Characterization of multidrug-resistant Gram-negative bacilli isolated from hospitals effluents: first report of a *bla*_{OXA-48}-like in *Klebsiella oxytoca*, Algeria

Khadidja Yousfi^{1,2} · Abdelaziz Touati¹ · Brigitte Lefebvre² · Philippe Garneau³ · Soumia Brahmi¹ · Alima Gharout-Sait¹ · Josée Harel³ · Sadjia Bekal^{2,4}

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Abstract

The antibiotic susceptibility profile and antimicrobial resistance determinants were characterized on Gram-negative bacilli (GNB) isolated from Algerian hospital effluents. Among the 94 isolates, *Enterobacteriaceae* was the predominant family, with *Escherichia coli* and *Klebsiella pneumoniae* being the most isolated species. In non-*Enterobacteriaceae*, *Acinetobacter* and *Aeromonas* were the predominant species followed by *Pseudomonas*, *Comamonas*, *Pasteurella*, and *Shewanella* spp. The majority of the isolates were multidrug-resistant (MDR) and carried different antimicrobial resistance genes including *bla*_{CTX-M}, *bla*_{TEM}, *bla*_{SHV}, *bla*_{OXA-48-like}, *bla*_{OXA-23}, *bla*_{OXA-51}, *qnrB*, *qnrS*, *tet(A)*, *tet(B)*, *tet(C)*, *dfrA1*, *aac(3)-IIc* (*aacC2*), *aac(6')-Ib*, *sul1*, and *sul2*. The *qacEΔ1-sul1* and *intI2* signatures of class 1 and class 2 integrons, respectively, were also detected. Microarray hybridization on MDR *E. coli* revealed additional resistance genes (*aadA1* and *aph3strA*, *tet30*, *mphA*, *dfrA12*, *bla*_{cmy2}, *bla*_{ROB1}, and *cmlA1*) and classified the tested strains as commensals, thus highlighting the potential role of humans in antibiotic resistance dissemination. This study is the first report of *bla*_{OXA-48-like} in *Klebsiella oxytoca* in Algeria and *bla*_{OXA-23} in *A. baumannii* in Algerian hospital effluents. The presence of these bacteria and resistance genes in hospital effluents represents a serious public health concern since they can be disseminated in the environment and can colonize other hosts.

Keywords Multidrug resistance · Hospital effluents · Gram-negative bacilli · Algeria

Introduction

Antimicrobial resistance resulting from intensive and inappropriate use of antibiotics represents an increasing challenge for health care services worldwide. In Gram-negative

bacilli (GNB), this resistance is a cause for concern, owing to the emergence and dissemination of different resistance genes identified either in clinical setting or in the environment. Within the environment, aquatic ecosystems have been documented as an important vehicle and reservoir of antibiotic resistance [1]. Different studies have shown that hospital effluents contain high levels of antibiotic-resistant bacteria and contribute to antibiotic resistance dissemination in the environment [2, 3]. Moreover, the non-metabolized fraction of consumed antibiotics excreted directly in wastewater can exert a selective pressure promoting the selection of these resistant bacteria [2]. Generally, hospital effluents are discharged into municipal sewage and collected via wastewater treatment plants (WWTP) or eliminated directly into the environment without any treatments. The latter practice has been criticized and a pre-treatment has been recommended [4].

The resistant bacteria present in hospital wastewater could be a source of genes encoding various resistance mechanisms including extended spectrum β -lactamases (ESBLs),

Responsible Editor: Jorge Sampaio

✉ Sadjia Bekal
sadjia.bekal@inspq.qc.ca

¹ Laboratoire d'Écologie Microbienne, FSNV, Université de Béjaia, 06000 Béjaia, Algeria

² Laboratoire de santé publique du Québec, Institut national de santé publique du Québec, Sainte-Anne-de-Bellevue, QC H9X 3R5, Canada

³ Faculté de Médecine Vétérinaire, Centre de Recherche en Infectiologie Porcine, Université de Montréal, St-Hyacinthe, QC, Canada

⁴ Département de Microbiologie, Infectiologie et Immunologie, Université de Montréal, Montréal, QC, Canada

carbapenemases, and plasmid mediated quinolones resistance (PMQRs) [3, 5, 6]. These genes may be transmitted to other bacteria present in sewage and the environment via the aquatic system and the food chain. The spread of these bacteria in the natural environment can have significant consequences on ecological balance and animal and public health [2]. The present study aimed to identify and characterize resistance mechanisms of multidrug-resistant GNB found in hospital effluents and to improve our understanding on the role of hospital effluents as a source of a large diversity of antibiotic resistance genes.

Material and methods

Bacterial sampling

Twenty wastewater samples were collected from untreated effluents from four hospitals in Algeria: two in Bejaia and two in Tizi Ouzou provinces. Sampling was carried out during the period of maximum hospital activity, in 2011 and 2012 in Bejaia and Tizi Ouzou, respectively. In Bejaia, the samples were collected from non-functional WWTP and sewer pipes from Khelil Amrane (H1) and Amizour (H2) hospitals, respectively. In 2011, the H1 hospital had an annual capacity of 160 beds and admitted 5255 patients and the H2 hospital had a capacity of 209 beds and admitted 9360 patients. In Tizi Ouzou, samples were collected from sewer pipes from Nadir Mohammed hospital (H3) and Tigzirt hospital (H4). In 2012, the H3 hospital had 1042 beds and admitted 40,170 patients and the H4 hospital was the only establishment located in a rural region. It had a capacity of 35 beds and admitted 638 patients annually. All the hospitals included in this study were located close to the Mediterranean Sea with approximate distances of 0.4, 8, 33, and 44 km for H4, H1, H2, and H3, respectively. In addition, the H3 hospital was located at about 15 km from Oued Sebaou and the H2 at around 15 km from Oued Soummam, the main rivers of the provinces of Tizi Ouzou and Bejaia, respectively, which flow into the Mediterranean Sea.

