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Original article

Colistin and carbapenem resistance Among *Pseudomonas* and *Acinetobacter* clinical isolates in Menoufia University Hospitals

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ABSTRACT

Background and aim: Colistin and carbapenem-resistant *Pseudomonas* and *Acinetobacter* isolates can cause severe infections. We aimed to determine the prevalence of colistin and carbapenem resistance in *Pseudomonas* and *Acinetobacter* nosocomial isolates and investigate the underlying mechanisms. **Methods:** The antimicrobial susceptibility of *Pseudomonas* and *Acinetobacter* isolates was tested through disk diffusion method, while colistin resistance was tested by agar dilution method. Extended spectrum-β-lactamases (ESβLs) production was tested using the combined disk method, and carbapenemase production was tested phenotypically (using modified carbapenem inactivation method (mCIM) and Imipenem/EDTA combined disc diffusion test). Genotypic analysis detected carbapenemase genes. Colistin-resistant isolates were investigated for efflux pump mechanisms using the carbonyl cyanide 3-chlorophenylhydrazone (CCCP). **Results**: Fifty *Pseudomonas* and thirty *Acinetobacter* isolates were isolated from the collected samples. Approximately, 44% of *Pseudomonas* and 43.3% of *Acinetobacter* isolates produced ESβLs. Carbapenemase production was found in 38% of *Pseudomonas* and 40% of *Acinetobacter* isolates while 28% and 23.3% produced metallo-β-Lactamases (MβLs). Colistin resistance was detected in 14% of *Pseudomonas* and 10% of *Acinetobacter* isolates. CCCP reduced colistin MIC by ≥8 folds in 85.7% and 100% of colistin-resistant *Pseudomonas* and *Acinetobacter* isolates, respectively. The carbapenemase genes *bla* NDM, *bla* VIM-2 and *bla* IMP-1 were found in 33.3%, 16.7% and 6.7% of *Pseudomonas* isolates, and in 25%, 15% and 5% of *Acinetobacter* isolates, respectively. **Conclusion:** *Pseudomonas* and *Acinetobacter* isolates showed resistance to multiple antibiotics. Carbapenemase production shows challenges for effective treatment. Efflux pump inhibitors exhibited potential in reversing colistin resistance emphasizing the need to avoid unnecessary clinical use of colistin.

Introduction

Antibiotics are at the forefront of the battle against infectious bacteria. The use of antibiotics has changed the outcome of bacterial infections and saved millions of lives [1]. However, disease-causing microbes have become increasingly resistant to the commonly used antibiotics so antimicrobial resistance has become a global crisis [2].

The microorganisms that are mainly involved in antibiotic resistance called ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus*

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aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and *Enterobacteriaceae*) which are capable of "escaping" from common antibacterial treatments. *Pseudomonas* and *Acinetobacter* spp. are the most challenging due to their particular antibiotic resistance characteristics [3].

Carbapenems have broad-spectrum activity against various bacteria and are widely regarded as the last resort for treatment of infections with multidrug-resistant (MDR) pathogens. However, resistance to carbapenems has dramatically increased worldwide causing a rise in healthcare costs and worsening clinical outcome [4]. The main mechanism of carbapenem resistance is hydrolysis of carbapenems with carbapenem-hydrolyzing enzymes, carbapenemases like MβLs, and oxacillinases. MβLs are classes of powerful enzymes called carbapenemases responsible for carbapenem-antibiotic resistance. Four groups of these enzymes have been described in *Pseudomonas* and *Acinetobacter*, including IMP-like, SIM-1, NDM-type, and VIM-like carbapenemases. MβLs-encoding genes are located on integrons that can be transmitted from one bacterial species to another [5].

As a result of spread of carbapenem resistance, colistin sulphate became the last resort for treatment of infections with carbapenemase-producing bacteria. Therefore, the world health organization (WHO) classified colistin as an important for human medicine [6]. Colistin resistance mechanisms are complex and involve many genes that have not yet been fully identified. One of these mechanisms is efflux pump mechanisms. Many efflux pumps have been identified as reducing colistin susceptibility, such as KpnEF and the AcrAB–TolC complex. Various efflux pump inhibitors (EPIs) have been tested on Gram-negative bacteria such as CCCP which acts as an uncoupler of oxidative phosphorylation which disrupts the ionic gradient of bacterial membranes. This inhibitor compound has been effectively used in addition to colistin to improve susceptibility to this antibiotic. It also makes the bacterial cells metabolically inactive giving rise to the debate whether the synergistic effect that CCCP shows with a range of antibiotics is actually a consequence of efflux pump inactivity or metabolic inactivity of the cells [7, 8].

Our study aimed to determine the prevalence of carbapenem and colistin resistance in hospital-acquired *Pseudomonas* and *Acinetobacter* isolates and investigate the underlying mechanisms.

