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Integron occurrence is linked to reduced biocide susceptibility in multidrug resistant *Pseudomonas aeruginosa*

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ABSTRACT

Objective: Integrons are gene acquisition systems commonly found in bacterial genomes that play a major role in the dissemination of resistance to antibiotics. This work aimed to study the relationship between the presence of integrons and the reduced susceptibility of multidrug-resistant (MDR) *Pseudomonas aeruginosa* isolates towards different groups of biocides.

Methods: The antimicrobial susceptibility patterns of 104 clinical isolates were determined against different antibiotics by the disk diffusion method. The isolates were also tested for their susceptibility to six biocides (glutaraldehyde, benzalkonium chloride, cetrimide, chlorhexidine gluconate, chlorocresol and gluconate, and phenyl mercuric nitrate) by agar dilution. The presence of integrons and resistance genes in MDR isolates were detected by polymerase chain reaction.

Results: Thirty-six *Pseudomonas* isolates were MDR, and the majority of these isolates showed reduced susceptibility to biocides. In the MDR isolates, Class I integron was detected in 22 isolates (61.1%), while Class II and III integrons were identified in only four isolates (11.1%), In addition, *aacA4* and *qacE* genes were detected in 22 (61.1%) and 11 (30.5%) isolates, respectively. Integron I-positive isolates showed reduced susceptibility to tested biocides.

Conclusions: The current study reveals the presence of different classes of integrons, with class I being predominant. Class I integron may be responsible for generating MDR *P. aeruginosa* isolates with reduced susceptibility to biocides. This linkage between integrons and biocide resistance in MDR-*Pseudomonas* isolates is notable and could be clinically important. Strict antibiotic prescription policies and the adequate use of biocides could help in controlling this problem.

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Introduction

Pseudomonas aeruginosa is an opportunistic, nosocomial pathogen that is isolated mostly from patients with urinary tract and wound infections. Infections from *P. aeruginosa* can cause serious problems in hospitalised patients with cystic fibrosis, cancer and burns.[1–3] These infections are problematic due to the strong resistance of *P. aeruginosa* to antimicrobials.[2] Nosocomial infections caused by multidrug-resistant (MDR) *P. aeruginosa* represent a great threat, responsible for high morbidity and mortality among hospitalised patients.[3]

Biocides (disinfectants and antiseptics) are used extensively in hospitals and their effectiveness is very important for controlling microbial populations and preventing the transmission of infections in hospitals.[4] The risk of *P. aeruginosa* infection is increased with poor hospital hygiene, as associated strains can persist even in water systems for long periods.[5] *P. aeruginosa* tolerates a wide variety of physical conditions as well as many antibiotics [6] and has a remarkable ability to persist and multiply in incorrectly diluted and improperly stored biocide preparations.[2, 7]

Genes that confer resistance to biocides may be linked to antibiotic resistance genes based on their proximity on mobile genetic elements such as plasmids.[8] Integrons are efficient recombination and expression systems that are able to capture exogenous gene cassettes. Many integrons are linked to mobile genetic elements, such as plasmids, other integrons are located on chromosomes.[9] The most important components of any integron include the integrase gene (*intI*), the promoter and the adjacent recombination site (*attI*).[10] The integron classes that mostly contribute to the problem of MDR are class I, II and III.[11] Many of the antibiotic resistance genes found in MDR isolates of Gram-negative bacteria are part of the integron.[12] Classical mobile integrons might contain genes that are responsible for resistance to aminoglycosides (e.g. *aacA4*), β-lactams (e.g. *oxa9*), chloramphenicol (e.g. *cmlA2*) and sulphonamide (e.g. *sul1*), as well as genes that confer resistance to antiseptics and disinfectants (e.g. *qacE* and *qacEΔ*).[9, 12–14] In Gram-negative bacteria, the *qac* genes are often linked with class 1 integrons that carry a variety of antibiotic resistance genes.[15]

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The aim of the present study was to determine any potential link between different types of integrons from local MDR- *P. aeruginosa* isolates and the reduced susceptibility of these isolates to biocides.