Microbiological analysis

A volume of 200 ml of each sample was collected in sterile glass flasks and transported to the laboratory in a cooler, and samples were processed within 2 h upon arrival. Serial dilutions of 10^{-1} to 10^{-4} of each sample were performed. One hundred microliters of each sample and their corresponding dilutions were plated on MacConkey agar supplemented with 2 µg/ml of cefotaxime followed by incubation at 37 °C for 18–24 h. A single colony from colonies sharing the same appearance by visual inspection (morphology, color, shape, size) was selected and purified. After purification, the isolates

were stored in a nutrient broth containing 10% glycerol (v/v) at –80 °C. Rapid identification was performed using the API 20E test strip (Biomérieux, France) for *Enterobacteriaceae* according to the manufacturer's instructions in conjunction with conventional biochemical testing [7]. Non-*Enterobacteriaceae* bacteria were identified by sequencing the 16S rRNA and *rpoB* according to the Laboratoire de Santé Publique du Québec (LSPQ) reference methods or *gyrB* for *Shewanella* spp. [6].

The bacterial diversity of different genera of each hospital was evaluated using Shannon diversity index. The BPMSG diversity online calculator website <https://bpmsg.com/academic/div-calc.php> was used to calculate this index.

Antimicrobial susceptibility testing and extended-spectrum β -lactamase (ESBL) detection

Antimicrobial susceptibility was performed using the disk diffusion method and the results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI, 2014) guidelines [8]. The antibiotics (Bio-Rad, France) tested were as follows: ticarcillin (TIC, 75 µg), ticarcillin-clavulanic acid (TIM, 75/10 µg), amoxicillin-clavulanic acid (AMC, 20/10 µg), ertapenem (ETP, 10 µg), imipenem (IPM, 10 µg), ceftazidime (CAZ, 30 µg), cefepime (FEP, 30 µg), aztreonam (ATM, 30 µg), gentamicin (GEN, 10 µg), amikacin (AMK, 30 µg), ciprofloxacin (CIP, 5 µg), tetracycline (TET, 30 µg), and trimethoprim-sulfamethoxazole (SXT, 1.25/23.75 µg). The ESBL production was detected by the CLSI confirmatory test using both CTX (30 µg) and CAZ (30 µg) disks alone and in combination with clavulanic acid (CA, 10 µg) [8].

Detection of antibiotic resistance genes

DNA extraction was performed by the MagAttract® DNA Mini M48 kit using the biorobot M48 (Qiagen, USA) according to the manufacturer's instructions. For β -lactam resistance genes, PCR amplification targeting *bla*_{CTX-M}, *bla*_{SHV}, and *bla*_{TEM} genes was carried out in a 50 µl final volume using Phusion DNA Polymerase (New England Biolabs, USA) [9, 10]. For the carbapenemases encoding genes which included *bla*_{OXA-23}, *bla*_{OXA-24}, *bla*_{OXA-51}, and *bla*_{OXA-58}, multiplex PCR was performed in a 50 µl final volume using HotStar Taq enzyme (Qiagen, USA) [11, 12]. The *bla*_{OXA-48}-like genes were amplified using simplex PCR with the same amplification conditions.

Genes encoding resistance to non- β -lactam antibiotics were amplified in a 25 µl volume using AmpliTaq DNA polymerase (ThermoFisher scientific, USA) according to the manufacturer's guidelines. The non- β -lactam resistance genes included *qnrA*, *qnrB*, and *qnrS* encoding resistance to quinolones [13, 14], *acc(6')-Ib* and *aac(3)-IIc* (*aacC2*) encoding resistance to aminoglycosides, *dfrA* encoding resistance to

trimethoprim, *tet(A)*, *tet(B)*, and *tet(C)* encoding resistance to tetracycline, and *sul1*, *sul2*, and *sul3* encoding resistance to sulfonamides [15, 16]. In addition, *qacEΔ1-sulI* and *intI2* genetic markers for class 1 and class 2 integrons, respectively, were also amplified in the same conditions [17, 18]. The primers used in this study and PCR conditions are listed in Table 1.

DNA microarray analysis of *Escherichia coli* isolates

Nine representative multidrug-resistant (MDR) *E. coli* isolates were analyzed by microarray hybridization. The *E. coli* Maxivirulence version 3.1 microarray was used as previously described [19]. This microarray can detect 348 virulence genes and 98 antibiotic resistance genes and can determine putative *E. coli* pathotypes based on virulence gene profiles [20] and phylogenetic groups (A1, B1, B2, and D) based on the pattern of presence or absence of *chuA*, TspE4.C2, and *yjaA* [21].