Patients and methods

This cross sectional study was conducted in the Medical Microbiology and Immunology department, Faculty of Medicine, Menoufia University during the period from April, 2021 till March, 2023. Ethical approval was obtained from the local Ethics Committee of the Faculty of medicine, Menoufia University (3/2021 MICR30). Written informed consents were obtained from the study participants before involvement in the study. The study was conducted according to Helsinki Declaration. Full patient history (especially duration of hospitalization, previous antibiotic administration or exposure to invasive procedure and associated co-morbidities) was obtained from the patients admitted to different hospital departments and ICUs with different types of infections that manifested after 48 hours of admission.

The sample size is calculated as a total of 45 samples of *Pseudomonas* isolates are required. Accounting for a drop-out of 10%, a total of 50 specimens are required.

Specimens' collection and isolation of *Pseudomonas and Acinetobacter* species

A total of 285 clinical samples (urine, pus, sputum, endotracheal aspirate, burn and wound swabs and blood) were collected, processed and cultured on standard commonly-used bacteriological media. *Pseudomonas* and *Acinetobacter* isolates were identified by standard microbiological methods and confirmed up to species level by Vitek2 compact system [9].

Inclusion and exclusion criteria

The study included patients of all ages and genders who had been admitted for more than 48 hours and were suspected of having a nosocomial infection (such as wound infections, urinary tract infections, blood stream infections and respiratory infections). Patients who did not provide complete data and acceptable specimens, as well as those who had insufficient volume of specimens were excluded.

All the obtained *Pseudomonas* and *Acinetobacter* species were subjected to:

1-Antimicrobial susceptibility test

Antimicrobial susceptibility test was performed for all *Pseudomonas* and *Acinetobacter* isolates using the Kirby-Bauer technique (disk diffusion method) on Muller-Hinton agar (MHA) plates against

different antimicrobial agents and interpreted according to the guidelines of CLSI, 2022 [10]. The following antimicrobial agents (Oxoid, England) were used: piperacillin (PRL 100 µg), piperacillin/tazobactam (TZP, 100/10 µg), ceftazidime (CAZ, 30 µg), ceftazidime-clavulanic acid (CAC, 30/10µg), ceftriaxone (CRO, 30 µg), cefotaxime (CTX, 30 µg), cefepime (CPM, 30 µg), meropenem (MEM, 10 µg), imipenem (IPM, 10 µg), doripenem (DOR, 10µg), aztreonam (ATM, 30µg), amikacin (AK, 30 µg), gentamicin (GM, 10 µg), tobramycin (TOB, 10 µg), doxycycline (DOX, 30 µg), ciprofloxacin (CIP, 5 µg), levofloxacin (LEV, 5 µg), norfloxacin (NOR, 5 µg), ofloxacin (OFX, 5 µg), ampicillin- sulbactam (SAM, 10/10 µg).

2-Phenotypic detection of extended-spectrum beta-lactamase (ESβL)-producing isolates

All *Pseudomonas* and *Acinetobacter* isolates were phenotypically screened for ESβL production and confirmed by combined disc method. In cases where the inhibition zone around ceftazidime-clavulanic acid discs was at least 5 mm greater than the one around the ceftazidime discs without clavulanic acid, the relevant isolate was considered as ESβL-producing strain [11].

3-Phenotypic detection of carbapenemase production

a-Modified carbapenem inactivation method (mCIM)

The modified carbapenem inactivation method (mCIM) was applied to confirm carbapenemase production when the tested isolate was non-susceptible to at least one of the carbapenems. The mCIM-positive results were interpreted by measuring the zone of inhibition around the meropenem disk. Results were considered positive if the zone of inhibition was 6 to 15mm or 16 to 18mm with pinpoint colonies within the zone. If the zone of inhibition was 16 to 18mm or ≥19mm in diameter, mCIM was considered indeterminate or negative, respectively [10, 12, 13].

b-Imipenem/EDTA combined disk diffusion test

Imipenem/EDTA combined disk diffusion test was used to detect class B carbapenemase (MβLs). After adjusting the turbidity of the tested organism to 0.5 McFarland standard, it was spread on the surface of MHA plates. Then, two imipenem disks (10 µg; one of them contained10µl of 0.5 M EDTA) were placed on the agar 15 mm apart (center-to-center). After incubating at 37°C overnight, an increase in inhibition zone diameter of ≥7 mm in the EDTA-supplemented disk was interpreted as positive for metallo-β-lactamase production [14, 15].

4-Effect of efflux pump inhibitor (CCCP) on colistin MIC by using the agar dilution method

Two sets of MHA (Oxoid, England) were prepared: the first with colistin only (Sigma Aldrich; code: C4461-100MG), the second with colistin and CCCP (Sigma Aldrich; code: C2759). Colistin concentrations ranged from 0.125 to 128µg/ml. Resistance to colistin was considered if the MIC is ≥ 4µg/mL according to the standard guidelines of CLSI [10].

A stock solution of CCCP was prepared at 5mg/mL in DMSO. The final concentration of CCCP in the MHA was 0.01mg/mL with a DMSO concentration of 0.2%. The resulting MIC fold changes after the addition of CCCP were calculated as the ratio of the CCCP-free antibiotic's MIC level to that of the CCCP-added antibiotic. The positive criterion for the presence of efflux pumps in isolates was an ≥ 8-fold decrease in colistin MIC after adding CCCP. The mean fold change was calculated [8].