Materials and methods

One hundred and four *P. aeruginosa* isolates were collected from Mansoura University hospital, Egypt, from different clinical samples (blood, urine, wound, burn, ear and endotracheal swabs). The isolates were identified using standard microbiological tests including: pyocyanin pigment production, growth at 42 °C, catalase, oxidase and oxidative–fermentative activity.[16] All the isolates were collected under approved ethical standards.

Antimicrobial susceptibility was determined by the disk diffusion method according to Clinical and laboratory Standard Institute (CLSI).[17] Susceptibility testing was performed using the following antibiotics: piperacillin (PRL, 100 µg), carbenicillin (CAR, 100 µg), cefotriaxone **(**CRO, 30 µg), ceftazidime (CAZ, 30 µg), imipenem (IPM, 10 µg), ciprofloxacin (CIP, 5 µg), amikacin (AK, 30 µg), gentamicin (CN, 10 µg), sulphamethoxazole/trimethoprime (SXT, 23.75/1.25 µg), and chloramphenicol (C, 30 µg). Antibiotic disks were obtained from Oxoid, Hampshire, England. The results were interpreted according to the criteria indicated in the CLSI guidelines.[17]

The minimum inhibitory concentrations (MIC) of six different biocides were determined by the agar dilution method.[18] The following biocides were tested: glutaraldehyde, benzalkonium chloride (El-Nasr Pharmaceutical Company, Cairo, Egypt), cetrimide, chlorhexidine gluconate, chlorocresol (Sigma Chemical Company, U.S.A), and phenyl mercuric nitrate (BDH, England), starting with concentrations recommended previously [19]. The biocides were incorporated into sterilised culture media to obtain the desired concentration ranges: 500–4000 mg l^{-1} (5–40 mmol) for glutaraldehyde, 100–800 mg l^{-1} (0.35–2.8 mmol) for benzalkonium chloride, 10–80 mg l^{-1} (0.02–0.16 mmol) for chlorhexidine, 750–2000 mg l^{-1} (5.3–14 mmol) for chlorocresol, 25–800 mg l^{-1} (0.07–2.35 mmol) for phenyl mercuric nitrate, and 500–4000 mg l^{-1} (1.5–12 mmol) for cetrimide. All biocides were added to the molten Mueller–Hinton agar medium (MHA) at 50 °C (Oxoid, Hampshire, England), except glutaraldehyde in which nutrient agar was used to minimise interaction with the medium. Biocide-containing agar media were mixed thoroughly and poured immediately into Petri dishes.

Overnight cultures of *Pseudomonas* isolates were adjusted to a turbidity of 0.5 McFarland standard and then diluted 1:10. Volumes of 5 µL from diluted bacterial suspensions (corresponding to about 10^4 CFU per spot) were spotted onto the surface of the agar plates containing various concentrations of the biocides and incubated overnight at 37 °C. The MIC was defined as the lowest concentration of biocide which inhibited the growth of tested isolates. The interpretation of the obtained MIC values was considered relative to the biocides MIC_{50}, where MICs > MIC_{50} were interpreted as reduced susceptible and MICs < MIC_{50} were interpreted as susceptible.[18]

Plasmid DNA was extracted using the Gene JET Plasmid Miniprep Kit (Thermoscientific, EU, Lithuania) according to the manufacturer's instructions. Different classes of integrons and resistance genes (*aacA4* and *qacE*) were detected by polymerase chain reaction (PCR) using the primers listed in Table 1. The Hep58 and hep59 primers were used to amplify the class I integron cassette region [20], while primers complementary to conserved segments of integrase genes were used to amplify the *intI2*, and *intI3* genes [21]. The *aacA4* [22] and *qacE* [13] genes were amplified from plasmid DNA of MDR isolates and also from the purified amplicons of Integron I for positive isolates using the Gene-JET Gel Extraction Kit.