Results

Bacterial diversity

A total of 94 Gram-negative bacilli were cultured from selective plates. The distribution of these isolates was as follows: 25 isolates were from H1, 20 from H2, 22 from H3, and 27 from H4. Among the 26 different species identified, *Enterobacteriaceae* was the most predominant family with a prevalence of 78% ($n = 73$). The identified species from this family included *E. coli* ($n = 19$), *Klebsiella pneumoniae* ($n = 14$), *Citrobacter freundii* ($n = 9$), *Klebsiella oxytoca* ($n = 7$), *Enterobacter cloacae* ($n = 5$), *Citrobacter braakii* ($n = 4$), *Morganella morganii* ($n = 3$), *Citrobacter youngae* ($n = 2$), *Proteus vulgaris* ($n = 2$), *Providencia rettgeri* ($n = 2$), *Cedecea lapagei* ($n = 1$), *Enterobacter aerogenes* ($n = 1$), *Enterobacter agglomerans* ($n = 1$), *Enterobacter asburiae* ($n = 1$), *Enterobacter sakazakii* ($n = 1$), and *Kluyvera* sp. ($n = 1$). The prevalence of non-*Enterobacteriaceae* GNB was 22% ($n = 21$). This group included *Acinetobacter baumannii* ($n = 5$), *Aeromonas hydrophila* ($n = 5$), *Aeromonas* sp. ($n = 3$), *Aeromonas veronii* ($n = 2$), *Pseudomonas aeruginosa* ($n = 2$), *Aeromonas sobria* ($n = 1$), *Comamonas aquatica* ($n = 1$), *Pasteurella* spp. ($n = 1$), and *Shewanella xiamenensis* ($n = 1$). The predominant species from these hospitals were *K. pneumoniae* (13/25), *E. coli* (13/20), *E. coli* (5/22), and *K. oxytoca* (6/27) for H1, H2, H3, and H4, respectively. In addition, 10/21 non-*Enterobacteriaceae* isolates (47.6%) were collected from Nadir Mohamed hospital (H3). Shannon diversity index was different between the effluents of different hospitals and

revealed greater genera diversity for effluents of H3 (2.07), followed by H4 (1.7), H1 (1.4), and H2 (1.2).

Antibiotic susceptibility of hospital effluents isolates

For β -lactam antibiotics, the highest resistance rate was observed with TIC representing 98.6% and 66.6% for *Enterobacteriaceae* and non-*Enterobacteriaceae*, respectively, followed by CAZ with 86.3% and 47.6% for *Enterobacteriaceae* and non-*Enterobacteriaceae*, respectively. For non- β -lactam antibiotics, the highest resistance rate observed with ciprofloxacin was 74% and 62% for *Enterobacteriaceae* and non-*Enterobacteriaceae*, respectively. All the isolates were resistant to at least two antibiotic classes and the MDR phenotype was observed in 91/94 (96.8%) isolates exhibiting resistance to 3 to 8 antibiotic classes. The MDR phenotype was observed in 72/73 isolates of the *Enterobacteriaceae* family (98.6%) and in 19/21 isolates in the non-*Enterobacteriaceae* families (90.5%) which were resistant to three or more antimicrobial classes. In addition, the ESBL phenotype was detected in 45/73 of *Enterobacteriaceae* (61.5%) and in 9/21 of non-*Enterobacteriaceae* GNB (42.8%).

Antibiotics resistance mechanisms

Among the genes encoding class A β -lactamases tested in this study, *bla*_{CTX-M}, *bla*_{TEM}, and *bla*_{SHV} were detected in 27/94 (28.7%), 17/94 (18.1%), and 14/94 (14.9%) isolates, respectively. The *bla*_{CTX-M} gene was detected only in the *Enterobacteriaceae* family including *E. coli* ($n = 7$) and *K. pneumoniae* ($n = 7$), *K. oxytoca* ($n = 3$), *C. youngae* ($n = 2$), *C. freundii* ($n = 2$), *C. braakii* ($n = 1$), *E. aerogenes* ($n = 1$), *E. agglomerans* ($n = 1$), *E. asburiae* ($n = 1$), *M. morganii* ($n = 1$), and *P. vulgaris* ($n = 1$). The *bla*_{TEM} gene was also detected in this family and was detected in *E. coli* ($n = 5$) and *K. pneumoniae* ($n = 3$) followed by *K. oxytoca* ($n = 1$), *C. Youngae* ($n = 1$), *E. agglomerans* ($n = 1$), and *P. rettgeri* ($n = 1$). The *bla*_{SHV} gene was detected in *K. pneumoniae* ($n = 9$), *E. cloacae* ($n = 3$), *E. agglomerans* ($n = 1$), and *E. coli* ($n = 1$). Of the 21 non-*Enterobacteriaceae* isolates, only *bla*_{TEM} was detected in five isolates including *A. hydrophila* ($n = 2$), *Aeromonas* spp. ($n = 1$), *A. veronii* ($n = 1$), and *A. baumannii* ($n = 1$).

Regarding carbapenemases encoding genes, *bla*_{OXA-48}-like was detected in *K. oxytoca* ($n = 2$). The *bla*_{OXA-416} gene was also detected in *S. xiamenensis* isolate and the genome was fully sequenced and documented in a separate study [6]. The *bla*_{OXA-23} gene was detected in two *A. baumannii* isolates whereas the *bla*_{OXA-51} gene was detected in all the five isolates. The *bla*_{OXA-24} and *bla*_{OXA-58} genes tested negative in all isolates.

