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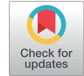
Biocide tolerance and antibiotic resistance of *Enterobacter* spp. isolated from an Algerian hospital environment



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ABSTRACT

Objectives: In this study, 77 *Enterobacter* spp. isolates from a collection of 175 Gram-negative bacilli isolated from Tlemcen University Hospital Center (North-West of Algeria) were tested for antibiotic resistance, biocide tolerance and genetic determinants of antimicrobial resistance.
Methods: The isolates were identified by 16S rDNA gene sequencing. Biocide tolerance was determined by broth microdilution, and antibiotic resistance was determined by disk diffusion. Genetic determinants of resistance were studied by PCR amplification using suitable primers.
Results: The most common *Enterobacter* species was *Enterobacter cloacae* (58.4%), followed by *Enterobacter hormaechei* (24.7%). The most common antibiotic resistance was to ticarcillin either alone or in combination with clavulanic acid (70.1%), followed by cefepime (68.8%), cefotaxime (63.6%), ceftazidime (54.5%) and gentamicin (54.5%). Tobramycin was active against 87.0% of the isolates. Levels of biocide tolerance were high for hexachlorophene and to a lesser extent for benzalkonium chloride. The extended-spectrum β-lactamase genes bla_{TEM} and $bla_{CTX\text{-}M}$ were detected in 44.2% and 36.4% of isolates, respectively. Other antimicrobial resistance genes (ARGs) frequently detected were *aac(6′)-Ib* (57.1%) and *sul2* (50.6%). Multidrug-resistant isolates carrying several ARGs were common. Significant positive correlations were detected for efflux pump genes with ARGs and also between ARGs.
Conclusion: The results of this study reveal that*Enterobacter spp.* isolates from hospital settings are both resistant to clinically-used antibiotics and tolerant to biocides. Biocide tolerance could be an advantage for antibiotic-resistant strains in hospitals.
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1. Introduction

Nosocomial infections are responsible for significant morbidity and mortality in the hospital environment [1]. It has been estimated that 20–40% of healthcare-related infections are due to cross-transmission via the hands of caregivers contaminated by direct contact with patients or indirectly by touching contaminated environmental surfaces [2]. In recent years, considerable efforts have been made to improve control practices for these infections, which has led to increased use of biocides [3]. The latter can often lead to the appearance of the adaptation phenomenon and consequently to resistance to biocide molecules [4], which regularly affects new species [5,6]. Although bacterial insensitivity to biocides was described in the 1950s and 1960s and appears to be increasing [7], debate is currently ongoing regarding the possible emergence of biocide tolerance in hospitals and whether this tolerance may be accompanied by antibiotic resistance. Although few studies have confirmed this co-resistance [8,9], some authors have reported that Gram-negative bacteria that have developed tolerance to certain biocidal compounds may also be non-susceptible to certain antibiotics [10].

Enterobacter spp. are frequently found in natural environments such as water, wastewater, vegetables and soil [11]. These bacteria are a very common pathogen and are frequently isolated in hospitals [12,13]. They are therefore often involved in healthcare-related infections, causing 8% of nosocomial bacteraemia cases, and are also the second most common Gram-negative pathogen of pneumonia in intensive care unit patients [14]. The presence of *Enterobacter* spp. in hospital settings is increasingly alarming because of its rapid development of antimicrobial resistance [11].

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Some species belonging to this genus are capable of acquiring many mechanisms that contribute significantly to antimicrobial resistance, which considerably reduces therapeutic choices [14,15].

Since no report regarding the status of biocide tolerance in Algeria is available, the aim of the current study was to determine the efficacy of biocides on a collection of *Enterobacter* spp. isolates of hospital origin and to explore the possible correlation between tolerance to biocides and antibiotic resistance as well as to characterise the genetic background of this antimicrobial resistance.

2. Materials and methods

2.1. Sampling and bacterial isolation

A total of 256 samples were obtained over 5 months (November 2015 to March 2016) in the operating theatres of the targeted services (surgery A, orthopaedics and traumatology, and medical–surgical emergencies) at Tlemcen University Hospital Center (North-West of Algeria). The samples were taken by the swabbing technique on different surfaces. Following enrichment in brain–heart infusion broth (Liofilchem, Roseto degli Abruzzi, Italy) for 24 h at 37 °C, bacteria were isolated and purified on MacConkey agar (Liofilchem).