5-Molecular characterization of carbapenem resistance (*bla*$_{\text{NDM-1}}$, *bla*$_{\text{IMP-1}}$, *bla*$_{\text{VIM-2}}$ genes) by multiplex PCR

Bacterial DNA was extracted and purified from 30 *Pseudomonas* and 20 *Acinetobacter* isolates (30 *Pseudomonas* isolates: 19 positive mCIM and 11 sensitive to carbapenems, 20 *Acinetobacter* isolates: 12 positive mCIM and 8 sensitive to carbapenems) using the gene JET™ genomic DNA purification kit (Thermo Fisher Scientific, UK). The used primers (Invitrogen, Thermo Fisher, UK) sequences and amplicon sizes are illustrated in the following table:

The PCR program for the amplification of these genes included the initial denaturation at 95oC for 10 min and 35 cycles of 94oC for 1.5 min, primer annealing at 58oC for 1min and polymerization at 72 °C for 1min. A final polymerization step was set at 72 °C for 7 min [17]. The products were visualized by electrophoresis on ethidium bromide-stained (Sigma, USA) 1.5% agarose gel (Thermo Fisher scientific, USA). The products were visualized by UV transilluminator and compared with 100-1000 bp ladder (Thermo Fisher scientific, USA) [19].

Statistical analysis

Data were collected, tabulated and analyzed by statistical package for the social science (SPSS version 20, SPSS inc., Chicago, Illinois, USA) software. Chi-square test (X2) at 5% level of

significance and Kappa test was done substantial agreement at 0.61 – 0.80. Accuracy was represented using the terms sensitivity, specificity, positive predictive value, negative predictive value and overall accuracy.

Results

A total of 50 *Pseudomonas* and 30 *Acinetobacter* isolates were collected from 285 different clinical samples collected from patients with different types of hospital-acquired infections. The highest isolation rate of *Pseudomonas* spp. was from burn swabs (38%) followed by urine samples (34%), while *Acinetobacter* was mostly isolated from urine samples (33.3%), followed by burn swabs (23.3%) as shown in **figure (1).** Also, the highest isolation rates of *Pseudomonas* spp. were from burn unit (34%), followed by ICUs (28%) while the highest isolation rates of *Acinetobacter* spp. were from ICUs (33.3%), followed by burn unit (20%) as shown in **figure (2).**

By Vitek2 compact system, *P. aeruginosa* was the predominant *Pseudomonas* spp. representing 90% (45/50) followed by *P. putida* (8%) and *P. protegens* (2%). While *Acinetobacter baumannii (A. baumannii)* was the most predominant spp. representing 93.3% (28/30) followed by *A. pitti* 6.7% (2/30).

Both *Pseudomonas* and *Acinetobacter* spp. exhibited high antimicrobial resistance rates to almost all the tested antimicrobial drugs as shown in **table (1).** *Pseudomonas* isolates were highly resistant to both norfloxacin and ofloxacin (76%, for each), while *Acinetobacter* isolates showed the highest resistance to piperacillin (83.3%) followed by levofloxacin (73.3%). On the other hand, the lowest resistance rate for both *Pseudomonas* and *Acinetobacter* isolates was against meropenem (36% and 40%, respectively) as shown in **table (1).**

In our study, 24/50 (48%) of *Pseudomonas* spp. and 12/30 (40%) of *Acinetobacter* spp. were MDR while 13/50 (26%) of *Pseudomonas* and 11/30 (36.7%) of *Acinetobacter* were XDR.

ESβL production was phenotypically confirmed by cephalosporin/clavulanate combination disk test in 44% and 43.3% of *Pseudomonas* and *Acinetobacter* isolates, respectively as shown in table (1). Regarding carbapenemase production, disk diffusion screening method revealed that 24/50 (48%) of *Pseudomonas* isolates were carbapenemase-producers and 15 out of 30 (50%) of *Acinetobacter* isolates were carbapenemase-producers. On the other hand, only 19 (38%) and 12 (40%) of *Pseudomonas* and *Acinetobacter* isolates were confirmed by modified carbapenem inactivation test to be carbapenemase-producers. There was substantial agreement (Kappa test= 0.80) between the two methods in *Pseudomonas* and *Acinetobacter* isolates **(Table 2).** Compared to the screening test, MβLs production was confirmed in only 14 (28%) and 7 (23.3%) of *Pseudomonas* and *Acinetobacter* isolates, respectively by the combined imipenem/EDTA synergy disk test as shown in **table (1).**

MIC of colistin sulphate by agar dilution method showed that only 14% and 10% of *Pseudomonas* and *Acinetobacter* isolates, respectively were resistant to colistin with MIC ranging from 4-64 μg/ml for *Pseudomonas* and 8-64 μg/ml for *Acinetobacter* isolates **(Table 3).** The addition of CCCP efflux pump inhibitor decreased MIC of colistin by ≥8 folds in 85.7% and 100% of colistin-resistant *Pseudomonas* and *Acinetobacter* isolates, respectively. The mean MIC of colistin before and after CCCP addition was (26.9 and 0.61) for *Pseudomonas* and (34.7 and 0.3) for *Acinetobacter* isolates as shown in **table (2).**

