

ORIGINAL ARTICLE

Relationship between Biofilm Formation and Production of Extended-Spectrum β -lactamases and Metallo- β -lactamases among Gram-negative Bacilli

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ABSTRACT

Key words:

ESBL; MBL; antibiotic resistance; biofilm; Gram-negative

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Background: Gram-negative bacilli are important pathogens of hospital-acquired infections as they can survive in hospital environment. They show a wide range of antibiotic resistance. **Objectives:** To detect biofilm, ESBL and MBL production among Gram-negative bacilli, to correlate their relation together and assess their antibiotic resistance pattern. **Methodology:** Two hundred isolates of Gram-negative bacilli, collected from different clinical samples of hospitalized patients at Cairo University Hospitals, were subcultured on MacConkey's agar and identified by conventional methods. Antibiotic susceptibility testing was performed using Kirby-Bauer disc diffusion method. The ability of biofilm production was assessed using microtiter plate method. **Results:** Gram-negative bacilli isolates were most frequently recovered from urine samples (53%). Biofilm formation was found in 47% of the isolates. Furthermore, strong biofilm-forming category was detected in 2% of the isolates, while moderate and weak biofilm-forming categories were detected in 13% and 85% respectively. Production of ESBL and MBL among Gram-negative bacilli isolates was 39.5 % and 49% respectively. **Conclusions:** Biofilm formation was associated with MBL production among Gram-negative isolates. Moreover, ESBL and MBL producers were more resistant to antibiotics than non ESBL and non MBL producers.

INTRODUCTION

Among the different virulence factors of Gram-negative bacilli (GNB), the most important one is biofilm formation, Extended spectrum β -lactamases (ESBL) and Metallo- β -lactamases (MBL) which could be related to their high degree of antibiotic resistance¹. Within biofilm, the bacteria are more resistant to antibiotics owing to their environmental adaptations e.g. metabolic alteration².

ESBL are rapidly emerging group of β -lactamases that give rise to resistance to most beta-lactam antibiotics (penicillins, cephalosporins and monobactams) but not carbapenems and cephamycins³. MBL are β -lactamases that hydrolyze most β -lactam agents, especially carbapenems, but not monobactams, and are β -lactamase inhibitors resistant⁴.

In GNB, beta-lactamase production is the most important mechanism of beta-lactam resistance. Spread of ESBL and MBL production is increasing worldwide as well as biofilm formation, contributing to development of MDR organism¹.

The objectives of this research were to: study biofilm formation, ESBL and MBL production by Gram-negative bacilli recovered from different clinical samples, assess these clinical isolates antibiotic resistance pattern, assess the relation between biofilm

formation and ESBL & MBL production and investigate biofilm formation impact and/or ESBL and MBL production on antibiotic resistance.

METHODOLOGY

Ethical consideration, study design and setting:

This cross-sectional analytic study was conducted over a period of 6 months during 2020 at Microbiology and Immunology Department, Faculty of Medicine, Cairo University. It was approved by the research ethics committee of the institutional board review, Faculty of Medicine, Cairo University, Egypt (Approval no. MS-43 -2020 on 16-4-2020).

Two hundred Gram-negative bacilli isolates were recovered from clinical isolates of different sample sources (e.g. urine, sputum, etc...) of hospitalized patients at Cairo University Hospitals.

Laboratory procedures and bacterial identification:

All isolates were subcultured on MacConkey agar [NO.3, Code: CM0115 (Oxoid, Hampshire, UK)] and incubated for 24 h at 37 °C aerobically. They were stored at -80°C by emulsifying a loopful of bacterial colonies in 500 μ l of 50% glycerol broth⁵. Isolates were identified by conventional methods: colony morphology, microscopic examination and biochemical reactions on day 2⁶.

Oxidase test differentiated colonies into two groups *Enterobacteriaceae* and non-fermentative Gram-negative bacilli (NFGNB), then further identified by these biochemical tests: triple sugar iron, urease, citrate, lysine decarboxylase and motility- indole-ornithine (Oxoid, UK).

Antibiotic susceptibility testing:

Kirby-Bauer disc diffusion method was used on Mueller Hinton agar (MHA) (Oxoid, Hampshire, UK) on day 2⁷. The following discs were applied (Himedia, India) and incubated for 18 h and inhibition zones diameter was measured and interpreted using CLSI 2019 guidelines⁸: Ampicillin (AMP 10µg), Ampicillin/sulbactam (AS 10µg/10µg), Amoxicillin/ clavulanate (AMC 20µg/10µg), Piperacillin/ tazobactam (PIT 100/10µg), Cefuroxime (CXM 30µg), Cefazolin (CZ 30µg), Cefoxitin (CX 30µg), Ceftazidime (CAZ 30µg), Ceftazidime-clavulanate (CAC 30/10µg), Cefotaxime (CTX 30µg), Ceftriaxone (CTR 30µg), Cefepime (CPM 30µg), Imipenem (IPM 10µg), Imipenem EDTA (IE 10/750µg), Meropenem (MRP 10µg), Ertapenem (ETP 10µg), Amikacin (AK 30µg), Tobramycin (TOB 10µg), Gentamicin (GEN 10µg), Ciprofloxacin (CIP 5µg), Levofloxacin (LE 5µg), Cotrimoxazole (COT 25µg).