Each PCR reaction consisted of 25 µL of dream Taq green PCR master mix **(**Thermoscientific**)**, 2 µL of each primer, 2µL of template DNA and nuclease-free water up to 50 µL. The amplification programme of integrons was as follows: initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing for 45 s at 55 °C, 57 °C or 59 °C for integron I, II and III, respectively, extension at 72 °C for 1 min and final extension at 72 °C for 7 min. Amplification for *aacA4* was as follows: initial denaturation at 94 °C for 5 min, 35 cycles of 30 s at 94 °C, 45 s at 60 °C and 20 s at 72 °C and final extension for 10 min at 72 °C. The PCR of the *qacE* gene was performed with 35 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min and extension at 72 °C for 1 min.

Table 1. Primers used and amplicon sizes.

Target sequence	Primers	Sequence 5′-3′	Product size (bp)	Reference
IntI	hep58-F	TCATGGCTTGTTATGACTGT	Variable (1600 or 1200)	20
	hep59-R	GTAGGGCTTATTATGCACGC		
IntII	intI2L-F	CACGGATATGCGACAAAAAGGT	789	21
	intI2-R	GTAGCAAACGAGTGACGAAATG		
IntIII	intI3L-F	GCCTCCGGCAGCGACTTTCAG	980	21
	intI3-R	ACGGATCTGCCAAACCTGACT		
AccA4	*accA4*-F	GCTCTTGGAAGCGGGGACGG	300	22
	accA4-R	TCGCTCGAATGCCTGGCGTG		
qacE	*qacE*-F	GGGAATTCGCCCTACACAAATTGGGAGA	350	13
	qacE-R	TACTCGAGTTAGTGGGCACTTGCTTTGG		

Results

All of the 104 *P. aeruginosa* isolates were resistant to carbenicillin. Susceptibility to other antiboitics are shown in Table 2. The antimicrobial susceptibility data revealed that 36 (34.6%) isolates were MDR, being resistant to three or more classes of antimicrobial agents (1, 16, and 19 isolates were resistant to 3, 4 or 5 classes, respectively).

The MICs of six different biocides were determined for all *P. aeruginosa* isolates. In case of MDR isolates, 4000 mg l^{-1} of glutaraldehyde and cetrimide was inhibitory to all MDR isolates (the MIC_{50}s were 1200 and 1750, respectively). For chlorocresol, 2000 mg l^{-1} inhibited the growth of all MDR isolates (MIC_{50} was 1250). While in case of benzalkonium chloride and phenyl mercuric nitrate, 800 mg l^{-1} inhibited the growth of all MDR isolates (MIC_{50}s were 150 and 300, respectively). For chlorhexidine, 80 mg l^{-1} made complete inhibition to all MDR isolates and the MIC_{50} was 30 mg l^{-1}. The MICs for the non-MDR isolates were lower than the MDR isolates and the MIC_{50}s were as follows: 750 mg l^{-1} for glutaraldehyde, 850 mg l^{-1} for cetrimide, 900 mg l^{-1} for chlorocresol, 60 mg l^{-1} for benzalkonium chloride, 50 mg l^{-1} for phenyl mercuric nitrate and 10 mg l^{-1} for chlorhexidine.

PCR amplification of the integron I variable region revealed that only 22 (61.1%) of the MDR isolates were integron I positive. Seventeen isolates that were integron I positive had amplicon sizes of 1200 bp, while the remaining five isolates had amplicon sizes of 1600 bp (Figure 1(a)). For integron II, only four MDR isolates (11.1%) were integron II positive with amplicon sizes of 800 bp (Figure 1(b)). In the case of integron III, only four of the MDR isolates (11.1%) were integron III positive having amplicon sizes of 1000 bp (Figure 1(c)). The *aacA4* gene was detected in 22 (61.1%) of the MDR isolates with amplicon sizes of 300 bp (Figure 1(d)), 16 of them (44.4%) were positive for integrons and *aacA4* (Table 3). The *qacE* gene was detected in 11 (30.5%) of the MDR isolates with amplicon sizes of 350 bp, and was only found in integron I-positive isolates (Figure 1(e), Table 3).