2.2. Identification of bacterial isolates

Isolates were first identified by conventional tests (Gram stain, catalase test and oxidase test) and were confirmed using an API 20E system (bioMérieux, Marcy-l'Étoile, France). For molecular identification, amplification of the 16S rDNA gene was performed as previously described [16,17]. Following DNA extraction using a Bacterial Genomic DNA Extraction Kit (Xtrem Biotech, SL, Granada, Spain) according to the manufacturer's instructions, amplicons were purified using an Illustra™ ExoProStar 1-step Purification Kit (GE Healthcare, Little Chalfont, UK) and were then sequenced by the sequencing centre Sistemas Genómicos (Valencia, Spain). A search for homology of the DNA sequences was performed using the BLAST algorithm available at the National Center for Biotechnology Information (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.3. Antimicrobial susceptibility testing

Isolates identified as *Enterobacter* spp. were selected for further study. The susceptibility of *Enterobacter* spp. isolates to the antibiotics ticarcillin, ticarcillin/clavulanic acid, piperacillin/tazobactam, ceftazidime, cefotaxime, cefepime, imipenem, trimethoprim/sulfamethoxazole, chloramphenicol, ciprofloxacin, ofloxacin, amikacin, gentamicin, fosfomycin, nalidixic acid, tobramycin, colistin and aztreonam was tested by the disk diffusion method on Mueller–Hinton agar (Liofilchem) according to Clinical and Laboratory Standards Institute (CLSI) guidelines [18].

2.4. Determination of biocide tolerance

Minimum inhibitory concentrations (MICs) were determined by the broth microdilution method for the following five biocides: benzalkonium chloride (BC); cetrimide; chlorhexidine; hexadecylpyridinium chloride; and hexachlorophene (CF). CF was dissolved in ethanol (10% w/v). The remaining biocides were aseptically dissolved in tryptic soy broth (Liofilchem) to achieve final concentrations ranging from 8–2048 mg/L. Then, 180 μL of each biocide dilution was incubated with a bacterial suspension adjusted to a density equivalent to 5×10^5 CFU/mL.

Positive controls (broth + bacterial suspension) and negative controls (broth + biocide) were included in the microplate. Incubation was performed for 24 h at 37 °C and the optical density reading was carried out using a ELx808 microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

2.5. Detection of antimicrobial resistance genes (ARGs) by PCR

Genes related to antimicrobial resistance were investigated by PCR using the primers indicated in Supplementary Table S1. The efflux pump genes searched for were *mdfA* [19] and *oqxA* of the OqxAB multidrug efflux pump [20].

The investigated genes encoding β-lactam resistance were bla_{TEM} [21], bla_{PSE} [22], $bla_{CTX\text{-}M}$ and $bla_{CTX\text{-}M\text{-}2}$ [23], $bla_{NDM\text{-}1}$, bla_{IMP}, $bla_{OXA\text{-}23}$ and $bla_{VIM\text{-}2}$ [24]. Other genetic determinants investigated were as follows: the tetracycline resistance genes *tet*(B), *tet*(C) and *tet*(D) [25]; the aminoglycoside resistance gene *aac(6')-Ib* [26]; the streptomycin resistance gene *aadA* [27]; the phenicol resistance gene *floR* [22]; the trimethoprim resistance genes *dfrA12* and *dfrA15* [27]; and the sulfonamide resistance genes *sul1*, *sul2* and *sul3* [21]. The integrase gene *intI1* was investigated using the primers intF and intR [28].

2.6. Statistical analysis

Statistical analysis, including the correlation matrix, was performed using IBM SPSS Statistics for Windows v.22 (IBM Corp., Armonk, NY). Positive correlations were defined as very weak (0.00–0.19), weak (0.20–0.39), moderate (0.40–0.59), strong (0.60–0.79) or very strong (0.80–0.99), with significant *P*-values of <0.05.

3. Results

3.1. Bacterial identification

A total of 175 Gram-negative bacilli were recovered from the hospital samples. 16S rDNA gene sequencing allowed the identification of 77 *Enterobacter* spp. isolates, representing a prevalence of 44.0%. *Enterobacter cloacae* was the most common species (45/77; 58.4%), followed by *Enterobacter hormaechei* (19/77; 24.7%). Other species identified were *Enterobacter xiangfangensis* (4/77; 5.2%), *Enterobacter cancerogenus* (1/77; 1.3%) and *Enterobacter* (*Cronobacter*) *sakazakii* (1/77; 1.3%).

3.2. Antimicrobial susceptibility

Among the β-lactams tested, imipenem was the most efficient, with only six resistant isolates. Some β-lactam antibiotics were not effective, e.g. a resistance rate of approximately 70% for ticarcillin; combination of ticarcillin with clavulanic acid did not significantly restore its effectiveness. The tested cephalosporins (cefepime, cefotaxime and ceftazidime) and monobactams (aztreonam) were moderately effective, with resistance rates of 68.8%, 63.6%, 54.5% and 41.6%, respectively. The activity of quinolones differed depending on the molecule used, with recorded resistance rates of 46.8% for nalidixic acid, 20.8% for ciprofloxacin and 11.7% for ofloxacin. Aminoglycosides were not spared of the resistance phenomenon, although tobramycin was active against 870.% of the isolates. Gentamicin was ineffective against 54.5% of the isolates. It should be noted that no colistin-resistant isolates were identified during the study.

3.3. Biocide tolerance

The MICs of the five biocides are shown in Table 1. MIC distributions for the predominant species found in the study (*E. cloacae* and *E. hormaechei*) are shown in Fig. 1. The activity of the biocides differed according to the molecules tested. Thus, some biocides were distinguished by high MICs (up to 128 mg/L for CF),

Table 1
Antibiotic resistance, biocide tolerance and genetic determinants of resistance detected in *Enterobacter* spp. isolates from an Algerian hospital environment ($n = 77$).