Screening for ESBL and MBL production:

ESBL and MBL production by isolates were tested using confirmatory CLSI test and double disc synergy test. Ceftazidime (CAZ 30 µg), Ceftazidime-clavulanate (CAC 30/ 10 µg), Imipenem (IPM) and Imipenem-EDTA (IE 10/750µg) discs were put over MHA plate with other discs. On day 3, results were interpreted after incubation for 18 h. An increase of ≥ 5 mm in zone diameter for Ceftazidime with clavulanate versus zone diameter of Ceftazidime alone confirmed ESBL production⁸. An increase of ≥ 7 mm in zone diameter for Imipenem-EDTA versus zone diameter of Imipenem only indicated MBL production¹.

Detection of biofilm formation:

Isolates were examined for biofilm formation by the gold standard semi-quantitative method known as tissue culture plate (TCP) method using 96-well flat-bottomed sterile polystyrene plates (Nunc, Roskilde, Denmark) on day 2^{9,10,11}. Bacterial suspensions were prepared from each isolate in 2 ml of sterile trypticase soy broth (TSB) (Oxoid, Hampshire, UK) with 1% glucose, adjusting turbidity (0.5 McFarland) then diluted 1:100 with TSB.

200µl of diluted suspension were dispensed into the wells of TCP in duplicate. The positive control organism (*Acinetobacter* spp, biofilm-former) was a clinical isolate stored in strain bank of Medical Microbiology and Immunology Department, Faculty of Medicine, Cairo University. Six wells (sterile TSB) served as negative control. The positive control was prepared as the isolates and tested in 2 wells. Plates were covered then incubated at 37°C overnight. The contents of wells were decanted into a discard container

then washed three times with 200 µL of phosphate buffer saline (PBS) (Oxford, India) to remove any free-floating bacteria. 150 µl of 100% methanol (Biotech, India) were added to wells and left for 20 minutes room temperature to allow fixation of the remaining attached bacteria, then decanted and 150 µl of 1% crystal violet solution (Merk, Germany) were used as a stain for 15 minutes. Excess stain was removed using sterile deionized water (El Gomhoreya, Egypt) until washing was free of stain. Plates were air dried for 15 min. Finally, 150 µl of 33% glacial acetic acid (Piochem, Egypt) were added to wells to allow resolubilization of dye (Figure 2).

Micro ELISA autoreader Stat Fax-2100 (Awareness Technology, US) measured optical density (OD) at 490 nm for each well at Medical Microbiology and Immunology department [Faculty of Medicine, Cairo University]. Results were interpreted according to Stepanovic *et al.*¹⁰ (Table 1) on day 3.

Table 1: Interpretation of biofilm formation using TCP method¹⁰

Mean OD value of each isolate	Biofilm formation
1- Isolate OD \leq ODC	Non biofilm former
2- Isolate OD $>$ ODC	Biofilm former
a) If isolate OD $>$ ODC and ≤ 2 ODC	Weak biofilm former
b) If isolate OD $>$ 2ODC and ≤ 4 ODC	Moderate biofilm former
c) Isolate OD $>$ 4ODC	Strong biofilm former

Optical density cut-off value (ODC) = average OD of negative control + 3×standard deviation (SD) of negative control¹⁰

Statistical analysis of data:

Data were analyzed using IBM Statistical Package for Social Sciences (SPSS) version 26 (Armonk, NY, USA). Data were described as frequency (count) and relative frequency (percentage) for categorical data. Chi square (χ^2) test was used to compare categorical data. Exact test was performed instead when the expected frequency is less than 5¹². P-value less than 0.05 were considered statistically significant.

RESULTS

Types of samples:

Two hundred Gram-negative isolates were retrieved as follows regarding samples: 106 urine (53%), 39 wound (19.5%), 21 sputum (10.5%), 20 pus (10%) and 14 blood (7%) samples.

Isolates identification:

Gram-negative bacilli were identified to genus level: 88 *E.coli* (44%), 66 *Klebsiella* spp. (33%), 37 *Pseudomonas* spp. (18.5%), 6 *Acinetobacter* spp. (3%) and 3 *Proteus* spp. (1.5%) isolates (figure 1).