The presence of different classes of integrons, resistance genes, as well as the reduced susceptibility to biocides and antibiotic resistance profile are shown in Table 3. The data showed that almost all the integron I-positive isolates had reduced susceptibility to the tested biocides.

Table 2. Susceptibility pattern of P. aeruginosa isolates to different antibiotics.

	Pseudomonas spp (n = 104)		
Antibiotic disk	*R* (%)	*I* (%)	*S* (%)
Imipenem	19.2	2	78.8
Piperacillin	20.2	0	79.8
Amikacin	24	3	73
Gentamicin	26.9	3.9	69.2
Ciprofloxacin	30.8	6.7	62.5
Chloramphenicol	38.5	20.2	41.3
Sulphamethoxazole/ Trimethoprime	56.7	30.8	12.5
Ceftazidime	69.2	17.3	13.5
Ceftriaxone	83.6	2.9	13.5
Carbenicillin	100	0	0

R = resistant, I = intermediate, and S = sensitive.

Discussion

In the recent decades, there has been debate over the selection of resistance between antibiotics and biocides. [8] The current study aimed to elucidate the relationship between integrons and the reduced susceptibility to biocides in MDR-*P. aeruginosa*.

Among the 104 *P. aeruginosa* isolates in this study, 36 (34.6%) were MDR. Previous local studies showed higher resistance rates, with more than 50% of isolates being MDR,[23, 24] but other global studies showed lower MDR rates.[25, 26] This could be attributed to several factors including the misuse of antibiotics in local hospitals. The data revealed that imipenem, piperacillin, amikacin, gentamicin and ciprofloxacin were the best therapeutic options for treating *P. aeruginosa* infections. Here, we show that 19.2% of the isolates were resistant to imipenem, lower than those (39.3% and 41.4%) previously reported regionally.[27, 28] Prior studies are consistent with our data.[29, 30] Increased antimicrobial use is the main driving force leading to the evolution of drug-resistant bacteria.[31] Therefore, strict policies are urgently required to limit the unnecessary use of carbapenems to minimise the emergence of resistance to these valuable antibiotics.

Although the CLSI has published guidelines on the classifying of strains as sensitive or resistant to antibiotics, there is no equivalent guidelines for biocides [32]. However, some researchers have analysed the MIC values of biocides using different protocols and provided limited data relating to industrial and clinical strains [18, 32–34]. The MIC for glutraldhyde in our study was lower than previously recommended 2% concentration,[19] comparable to previously reported results. [33] The MIC for chlorhexidine was slightly higher than that reported previously,[32, 34] but lower than results of other researchers.[18, 33] The MIC of benzalkonium chloride was higher than the previously reported for *P*seudomonas and *Acinetobacter* strains.[18, 32, 34] For phenyl mercuric nitrate and cetrimide, the MICs were also higher than the previously reported.[33] The MIC for chlorocresol was higher than the 0.05% suggested by the United States Pharmacopeia (USP).[19] It has been reported that contact of microbes with sublethal concentrations of biocides can select for strains with an increased MIC,[34, 35] indicating that biocides should be only used at appropriate concentrations to prevent the survival of the MDR strains in the hospital environment.

The frequency of class I integron in the present study (61.1%) is relatively higher than previously reported rates of 57.4% from Nigeria [36] and 45.8% from China.[37] However, the rate is lower than the 69.2% reported from

