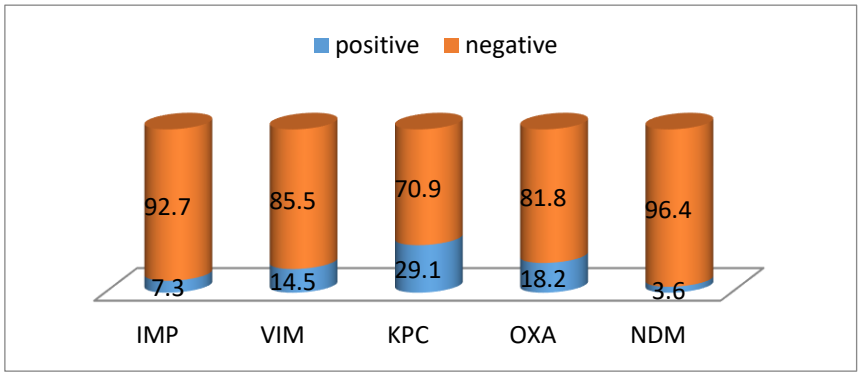

Figure (1): Bar chart showing the distribution of different carbapenemases genes among MDR GNB

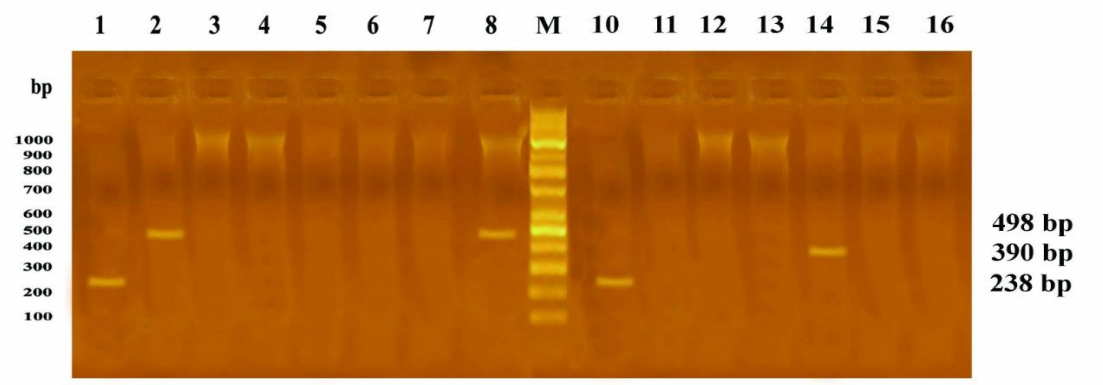

Figure (2): Gel electrophoresis of PCR products demonstrating bla_{KPC}, bla_{OXA}, and bla_{VIM} in MDR isolates.

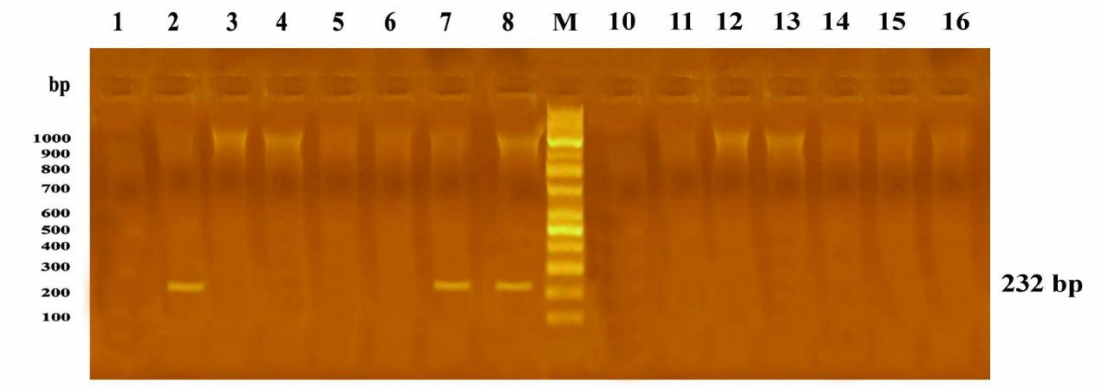

Figure (3): Gel electrophoresis of PCR products demonstrating bla_{IMP} in three three MDR isolates.

DISCUSSION

Over a period of three months, a total of 95 GNB were isolated from an equal number of ICU patients with different HAIs. A total of 57.9% of infections were noted in female patients compared to 42.1% in male patients. Higher male ratios ranging from 50.6% to 80% were noticed among ICU infections in previous studies **(18, 19, 20)**. The high incidence in females in the current study may be due to the high number of isolates from UTIs (46.3%) with a known higher prevalence in females. In addition, a higher number of females admitted to the ICU during the study period may have also participated.