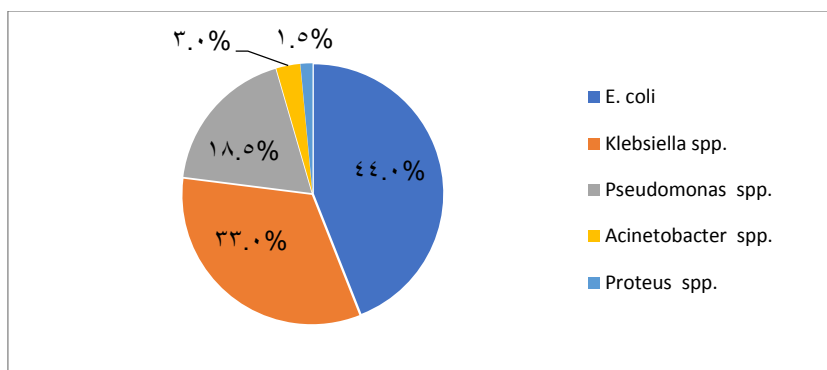


Fig. 1: Isolated Gram-negative bacilli

Antibiotic resistance pattern of isolated organisms:

Table 2: Antibiotic resistance pattern among isolated organisms

Antibiotic tested	<i>E. coli</i> (88) No. (%)	P value	<i>Klebsiella</i> (66) No. (%)	P value	<i>Proteus</i> (3) No. (%)	P value	<i>Pseudomonas</i> (37) No. (%)	P value	<i>Acinetobacter</i> (6) No. (%)	P value
Ampicillin (AMP)	74 (84%)	0.002	65 (98%)	0.004	3 (100%)	1	-	-	-	-
Ampicillin/ sulbactam	22 (25%)	< 0.001	40 (61%)	< 0.001	2 (67%)	0.570	-	-	3 (50%)	0.693
Amoxicillin/ clavulanate	38 (43%)	0.003	46 (70%)	0.001	1 (33%)	0.596	-	-	-	-
Piperacillin/ tazobactam	32 (36%)	< 0.001	47 (71%)	< 0.001	1 (33%)	0.619	17 (46%)	0.539	4 (67%)	0.683
Cefoxitin (CX)	24 (27%)	< 0.001	45 (68%)	< 0.001	2 (67%)	0.592	-	-	-	-
Ceftazidime (CAZ)	52 (59%)	0.003	53 (80%)	0.026	3 (33%)	0.556	28 (76%)	0.404	4 (67%)	1
Cefotaxime (CTX)	61 (69%)	0.231	52 (79%)	0.183	2 (67%)	1	-	-	4 (67%)	0.660
Ceftriaxone (CTR)	57 (65%)	0.024	55 (83%)	0.009	2 (67%)	1	-	-	4 (67%)	0.671
Cefepime (CPM)	52 (59%)	0.310	48 (73%)	0.046	2 (67%)	1	20 (54%)	0.212	4 (67%)	1
Cefuroxime (CXM)	63 (71.5%)	0.151	53 (80%)	0.278	3 (33%)	1	-	-	-	-
Gentamicin (GEN)	27 (31%)	0.003	31 (47%)	0.370	0 (0%)	0.263	23 (62%)	0.007	4 (67%)	0.405
Tobramycin (TOB)	21 (24%)	< 0.001	33 (50%)	0.019	0 (0%)	0.286	19 (51%)	0.075	4 (67%)	0.207
Amikacin (AK)	8 (9%)	< 0.001	30 (45%)	0.001	1 (33%)	1	16 (43%)	0.042	4 (67%)	0.064
Cotrimoxazole (COT)	50 (57%)	0.177	43 (65%)	0.441	3 (100%)	0.286	-	-	4 (67%)	1
Ciprofloxacin (CIP)	60 (68%)	0.357	55 (83%)	0.009	3 (100%)	0.559	21 (57%)	0.028	4 (67%)	1
Levofloxacin (LEV)	56 (64%)	0.972	40 (61%)	0.551	3 (100%)	0.301	24 (65%)	0.849	4 (67%)	1
Cefazolin (CZ)	72 (82%)	0.240	58 (88%)	0.363	3 (100%)	1	-	-	-	-
Imipenem (IMP)	26 (29.5%)	< 0.001	44 (67%)	0.012	2 (67%)	1	31 (84%)	< 0.001	5 (83%)	0.221
Meropenem (MRP)	81 (92%)	0.023	66 (100%)	0.043	3 (100%)	1	36 (97%)	1	6 (100%)	1
Ertapenem (ETP)	21 (24%)	< 0.001	38 (57.5%)	< 0.001	2 (67%)	0.563	-	-	-	-

P value <0.05 is considered statistically significant

E. coli isolates were statistically significantly resistant to AMP, CAZ, CTR and MRP and statistically significantly sensitive to AS, AMC, PIT, CX, GEN, TOB, AK, IPM and ETP. *Klebsiella* spp. isolates were statistically significantly resistant to AMP, AS, AMC, PIT, CX, CAZ, CTR, CPM, CIP, IPM, MRP and ETP

and statistically significantly sensitive to AK. Although *Proteus* spp. and *Acinetobacter* spp. showed high resistance to the previous antibiotics, no statistical significance was found. *Pseudomonas* spp. had statistically significant resistance to GEN and IPM but sensitivity to AK (Table 2).