Enterobacter spp.[a]	Detected ARG(s)	Antibiotic resistance[b]	Biocide MIC (mg/L)				
			HDP	BC	CF	CT	CH
E. cancerogenus zb01	*aac(6′)-Ib*	TIC, TCC, CTX, FEP, CIP	32	32	64	64	32
E. cloacae zb01	*aac(6′)-Ib*, *dfrA12*, *sul2*, bla_{TEM}, bla_{VIM-2}	TIC, TCC, TZP, CAZ, CTX, FEP, IPM, SXT, CHL, CIP, OFX, AMK, GEN, NAL	32	64	128	32	16
E. cloacae zb02	*dfrA12*, *sul2*, *aac(6′)-Ib*, bla_{TEM}	TIC, TCC, CAZ, CTX, FEP, SXT, CHL, CIP, GEN, NAL	32	32	128	64	8
E. cloacae zb03	*aac(6′)-Ib*, *sul2*, bla_{CTX-M}, bla_{TEM}	TIC, TCC, CAZ, CTX, FEP, SXT, CHL, GEN, ATM	32	32	128	32	8
E. cloacae zb04	*intI1*	TIC, TCC, TZP, CAZ, CTX, FEP, SXT, CHL, CIP, OFX, AMK, GEN, NAL, TOB, ATM	64	32	128	64	16
E. cloacae zb05	*dfrA12*	FOS	64	32	128	64	16
E. cloacae zb06		CTX	64	32	128	32	32
E. cloacae zb07	*sul2*, *tet*(B), *floR*, bla_{NDM-1}, bla_{OXA-23}	TIC, TCC, TZP, CAZ, CTX, FEP, IPM, SXT, CHL, CIP, OFX, AMK, GEN, NAL, TOB, ATM	64	64	128	32	16
E. cloacae zb08	*aac(6′)-Ib*, *dfrA12*, *sul2*, bla_{CTX-M}, bla_{TEM}	TIC, TCC, TZP, CAZ, CTX, FEP, SXT, CHL, GEN, NAL, TOB, ATM	64	64	128	64	32
E. cloacae zb09	*aac(6′)-Ib*, *intI1*	TIC, CHL	32	64	128	64	8
E. cloacae zb10	*dfrA12*, *aadA*, *aac(6′)-Ib*, bla_{CTX-M}, $bla_{CTX-M-2}$, bla_{TEM}	TIC, TCC, TZP, CAZ, CTX, FEP, SXT, GEN, NAL, ATM	32	64	128	64	16
E. cloacae zb11	*dfrA12*, *sul1*, *aac(6′)-Ib*	TIC, TCC, CHL, ATM	64	64	128	32	32
E. cloacae zb12	*dfrA12*, *mdfA*, *sul2*, *tet*(B), *aac(6′)-Ib*, bla_{CTX-M}, bla_{TEM}	TIC, TCC, CAZ, CTX, FEP, SXT, CHL, GEN, ATM	16	32	64	32	32
E. cloacae zb13	*dfrA12*, *aadA*, *aac(6′)-Ib*, bla_{CTX-M}, $bla_{CTX-M-2}$, bla_{TEM}	TIC, TCC, TZP, FEP	64	32	128	32	16
E. cloacae zb14	*dfrA12*		32	32	128	32	32
E. cloacae zb15	*intI1*, $bla_{CTX-M-2}$	ATM	16	32	128	64	32
E. cloacae zb16	*sul2*, *aadA*, *aac(6′)-Ib*, bla_{TEM}	TIC, TCC, TZP, CAZ, CTX, FEP, SXT, CHL, GEN, NAL, ATM	128	16	64	32	32
E. cloacae zb17	*sul2*, *aac(6′)-Ib*, bla_{CTX-M}	TIC, TCC, TZP, CAZ, CTX, FEP, IPM, SXT, CHL, CIP, OFX, AMK, GEN, NAL, TOB, ATM	32	32	128	64	32
E. cloacae zb18	*oqxA*, *floR*	FEP, FOS	<512	64	64	64	32
E. cloacae zb19	*aadA*, *aac(6′)-Ib*	FEP	64	32	128	32	32
E. cloacae zb20	*dfrA12*, *aac(6′)-Ib*		64	32	64	32	32
E. cloacae zb21	*intI1*, *aac(6′)-Ib*, *floR*	TZP, CTX, FEP	<512	64	64	64	32
E. cloacae zb22	bla_{CTX-M}, bla_{IMP}	TIC, TCC, CAZ, CTX, FEP, IPM, GEN, ATM	<512	128	64	128	64
E. cloacae zb23	*sul1*, *sul2*, *aadA*, bla_{IMP}	TIC, TCC, CAZ, CTX, FEP, IPM, SXT, CHL, GEN, NAL, ATM	32	128	128	64	32
E. cloacae zb24	*dfrA12*, *sul2*, *aac(6′)-Ib*, bla_{TEM}	TIC, TCC, CAZ, CTX, FEP, SXT, CHL, GEN, NAL, ATM	64	32	64	32	16
E. cloacae zb25	*dfrA12*, *sul2*, *aac(6′)-Ib*, bla_{TEM}	TIC, TCC, CAZ, CTX, FEP, SXT, CHL, CIP, GEN, NAL	64	32	128	32	16
E. cloacae zb26	bla_{CTX-M}	FOS	64	32	128	64	16
E. cloacae zb27	*floR*	TIC, TCC, CAZ	8	8	128	32	4
E. cloacae zb28	*dfrA12*, *aadA*, *aac(6′)-Ib*	TIC, TCC, FEP, SXT, CHL, OFX, NAL	64	32	128	32	16
E. cloacae zb29	*dfrA12*, *aac(6′)-Ib*	TIC, TCC, CTX, FEP, SXT, CHL, CIP, OFX, NAL	64	32	128	32	16
E. cloacae zb30	*intI1*, *sul2*	TIC, TCC, CAZ, CTX, FEP, AMK, GEN, ATM	32	32	64	64	32
