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ORIGINAL ARTICLE

Prevalence of Class 1, 2, and 3 Integrons and Carbapenem-Resistance in Gram-negative bacteria

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Key words:

Integrons; Gram-negative bacteria; antimicrobial resistance; carbapenems

ABSTRACT

Background: *Resistance to various antimicrobial agents in gram-negative bacteria has become an important health threat with some acquired through integrons.* ***Objectives:*** *The present study aims to evaluate the resistance profiles, and the prevalance of integrases in gram-negative isolates.* ***Methodology:*** *We examined 70 gram negative isolates comprising 35 carbapenem-susceptible and 35 carbapenem-nonsusceptible isolates for antimicrobial resistance patterns against 13 antibiotics and the screening for integrases by polymerase chain reaction.* ***Results:*** *Isolates comprised 41 Enterobacteriaceae, 17 Pseudomonas aeruginosa, and 12 Acinetobacter baumannii. Pseudomonas aeruginosa was the most common detected among our isolates, followed by Escherichia coli and Acinetobacter baumanniii. Carbapenem-nonsusceptible gram-negative isolates showed significant higher rates of resistance to all tested antibiotics except for aztreonam. Class 1 were found among 12 and 29 isolates, while class 2 were detected in two and 9 isolates of carbapenem-susceptible and non-susceptible isolates, respectively with significant differences between both groups. None of the isolates expressed class 3 integrons. Class 1 integrons were mostly detected among Pseudomonas aeruginosa, Acinetobacter baumanniii, and Escherichia coli in both groups.* ***Conclusion:*** *Integrases were common among our gram-negative isolates. Their high prevalence denotes that the hospital environment may favor the formation of resistant gene cassettes that necessitate the application of effective measures to fight them.*

INTRODUCTION

Resistance to antimicrobial agents in Gram-negative bacteria has become an important public health problem. Isolates are categorized as: pandrug resistant (PDR), extensive drug-resistant (XDR), multidrug resistant (MDR), and non-multiresistant, if resistance is found to all, to all except 1 or 2, to ≥ 3, and to < 3 antibiotic classes, respectively [1]. Bacteria can obtain resistance through the transfer of resistance genes located on integrons [2]. Integrons are a major mechanism for the spread of multidrug resistance [3], where they are able to capture, express and excise resistance genes[4]. Each integron composed of an integrase (*intI*), a recombination site (*attI*), and a promoter (PC) [5]. The amino acid sequences of *IntI* integrases was used for dividing integrons into classes [6]. Class 1 integrons are the most important and clinically relevant, with a wide distribution among bacterial species as *Acinetobacter*, *Vibrio*, *Aeromonas*, *Proteus*, *Burkholderia*, *Alcaligenes*, *Campylobacter*, *Enterobacter*, *Citrobacter*, *Klebsiella*, *Mycobacterium, Pseudomonas, Serratia*, *Salmonella*, *Shigella*, and *Escherichia* [7]. Class 2 integrons were found in *Acinetobacter*, *Aeromonas*, *Morganella,* and *Enterobacteriacae*[8]. Class 3 have been found in *Acinetobacter spp.*, *Pseudomonas aeruginosa*, and *Enterobacteriacae* [7, 8].

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METHODOLOGY

1. Bacterial strains

During a period from May 2015 to August 2015, a total of 70 non-repetitive Gram-negative isolates were collected from patients with multiple types of infection at Assiut University Hospitals. All isolates were identified by the standard methods and the API20E system (Biomerieux, France). The study was approved by the Ethical Committee of our university. An informed consent was obtained from all participants.