ESBL, MBL and biofilm production distribution:

Table 3: Distribution of ESBL, MBL and biofilm producers among isolated organisms

Organism (No.)	Biofilm producers No. (%)	P value	ESBL producers No. (%)	P value	MBL producers No. (%)	P value	ESBL & MBL producers No. (%)	P value	Biofilm & ESBL producers No. (%)	P value	Biofilm & MBL producers No. (%)	P value	Biofilm, ESBL & MBL producers No. (%)	P value
<i>E. coli</i> (88)	29 (33%)	≤0.001	46 (52%)	0.001	25 (28%)	≤0.001	7 (8%)	0.298	10 (11%)	0.542	21 (24%)	0.003	6 (7%)	0.585
<i>Klebsiella</i> (66)	38 (57.5%)	0.035	24 (36%)	0.524	36 (54.5%)	0.271	8 (12%)	0.600	10 (15%)	0.525	27 (41%)	0.219	5 (7.5%)	0.877
<i>Pseudo-monas</i> (37)	20 (54%)	0.341	8 (22%)	0.014	29 (78%)	≤0.001	5 (13.5%)	0.552	5 (13.5%)	1	16 (43%)	0.244	4 (11%)	0.504
<i>Acineto-bacter</i> (6)	4 (67%)	0.423	1 (17%)	0.406	6 (100%)	0.013	1 (17%)	0.491	1 (17%)	0.571	4 (67%)	0.186	1 (17%)	0.398
<i>Proteus</i> (3)	3 (100%)	0.102	0 (0%)	0.279	2 (67%)	0.616	0 (0%)	1	0 (0%)	1	2 (67%)	0.281	0 (0%)	1
Total (200)	94 (47%)		79 (39.5%)		98 (49%)		21 (10.5%)		26 (13%)		70 (35%)		16 (8%)	

P value <0.05 is considered statistically significant

Among *E. coli* isolates: There was statistically significant positive association with ESBL production (46/88, 52%). However, statistically significant negative association with biofilm production (29/88, 33%), MBL production (25/88, 28%) and co-production of biofilm and MBL (21/88, 24%) was detected (Table 3).

Among *Klebsiella* spp. isolates: There was statistically significant positive association with biofilm production (38/66, 57.5%) (Table 3).

Among *Pseudomonas* spp. isolates: There was statistically significant positive association with MBL production (29/37, 78%) and statistically significant negative association with ESBL production (8/37, 22%) (Table 3).

Among *Acinetobacter* spp. isolates: There was statistically significant positive association with MBL production (6/6, 100%) (Table 3).

Among *Proteus* spp. isolates: There was no statistically significant association with biofilm, ESBL and MBL production. Biofilm production was statistically significant in *Klebsiella* spp. (P value = 0.035). Although the majority of *Proteus* spp, *Acinetobacter* spp. and *Pseudomonas* spp. were biofilm producers, no statistical significance was found (Table 3).

ESBL production was statistically significant in *E. coli* (P value = 0.001). MBL production was statistically significant in *Pseudomonas* spp. (P value ≤ 0.001) and *Acinetobacter* spp. (P value = 0.013) (Table 3).

Table 4: ESBL and MBL distribution among biofilm producing isolates

Biofilm production	Biofilm producers 94 No. (%)	Non biofilm producers 106 No. (%)	P value
β-lactamase production			
79 ESBL producers	26 (33%)	53 (67%)	0.001
98 MBL producers	70 (71%)	28 (29%)	≤0.001
21 ESBL and MBL producers*	16 (76%)	5 (24%)	0.005
44 Non ESBL and Non MBL producers	14 (32%)	30 (68%)	0.005

(P value <0.05 is considered statistically significant)

*Number of ESBL and MBL co-producers are included in either ESBL producers or MBL producers

Relation between biofilm degree and β-lactamase production:

Regarding biofilm producers, there were strong (2/94, 2%), moderate (12/94, 13%) and weak (80/94, 85%) biofilm producers. Weak biofilm producers showed statistically significant positive association with MBL production (62/98, 63%), ESBL and MBL co-production (14/21, 67%). However, weak biofilm

producers showed statistically significant negative association with ESBL production (20/79, 25%). Non biofilm producers showed statistically significant positive association with ESBL production (53/79, 67%) and negative association with MBL production (28/98, 28.5%) and ESBL and MBL co-production (5/21, 24%) as shown in table 5.

Table 5: Association between the degree of biofilm production and ESBL and MBL production among isolated organisms

Degree of biofilm production (No.) \ Type of β-lactamase (Total No.)	ESBL (79) No. (%)	P value	MBL (98) No. (%)	P value	ESBL & MBL (21) No. (%)	P value
Strong biofilm producers (2)	2 (2.5%)	0.155	0 (0%)	0.498	0 (0%)	1
Moderate biofilm producers (12)	4 (5%)	0.767	8 (8%)	0.207	2 (9.5%)	0.366
Weak biofilm producers (80)	20 (25%)	0.001	62 (63%)	≤ 0.001	14 (67%)	0.008
Non biofilm producers (106)	53 (67%)	0.001	28 (28.5%)	≤ 0.001	5 (24%)	0.005

P value <0.05 is considered statistically significant

Biofilm degree among isolated organisms:

Among *E. coli*, there was statistically significant negative association with weak production of biofilm (26/88, 29.5%) and positive association with non-biofilm production (59/88, 67%).