Multiplex PCR showed that *bla* NDM, *bla* VIM-2 and *bla* IMP-1 genes were detected in 33.3%, 16.7% and 6.7%, respectively from the tested *Pseudomonas* isolates and 25%, 15% and 5% in *Acinetobacter*, respectively **(Figure 3 and Table 3).**

Considering PCR as the gold standard, the modified carbapenem inactivation test in *Pseudomonas* isolates, had sensitivity, specificity, PPV, NPV and accuracy of 93.33%, 66.67%, 73.68%, 90.91% and 80%, respectively, while in *Acinetobacter* isolates, the percentages were 87.5%, 58.33%, 58.33%, 87.5% and 70%, respectively. On the other hand, the combined imipenem/EDTA synergy test had (86.67%, 87.5%) sensitivity, (93.33%, 100%) specificity, (92.86%, 100%) PPV, (87.5%, 92.31%) NPV and (90%, 95%) accuracy in detecting MβLs-production in *Pseudomonas* and *Acinetobacter* isolates, respectively as shown in **table (4).**

Table 1. Primer sequence and amplicon sizes

Primer name	Sequence	Product size (bp)	Reference
***bla*NDM-1**	F : TTGGCGATCTGGTTTTCC R : GGTTGATCTCCTGCTTGA	195	[16]
***bla*IMP-1**	F: ACC-GCA-GCA-GAC-TCT-TTG-CC R: ACA-ACC-AGT-TTT-GCC-TTA-CC	587	[17]
***bla*VIM-2**	F: GATGGTGTTTGGTCGCATA R: CGAATGCGCAGCACCAG	390	[18]

Table 2. Antimicrobial susceptibility patterns and screening and confirmatory phenotypic methods used for detection of ESβLs and carbapenemase production among *Pseudomonas* and *Acinetobacter* isolates.

	***Pseudomonas* isolates (No = 50)**						***Acinetobacter isolates* (No = 30)**					
1- Disk diffusion method	**S***		**I***		**R***		**S***		**I***		**R***	
	NO.	**%**	**NO.**	**%**	**NO.**	**%**	**NO.**	**%)(**	**NO.**	**%**	**NO.**	**%**
Piperacillin	13	26	7	14	30	60	3	(10)	2	6.7	25	83.3
Piperacillin-tazobactam	20	40	6	12	24	48	8	(26.7)	6	20	16	53.3
Ampicillin-sulbactam	-	-	-	-	-	-	6	20	5	16.7	19	63.3
Ceftazidime	14	28	0	0	36	72	10	33.3	2	6.7	18	60
Cefipime	19	38	5	10	26	52	5	16.7	5	16.7	20	66.7
Cefotaxime	-	-	-	-	-	-	7	23.3	6	20	17	56.6
Ceftriaxone	-	-	-	-	-	-	9	30	0	0	21	70
Aztreonam	19	38	10	20	21	42	-	-	-	-	-	-
Doripenem	19	38	5	10	26	52	11	36.7	2	6.7	17	56.7
Imipenem	21	42	5	10	24	48	12	40	3	10	15	50
Meropenem	26	52	6	12	18	36	14	46.7	4	13.3	12	40
Gentamicin	12	24	1	2	37	74	7	23.3	3	10	20	66.7
Tobramycin	14	28	3	6	33	66	7	23.3	4	13.3	19	63.3
Amikacin	18	36	5	10	27	54	11	36.7	1	3.3	18	60
Ciprofloxacin	8	16	7	14	35	70	7	23.3	2	6.7	21	70
Levofloxacin	9	18	4	8	37	74	8	26.7	0	0	22	73.3
Norfloxacin	9	18	3	6	38	76	-	-	-	-	-	-
Ofloxacin	8	16	4	8	38	76	-	-	-	-	-	-
Doxycycline	-	-	-	-	-	-	12	40	5	16.7	13	43.3
2- Phenotypic tests to detect ESβL production	**Positive**		**Negative**				**Positive**		**Negative**			
	NO.	**%**	**NO.**	**%**			**NO.**	**%**	**NO.**	**%**		
Disc diffusion method	36	72	14	28			21	70	9	30		
Combined disc test	22	44	28	56			13	43.3	17	56.7		
X^2	**12.54**						**4.34**					
P value	**<0.001**						**0.04 (<0.05)**					
3- Phenotypic tests to detect carbapenemase production	**Positive**		**Negative**				**Positive**		**Negative**			
	NO.	**%**	**NO.**	**%**			**NO.**	**%**	**NO.**	**%**		
Disk diffusion method	24	48	26	52			15	50	15	50		
Modified carbapenem inactivation test	19	38	31	62			12	40	18	60		
Symmetrical measurement	**Kappa test =0.80**						**Kappa test =0.80**					

*Ampicillin-sulbactam, cefotaxime, ceftriaxone and doxycycline are recommended by CLSI 2022 for *Acinetobacter* but not for *Pseudomonas.*

*Azetreonam, norfloxacin and ofloxacin are recommended by CLSI 2022 for *Pseudomonas* but not for *Acinetobacter* .