Table 1 Primers and PCR conditions used in this study

Primer	Target	Control strain	Primer sequence (5'–3')	PCR conditions	Amplicon size (pb)	Reference
SHVF2	<i>bla_{SHV}</i>	ATCC BAA200	GGTTAT GCG TTA TAT TCG CC	40 cycles of 30 s at 98 °C, 10 s at 98 °C, 30 s at 65 °C, 30 s at 72 °C; 10 min at 72 °C; 4 °C	867	[9]
SHVR			TTA GCG TTG CCA GTG CTC			
TEMF	<i>bla_{TEM}</i>	ATCC BAA197	ATG AGT ATT CAA CAT TTC CG	30 cycles of 30 s at 98 °C, 10 s at 98 °C, 30 s at 58 °C, 30 s at 72 °C; 10 min at 72 °C; 4 °C	867	[9]
TEMR			CTG ACA GTT ACC AAT GCT TA			
CTX-M-U1	<i>bla_{CTX-M}</i>	ID098246	ATG TGC AGY ACC AGT AAR GTK ATG GC	30 cycles of 30 s at 98 °C, 10 s at 98 °C, 30 s at 65 °C, 30 s at 72 °C; 10 min at 72 °C; 4 °C	593	[10]
CTX-M-U2			TGG GTR AAR TAR GTS ACC AGA AYC AGC GG			
OXA-P1	<i>bla_{OXA-48}</i>	LSPQ4078	ATG CGT GTA TTA GCC TTA TC	10 min at 20 °C; 30 cycles of 15 min at 95 °C, 60 s at 94 °C, 60 s at 57 °C; 10 min at 72 °C; 4 °C	759	This study
OXA-P2			CTA GGG AAT AAT TTT NTC CT			
OXA-23-like-FWD	<i>bla_{OXA-23}</i>	MA092246	GAT CGG ATT GGA GAA CCAGA		501	[11]
OXA-23-like-REV			ATT TCT GAC CGC ATT TCC A			
OXA-24-like-FWD	<i>bla_{OXA-24}</i>	MA09250	GGT TAG TTG GCC CCC TTA AA		246	[11]
OXA-24-like-REV			AGT TGA GCG AAA AGG GGA TT			
OXA-51-like-FWD	<i>bla_{OXA-51}</i>	LSPQ4139	TAA TGC TTT GAT CGG CCT TG		353	[12]
OXA-51-like-REV			TGG ATT GCA CTT CAT CTT GG			
OXA-58-like-FWD	<i>bla_{OXA-58}</i>	LSPQ4139	AAG TAT TGG GGC TTG TGC TG		599	[11]
OXA-58-like-REV			CCC CTC TGC GCT CTA CAT AC			
QnrAm-F	<i>qnrA</i>	J53pMG252	AGA GGA TTT CTC ACG CCA GG	10 min at 95 °C and 35 cycles of amplification consisting of 1 min at 95 °C, 1 min at 54 °C and 1 min at 72 °C and 10 min at 72 °C	580	[13]
QnrAm-R			TGC CAG GCA CAG ATC TTG AC			
qnrB-forward	<i>qnrB</i>	J53pMG298	GAT CGT GAA AGC CAG AAA GG	94 °C for 4 min, 35 cycles of 94 °C of 30 s, 58 °C for 30 s, and 68 °C for 45 s, and a final step of 68 °C for 10 min	469	[14]
qnrB-reverse			ACG ATG CCT GGT AGT TGT CC			
qnrS-forward	<i>qnrS</i>	J53pMG306	ACG ACA TTC GTC AAC TGC AA		417	[14]
qnrS-reverse			TAA ATT GGC ACC CTG TAG GC			
aac-forward	<i>acc (σ')-lb</i>	Salmonella SA20042859	TTG CGA TGC TCT ATG AGT GGC TA		482	[15]
aac-reverse			CTC GAA TGC CTG GCG TGT TT			
dfpA1	<i>dfpA1</i>	S17-1 lamda pir	AAG AAT GGA GTT ATC GGG AAT G		391	[16]
rDFRa			GGG TAA AAA CTG GCC TAA AAT TG			
fAACa	<i>aacC2(aac(3)-Ila)</i>	R176	CGG AAG GCA ATA ACG GAG		740	[16]
rAACa			TCG AAC AGG TAG CAC TGA G			
fTETa	<i>tet(A)</i>	SAS1393	GTG AAA CCC AAC ATA CCC C		888	[16]
rTETa			GAA GGC AAG CAG GAT GTA G			
fTETb	<i>tet(B)</i>	CT4afoob	CCT TAT CAT GCC AGT CTT GC		774	[16]
rTETb			ACT GCC GTT TTT TCG CC			
fTETc	<i>tet(C)</i>	pBR322	ACT TGG AGC CAC TAT CGA C		881	[16]
rTETc			CTA CAA TCC ATG CCA ACC C			
fSULa	<i>sulI</i>	SAS1393	TTC GGC ATT CTG AAT CTC AC		822	[16]
rSULa			ATG ATC TAA CCC TCG GTC TC			
fSULb	<i>sulII</i>	RSF1010	CGG CAT CGT CAA CAT AAC C		722	[16]
rSULb			GTG TGC GGA TGA AGT CAG			
fsul3	<i>sulIII</i>	sul3	GAG CAA GAT TTT TGG AAT CG		790	This study
rsul3			CTA ACC TAG GGC TTT GGA			
fQACe	<i>qacEΔI-sulI</i>	SAS1393	ATC GCA ATA GTT GGC GAA GT		797	[17]
rSUL1			GCA AGG CGG AAA CCC GCG CC			
int2L	<i>int2</i>	PT508-124.61	CAC GGA TAT GCG ACA AAA AGG T		788	[18]
int2R			GTA GCA AAC GAG TGA CGA AAT G			

Table 2 Antimicrobial resistance profiles and resistance genes detected in MDR *Enterobacteriaceae* isolates