Among *Klebsiella* spp, there was statistically significant negative association with non-biofilm production (28/66, 42.5%) (Table 6). Figure 2 illustrates TCP method for biofilm detection.

Table 6: Degree of biofilm production among different isolated organisms

Degree of biofilm production (No.) \ Organism (Total No.)	<i>E. coli</i> (88) No. (%)	P value	<i>Klebsiella</i> (66) No. (%)	P value	<i>Proteus</i> (3) No. (%)	P value	<i>Pseudomonas</i> (37) No. (%)	P value	<i>Acinetobacter</i> (6) No. (%)	P value
Strong biofilm producers (2)	0 (0%)	0.505	1 (1.5%)	0.552	0 (0%)	1	1 (2.7%)	0.337	0 (0%)	1
Moderate biofilm producers (12)	3 (3.5%)	0.171	7 (11%)	0.064	0 (0%)	1	2 (5.4%)	1	0 (0%)	1
Weak biofilm producers (80)	26 (29.5%)	0.007	30 (45%)	0.269	3 (100%)	0.063	17 (45.9%)	0.413	4 (66.6%)	0.220
Non biofilm producers (106)	59 (67%)	≤ 0.001	28 (42.5%)	0.035	0 (0%)	0.102	17 (45.9%)	0.341	2 (33.3%)	0.423

P value <0.05 is considered statistically significant

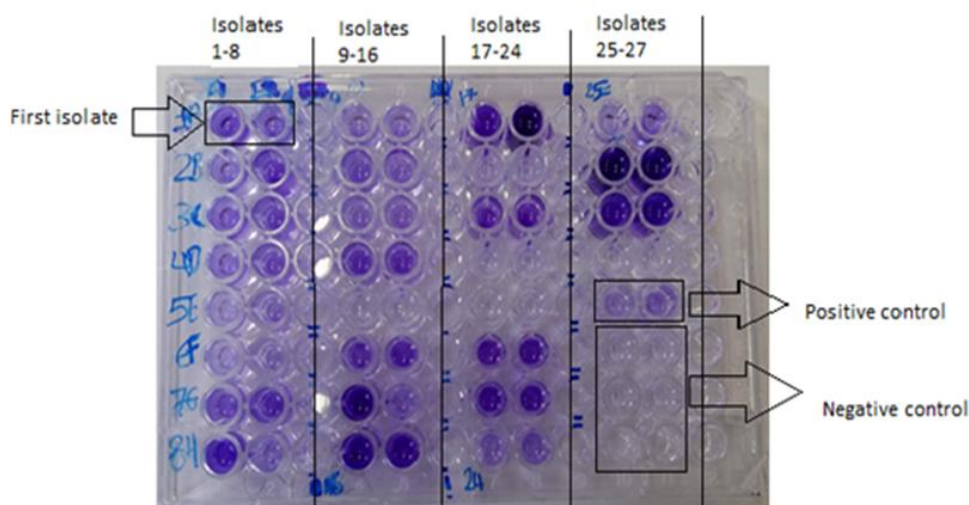


Fig. 2: A flat-bottomed tissue culture plate (96-well) for detecting biofilm formation among different isolates
 A non-biofilm-forming isolate (wells no. D7, D8)-A strong biofilm-forming isolate (wells no. B10, B11)-A moderate biofilm-forming isolate (wells no. A4, A5)-A weak biofilm-forming isolate (wells no. E1, F1)

Distribution of ESBL and MBL producers according to sample type:

A statistically significant negative association between *E. coli* and ESBL production (33/72, 46%) in urine sample was detected (figure 3).

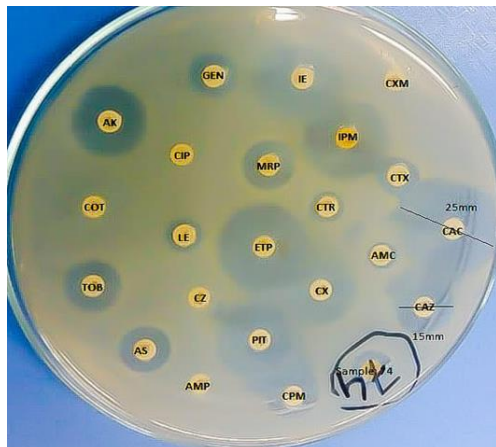


Fig. 3: Antibiotic susceptibility test of *E. coli* showing ESBL production on a 25 cm plate of MHA

Biofilm production degree in relation to sample type:

No statistically significant differences were found in frequency and degree of biofilm production regarding different samples enrolled (urine, wound, sputum, pus and blood).