2. Antimicrobial susceptibility testing

Susceptibilities of the isolated bacterial strains were determined by the disk diffusion method on Muller Hinton agar medium as recommended by the Clinical and Laboratory Standards Institute (CLSI) [9] to 13 antimicrobial agents: piperacillin (100µg), amoxycillin/clavulanic acid (20/10µg), chloramphenicol (30µg), quinolones [ciprofloxacin (5µg) and levofloxacin (30 µg)], aminoglycosides [gentamicin (10 µg) and amikacin (30 µg)], cephalosporin [cefotaxime (30µg), ceftazidime (30µg), cefepime (30µg)], aztreonam (10µg), (Bioanalyse, Turkey), and carbapenems [meropenem (10µg), and imipenem (10µg)] (Toxoid, England).

3. Detection of class 1, 2, and 3 integrons by PCR

DNA was extracted by the boiling method as described previously[10]. Bacteria were cultured overnight (200 µL) and then mixed with 800 µL sterile distilled water and then boiled for 10 min. The resulting solution was centrifuged at 12,000 ×g for 2 min and then the supernatant was decanted and the pellet was resuspended in 300–400 mL of sterile distilled water and stored at –20°C, which were used as DNA templates. The specific primers for detecting intI genes [11] are shown in table 1. PCR was performed for each gene in a 50 µl final volume containing 200 µM deoxynucleoside triphosphates, 1 µM each primer, 1.5 mM MgCl, 2 U Taq DNA polymerase and 2 µl of genomic DNA of the test strain, in a thermal cycler (Biorad, USA) using the following conditions: a denaturation step at for 2 min at 94°C, then 30 cycles were repeated that consisted of; 94°C for 60 sec, 55°C for 60 sec and 72°C for 90 sec. This was followed by a final extension step at 72ºC for 7 min. In the amplification of *intI3* gene, the annealing step was carried out at 57°C. PCR products were visualized under UV light after staining with ethidium bromide.

4. Statistical analysis

SPSS 20.0 package were used for the statistical analysis of data. Statistical significance was assessed via $\chi 2$ or Fisher's exact test for categorial variables and Student's *t*-test for continuous variables. A statistical significant difference was considered if P value <0.05.

RESULTS

Patient's characteristics, gram-negative isolates and antimicrobial resistance

For comparing prevalence of integrons, we examined 35 carbapenem-susceptible and 35 non-susceptible gram-negative isolates. These isolates comprised 41 *Enterobacteriaceae*, 17 *Pseudomonas aeruginosa* (*P. aeruginosa*), and 12 *Acinetobacter baumannii* (*A. baumannii*). *P. aeruginosa* was the mostly detected among our isolates, followed by *Escherichia coli* (*E. coli*) and *A. baumannii*. For carbapenem-susceptible isolates; *E. coli* was the mostly detected isolates, while for carbapenem-nonsusceptible isolates; *P. aeruginosa* was the mostly detected strains (Fig. 1). A statistical significant difference was found between number of bacterial isolates for both groups ($\chi 2$; P=0.004). Clinical characteristics of patients are shown in table 2. The mean ages of patients were 36.85±13.3 and 44.7 ± 14.00 years with no significant difference. For both carbapenem-susceptible and non-susceptible bacteria, males were the mostly affected with male-female ratios were 1.7:1 and 3.4:1, respectively. Patients were admitted mainly to the Internal Medicine department followed by the Chest Department and Chest ICU. Carbapenem-susceptible and nonsusceptible isolates showed significant difference regarding the place of admission (Fisher's exact test; P=0.024) where, carbapenem-susceptible strains were mainly isolated from patients admitted to the Internal Medicine Department, while carbapenem-nonsusceptible isolates were mainly from patients admitted to the Chest ICU. Our samples were mainly sputum, wound, and urine with significant differences between both groups (Fisher's exact test; P=0.035). Some of our patients had comorbidities. IV and urinary catheters were mostly fixed to patients infected by carbapenem-nonsusceptible isolates (Fisher's exact test; P<0.001 and =0.045, respectively). Those infected by carbapenem-nonsusceptible isolates were under mechanical ventilation (Fisher's exact test; P=0.028). While, no significant differences were found between the two groups regarding the history of previous surgery or the immunosuppressive therapy (Table 2). The resistance patterns of gram negative isolates are shown in table 3. Carbapenem-susceptible and nonsusceptible isolates showed highly significant differences in accordance to antibiotic resistance. Except for aztreonam, where resistance was similar for both groups, resistance rates were significantly higher to all other antibiotics (Fisher's exact test; P values <0.004). Non-MDR was detected among 57% and 20% of carbapenem-susceptible and nonsusceptible isolates (P=0.003), while PDR were found among 14% and 57% of both groups, respectively (P<0.001). MDR and XDR patterns showed no significant differences between both groups. (Fig. 1, Table 1, 2)