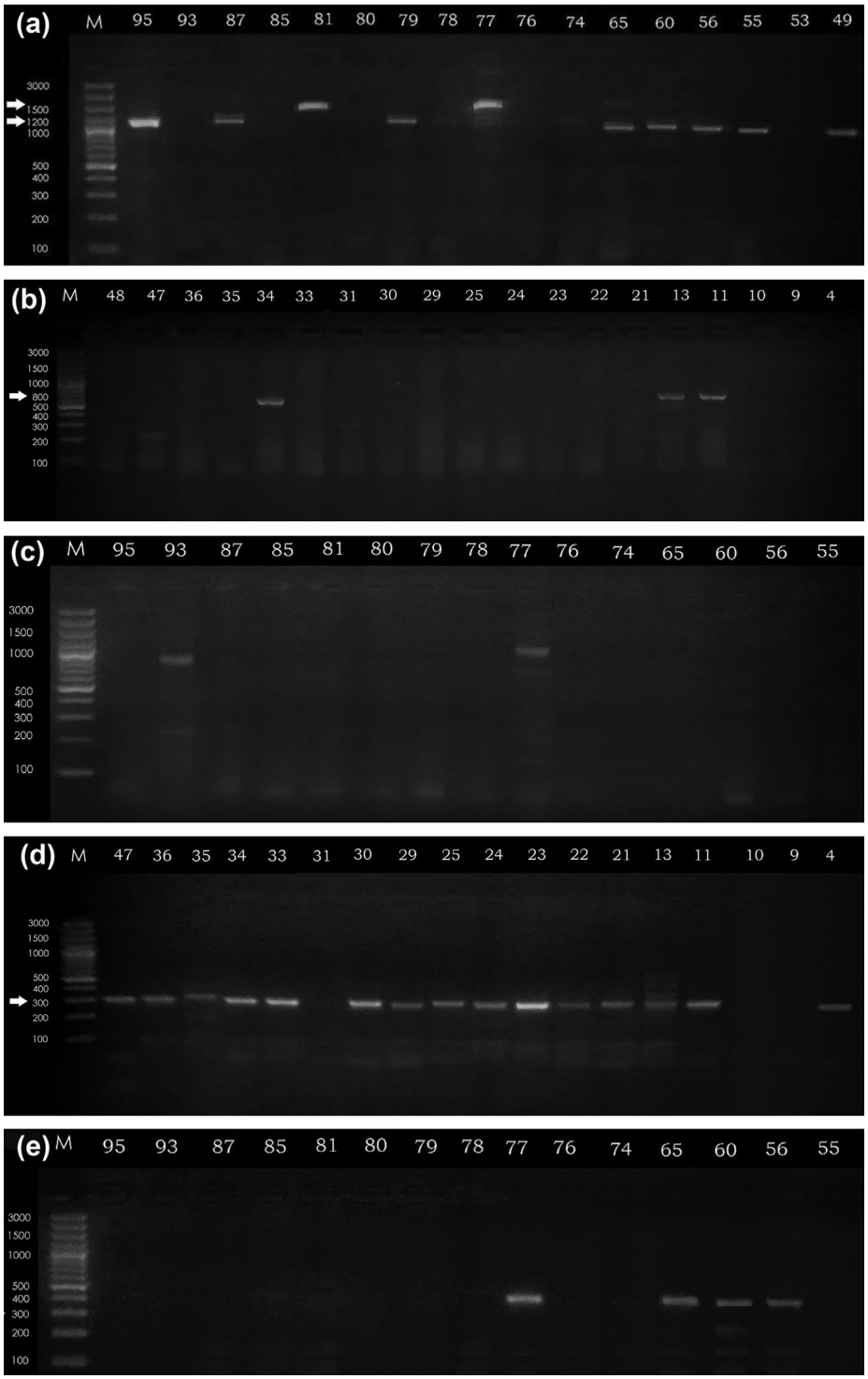

Figure 1. Gel electrophoresis of PCR products for detection of (a) Integron I: most of the Integron I positive strains gave 1200 bp bands, only isolates 77 and 81 gave 1600 bp bands, (b) Integron II: positive isolates with amplicon sizes of approximately 800 bp. (c) Integron III: positive isolates with amplicon sizes of approximately 1000 bp. (d) *aacA4* gene: positive isolates with amplicon sizes of approximately 300 bp. (e) *qacE* gene: positive isolates with amplicon sizes of approximately 350 bp.