Biofilm production degree in relation to sample type and isolated organisms:

Degree of biofilm production among isolates recovered from different samples was statistically insignificant as regards isolated organisms.

Relation between biofilm degree and β -lactamase production with respect to sample type:

In urine samples, weak biofilm producers were statistically significantly positively associated with MBL production (29/41, 70.5%) as well as ESBL and MBL co-production (7/10, 70%) and negative association between ESBL production and biofilm formation (14/45, 31%) in urine samples. In pus samples, a statistically significant negative association was found between weak biofilm producers and ESBL production (2/12, 16%).

Effect of biofilm and β -lactamase production on antibiotic resistance profile of isolated organisms:

ESBL producers showed statistically significant resistance to AMP, CAZ, CTX, CTR, CPM, CXM, CIP and CZ in relation to non ESBL producers. ESBL producers showed statistically significant susceptibility to AS, AMC, PIT, CX, AK, IPM and ETP in relation to non ESBL producers. MBL producers showed statistically significant resistance to AS, AMC, PIT, CX, CAZ, GEN, TOB, AK, IPM and ETP in relation to non MBL producers. Biofilm producers showed statistically significant resistance to AS, CX, AK, IPM and ETP in relation to non-biofilm producers.

DISCUSSION

Significant community and hospital-acquired infections are caused by GNB. They can cause various infections including pneumonia, UTI, meningitis, sepsis and wound infections²³.

In this research, urine (53%) samples were the most common collected samples from which GNB were

recovered followed by wound swabs (19.5%), sputum (10.5%), pus (10%) and blood (7%) samples. Similarly, Agyepong *et al.*¹³ showed the order of sample source was as follows: urine (48.5%), wound (23.5%) and sputum (11.5%) samples. Differences in number of samples between this study and A Abdel Salam and Hager¹⁴ could be due to difference in the rate of infection resulting from different settings in which samples were collected. A Abdel Salam and Hager¹⁴ collected samples from ICU patients not from hospital wards.

In this study, *E. coli* (44%) were the most common isolated GNB (figure 1) in agreement with Veeraraghavan *et al.*¹⁵ followed by other GNB. Dissimilarly, different distribution of isolated GNB was found in A Abdel Salam and Hager¹⁴ study as the majority of their collected samples were blood and sputum samples unlike this study which were urine samples.

In this research, *E. coli* resistance (table 2) was agreeing with Gashe *et al.*¹⁶ and Jamil *et al.*¹⁷ who revealed close resistance results to some antibiotics used. *Klebsiella* spp. resistance (table 2) was in line with Effah *et al.*¹⁸ who reported that resistance of *Klebsiella* spp. to CAZ, CIP, CPM and IPM was high in Asia. Dumaru *et al.*¹ and Effah *et al.*¹⁸ revealed that resistance of *Klebsiella* spp. to AK was low. In contrast, Chong *et al.*¹⁹ showed that *Klebsiella* spp. exhibited lower resistance rates to CPM.

In the current study, *Pseudomonas* spp. resistance to IPM was in concordance with Hu *et al.*²⁰ reported high resistance rate of *Pseudomonas* spp. to IPM. Haghi *et al.*²¹ showed similar results to GEN. Dumaru *et al.*¹ reported lower resistance rates of *Pseudomonas* spp. to GEN and IPM. Antibiotic resistance high prevalence in this study and other studies could be explained by that hospitalized patients were the source of the isolates recovered. However, variations in antibiotic resistance pattern of different isolated organisms between this study and other studies could be due to different antibiotic policies used in each hospital.

Biofilm formation was detected in (94/200, 47%) of GNB in the present study. There was significant high prevalence of biofilm formation among *Klebsiella* spp. (38/66, 57.5%), while biofilm formation prevalence was significantly low among *E. coli* (29/88, 33%). Consistently, Raya *et al.*²² showed close results where 44.6% of GNB, 64.2% of *Klebsiella* spp. and 42.1% of *E. coli* were biofilm formers. Besides, Cepas *et al.*²³ reported 49.3% of GNB isolates were biofilm-formers and 30.3% of *E. coli* were biofilm formers. Similarity in biofilm forming ability of GNB, *E. coli* and *Klebsiella* spp. may be because that this study, Raya *et al.*²² and Cepas *et al.*²³ used TCP method for detecting biofilm formation. However, Dumaru *et al.*¹ detected higher prevalence rate of biofilm formation detected among GNB (62.7%) where 77.55% of *Klebsiella* spp. were

biofilm formers. This might return to that Dumaru *et al.*¹ used Congo red and tube adherence methods to detect biofilm formation. Karigoudar *et al.*²⁴ found that 89.7% of *E. coli* were biofilm formers. This disagrees with our study. They estimated biofilm forming ability of *E. coli* recovered from urine samples of catheterized patients which could explain the significant higher biofilm formation.