Species	Resistance genes and integrons	Isolate	CTX	CAZ	FEP	AMC	IMP	ERT	TIC	ATM	CIP	AMK	GEN	SXT	TET
<i>Citrobacter youngiae</i>	<i>bla</i> _{CTX-M} / <i>bla</i> _{TEM} / <i>qnrB</i> / <i>aac</i> (6)- <i>lb</i> / <i>aac</i> C2/ <i>sul</i> 2/ <i>tet</i> (A)/ <i>qac</i> EAJ- <i>sul</i> 1	M18													
	<i>bla</i> _{CTX-M} / <i>qnrB</i> / <i>aac</i> (6)- <i>lb</i> / <i>aac</i> C2/ <i>sul</i> 2/ <i>tet</i> (A)/ <i>qac</i> EAJ- <i>sul</i> 1	M27													
<i>Citrobacter braakii</i>	<i>bla</i> _{CTX-M} / <i>aac</i> C2/ <i>aac</i> (6)- <i>lb</i> / <i>sul</i> 1/ <i>sul</i> 2	M25-1													
	<i>aac</i> C2/ <i>aac</i> (6)- <i>lb</i> / <i>sul</i> 1/ <i>sul</i> 2	M22													
		M26													
	<i>aac</i> (6)- <i>lb</i>	M25													
<i>Citrobacter freundii</i>	<i>bla</i> _{CTX-M} / <i>qnrB</i> / <i>aac</i> (6)- <i>lb</i> / <i>aac</i> C2/ <i>tet</i> (C)/ <i>dfr</i> A1/ <i>int</i> 2	M23													
	<i>bla</i> _{CTX-M} / <i>qnrB</i> / <i>aac</i> (6)- <i>lb</i>	M31													
	<i>qnrB</i> / <i>aac</i> (6)- <i>lb</i> / <i>aac</i> C2/ <i>sul</i> 1/ <i>sul</i> 2/ <i>tet</i> (A)/ <i>dfr</i> A1/ <i>qac</i> EAJ- <i>sul</i> 1/ <i>int</i> 2	T11													
	<i>qnrB</i> / <i>aac</i> (6)- <i>lb</i> / <i>aac</i> C2/ <i>sul</i> 2/ <i>dfr</i> A1/ <i>qac</i> EAJ- <i>sul</i> 1	M2													
	<i>qnrB</i>	T12													
	<i>sul</i> 2	H2													
<i>Escherichia coli</i>	<i>bla</i> _{CTX-M} / <i>qnrB</i> / <i>aac</i> (6)- <i>lb</i> / <i>aac</i> C2/ <i>sul</i> 2/ <i>tet</i> (A)/ <i>qac</i> EAJ- <i>sul</i> 1	T15													
	<i>bla</i> _{CTX-M} / <i>qnrB</i> / <i>aac</i> (6)- <i>lb</i> / <i>sul</i> 2/ <i>tet</i> (A)/ <i>qac</i> EAJ- <i>sul</i> 1	T18													
	<i>bla</i> _{CTX-M} / <i>aac</i> (6)- <i>lb</i> / <i>aac</i> C2/ <i>tet</i> (A)/ <i>sul</i> 1	T3													
	<i>bla</i> _{CTX-M} / <i>aac</i> (6)- <i>lb</i> / <i>aac</i> C2/ <i>tet</i> (B)/ <i>sul</i> 1	T10													
	<i>bla</i> _{CTX-M} / <i>aac</i> (6)- <i>lb</i> / <i>aac</i> C2/ <i>sul</i> 2	A11													
	<i>bla</i> _{CTX-M} / <i>aac</i> (6)- <i>lb</i> / <i>tet</i> (B)	T9													
	<i>bla</i> _{CTX-M}	W30													
		W1													
	<i>bla</i> _{TEM} / <i>qnrB</i> / <i>dfr</i> A1/ <i>sul</i> 2/ <i>int</i> 2	W4													
		W15													
	<i>bla</i> _{TEM} / <i>aac</i> C2	W5													
	<i>bla</i> _{TEM} / <i>sul</i> 2	W12													
	<i>bla</i> _{SHV} / <i>sul</i> 1	W34													
	<i>aac</i> C2/ <i>dfr</i> A1/ <i>sul</i> 1/ <i>sul</i> 2	W35													
	<i>aac</i> C2/ <i>sul</i> 1/ <i>sul</i> 2	W28													
	<i>qnrB</i> / <i>aac</i> C2	W24													
	<i>aac</i> C2	W10													
	<i>sul</i> 2	W11													
		W6													
<i>Enterobacter aerogenes</i>	<i>bla</i> _{CTX-M} / <i>qnrB</i> / <i>aac</i> C2/ <i>aac</i> (6)- <i>lb</i> / <i>sul</i> 2	W27													
<i>Enterobacter asburiae</i>	<i>bla</i> _{CTX-M} / <i>qnrB</i> / <i>aac</i> C2/ <i>aac</i> (6)- <i>lb</i> / <i>sul</i> 2/ <i>tet</i> (A)/ <i>qac</i> EAJ- <i>sul</i> 1	M1													
<i>Enterobacter colossae</i>	<i>bla</i> _{qiv} / <i>qnrB</i>	A18													
		A22													
	<i>bla</i> _{qiv}	A13													
<i>Enterobacter agglomerans</i>	<i>bla</i> _{CTX-M} / <i>bla</i> _{TEM} / <i>bla</i> _{qiv} / <i>aac</i> C2/ <i>sul</i> 2	W14-2													
<i>Klebsiella oxytoca</i>	<i>bla</i> _{CTX-M} / <i>qnrB</i> / <i>aac</i> (6)- <i>lb</i> / <i>aac</i> C2/ <i>qac</i> EAJ- <i>sul</i> 1	M5													
	<i>bla</i> _{CTX-M} / <i>bla</i> _{TEM} -48/ <i>qnrB</i> / <i>aac</i> (6)- <i>lb</i> / <i>aac</i> C2/ <i>sul</i> 1/ <i>sul</i> 2	M20-1													
	<i>bla</i> _{CTX-M} / <i>bla</i> _{TEM} / <i>bla</i> _{TEM} -48/ <i>qnrB</i> / <i>aac</i> (6)- <i>lb</i> / <i>aac</i> C2/ <i>sul</i> 1/ <i>sul</i> 2	M21													
	<i>qnrS</i> / <i>aac</i> C2/ <i>sul</i> 2	T20													
	<i>aac</i> C2/ <i>dfr</i> A1/ <i>int</i> 2	M8													
<i>Klebsiella pneumoniae</i>	<i>bla</i> _{CTX-M} / <i>bla</i> _{TEM} / <i>bla</i> _{qiv} / <i>qnrB</i> / <i>aac</i> (6)- <i>lb</i> / <i>aac</i> C2/ <i>sul</i> 2	H6													
	<i>bla</i> _{CTX-M} / <i>bla</i> _{qiv} / <i>qnrB</i> / <i>aac</i> (6)- <i>lb</i> / <i>aac</i> C2/ <i>sul</i> 2	A2													
		A14													
	<i>bla</i> _{CTX-M} / <i>bla</i> _{qiv} / <i>aac</i> (6)- <i>lb</i> / <i>aac</i> C2/ <i>sul</i> 2	A21													
	<i>bla</i> _{CTX-M} / <i>bla</i> _{TEM} / <i>bla</i> _{qiv} / <i>aac</i> C2/ <i>sul</i> 2	A20													
		H5													
	<i>bla</i> _{CTX-M} / <i>bla</i> _{qiv} / <i>qnrB</i> / <i>aac</i> (6)- <i>lb</i> / <i>sul</i> 2	H7													
	<i>aac</i> (6)- <i>lb</i> / <i>sul</i> 2	W22													
<i>Kluyvera</i> sp.	<i>bla</i> _{qiv}	A7													
		H8													
	<i>tet</i> (C)	M11													
<i>Morganella morganii</i>	<i>bla</i> _{CTX-M} / <i>qnrB</i> / <i>aac</i> (6)- <i>lb</i> / <i>aac</i> C2	T19-1													
	<i>aac</i> C2/ <i>sul</i> 1	T1-1													
	<i>sul</i> 1	W2													
<i>Proteus vulgaris</i>	<i>bla</i> _{CTX-M} / <i>aac</i> (6)- <i>lb</i> / <i>aac</i> C2/ <i>sul</i> 2/ <i>tet</i> (A)/ <i>qac</i> EAJ- <i>sul</i> 1	T25													
	<i>qnrB</i> / <i>dfr</i> A1/ <i>sul</i> 1	T23-1													
<i>Providencia rettgeri</i>	<i>bla</i> _{TEM} / <i>aac</i> C2/ <i>dfr</i> A1	M35													
	<i>aac</i> C2/ <i>dfr</i> A1/ <i>int</i> 2	M8-1