Table 3. Effect of efflux pump inhibitor (CCCP) on MIC of colistin-resistant *Pseudomonas* and *Acinetobacter* isolates.

No.	Bacterial spp.	MIC of colistin	MIC of colistin + CCCP	*MIC fold change
Colistin-resistant *Pseudomonas* isolates (NO.=7)				
1-	***P. aeruginosa***	4	≤ 0.125	≥32
2-	***P. aeruginosa***	8	2	4
3-	***P. aeruginosa***	16	≤ 0.125	≥ 128
4-	***P. aeruginosa***	16	0.25	64
5-	***P. aeruginosa***	16	0.25	64
6-	***P. aeruginosa***	64	1	64
7-	***P. aeruginosa***	64	0.5	128
Mean MIC		26.9	0.61	69.1
decreased MIC by ≥8 folds		**6 isolates (85.7%)**		
Colistin-resistant *Acinetobacter* isolates (NO.= 3)				
1-	***A. baumanii***	8	≤ 0.125	64
2-	***A. baumanii***	32	0. 5	64
3-	***A. baumanii***	64	0.25	256
Mean MIC		34.7	0.3	128
decreased MIC by ≥8 folds		**3 isolates (100%)**		

*The MIC fold changes was calculated as the ratio of the CCCP-free antibiotic's MIC level to that of the CCCP-added antibiotic.
-The mean fold change was calculated by the following equation:
{1/total sample size (n)} x sum (MIC fold change x frequency of fold change ([10].

Table 4. Detection rate of bla_{NDM}, bla_{VIM-2} and bla_{IMP-1} among the tested *Pseudomonas* and *Acinetobacter* isolates by conventional multiplex PCR.

Genotype	Tested *Pseudomonas* isolates (NO.= 30)		Tested *Acinetobacter* isolates (NO.=20)	
	Positive NO. (%)	**Negative NO. (%)**	**Positive NO. (%)**	**Negative NO. (%)**
Single gene				
***bla*NDM**	8 (26.7)	22 (73.3)	4 (20)	16 (80)
***bla*VIM-2**	4 (13.3)	26 (86.7)	2 (10)	18 (90)
***bla*IMP-1**	1 (3.3)	29 (96.7)	1 (5)	19 (95)
Combined genes				
***bla*NDM** and ***bla*VIM-2**	1 (3.3)	29 (96.7)	1(5)	19 (95)
***bla*NDM** and ***bla*IMP-1**	1(3.3)	29 (96.7)	0	20 (100)
Total detected genes				
***bla*NDM**	10 (33.3)	20 (66.7)	5 (25)	15 (75)
***bla*VIM-2**	5 (16.7)	25 (83.3)	3 (15)	17 (85)
***bla* IMP-1**	2 (6.7)	28 (93.3)	1(5)	19 (95)

Table 5. Sensitivity and specificity of combined imipenem/EDTA Synergy and modified carbapenem inactivation test in relation to PCR among *Pseudomonas* and *Acinetobacter* isolates.

imipenem/EDTA synergy test	Multiplex PCR for detection of MβL production					
	***Pseudomonas* isolates (NO.=30)**			***Acinetobacter* isolates (NO.=20)**		
	total NO (30)	**Positive (15)**	**Negative (15)**	**total NO. (20)**	**Positive (8)**	**Negative (12)**
		NO.	**NO.**		**NO.**	**NO.**
Positive	14	13	1	7	7	0
Negative	16	2	14	13	1	12
Sensitivity	86.67%			87.5%		
Specificity	93.33%			100%		
Positive predictive value (PPV)	92.86%			100%		
Negative predictive value (NPV)	87.5%			92.31%		
Accuracy	90%			95%		
Modified carbapenem inactivation test	***Pseudomonas* isolates (NO. =30)**			***Acinetobacter* isolates (NO. =20)**		
	Total NO. (30)	**Positive (15)**	**Negative (15)**	**Total NO. (20)**	**Positive (8)**	**Negative (12)**
		NO.	**NO.**		**NO.**	**NO.**
Positive	19	14	5	12	7	5
Negative	11	1	10	8	1	7
Sensitivity	93.33%			87.5%		
Specificity	66.67%			58.33 %		
Positive predictive value (PPV)	73.68%			58.33%		
Negative predictive value (NPV)	90.91%			87.5%		
Accuracy	80%			70%		

Figure 1. Distribution of *Pseudomonas* and *Acinetobacter* spp. according to type of clinical samples