Lack of application of antimicrobial stewardship and excessive use of the over counter antibiotics contributed to the increasingly emerging ESBL infections. Empiric use of cephalosporins and length of stay in hospitals especially ICUs have been directly related to increased number of hospital-acquired infections caused by ESBL producing bacteria. In the current study, ESBL production was detected in 39.5% of GNB. ESBL production prevalence was significantly high among *E. coli* isolates (52%) and low among *Pseudomonas* spp. (22%). In agreement, Shrestha *et al.*²⁵ reported 50.9% of *E. coli* were ESBL producers in Nepal. Higher ESBL production was detected among *E. coli* in Adabara *et al.*²⁶ in Nigeria (88.5%) who used double disc synergy test (DDST) for screening ESBL production. The present study and Shrestha *et al.*²⁵ used CLSI confirmatory test. Amirkamali *et al.*²⁷ in Tehran used CLSI confirmatory method and found that ESBL production among *Pseudomonas* spp. was 27.5%. Dissimilarly, Farhan *et al.*²⁸ in Egypt used CLSI confirmatory test and revealed that 61.6% of *Pseudomonas* spp. were ESBL producers. This higher rate may be due to Farhan *et al.*²⁸ studied MDR *Pseudomonas* spp. Geographical distribution of ESBL producers may be the cause of variations in ESBL production rate detected by different studies. ESBL producers' prevalence varies considerably worldwide and changes over time.

In this study, MBL production was detected in (98/200, 49%) of GNB. It was significantly high among *Acinetobacter* spp. (100%) then *Pseudomonas* spp. (78%) and significantly low among *E. coli* (28%). In concordance, Lee *et al.*²⁹ in Taiwan reported that 47.3% of GNB were MBL producers. 99% of *Acinetobacter* spp. were MBL producers in the Iranian study of Safari *et al.*³⁰. In concordance, the Egyptian study of Diab *et al.*³¹ reported high MBL production among *Pseudomonas* spp. (82%). In contrast, Dumaru *et al.*¹ in India revealed lower rate of MBL production among *Acinetobacter* spp. (20.63%) and among *Pseudomonas* spp. (26.31%). Javed *et al.*³² study in Pakistan revealed close results where production of MBL was detected in 33% of *E. coli*. On the contrary, Nepal *et al.*³³ study in South Asia showed 66.6% of *E. coli* were MBL producers. Differences between this study and other studies in geographical distribution contributed to variations in MBL production prevalence.

In the current research, biofilm formation prevalence rate among MBL producing *E. coli* was significantly

low (21/88, 24%). Similarly, a negative correlation between biofilm formation and MBL production among *E. coli* was stated in Dumaru *et al.*¹ but at a lower prevalence rate (2.48%).

In this study, a positive correlation was found between biofilm formation and MBL production among GNB (74%) as well as ESBL and MBL co-production (17%). Similarly, Heydari & Eftekhari³⁴ reported that among biofilm formers, production of MBL was significantly higher (70.3%) compared to non-biofilm formers (31.4%). Moreover, Dumaru *et al.*¹ reported that a statistically significant positive association between MBL production and biofilm formation was found. Agreement between results is an alarming sign since overlapping of MBL and biofilm production can render the bacteria more highly resistant to antibiotics. In contrast, no statistically significant association was established between biofilm formation and MBL production in Baniya *et al.*³⁵. Different population, different sample size and different infections could explain this.

In our study, a negative correlation between biofilm formation and ESBL production (28%) was found. However, Heydari and Eftekhari³⁴ and Dumaru *et al.*¹ reported that no statistically significant correlation as regards biofilm formation and ESBL production was detected. Disagreement between our study and other studies can be attributed to differences in sample size as well as in prevalence of ESBL producers and biofilm formers. Dissimilarly, Shrestha *et al.*²⁵ found positive correlation concerning biofilm formation and ESBL production (56%).

In the present study, weak biofilm-formers were positively associated with MBL production (63%) as well as ESBL and MBL co-production (67%). Weak biofilm formers were negatively associated with ESBL production (25%). Similarly, Shrestha *et al.*²⁵ found that 23.5% of ESBL producers were weak biofilm formers and negatively associated. In disagreement, Heydari & Eftekhari³⁴ revealed that isolates producing only one β -lactamase either ESBL or MBL were weak biofilm formers. The differences in results could be differences in study population.

In the current research, when different isolated organisms were considered, there was a statistically significant negative correlation between weak degree of biofilm formation and *E. coli* (29.5%) in concordance with Shrestha *et al.*²⁵ (22%). In disagreement, Tajbakhsh *et al.*³⁶ stated that weak biofilm formers were 56.25% of biofilm forming *E. coli* and this was significant. This could be explained by using Congo red agar method for biofilm formation degree detection.