E. cloacae zb31	*sul2*, *aac(6′)-Ib*, bla_{CTX-M}, bla_{TEM}	TIC, TCC, CAZ, CTX, FEP, SXT, CHL, GEN	64	64	128	32	32
E. cloacae zb32	*aac(6′)-Ib*	CHL, ATM	64	32	128	32	32
E. cloacae zb33	*aac(6′)-Ib*, bla_{CTX-M}	TIC, TCC, CAZ, CTX, SXT, AMK, GEN, NAL, TOB, ATM	32	32	128	32	32
E. cloacae zb34	*dfrA12*, *oqxA*, *sul2*, *aadA*, *aac(6′)-Ib*, bla_{CTX-M}, bla_{TEM}	TIC, TCC, TZP, CAZ, CTX, FEP, SXT, CHL, CIP, GEN, NAL	64	64	128	32	32
E. cloacae zb35	*dfrA12*, *aadA*, bla_{CTX-M}, bla_{TEM}	TIC, TCC, TZP, CAZ, CTX, FEP, SXT, CHL, GEN, NAL	32	32	128	16	32
E. cloacae zb36	bla_{CTX-M}		256	64	128	32	8
E. cloacae zb37	*sul1*, *aadA*, *aac(6′)-Ib*	CTX, CHL, NAL	32	16	128	32	32
E. cloacae zb38	*sul1*, *aadA*	TIC, CTX, FEP, CHL, AMK, GEN, FOS	32	32	128	32	32
E. cloacae zb39	*dfrA12*, *sul2*, *aadA*, bla_{CTX-M}, bla_{TEM}	TIC, TCC, TZP, CAZ, CTX, FEP, SXT, CHL, GEN, NAL	32	32	128	32	32
E. cloacae zb40	*dfrA12*, bla_{CTX-M}		32	32	64	64	32
E. cloacae zb41	*dfrA12*, *sul2*, *aadA*, *aac(6′)-Ib*, bla_{CTX-M}, bla_{TEM}	TIC, TCC, TZP, CAZ, CTX, FEP, SXT, CHL, GEN, NAL, ATM	64	32	128	32	16
E. cloacae zb42	*intI1*, *sul2*, *aac(6′)-Ib*, bla_{CTX-M}	TIC, TCC, TZP, CAZ, CTX, FEP, SXT, CHL, GEN, NAL, ATM	32	32	128	64	32
E. cloacae zb43	*sul1*, *sul2*, *sul3*, bla_{TEM}	TIC, TCC, TZP, CAZ, CTX, FEP, SXT, CHL, CIP, OFX, AMK, GEN, NAL, TOB, ATM	32	32	128	32	32
E. cloacae zb44	*intI1*, *sul2*		64	32	128	64	32
E. cloacae zb45	*intI1*, *dfrA12*, *sul2*, *aadA*, *aac(6′)-Ib*, bla_{CTX-M}, bla_{TEM}	TIC, TCC, TZP, CAZ, CTX, FEP, SXT, CHL, CIP, GEN, NAL	64	32	128	32	32
E. hormaechei zb01	*intI1*, *sul2*, *aadA*, *floR*, bla_{TEM}		32	32	128	32	16
E. hormaechei zb02	*dfrA12*, *sul2*, *aac(6′)-Ib*, bla_{TEM}	TIC, TCC, CAZ, CTX, FEP, SXT, CHL, AMK, GEN, NAL	8	32	128	32	32
E. hormaechei zb03			64	32	128	64	64
E. hormaechei zb04		TIC, TCC, FEP	64	32	64	32	32
E. hormaechei zb05	*intI1*, *sul1*	TIC, TCC, CAZ, CTX, FEP, SXT, CHL, GEN	32	64	64	64	32
E. hormaechei zb06	*tet*(D)	TIC, TCC, TZP, CAZ, CTX, FEP, SXT, CIP, OFX, AMK, GEN, NAL, TOB, ATM	64	64	128	32	16
E. hormaechei zb07	*sul2*, *aadA*, *aac(6′)-Ib*, bla_{CTX-M}, bla_{TEM}	TIC, TCC, CAZ, CTX, FEP, SXT, CHL, GEN, NAL, ATM	128	32	128	32	32
E. hormaechei zb08	*sul2*, *aadA*, *aac(6′)-Ib*, bla_{CTX-M}, bla_{TEM}	TIC, TCC, TZP, CAZ, CTX, FEP, CHL, CIP, GEN, TOB, ATM	128	16	64	32	32
E. hormaechei zb09	*sul2*, *aac(6′)-Ib*, bla_{CTX-M}	TIC, TCC, CAZ, CTX, FEP, AMK, GEN, ATM	32	32	128	64	32
E. hormaechei zb10	*dfrA12*, *sul2*, *aadA*, *aac(6′)-Ib*, bla_{CTX-M}, bla_{TEM}	TIC, TCC, TZP, CAZ, CTX, FEP, SXT, GEN, NAL, ATM	128	64	128	64	64
E. hormaechei zb11	*sul1*, *sul2*, *aac(6′)-Ib*, bla_{TEM}	TIC, TCC, CAZ, CTX, FEP, SXT, CHL, CIP, AMK, GEN, NAL, ATM	32	32	128	32	32
E. hormaechei zb12	*dfrA12*, *sul2*, *aac(6′)-Ib*, bla_{TEM}	TIC, TCC, TZP, CAZ, CTX, FEP, SXT, CHL, GEN, NAL, TOB, ATM	64	32	128	32	32
E. hormaechei zb13		TIC, TCC, FEP, AMK	64	32	64	64	32
E. hormaechei zb14	*dfrA12*		32	16	128	64	16
E. hormaechei zb15	*sul2*, *aac(6′)-Ib*, bla_{TEM}	FEP	128	32	128	64	32
E. hormaechei zb16	*intI1*, *sul2*, *aac(6′)-Ib*, bla_{CTX-M}, bla_{TEM}	TIC, TCC, CAZ, CTX, FEP, SXT, CHL, GEN, NAL, ATM	64	64	128	32	32
E. hormaechei zb17	*sul2*		32	32	64	64	16