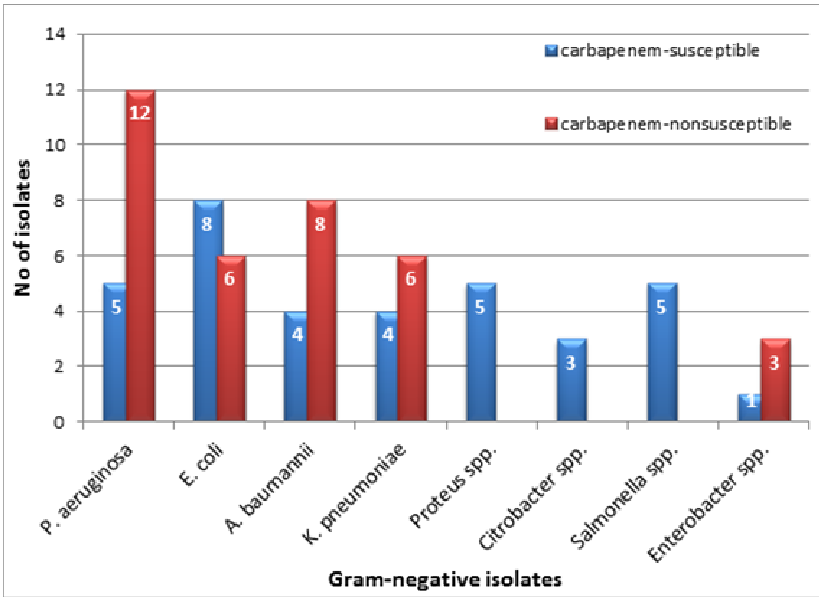

Fig. 1: Frequency distribution of bacterial strains among carbapenem-susceptible and nonsusceptible isolates

Table 1: Oligonucleotide primers used in the PCR analysis

Primer	Oligoneucleotide sequence	Amplicon size (bp)	Reference
intI1 F	5′-GCATCCTCGGTTTTCTGG-3′	457	[11]
intI1 R	5′-GGTGTGGCGGGCTTCGTG-3′		
intI2 F	5′-CACGGATATGCGACAAAAAGGT-3′	789	[11]
IntI2 R	5 ′-GTAGCAAACGAGTGACGAAATG-3′		
intI3 F	5 ′-ATCTGCCAAACCTGACTG-3′	922	[11]
IntI3 R	5 ′-CGAATGCCCCAACAACTC-3′		

Table 2: Demographic and clinical data of carbapenem-susceptible and non-susceptible Gram-negative isolates (n=70)