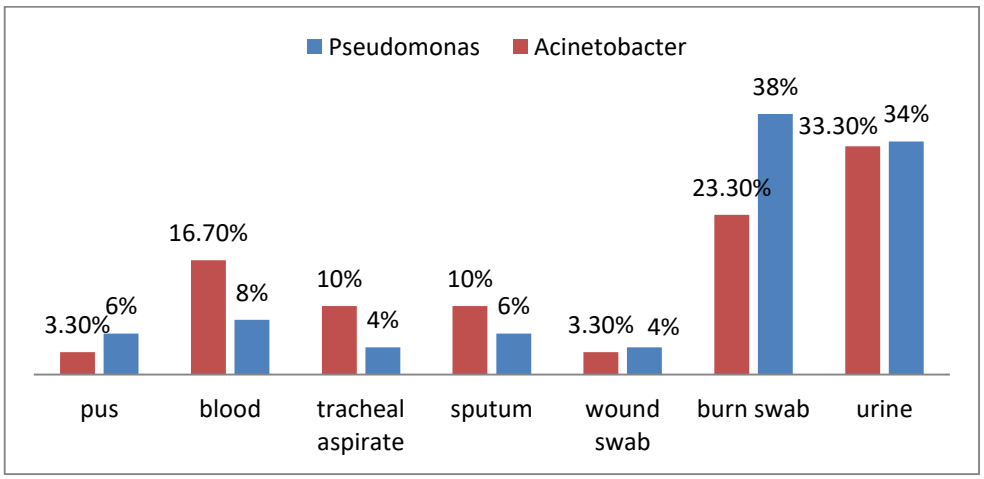

Figure 2. Distribution of *Pseudomonas* and *Acinetobacter* spp. among different hospital departments.

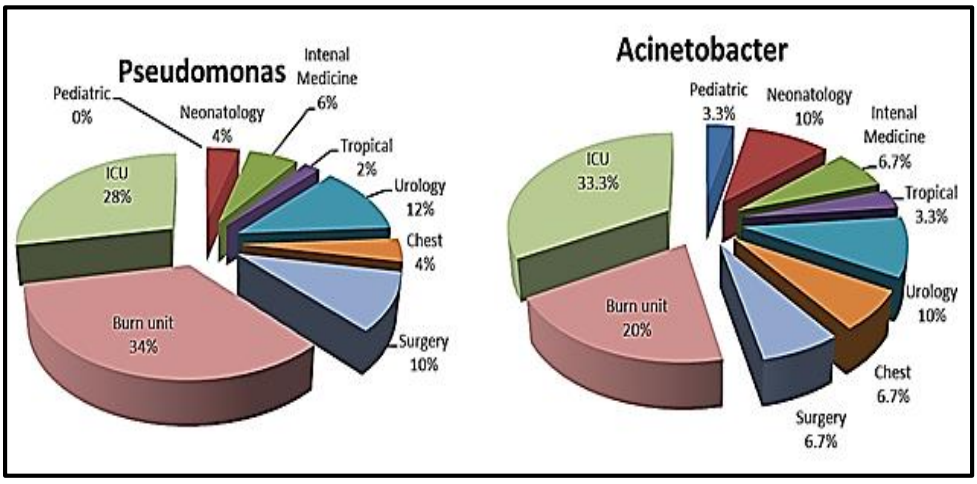

Figure 3. Agarose gel electrophoresis for the amplified products of *bla* NDM, *bla* VIM-2 and *bla* IMP-1 genes.

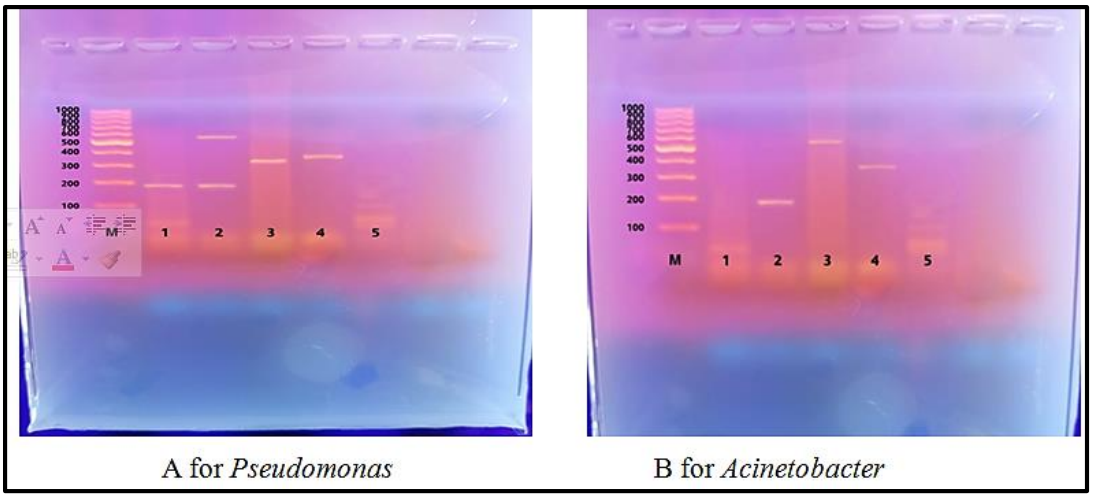

Figure (A): Multiplex PCR-amplified products of *Pseudomonas* isolates. Lane M (ladder): DNA molecular size marker (100-1000 bp). Lane1 was positive for *bla* NDM (bp 195). Lane 2 was positive for both bla_{NDM} (bp 195) & bla_{IMP-1}(bp 587). Lane 3 was positive for mcr-1 colistin resistance gene for further investigations. Lane 4 was positive for bla_{VIM-2} (bp 390). Lane 5 was negative.
Figure (B): Multiplex PCR-amplified products of *Acinetobacter* isolates. Lane 2 was positive for bla_{NDM} (bp 195). Lane 3 was positive for bla_{IMP-1} (bp 587). Lane 4 was positive for bla_{VIM-2} (bp 390). Lanes 1 and 5 were negative.