There was significant negative association between *E. coli* and ESBL production (33/72, 46%) in our study regarding urine samples. In line, Yang and Zhang³⁷ found that the rates of ESBL producing bacteria recovered from urine samples were close to those

recovered from other different samples and that non ESBL producers were higher than ESBL producers in all samples. In disagreement, Nepal *et al.*³³ reported that urine was the principal source of ESBL producing isolates may be because he studied larger sample size on a wide scale.

In the present research, no significant differences statistically in frequency and degree of biofilm production were found in relation to different samples enrolled. Similarly, Cepas *et al.*²³ reported no significant differences in frequency of biofilm formers with respect to different sample types were detected. A strong correlation between biofilm formation and site of sample was found in Sanchez *et al.*³⁸ study which stated isolates recovered from non-fluid sites showed a significantly higher proportion of biofilm formers in relation to those from fluid sites. Disagreement in results could be explained that Sanchez *et al.*³⁸ used scanning electron microscopy to examine biofilm formation.

In our study, a significant positive association between weak biofilm formation and MBL production (70.5%) was detected as well as ESBL and MBL co-production (70%) in urine samples compared to other samples. In contrast, Cepas *et al.*²³ stated that no statistically significant differences between biofilm formation degree and sample site were found.

In this research, ESBL producers were more resistant to AMP (100%), CAZ (85%), CTX (85%), CTR (83.5%), CPM (78%), CXM (83.5%), CIP (80%) and CZ (87%) compared to non ESBL producers. ESBL producers were less resistant to AS (37%), AMC (51%), PIT (35%), CX (30%), AK (18%), IPM (37%) and ETP (25%). High resistance to AMP (100%), CAZ (87.14%), CTX (90%), CTR (82.86%) and CIP (74.29%) among ESBL producers was reported in Nwafia *et al.*³⁹. In agreement with Dumaru *et al.*¹ where a significant association between ESBL production and antibiotic resistance was found. The significantly higher antibiotic resistance among ESBL producers could return to the fact that genes encoding ESBL are present on transferable plasmids that may be carrying other resistance genes. Nwafia *et al.*³⁹ showed close results in low resistance of ESBL producers to PIT (34.29%), AK (45.43%), IPM (1.43%) and ETP (7.14%).

In the current study, MBL producers were significantly resistant to AS (42%), AMC (84%), PIT (61%), CX (44%), CAZ (76.5%), GEN (52%), TOB (49%), AK (43%), IPM (79.5%) and ETP (36%) compared to non MBL producers. Dumaru *et al.*¹ revealed that MBL production and antibiotic resistance association was significant statistically which agreed with this study. High antibiotic resistance of MBL in this research and other studies could be explained by that transferable MBL is encoded by *bla* genes which are found as gene cassettes in class 1 integrons. They

spread between bacteria by plasmids and on transposons.

In this study, biofilm formers were significantly resistant to only 5 antibiotics: AS (42.5%), CX (47.8%), AK (37%), IPM (69%) and ETP (38%) in comparison with non-biofilm formers. In concordance, Neupane *et al.*⁴⁰ revealed that antibiotic resistance of biofilm forming strains was higher than non-biofilm forming and the correlation between biofilm formation and antibiotic resistance was statistically significant. Higher antibiotic resistance among biofilm formers, as regards to non biofilm formers, can be due to that biofilm residing bacteria have intrinsic resistance to many antibiotics raising resistance up to 1000 folds. Cepas *et al.*²³ assessed the relation between biofilm formation and MDR GNB and found that there was a comparable level of biofilm formation between MDR and non MDR with no significant differences between the two groups.

CONCLUSIONS

In this research, *E. coli* and *Klebsiella* spp. were the most common isolated GNB from urine samples. A high prevalence rate of antibiotic resistance was found among the studied GNB. The prevalence rate of biofilm formation is significantly high among the studied *Klebsiella* spp. A high prevalence rate of ESBL production among *E. coli* isolates and a high prevalence rate of MBL production among *Acinetobacter* spp. and *Pseudomonas* spp. isolates was reported. Weak biofilm formation is significantly positively associated with MBL production as well as ESBL and MBL co-production among the isolated GNB. No biofilm formation is significantly positively associated with ESBL production. β -lactamase producers show significantly higher resistance to antibiotics used. This necessitates adherence to the antibiotic regimen by clinicians regarding the antibiotic stewardship guidelines to avoid emergence of MDR organisms as ESBLs and MBLs. Continuous application of antibiotic susceptibility surveillance is mandatory for the usefulness of these drugs. Strategies must be applied in hospitals to minimize β -lactamase producing organisms spread by applying infection control policies to prevent their dissemination.

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Authors' contributions

Nermin Samir: providing the study conception and design, supervision, data analysis, and writing the original draft of the manuscript. Nadia Hafez: supervision and revision of the manuscript. Maha Elnaggar: material preparation, performance of the experiments, data collection, and analysis. All authors provided critical feedback and took part in reviewing and revising the intellectual as well as the technical parts of the manuscript. All authors read and approved the final manuscript.

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