Table 1 (*Continued*)

Enterobacter spp.[a]	Detected ARG(s)	Antibiotic resistance[b]	Biocide MIC (mg/L)				
			HDP	BC	CF	CT	CH
E. hormaechei zb18	*dfrA12*, *sul2*, *aadA*, *aac(6′)-Ib*, bla_{CTX-M}, bla_{TEM}	TIC, TCC, CAZ, CTX, FEP, SXT, CHL, AMK, GEN, NAL, ATM	64	32	128	32	16
E. hormaechei zb19	*intI1*, *dfrA12*, *sul2*, *aadA*, *aac(6′)-Ib*, bla_{TEM}, bla_{IMP}	TIC, TCC, TZP, CAZ, CTX, FEP, SXT, CHL, GEN, NAL, ATM	32	64	64	64	32
E. (Cronobacter) sakazakii zb01	*mdfA*, *sul2*, *tet*(B), *aac(6′)-Ib*, *floR*	TIC, TCC, CAZ, CTX, FEP, SXT, CIP, OFX, AMK, GEN, NAL, TOB, ATM	32	64	128	32	32
Enterobacter sp. zb01	*oqxA*, *aac(6′)-Ib*, *floR*, bla_{TEM}	FOS	<512	64	128	64	32
Enterobacter sp. zb02	*aadA*, bla_{TEM}		128	32	128	64	32
Enterobacter sp. zb03	*mdfA*, *tet*(B), *aadA*, *floR*	TIC, TCC, TZP, FEP, CHL, NAL	32	32	128	32	4
Enterobacter sp. zb04	*dfrA12*, *sul2*, *aac(6′)-Ib*, bla_{TEM}	TIC, TCC, TZP, CAZ, CTX, FEP, SXT, CHL, GEN, NAL	64	32	64	32	16
Enterobacter sp. zb05	*dfrA12*, *sul2*, *aac(6′)-Ib*, bla_{CTX-M}, bla_{TEM}	TIC, TCC, CAZ, CTX	16	32	128	32	32
Enterobacter sp. zb06		TZP, FEP, FOS	64	32	128	32	16
Enterobacter sp. zb07	*dfrA12*, *sul1*, $bla_{CTX-M-2}$	TIC, TCC, CAZ, CTX, FEP, CIP, GEN, NAL	32	32	128	64	16
E. xiangfangensis zb01	*dfrA12*, *sul2*, bla_{CTX-M}	TIC, TCC, TZP, CTX, FEP, SXT, CHL, GEN, NAL, ATM	64	32	128	64	32
E. xiangfangensis zb02	*mdfA*, *sul1*, *sul2*, *tet*(B), bla_{CTX-M}, bla_{TEM}	TIC, TCC, CTX, CHL	64	64	128	32	32
E. xiangfangensis zb03	*aac(6′)-Ib*		32	32	128	64	8
E. xiangfangensis zb04	*intI1*, *mdfA*, *sul2*, *tet*(B), *aadA*, *aac(6′)-Ib*, bla_{CTX-M}, bla_{TEM}, bla_{OXA-23}	TIC, TCC, CAZ, CTX, FEP, IPM, GEN, ATM	64	32	128	64	32

ARG, antimicrobial resistance gene; MIC, minimum inhibitory concentration; HDP, hexadecylpyridinium chloride; BC, benzalkonium chloride; CF, hexachlorophene; CT, cetrimide; CH, chlorhexidine.