The mean age of infected patients in the current study was 42.91±16.85 years. Similar or even younger ages (47.37, 38.7±14.9 years) have been reported previously **(21, 22)**. However, older ages (52.06±17.4, 63.1±16.5 years) were reported in other studies **(19, 20)**. This may reflect the population's health status as well as the provided health care in different studied localities.

Prolonged ICU stay can adversely affect the health status of patients and increase the rate of infections **(23)**. Most patients in this study (72.6%) were hospitalized for more than 14 days which is consistent with previous reports **(20, 24, 25)**.

Assessment of patients' comorbidity in the current study revealed that most of them were diabetic (75.8%) and/or hepatic (68.4%). Renal disease was detected in 13.7% of them. Diabetes and liver cirrhosis were the most frequent underlying medical conditions in bloodstream infection (23.1% and17.3%, respectively) and ventilator-associated pneumonia (33.3% and 10.4%, respectively) among HAIs in 13 ICUs from four tertiary care hospitals in Egypt **(26)**. A similar finding has been reported by (**20**). Chronic renal disease was reported as an important factor associated with ICU-acquired Gram-negative infections being noticed in 23.4% of infected patients in another study **(27)**.

The findings of the current study revealed that prolonged stay in the ICU (> 14 days) and ventilator use were significant risk factors for MDR GNB acquisition after multivariate regression analysis. A similar finding was reported previously **(22, 28)**. However, other factors such as antibiotic usage one month prior to ICU admission, urethral catheterization, tracheal intubation, weakened immune response, and old age > 60 years have been also reported **(29)**.

The distribution of the isolated GNB species according to the anatomical site of infection in the current study revealed that E. coli isolates were mostly obtained (84.8%) from UTI cases, Klebsiella spp. from LRTI (40.7%), Pseudomonas spp. from both LRTI and wound infection (38.5% for each), Citrobacter and Proteus spp. from UTI (33.3% and 50%, respectively), and Acinetobacter spp. from LRTI (75%).

The contribution of GNB species to different ICU infections varies slightly among different reports reflecting the effect of different ICU environments and patients' conditions on the endemicity and transmissibility of GNB. However, and consistent with the current findings, the highest isolation of E. coli from UTI in ICU patients was previously reported in several studies **(30, 31, 32)**. Similarly, the isolation of Klebsiella and Acinetobacter spp. from respiratory infections has been documented by **(33, 34)** respectively.

The MDR phenotype was noticed mostly in Enterobacter spp. (100%) in the current study, followed by Acinetobacter and Proteus spp. (75% for each), Citrobacter spp. (58.3%), E. coli (57.6%), Klebsiella spp. (55.6%) and the lowest rate was noticed with Pseudomonas isolates (46.2%). A comparable finding was reported in Nepal where all Enterobacter and Citrobacter isolates exhibited MDR phenotype, followed by Acinetobacter spp. (93%), Klebsiella spp. (86%), Pseudomonas spp. (84%), and then E. coli (64%) **(35)**. Acinetobacter spp. was the most frequently reported MDR GNB in other studies **(18, 36)**. However, in contrast to our finding, Pseudomonas isolates were reported to be the most frequent MDR GNB in the report of **(37)**.

Three antimicrobial combinations have been tested in the current study, among them, the combination ceftazidime/avibactam inhibited more than half of MDR isolates recording a 61.8% sensitivity ratio. Ceftazidime has a broad in-vitro activity against P. aeruginosa and Enterobacterales. However, its effect has become compromised by the increasing prevalence of isolates expressing ESBL, AmpC cephalosporinases, KPC, and MBL. The addition of avibactam, a non-beta lactam beta-lactamase inhibitor, restores the activity of the drug in-vitro against isolates expressing Ambler class A, C, and D beta-lactamases **(38, 39)**.

Sader and coworkers (40) evaluated the effect of ceftazidime/avibactam alone and in combination with other antibiotics and found that the drug alone exhibited a synergistic effect on 97.14% of KPC-producing K. pneumoniae. In accordance with the current study, ceftazidime/avibactam demonstrated a potent activity with 99.2% and 97.5% susceptibility ratios against MDR Enterobacteriaceae and carbapenem-resistant Enterobacteriaceae (CRE) isolates, respectively, and further demonstrated 86.5% susceptibility against MDR P. aeruginosa isolates.