Resistant
Intermediate
Susceptible

TIC ticarcillin, AMC amoxicillin-clavulanic acid, ETP ertapenem, IMP imipenem, CAZ ceftazidime, FEP cefepime, ATM aztreonam, GEN gentamicin, AMK amikacin, CIP ciprofloxacin, TET tetracycline, SXT trimethoprim-sulfamethoxazole

For genes conferring resistance to quinolones, the *qnrB* gene was detected in *E. coli* ($n = 6$), *C. freundii* ($n = 5$), *K. pneumoniae* ($n = 4$), *K. oxytoca* ($n = 3$), *C. youngae* ($n = 2$), *E. cloacae* ($n = 2$), *E. asburiae* ($n = 1$), *M. organii* ($n = 1$), *P. vulgaris* ($n = 1$), and *E. aerogenes* ($n = 1$). However, the *qnrS* gene was detected only in *K. oxytoca* ($n = 1$) and *A. baumannii* ($n = 2$) isolates, whereas the *qnrA* gene tested negative in all isolates. The *qnrVC6* variant was also detected in *S. xiamenensis* isolate as previously documented [6]. Genes conferring resistance to tetracycline *tet(A)*, *tet(B)*, and *tet(C)* were detected in 12/73 *Enterobacteriaceae* isolates (16.4%) and 8/21 non-*Enterobacteriaceae* isolates (38%). The *dfrA1* gene was found in *Enterobacteriaceae* isolates and distributed as follows: *C. freundii* ($n = 3$), *E. coli* ($n = 4$), *K. oxytoca* ($n = 1$), *P. vulgaris* ($n = 1$), and *P. rettgeri* ($n = 2$). In the non-*Enterobacteriaceae* isolates, this gene was detected in *A. hydrophila* ($n = 2$), *A. sobria* ($n = 1$), and *A. baumannii* ($n = 2$). The *sul1* gene was found in 14/73 *Enterobacteriaceae* isolates (19.2%) and in 10/21 non-*Enterobacteriaceae* isolates (47.6%), and the *sul2* gene was detected in 33 /73 *Enterobacteriaceae* isolates (45.2%) and in 3/21 non-*Enterobacteriaceae* isolates (14.3%).

With regard to genes conferring resistance to aminoglycosides, the *aac(3)-IIc* (*aacC2*) gene was detected in 36/73 *Enterobacteriaceae* isolates (49.3%) and 3/21 non-*Enterobacteriaceae* isolates (14.3%), and the *aac(6')-Ib* gene was found in 28/73 *Enterobacteriaceae* isolates (38.3%) and in 8/21 non-*Enterobacteriaceae* isolates (38%). The *qacEΔI-sul1* conserved segment of class 1 integron was detected in 9/73 *Enterobacteriaceae* isolates (12.3%) and in 1/21 non-

Enterobacteriaceae isolates (4.8%), and the *intI2* gene was found only in the *Enterobacteriaceae* group. The different gene combinations of MDR isolates are described in Table 2 and Table 3.