Iran,[38] and the 71.4% reported recently from Egypt.[24] On the other hand, class II and class III integrons were each detected in only four isolates, consistent with a previous study.[37] The class I integron remains the most common integron found in clinically important Gram-negative bacteria including *P. aeruginosa,* while the presence of class II and class III integrons are less frequently reported.[39]

The integron system has the ability to create novel combinations of resistance genes, leading to the evolution and spread of resistance.[9–11] In the current study, the aminoglycoside resistance gene (*aacA4*) was detected in 61.1% of the MDR isolates, comparable to the 58.3% previously reported.[22] The *aacA4* gene was found on integron I in 15 out of 22 *aacA4*-positive isolates. Also the *qacE* gene was detected in 11 (30.5%) of

Table 3. Incidence of integrons, resistance genes and reduced susceptibility to biocides in MDR Pseudomonas aeruginosa isolates.

Isolate No.	Integron type (bp)	Resistance genes	Biocides with reduced susceptibility *	Antibiotic(s) resistance profile
4	I (1200)	*aacA4*	GLU, BKC, CET, CC, CHX, PMN	CIP, AK, CN, C, SXT, CRO, CAZ, CAR
9	I (1200)		GLU, BKC, CET, CC, CHX, PMN	PRL, C, SXT, CRO, CAZ, CAR, CIP
10	–	–	–	CIP, C, SXT, CRO, CAZ, CAR
11	II & III	*aacA4*	–	CIP, CN, PRL, C, SXT, CRO, CAZ, CAR
13	I (1600), II	*aacA4, qacE*	GLU, BKC, CET, CC, CHX, PMN	CIP, C, SXT, CRO, CAZ, CAR
21	I (1200)	*aacA4, qacE*	GLU, BKC, CC, CHX, PMN	CIP, IPM, C, SXT, CRO, CAZ, CAR
22	–	*aacA4*	–	
23	I (1200)	*aacA4*	GLU, BKC, CET, CC, CHX, PMN	AK, CN, C, SXT, CAR
24	I (1600)	*aacA4, qacE*	GLU, BKC, CET, CC, CHX, PMN	CIP, IPM,, C, SXT, CAZ, CAR
25	I (1200)	*aacA4, qacE*	GLU, BKC, CET, CC, CHX, PMN	CIP, AK, CN, C, SXT, CRO, CAZ, CAR
29	–	*aacA4*	–	AK, CN, IPM, PRL, C, SXT, CRO, CAZ, CAR
30	I (1200)	*aacA4, qacE*	GLU, BKC, CET, CC, CHX, PMN	CIP, AK, CN, PRL, SXT, CAR
31	–	–	–	CIP, AK, CN, PRL, C, SXT, CRO, CAR
33	I (1200)–	*aacA4*	BKC, CET, CC, CHX, PMN	CIP, CN, IPM, PRL, C, SXT, CRO, CAZ, CAR
34	I (1200)II	*aacA4*	GLU, BKC, CC, PMN	CIP, AK, CN, C, SXT, CRO, CAZ, CAR
35	–	*aacA4*	–	CIP, IPM,, C, SXT, CAZ, CAR
36	I (1200), III	*aacA4*	GLU, BKC, CET, CC, CHX, PMN	AK, CN, IPM, PRL, CIP, SXT, CRO, CAZ, CAR, C
47		*aacA4*	–	CIP, AK, CN, PRL, C, SXT, CRO, CAZ, CAR
48	I (1600)	*qacE--*	GLU, BKC, CET, CC, CHX, PMN	
49	I (1200)	*qacE*	GLU, BKC, CET, CC, CHX, PMN	AK, CN, IPM, PRL, CIP, SXT, CRO, CAZ, CAR, C
53	–	*aacA4*	–	AK, CN, PRL, C, SXT, CRO, CAZ, CAR
55	I (1200)	*aacA4*	GLU	CIP, CN, C, SXT, CRO, CAZ, CAR
56	I (1200)	*aacA4, qacE*	GLU, BKC, CET, CC, CHX, PMN	
60	I (1200)	*aacA4, qacE*	GLU, BKC, CET, CC, CHX, PMN	CIP, AK, CN, IPM, PRL, SXT, CRO, CAZ, CAR
65	I (1200)	*aacA4, qacE*	GLU, BKC, CET, CC, CHX, PMN	
74	–	–	–	CIP, AK, CN, PRL, C, SXT, CRO, CAZ, CAR
76	–		–	CIP, AK, CN, C, SXT, CRO, CAZ, CAR
77	I(1600), III	*aacA4, qacE*	GLU, BKC, CET, CC, CHX, PMN	CIP, AK, CN, IPM, SXT, CRO, CAR
78	–	*aacA4*	–	CIP, AK, CN, IPM, C, SXT, CRO, CAZ, CAR
79	I (1200), II			CIP, AK, CN, IPM, PRL, SXT, CRO, CAZ, CAR
80	–		–	CIP, AK, CN, IPM, C, SXT, CRO, CAZ, CAR
81	I (1600)		GLU, CET, CC	CIP, AK, CN, SXT, CRO, CAR
85	–		–	CIP, AK, CN, IPM, C, SXT, CRO, CAZ, CAR
87	I (1200)		GLU, BKC, CET, CC, CHX, PMN	
93	III		–	CIP, C, SXT, CRO, CAR
95	I (1200)		BKC, CET, CC, CHX, PMN	C, SXT, CRO, CAZ, CAR