Discussion

Antibiotic resistance became a crisis that has been attributed to overuse and misuse of these medications, as well as lack of new drug development. Carbapenems are often the antimicrobials of last resort to treat infections due to ESβLs or AmpC-producing organisms. But unfortunately organisms began to develop resistance to them by several mechanisms like carbapenemase production. After emergence of carbapenem-resistant organisms, colistin became the main agent to treat these infections [20].

Our study indicated that the highest rate of *Pseudomonas* isolation was observed in burn swabs (38%), followed by urine samples (34%). *Pseudomonas aeruginosa* emerged as the predominant species of *Pseudomonas* accounting for 90% of the isolates. Other species were also identified as previously reported in other studies **Addis et al.** [21] and **Kumari et al.** [22]

For *Acinetobacter* spp., our study revealed that the highest isolation rate was found in urine samples (33.3%), followed by burn swabs (23.3%) and blood samples (16.7%). Similar findings were reported in Egypt **Hassan et al.** [23] and Iran **Moulana et al.** [24] Our study showed that *A. baumannii* was the most prevalent species, representing 93.3%, as previously demonstrated by Bitew [25] and **Kumari et al.** [26]

Regarding the antimicrobial susceptibility pattern, our study revealed high resistance rates of *Pseudomonas* isolates to both norfloxacin and ofloxacin (76% for each), while the lowest resistance rates were observed against aztreonam (42%) and meropenem (36%). These findings are consistent with that reported by Fahim [27] and **Shebl et al.** [28] in Egypt. Our study also showed that 83.3% and 73.3% of *Acinetobacter* isolates were resistant to piperacillin and levofloxacin, respectively, while only 40% of isolates were resistant to meropenem. Similar results were reported by Bitew [25] and **Halim et al.** [29]

About 44% of *Pseudomonas* isolates in this study were confirmed to be ESβL-producers. This finding is similar to that reported by **Tilahun et al.** [12] and Bitew [25], who observed that 43.5% and 53.8% of *Pseudomonas* isolates exhibited ESβl-production. However, a higher rate of ESβL production (59%) [11] was reported among *Pseudomonas* isolates. Regarding *Acinetobacter*, our study revealed that 43.3% of the isolated strains exhibited ESβL production. This finding aligns with that reported in Ethiopia by **Tilahun et al.** [12], who showed that 41.3% of *Acinetobacter* isolates were ESβLs-producers.

Carbapenemase-mediated carbapenem resistance poses a significant concern due to its profound impact on the available options for effective anti-infective strategies [30]. In accordance with previous studies, we found that 38% of *Pseudomonas* isolates and 40% of *Acinetobacter* isolates were carbapenemase producers by mCIM. Similarly, 41% of *Acinetobacter* isolates were reported as positive mCIM26. However, **Jing et al.** [31] did not identify any *Acinetobacter* isolate to be carbapenemase producers using the mCIM. To confirm the production of MβLs in this study, the imipenem/EDTA synergy test was performed as a phenotypic confirmatory assay. Our results demonstrated that 28% of *Pseudomonas* isolates were found to be MβL producers, a finding which is consistent with that reported by **Gautam et al.** [14] and **Kaur et al.** [15]. On the other side, 23.3% of *Acinetobacter* isolates were confirmed as MβL-producers by imipenem/ EDTA synergy test. Similar results were reported by Moulana et al. [24] who found that about 30% of *Acinetobacter* isolates were MBL producers.

Regarding colistin MIC, our findings revealed that 14% of *Pseudomonas* isolates were colistin-resistant by agar dilution method. Similar results were reported by **Abd El-Baky et al.** [32] in Egypt and **Ni et al.** [33] in China. However, higher rates were recorded by Ibrahim, [34] in Saudi Arabia. On the other hand, Mohamed et al. [35] in Egypt didn't report any colistin-resistant *Pseudomonas.* Resistance to colistin was found in only 10% of *Acinetobacter* isolates in our study, a result matched with that reported by **Moulana et al.** [24] and **Vakili et al.** [36]. The difference in colistin resistance rates results from the diversity of geographical regions, patients, general conditions, the study population, type of collected samples, adherence to infection control measures, implementation of antibiotic stewardship programs and the used antibiotic [37].