Phylogenetic groups and virulence profile of MDR *E. coli*

Escherichia coli isolates tested by microarray were classified as commensal and were assigned to the phylogenetic groups A ($n = 4$), D ($n = 3$), and B1 ($n = 2$). However, some virulence genes commonly found in *E. coli* pathotypes were detected [22]. They included *chuA*, *iha*, *cvaC*, *fimH*, *fyuA*, *iroN*, *iss*, *iutA*, *kpsM II*, *traT*, *ompA*, *ompT*, TSPE4.C2, and *yjaA*. This analysis also detected other resistance genes not tested by PCR. They included *aadA1* and *aph3strA* conferring resistance to aminoglycosides, *tet30* conferring resistance to tetracycline, *mphA* conferring resistance to macrolides, *dfrA12* conferring resistance to trimethoprim, *bla_{cmy2}* and *bla_{ROB1}* conferring resistance to β -lactams, and *cmlA1* conferring resistance to chloramphenicol. The detail microarray results are presented in Table 4.

Discussion

Hospital effluents represent an important source of antibiotics and MDR bacteria carrying several antimicrobial resistance genes and mobile genetic elements (MGEs). This poses a significant threat to public health and the environment,

Table 3 Antimicrobial resistance profile, resistance genes detected in MDR non-*Enterobacteriaceae* isolates

Species	Resistance genes and integrons	Isolate	CTX	CAZ	FEP	AMC	IPM	ERT	TIC	ATM	CIP	AMK	GEN	SXT	TET
<i>Aeromonas</i> sp	<i>bla_{TEM}/sul1</i>	H4													
<i>Aeromonas hydrophila</i>	<i>bla_{TEM}/aac(6')-Ib/aacC2/sul1/dfrA1/tet(A)/tet(C)</i>	M32													
	<i>bla_{TEM}/aac(6')-Ib/aacC2/sul1/tet(A)</i>	T8													
	<i>aac(6')-Ib/sul1/dfrA1/tet(C)</i>	T6-1													
	<i>dfrA1</i>	M15-1													
	<i>aac(6')-Ib</i>	T7													
<i>Aeromonas sobria</i>	<i>dfrA1/sul1/tet(A)</i>	M19													
<i>Aeromonas veronii</i>	<i>bla_{TEM}/aac(6')-Ib/aacC2</i>	M15													
	<i>aac(6')-Ib</i>	T5													
<i>Shewanella xiamenensis</i>	<i>bla_{OXA-51}/sul1/tet(C)/qacEΔI-sul1</i>	T17													
<i>Acinetobacter baumannii</i>	<i>bla_{TEM}/bla_{OXA-23}/bla_{OXA-51}/qnrS/sul1/sul2/dfrA1</i>	H13													
	<i>bla_{OXA-23}/bla_{OXA-51}/qnrS/aac(6')-Ib/sul1/sul2/tet(B)</i>	T6													
	<i>bla_{OXA-51}/aac(6')-Ib/sul1/tet(A)</i>	T19													
	<i>bla_{OXA-51}/sul1/dfrA1</i>	H12													
	<i>bla_{OXA-51}/sul1/sul2/tet(A)</i>	T23													
<i>Comamonas aquatica</i>	<i>aac(6')-Ib</i>	M6													

Resistant

Intermediate

Susceptible

TIC ticarcillin, AMC amoxicillin-clavulanic acid, ETP ertapenem, IPM imipenem, CAZ ceftazidime, FEP cefepime, ATM aztreonam, GEN gentamicin, AMK amikacin, CIP ciprofloxacin, TET tetracycline, SXT trimethoprim-sulfamethoxazole

Table 4 Phylogenetic groups, virulence genes, and antimicrobial resistance genes, detected by microarray, of tested *Escherichia coli* isolates

Isolate	PG	Virulence genes	Resistance genes
T3	B1	<i>fimH/kpsM II/traT/ompA/ompT/TSPE4.C2</i>	<i>bla_{CTX-M}/aacC2/sul1/tet(A)/tetR/tet30/mphA/dfpA12</i>
T9	A	<i>iutA/traT/ompA</i>	<i>bla_{CTX-M}/aacC2/tet30/tetR/mphA</i>
T10	D	<i>chuA/fimH/fyuA/iutA/traT/ompA/TSPE4.C2</i>	<i>bla_{CTX-M}/bla_{TEM}/bla_{qROB}/aacC2/sul1/mphA</i>
T18	A	<i>ompA</i>	<i>bla_{CTX-M}/qnrB/sul2/tet(A)/tet30/tetR/int1</i>
W1	D	<i>chuA/cvaC/fimH/iroN/iss/iutA/traT/ompA</i>	<i>bla_{TEM}/bla_{qROB}/aacC2/aadA1/dfpA1/sul2/tet(A)/tet30/mphA/tetR/int2</i>
W4	D	<i>chuA/iha/cvaC/fimH/iroN/iss/iutA/traT/ompA/ompT/ompA/TSPE4.C2</i>	<i>bla_{TEM}/bla_{qROB}/aacC2/aadA1/dfpA1/int1/sul1/sul2/tet(A)/tetR</i> <i>/aph3strA/mphA/cmlA/int2</i>
W30	A	<i>cvaC/fimH/traT/ompA/yjaA</i>	<i>bla_{CTX-M}</i>
T15	A	<i>fimH</i>	<i>bla_{TEM}/qnrB/aacC2/aadA1/bla_{CTX-M}/int1/sul2/tet(A)/tet30/tetR</i>
W34	B1	<i>cvaC/fimH/iroN/iss/traT/TSPE4.C2</i>	<i>bla_{qROB}/mphA/cmlA/dfpA12/int2</i>

PG phylogenetic group

especially when these effluents are discharged in the environment (rivers, lakes, and seas, etc.) without any prior treatments. In this context, our study investigated cephalosporin resistant *Enterobacteriaceae* and non-*Enterobacteriaceae* GNB isolated from hospital effluents in Algeria.