	carbapenem-susceptible (n=35)	***carbapenem-nonsusceptible (n=35)***	**P *value***
Mean age ± SD (range) (y)	36.85 ± 13.3	44.7 ± 14.00	**0.016**
Gender			
Female	13 (37%)	8 (23%)	0.297
Male	22 (63%)	27 (77%)	
Admission			**0.024**
Internal Medicine (20; 29%)	16 (46%)	4 (11%)	**0.003**
Chest department (19; 27%)	11 (31%)	8 (23%)	0.592
Chest ICU (17; 24%)	4 (11%)	13 (57%)	**0.024**
Nephrology unit (8; 11%)	3 (8.6%)	5 (14%)	0.710
Hematology unit (4; 6%)	1 (3%)	3 (8.6%)	0.614
Cardiology ICU (2; 3%)	0 (0%)	2 (6%)	0.493
Specimen source			**0.035**
Sputum (17; 24%)	13 (37%)	4 (11%)	**0.024**
Wound (16; 23%)	6 (26%)	10 (29%)	0.394
Urine (15; 21%)	10 (29%)	5 (14%)	0.244
Catheter (8; 11%)	2 (6%)	6 (26%)	0.259
Bronchial fluid (7; 10%)	2 (6%)	5 (14%)	**0.011**
Blood (5; 7%)	2 (6%)	3 (8.6%)	1.000
Pleural fluid (2; 3%)	0 (0%)	2 (6%)	0.493
Associated comorbidities			
IV catheter (21; 30%)	3 (8.6%)	18 (51%)	**<0.001**
Immunosuppressive therapy* (16; 23%)	5 (14%)	11 (31%)	0.153

Detection of class 1, 2, and 3 integrons in Gram-negative bacteria

Carbapenem-susceptible and non-susceptible gram negative isolates showed significant differences in the detection rates of class 1 and 2 integrons, where class 1 was found among 12 and 29 isolates, while class 2 was detected in two and 9 isolates, respectively (Fisher's exact test; $P<0.001$ and $=0.045$, respectively). None of the isolates expressed class 3 integrons (Table 3). The distribution of class 1 and 2 integrons among different bacterial strains is shown in figure 2. The detection of integrons by PCR is found in figures 3 and 4. Class 1 integrons were mostly detected among *P. aeruginosa*, *A. baumannii*, and *E. coli* in both carbapenem-susceptible and non-susceptible isolates, with significant difference between the detection rate among both groups (Fisher's exact test; $P=0.029$). Class 2 integrons were found in two isolates only among carbapenem-susceptible group (one *E. coli* and one *Salmonella* isolates) and among 9 isolates in carbapenem-nonsusceptible group with significant difference between both groups (Fisher's exact test; $P=0.009$).

Table 3. Antibiotic susceptibility in cabapenem-susceptible, nonsusceptible, and integron-positive Gram-negative isolates

Antibiotic	*Cabapenem-susceptible (n=35)*						*Cabapenem-nonsusceptible (n=35)*						P *value*
	Sensitive		Intermediate		Resistant		Sensitive		Intermediate		Resistant		
	No	%	No	%	No	%	No	%	No	%	No	%	
Piperacillin	14	40	2	6	19	54	0	0	0	0	35	100	**< 0.001**
Amox./clav.	22	63	3	8	10	29	6	17	2	6	27	74	**< 0.001**
Amikacin	20	57	1	3	14	40	7	17	1	3	27	74	**0.004**
Gentamicin	22	63	6	17	7	20	0	0	0	0	35	100	**< 0.001**
Aztreonam	16	46	2	6	17	48	9	26	3	8	23	66	0.232
chloramphenicol	23	69	5	14	7	17	7	17	4	11	24	68	**< 0.001**
Ceftazidime	26	74	3	8	6	17	5	14	3	8	27	74	**< 0.001**
Cefepime	24	68	2	6	9	26	8	23	1	3	26	74	**< 0.001**
Cefotaxime	23	66	6	17	6	17	8	23	7	17	20	57	**0.001**
Ciprofloxacin	27	74	2	6	6	20	10	29	1	3	24	68	**< 0.001**
Levofloxacin	29	83	1	3	5	14	12	34	2	6	21	60	**< 0.001**
Resistance pattern													
Non-MDR				20 (57%)						7 (20%)			**0.003**
MDR				6 (17%)						2 (6%)			0.259
XDR				4 (11%)						6 (17%)			0.734
PDR				5 (14%)						20 (57%)			**<0.001**
Integrons													
Class 1 integron gene				12 (34%)						29 (83%)			**<0.001**
Class 2 integron gene				2 (6%)						9 (26%)			**0.045**
Class 3 integron gene				0 (0%)						0 (0%)			NA**

Abbreviations: amox./clav.=amoxycillin/clavulanic acid; trim./sulfa.=trimethoprim/sulfamethoxazole. **NA=not applicable.