Because of the importance of efflux pumps in mediating antibiotic resistance, the efflux abolishment could be achieved by using chemical substances called efflux pump inhibitors [37]. Our results revealed that the use of CCCP decreased MIC of colistin by ≥8 folds in 85.7% and 100% of colistin-resistant *Pseudomonas* and *Acinetobacter* isolates, respectively confirming the presence of the efflux pump mechanism of colistin sulphate resistance. Likewise, the addition of CCCP led to reversed colistin resistance (MIC≤ 2mg/L) in all the colistin-resistant *Pseudomonas* and *Acinetobacter* studied strains. The mean fold change was (69.1 and 128) for *Pseudomonas* and *Acinetobacter* isolates, respectively. In agreement with this finding, one study reported that all the colistin-resistant *Pseudomonas* isolates became intermediate sensitive after adding CCCP where the mean fold change was 682.7% and all the tested isolates showed decreased MIC of colistin by ≥8 folds [38]. **Ni et al.** [33] in China also demonestrated that all the colistin-resistant *Pseudomonas* and *Acinetobacter* isolates became sensitive after adding CCCP. **Sekyere et al.** [8] in Ghana reported that CCCP reversed resistance to colistin in 94% of isolates and reduced colistin MIC by several folds in almost all the isolates and only 6% of isolates had no change at all after adding CCCP. However, a study in Egypt found that only 18.75% of colistin-resistant *Pseudomonas* isolates showed a reduction in the MICs of colistin ≥ 8-fold in the presence of CCCP [32].

Regarding screened antibiotic resistance genes in *Pseudomonas* isolates, our study revealed that 33.3% carried the bla_{NDM} gene, 16.7% carried the $bla_{\text{VIM-2}}$ gene and 6.7% carried the $bla_{\text{IMP-1}}$ gene.

These results align with that reported by **Ramadan et al.** [39] in Egypt who showed that the prevalence of *bla*$_{NDM}$ was 27.3% but higher rate of both *bla*$_{VIM-2}$ and *bla*$_{IMP-1}$ genes (50 and 18.2%, respectively). Similarly, **Kumari et al.** [26] in India found a prevalence of 20.8% for *bla*$_{NDM}$ and 12.5% for *bla*$_{VIM-2}$ in tested *Pseudomonas* isolates. In contrast, **Pragasam et al.** [40] in India detected a prevalence of 21% for *bla*$_{VIM-2}$ but lower rates of *bla*$_{NDM}$ and *bla*$_{IMP-1}$ (8% and 1%, respectively).

In this study, we investigated the presence of *bla*$_{NDM}$, *bla*$_{VIM-2}$ and *bla*$_{IMP-1}$ genes in *Acinetobacter* isolates using conventional PCR. We found that 25% of the tested strains carried *bla*$_{NDM}$, 15% carried *bla*$_{VIM-2}$ and 5% carried *bla*$_{IMP-1}$. Interestingly, one isolate (5%) was found to carry both *bla*$_{NDM}$ and *bla*$_{VIM-2}$. These findings are consistent with that reported by **Benmahmod et al.** [41] in Egypt, who found prevalence rates of 30%, 20% and 10% for *bla*$_{NDM}$, *bla*$_{VIM-2}$ and *bla*$_{IMP-1}$, respectively. Conversely, **Wasfi et al.** [42] in Egypt detected *bla*$_{IMP}$ in 5.8% of *Acinetobacter* isolates, while the prevalence of *bla*$_{NDM}$ and *bla*$_{VIM-2}$ was 67.7% and 0%, respectively. **Hassan et al.** [23] in Egypt reported lower prevalence rates of 11.7%, 0.5% and 0% to *bla*$_{NDM}$, *bla*$_{VIM-2}$ and *bla*$_{IMP-1}$, respectively. Notably, **Kumari et al.** [22] in India found higher rates with *bla*$_{NDM}$ and *bla*$_{VIM-2}$ prevalence of 40.5% and 48.6%, respectively, and the combined *bla*$_{NDM}$ and *bla*$_{VIM-2}$ genes prevalence of 21.6% in tested *Acinetobacter* isolates.

Considering PCR as the gold standard method in detecting carbapenemase production, the current study showed that the mCIM had 93.33% sensitivity, 66.67% specificity, 73.68% PPV, 90.91% NPV and 80% accuracy. These results agree with that reported by **Kumari et al.** [22] in India and **Gill et al.** [43]. For *Acinetobacter* isolates, the mCIM had 87.5% sensitivity and 58.33% specificity in relation to PCR, a finding which is similar to that reported by **Simner et al.** [44].

Conclusion

In conclusion, resistance to multiple antibiotics was observed in both *Pseudomonas* and *Acinetobacter* isolates. Carbapenemase-mediated resistance was detected by mCIM, which demonstrated good sensitivity. Colistin resistance was present in a small percentage of the isolates and efflux pump inhibitors showed promise in reversing colistin resistance. Antibiotic resistance genes, *bla*$_{NDM}$ and *bla*$_{VIM-2}$, were prevalent. These findings highlight the importance of antimicrobial stewardship in managing infections caused by these pathogens.

Limitation

The inability to perform genotypic analysis to all phenotypically identified *Pseudomonas* and *Acinetobacter* isolates due to financial constraints.

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Conflict of interest

Non declared.

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