[a] Isolates were identified by 16S rDNA gene sequencing.

[b] AMK, amikacin; ATM, aztreonam; CAZ, ceftazidime; CHL, chloramphenicol; CIP, ciprofloxacin; CTX, cefotaxime; FEP, cefepime; FOS, fosfomycin; GEN, gentamicin; IPM, imipenem; NAL, nalidixic acid; OFX, ofloxacin; SXT, trimethoprim/sulfamethoxazole; TCC, ticarcillin/clavulanic acid; TIC, ticarcillin; TOB, tobramycin; TZP, piperacillin/tazobactam.

reflecting both a possible tolerance of the *Enterobacter* isolates tested and a probable inefficiency of this type of product. Strains with high MICs for BC of 64 mg/L (24.7%) or 128 mg/L (2.6%) were also detected. Otherwise, molecules such as chlorhexidine were characterised by moderate MICs reaching 32 mg/L for 61% of isolates.

Results of biocide tolerance also revealed a strain-dependent effect in which some isolates were found to be tolerant to several antibiotics, reflecting a possible accumulation of resistance mechanisms.

3.4. Genetic determinants of resistance

Results of PCR amplification of genes encoding efflux pumps revealed the presence of the *mdfA* gene in 6.5% of isolates (5/77) (Table 1) and the *oqxA* gene in 3.9% (3/77).

Regarding resistance to β-lactams, the extended-spectrum β-lactamase (ESBL) genes bla_{TEM}, bla_{CTX-M} and $bla_{CTX-M-2}$ were detected in 44.2% (33/77), 36.4% (28/77) and 5.2% (4/77) of the isolates, respectively, and only two isolates carried these three genes together. The bla_{PSE} gene was not detected in any of the tested isolates.

The presence of genes responsible for the production of metallo-β-lactamases (MBLs) was also observed. The bla_{VIM-2} and bla_{NDM-1} genes were found in one isolate (1.3%) each, whereas this percentage was higher for bla_{IMP} (3/77; 3.9%) and bla_{OXA-23} (2/77; 2.6%).

The tetracycline resistance genes *tet*(B) and *tet*(D) were found with frequencies of 7.8% (6/77) and 1.3% (1/77), respectively, however the *tet*(C) gene was not detected. Unlike the *sul1* and *sul3* genes that were present with lower frequencies [11.7% (9/77) and 1.3% (1/77), respectively], 50.6% of isolates (39/77) carried the *sul2* gene. The integrase gene *intI1* of class 1 integrons associated with sulfonamide resistance genes was detected in 16.9% of isolates (13/77).

The *aac(6′)-Ib* gene involved in aminoglycoside resistance was the most predominant (57.1% of isolates; 44/77). The *aadA* gene responsible of streptomycin resistance was found in 28.6% of isolates (22/77), whilst the gene *floR* encoding resistance to phenicols was found in 10.4% (8/77). It is also worth noting that many isolates carried several ARGs, with up to eight ARGs in one strain (Table 1).

3.5. Correlation between the presence of different antimicrobial resistance genes

The efflux pump gene *oqxA* showed a weak (significant) positive correlation only with *floR* (Table 2), whilst the *mdfA* gene showed weak positive correlations with *floR* and bla_{OXA-23} and also a very strong positive correlation with the *tet*(B) tetracycline resistance gene. The *tet*(B) gene also showed significant positive correlations with bla_{OXA-23} and bla_{NDM-1}. Strong positive correlations were detected for the β-lactamase gene bla_{OXA-23} with bla_{NDM-1} and for bla_{TEM} with *sul2*. The *aac(6′)-Ib* gene showed moderate or weak positive correlations with bla_{TEM}, bla_{CTX-M}, *dfrA12* and *sul2*.

Moreover, the correlation was negative between some of the studied ARGs, particularly between *floR* and *dfrA12* and between *sul1* and bla_{CTX-M}.

4. Discussion

The presence of *Enterobacter* spp. in the hospital environment is alarming because it can cause serious infections especially when it accumulates antimicrobial resistance mechanisms [15]. In contrast to antibiotics, there is no consensus on the standardisation of susceptibility to biocides [29].