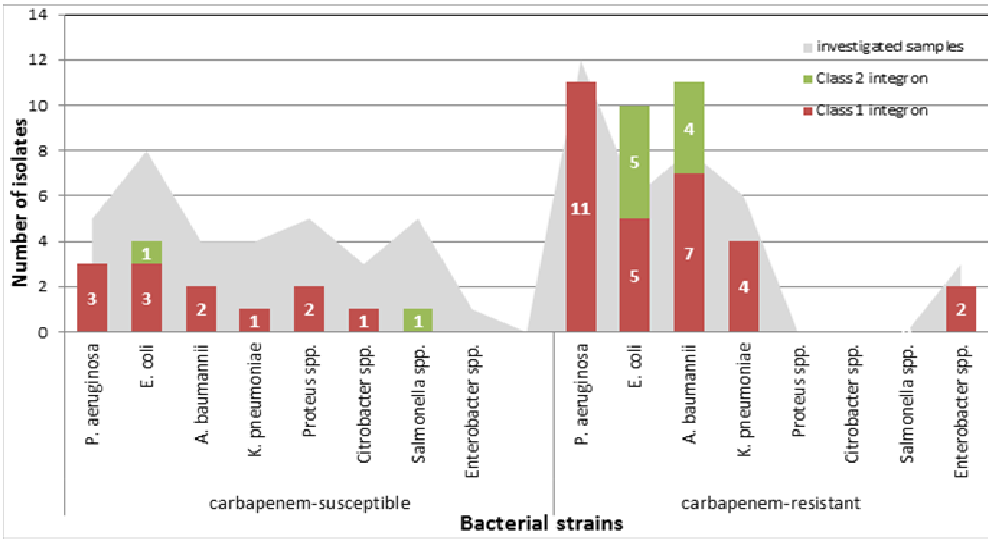

Fig. 2: Frequency distribution of class 1 and 2 integrons among carbapenem-susceptible and nonsusceptible Gram-negative bacteria

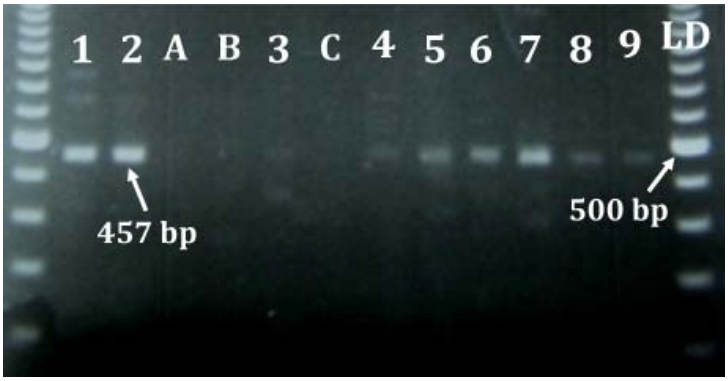

Fig. 3: PCR amplification of class 1 integron gene among some Gram-negative isolates on 1.3 % agarose gel. In all isolates, a fragment of 457 bp was detected. Lanes 1-9: positive isolates, lanes A, B, and C: negative samples, NC: negative control (distilled water), and LD: 100bp DNA ladder.

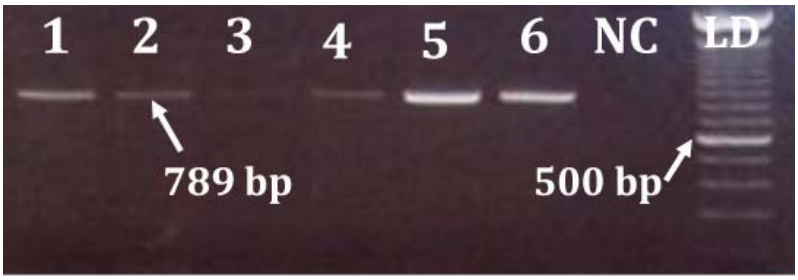

Fig. 4: PCR amplification of class 2 integron gene among some Gram-negative isolates on 1.3 % agarose gel. In all isolates, a fragment of 789 bp was detected. Lanes 1-6: positive isolates, NC: negative control (distilled water), and LD: 100bp DNA ladder.