In this work, the predominant species isolated were *E. coli* followed by *K. pneumoniae*. These bacteria are both pathogenic and commensal organisms and are frequent causes of nosocomial infections [23, 24]. In addition, *A. baumannii* and *A. hydrophila* were the predominant species among bacteria other than *Enterobacteriaceae* isolated in our study. *A. baumannii* has recently emerged as a leading cause of hospital associated outbreaks infections resulting from contamination and transmission in hospital environments [25]. *A. hydrophila* was also reported among the most frequent cause of human diseases among *Aeromonas* spp. [26]. The high levels of resistance to the β -lactam antibiotics (TIC and CAZ), and to the non- β -lactam-antibiotics (CIP and SXT), observed in this study, may be associated to the selective pressure exerted by these antibiotics commonly used in antibiotherapy. The fact that most of the isolates were MDR is worrisome. This is because MDR encoding genes are commonly carried on MGE such as plasmids, integrons, and transposons [27].

In our study, *bla_{CTX-M}* was the most predominant gene among the β -lactamase encoding genes detected. In fact, in the last decade, *bla_{CTX-M}* has explosively disseminated and becomes the most common resistance gene around the world [28]. Previous studies in Bejaia (site of our study) have also reported the dissemination of *bla_{CTX-M}* in patients [29] and in wild fish from the Mediterranean Sea [30]. This result is similar to that of some studies carried out on hospital effluents, particularly in Romania [31] and Poland [3]. However, the prevalence of these genes was lower compared to that reported in *Enterobacteriaceae* isolated from Zemirli hospital effluents, in Algeria [5].

Interestingly, genes encoding carbapenemases including *bla_{OXA-23}* and *bla_{OXA-48}*-like were detected for the first time in Algerian hospital effluents in *A. baumannii*, and *K. oxytoca* and *S. xiamenensis*, respectively. The emergence of such genes is considered as a threat in the treatment of GNB infections and can lead to MDR and extensive drug resistance; for example, MDR *A. baumannii* is recognized as the hardest nosocomial pathogen to control and treat [32]. The fact that *bla_{OXA-23}* was previously reported in *A. baumannii* isolated from hospitals located on the Mediterranean coast [33] and from seabream fish from the Mediterranean Sea [34] underlines the probable role of hospital effluents in the transmission of such genes from hospitals into the environment. These genes may also be derived from other sources such as municipal sewage and animal husbandry [2]. Interestingly, *S. xiamenensis* OXA-416 producing which is a known reservoir of antimicrobial resistance genes carried several other

resistance genes clustered on a transposon harbored by a pSx1 plasmid were detected and documented in a separate work [6]. The *bla*_{OXA-48} gene was also recently reported in *K. pneumoniae* outbreaks in Algeria [35] and this also indicates a potential dissemination between patients and the environment.

The genes *qnrB*, *qnrS*, *tet(A)*, *tet(B)*, *tet(C)*, *dfiA1*, *aacC2*, *aac(6′)-1b*, *sul1*, and *sul2* conferring resistance to non-β-lactam-antibiotics detected in this study are common in GNB and were also reported in different regions of Algeria [36]. The integrons were previously detected in high proportions of *Enterobacteriaceae* strains isolated from hospital effluents [37]. In our study, the identification of class 1 and class 2 integrons suggests that the resistance genes could be associated with MGEs and emphasizes their role in the dissemination of antibiotic resistance.

Microarray analysis performed on representative MDR *E. coli* isolates revealed the presence of additional resistance genes not tested by PCR. This highlights the importance of other methods such as microarray and genomic technology in the detection of resistance genes and MGEs. Previous studies on resistant *Enterobacteriaceae* isolates conducted on effluents from Algiers's hospital have reported the same resistance genes as in our study [5, 38] with the exception of *bla*_{ROB1}, *bla*_{OXA-48}, *bla*_{OXA-51}, *bla*_{OXA-23}, *mphA*, *tet30*, and *cmlA1*.

The *E. coli* phylogenetic groups A, B1, and D identified in this study had a low level of virulence and a high level of antimicrobial resistance, which is in agreement with previous studies [38]. This could be associated to the acquisition of antibiotic resistance [39]. The presence of these commensal MDR *E. coli* in hospital effluents underlines the role of humans as a source of antibiotic resistance in the environment.

In Algeria as well as many other developing countries, the use of antibiotics for human health and animal husbandry is not properly monitored and hospital effluents are continually discharged into the environment without pre-treatment. Selective pressure exerted by these antibiotics has several repercussions on public health and the environment, especially on the spread of antibiotic resistance. Our study revealed that Algerian hospital effluents carry MDR bacteria with several resistance genes, which could be transferred to different parts of the hydrological system such as surface water (used in agriculture) and sea water where they could reach aquatic animals as well as other neighboring countries. One cannot also exclude a possible transmission of MDR determinants to waterborne pathogens and their dissemination in the environment, which could lead to increased morbidity and mortality.

Owing to the global nature of the antimicrobial resistance problem, it is imperative for all countries to work together within a framework to better manage anthropogenic effluents, including hospital effluents, in order to limit the spread of MDR bacteria and antibiotic resistance genes.

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