The results of the present study suggest that a large percentage of *Enterobacter* spp. isolates tolerate high concentrations of biocides, specifically CF (up to 128 mg/L) and BC (64 mg/L or 128 mg/L). These results appear to disagree with those reported by Morrissey et al. [29] who estimated a MIC of 16 mg/L for BC and those of Fernández Fuentes et al. [30] who report MICs in the range of 10–100 mg/L for CF. This discrepancy could be the result of several factors, such as the selection pressure of biocide-tolerant mutants or modification of membrane rigidity [31].

Repeated exposure of a microbial community to biocides not only increases the selection of biocide-resistant bacteria but may also contribute to the expression and spread of antibiotic resistance mechanisms [31]. This phenomenon could be explained by the similarity of the resistance mechanisms [32]. Although most

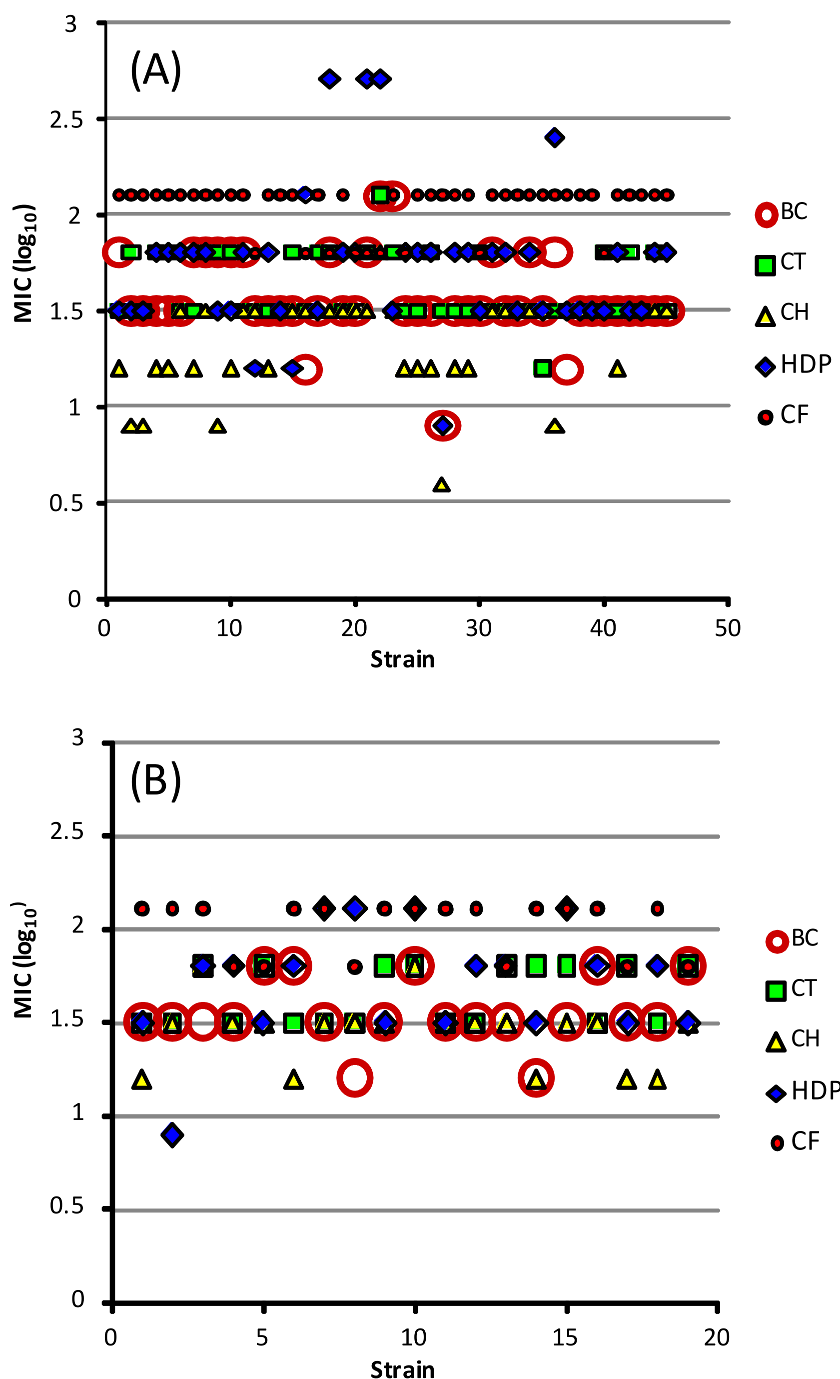

Fig. 1. Distribution of minimum inhibitory concentrations (MICs) for five different biocides against (A) *Enterobacter cloacae* and (B) *Enterobacter hormaechei* isolates from an Algerian hospital environment. BC, benzalkonium chloride; CT, cetrimide; CH, chlorhexidine; HDP, hexadecylpyridinium chloride; CF, hexachlorophene.

antibiotic resistance mechanisms are specific and result in resistance to a single molecule or class of antimicrobials, there are currently many examples of efflux systems that contribute to resistance to a wide range of structurally independent antimicrobials, including antibiotics and biocides [32].