DISCUSSION

Infections caused by resistant Gram-negative bacteria are becoming increasingly prevalent and now constitute a serious threat to public health worldwide because they are difficult to treat and are associated with high morbidity and mortality rates [12]. Integrons were recognized as an important cause of resistance among bacterial strains [13]. This study was designed to examine the antimicrobial resistance patterns and frequency of class 1, 2, and 3 integrons among groups of carbapenem-susceptible and nonsusceptiblae gram negative bacterial isolates in Assiut University Hospitals, Egypt. Both groups showed high resistance rate to most tested antibiotics in our work which is consistent with enormous previous reports [14-18].

Resistant gram negative bacterial isolates have increased markedly as a cause of several infections which implies a health problem worldwide[14]. In consistance with previous data[15,19], our patients that infected by carbapenem-nonsusceptible bacteria were admitted mainly to the ICU and were associated with significant co-morbidities. Patients who underwent surgical procedures or required invasive devices like IV or urinary catheters usually present with a more severe disease that may necessitate invasive medical procedures and long duration of hospital stay [19], especially for infections caused by the *Enterobacteriaceae*, *A. baumannii*, and *P. aeruginosa* [14]. In a previous PubMed search comprising 20 years from 1985 to 2005, identified 20 case-control studies demonstrated the most common risk factors associated with with drug-resistant *A. baumannii* infections were length of hospital stay, ICU admission, MV, invasive surgical procedures [15]. Class 1 integron was detected in most of our carbapenem-nonsusceptible isolates and in considerable number of the carbapenem-susceptible group, which is in accordance with previous findings[12,13,20]. About all carbapenem-non susceptible *P. aeruginosa* strains in our work expressed class 1 integrons. Class 1 integron were also mostly detected among *A. baumannii* and *E. coli* in both carbapenem-susceptible and non-susceptible isolates in our work. In previous data from China, results showed the wide expression of class 1 integrons in *P. aeruginosa* and *A. baumannii* strains [20], and also in *P. aeruginosa* isolates from Brazil [21], and in *A. baumannii* from the United Kingdom [22], in gram-negative isolates from Europe [23], in the *Enterobacteriaceae* group in The Netherlands [24] and France[25], and in *E. coli* strains in Taiwan [26]. In constrast to the high prevalence of class 1 integrons among our isolates, class 2 integrons were found among 6% and 26% of carbapenem-susceptible and nonsusceptible isolates, respectively, among *E. coli*, *A.*

baumannii, and *Salmonella* isolates. In consistance with our results, class 2 integrons were most frequently associated with members of the family *Enterobacteriaceae* and in *A. baumannii* [13]. Class 3 integrons were not detected among any of our isolates which related to previous findings [13, 27]. We compared susceptibility data and integron detection in carbapenem-suscepible and non-susceptible isolates, respectively. Isolates that expressed integron genes were significantly associated with high resistance rates to the tested antibiotics. This is due to the fact that, integrons carry many resistance genes to various groups of antibiotics [25]. Although there is a chance of finding the presence of integron elements in the absence of the antibiotic selection pressure, the high percent positive rate of integron integrase observed in this study indicates that there was already a strong selective pressure in the community. For combating gram-negative infections, we should be aware of the resistance patterns within the hospital institutions, and consider the clinical and demographic patient characteristics to guide us in the choice of empiric therapy.

CONCLUSION

Integrons were frequent among our Gram-negative bacteria. The presence of integrons necessitates the urgent demand for achieving effective measures to fight the dissemination of resistant bacterial strains.

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