However, other reports recorded higher resistance rates with XDR strains and strains resistant to carbapenems **(41, 42)**. A. baumannii exhibited much higher resistance compared to the other GNB particularly those obtained from ICUs (73.6% resistance rate) and even a complete resistance has been reported with A. baumannii that expresses bla_{OXA}-51-like beta-lactamase **(43, 44)**.

Colistin is a polypeptide antibiotic that affects gram-negative bacterial cell wall causing rapid bacterial killing in a concentration-dependent manner. However, major concerns exist about its safety **(45)**. Furthermore, its use as monotherapy could result in the emergence of heteroresistant phenotypes **(46)**. The addition of amikacin to colistin was found to augment the susceptibility to colistin in colistin-resistant E. coli and to increase the post-antibiotic effect of colistin when used against P. aeruginosa isolated from cystic fibrosis patients **(47, 48)**.

In the current study, the combination of colistin with amikacin was evaluated and found to have a synergistic effect on 66.7% of MDR Proteus isolates, 50% of MDR P. aeruginosa, and on nearly one third to one quarter of MDR Klebsiella and E. coli, respectively. However, no synergistic effect was recorded with Acinetobacter, Citrobacter, or Enterobacter isolates. Additionally, an antagonistic effect was detected with 33.3% of Acinetobacter isolates.

Colistin/amikacin combination was tested previously by **Banik and Shamsuzzaman (49)** on MDR E. coli both in-vivo and in-vitro. The in-vitro results demonstrated a synergistic effect on 75% of isolates while an additive effect was recorded with the remaining. Similarly, the combination has a

good synergistic effect against KPC-2-producing K. pneumoniae as well as against MDR K. pneumoniae isolates (72.72% synergistic effect) **(10, 50)**.

In the current study, the combination amikacin/aztreonam had a suboptimal synergistic effect on MDR isolates compared to the other two combinations. Compared to colistin/amikacin, it exhibited a lower synergistic effect on all examined isolates except with Citrobacter spp. where it had a synergistic effect on 28.6% of the isolates, whereas, colistin/amikacin combination did not record any synergistic effect with Citrobacter isolates.

The combination of aztreonam with aminoglycosides was evaluated in-vitro against MDR P. aeruginosa in Japan. The addition of aminoglycosides, including amikacin, was able to decrease the MIC of aztreonam in a dose-dependent manner and no apparent antagonism was detected **(51)**. In contrast to our finding, **(52)** reported that amikacin/aztreonam was the most effective two-drug combination inhibiting the proliferation of five of seven MDR P. aeruginosa isolates.

The difference in the method used to assess the antibiotic combination effect and strain-dependent factors may have contributed to the variabilities reported in the previous studies and the current study.

Due to high rate of carbapenem resistance, five carbapenemase genes have been screened in MDR isolates in the current study using PCR. They included the Ambler class A enzyme KPC, the MBL enzymes VIM, IMP, and NDM, and the class D enzyme OXA.

A total of 29 MDR isolates (52.7%) were found to harbor one or more genes. KPC and OXA were the most frequently detected carbapenemase genes in MDR isolates in the current study, recording frequencies of 29.1% and 18.2%, respectively. The relatively high ratio of MDR isolates having OXA gene is of great concern due to its difficult detection in phenotypic tests as well as its association with treatment failure. As expected, most of the isolates having OXA were belonging to Acinetobacter spp. (66.7%).KPC has been detected in MDR isolates in several previous reports including reports from Egypt **(53)**, however, most reports had found it one of the least detected genes in MDR GNB isolates **(54, 55)** which comes in contrast to the current study. In a previous study that involved three tertiary care hospitals in Egpyt, **El- Defrawy and coworkers (56)** reported that among carbapenem-resistant isolates, OXA-48 dominated in K. pneumoniae (40.6%) while NDM-5 gene was the most frequent among E. coli (9.6%).

A total of 9 MDR isolates (16.4%) harbored more than one carbapenemase gene in the current study. The coexistence of carbapenemase genes aggravates the therapeutic challenge as it limits the therapeutic options and is further associated with more horizontal spread among bacteria. The coexistence of carbapenemase genes was previously reported in different countries including Egypt **(56, 57)**.

However, this study is not without limitations as the lack of detection of other resistance mechanisms that are commonly associated with multidrug resistance in GNB as extended-spectrum beta-lactamases (EBSL) and the relatively small sample size.

CONCLUSION

The incidence of multi-drug resistant Gram-negative bacterial infections is remarkably high in Al-Ahrar intensive care unit with a high prevalence of carbapenemase genes. Compared to colistin/amikacin and amikacin/aztreonam, ceftazidime/avibactam had higher in-vitro performance and recorded higher sensitivity ratios with tested MDR GNB isolates.

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