Notes: IPM: imipenem, PRL: piperacillin, AK: amikacin, CN: gentamicin, CIP: ciprofloxacin, C: chloramphenicol SXT: sulphamethoxazole/trimethoprime, CAZ: ceftazidime, CRO: ceftriaxone, CAR: carbenicillin, GLU: glutaraldehyde BKC: Benzalkonium chloride CET: Cetrimide, CC: Chlorocresol CHX: Chlorhexidine PMN: Phenyl mercuric nitrate. *MIC > MIC_{50}.

the MDR isolates which were also integron I positive and showed reduced susceptibility to quaternary ammonium compounds (QAC). Kazama *et al.* detected the *qacE* gene at lower rates,[13], but a recent study reported a rate of 59% [40]. Other *qac* genes (e.g. *qacEΔ1*) [13, 40] or efflux pump overexpression (e.g. *Mex-Opr*) [35] could participate in reduced susceptibility to QAC in the rest of the isolates.

Different studies have confirmed the relationship between the presence of integron and resistance of *P. aeruginosa* to antibiotics.[36–38] In the present study, the occurrence of integron I could be linked with reduced susceptibility to biocide as the majority of integron I-positive isolates showed MIC values exceeding the MIC_{50} of each of the tested biocides. On the other hand, the isolates that only harboured integrons II or III were susceptible to all biocides under investigation.

In conclusion, the presence of integron I in MDR isolates could be responsible for the reduced biocide susceptibility in these isolates. This finding compounds the dilemma of eliminating resistant strains in hospitals. Biocides should always be used at adequate concentrations for maximising disinfection efficacy to control microbial populations and prevent the transmission of MDR isolates. Our results represent an advance in biomedical science as the first to report a link between integron I and biocide resistance in MDR-*P. aeruginosa*.

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Disclosure statement

No potential conflict of interest was reported by the authors.

Summary table

What is known about this subject:
- Integrons are gene acquisition systems commonly found in bacterial genomes.
- Integrons may have a role in dissemination of microbial resistance to chemotherapeutics
- The infections with MDR *P. aeruginosa* can cause serious problem especially in hospitalized patients

What this study adds:
- The current work shows an increased resistance of *Pseudomonas aeruginosa* isolates to different groups of antibiotics.
- A positive correlation was found between MDR resistance in isolates and low susceptibility to tested biocides
- Resistance to biocides was linked to presence of integron I that was found to be predominant in isolates showing high biocides' MICs.

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