Many of the isolates with high-level tolerance to CF were also multiply resistant to antibiotics, including fluoroquinolones such as ciprofloxacin and ofloxacin. This phenomenon of co-resistance was explained by Buffet-Bataillon et al. [33] who believe that the effects of non-antibiotic antimicrobials (quaternary ammonium compounds) have been so far underestimated, whilst various common mechanisms (including overexpression of efflux pumps, biofilm formation and spontaneous mutations) may contribute to co-resistance between antibiotic and biocides. These data can be extrapolated to other antimicrobial agents.

In addition, the current results also indicate a possible negative correlation between tolerance to certain biocides and resistance to certain antibiotics, which could be an excellent alternative in the choice of molecules adapted to our context.

The results showed a possible correlation between ARGs, thus the presence of the *aac(6′)-Ib* gene accompanied that of bla_{CTX-M} and bla_{TEM}. This same association has been described in Algeria for the first time in a strain of ESBL-producing *E. cloacae* harbouring both bla_{CTX-M} and bla_{TEM} genes [34]. This coexistence is not always obvious, because in another study in Algeria the *aac(6′)-Ib* gene was not found [35].

Table 2
Correlations between the detected antimicrobial resistance genes.

	aac(6')-Ib	*aadA*	bla_{CTX-M}	$bla_{CTX-M-2}$	bla_{IMP}	bla_{NDM-1}	bla_{OXA-23}	bla_{TEM}	bla_{VIM-2}	*floR*	*tet*(B)	*tet*(D)	*dfrA12*	*mdfA*	*oqxA*	*sul1*	*sul2*	*sul3*	*intI1*
aac(6')-Ib	1																		
aadA	0.147	1																	
bla_{CTX-M}	**0.288***	**0.234***	1																
$bla_{CTX-M-2}$	−0.040	0.103	0.073	1															
bla_{IMP}	−0.103	0.162	−0.007	−0.047	1														
bla_{NDM-1}	−0.136	−0.075	−0.084	−0.027	−0.023	1													
bla_{OXA-23}	−0.028	0.072	0.051	−0.038	−0.033	**0.702****	1												
bla_{TEM}	**0.485****	**0.391****	**0.333****	0.028	−0.044	−0.102	0.019	1											
bla_{VIM-2}	0.097	−0.075	−0.084	−0.027	−0.023	−0.013	−0.019	0.129	1										
floR	−0.145	−0.036	**−0.250***	−0.080	−0.069	**0.337****	0.212	−0.131	−0.039	1									
tet(B)	−0.050	0.128	−0.011	−0.068	−0.059	**0.395****	**0.562****	0.034	−0.033	**0.377****	1								
tet(D)	−0.136	−0.075	−0.084	−0.027	−0.023	−0.013	−0.019	−0.102	−0.013	−0.039	−0.033	1							
dfrA12	**0.295****	0.119	0.194	0.173	−0.023	−0.092	−0.130	**0.362****	0.144	**−0.272***	−0.133	−0.092	1						
mdfA	0.008	0.173	0.027	−0.062	−0.053	−0.030	**0.288***	0.084	−0.030	**0.256***	**0.907****	−0.030	−0.102	1					
oqxA	0.034	0.015	−0.007	−0.047	−0.041	−0.023	−0.033	0.091	−0.023	**0.371****	−0.059	−0.023	−0.023	−0.053	1				
sul1	−0.185	0.116	**−0.267***	0.097	0.136	−0.042	−0.059	−0.079	−0.042	−0.124	0.045	−0.042	−0.125	0.068	−0.073	1			
sul2	**0.380****	0.133	**0.290***	**−0.237***	0.065	0.113	0.161	**0.616****	0.113	−0.090	0.190	−0.116	0.149	0.155	−0.070	−0.045	1		
sul3	−0.136	−0.075	−0.084	−0.027	−0.023	−0.013	−0.019	0.129	−0.013	−0.039	−0.033	−0.013	−0.092	−0.030	−0.023	**0.315****	0.113	1	
intI1	−0.042	0.009	−0.041	0.051	0.088	−0.052	0.144	−0.052	−0.052	0.074	−0.002	−0.052	−0.218	0.022	−0.091	−0.056	0.098	−0.052	1

*,** Correlations were statistically significant at * $P < 0.05$ or ** $P < 0.001$.

Among the resistance genes investigated in this work, the *mdfA* gene was found in five isolates. Although this gene has been widely reported in *Escherichia coli* [36,37], other authors did not find it in *Enterobacter* spp. [38].

5. Conclusions

This work reveals worrisome tolerance to biocides in *Enterobacter* spp. isolates from an Algerian hospital environment. Biocide tolerance might be one of the factors for the co-selection of antibiotic resistance and the persistence of antibiotic-resistant strains in hospital settings. The results underline the needs to establish new strategies and to explore other alternatives against antibiotic-resistant bacteria, including new formulations or the use of bacteriophages, to fight these bacteria on surfaces before they can reach patients.

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Competing interests

None declared.

Ethical approval

Not required.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jgar.2019.04.005.

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